

# Increased Adipogenesis of Osteoporotic Human-Mesenchymal Stem Cells (MSCs) is Characterized by Impaired Leptin Action

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**Abstract** The bone marrow contains mesenchymal stem cells (MSCs) that differentiate to the osteogenic and adipogenic lineages. The fact that the decrease in bone volume of age-related osteoporosis is accompanied by an increase in marrow adipose tissue implies the importance that the adipogenic process may have in bone loss. We previously observed that MSCs from control and osteoporotic women showed differences in their capacity to differentiate into the osteogenic and adipogenic pathways. In vitro studies indicate that bone marrow stromal cells are responsive to leptin, which increases their proliferation, differentiation to osteoblasts, and the number of mineralized nodules, but inhibits their differentiation to adipocytes. The aim of the present report was to study the direct effect of leptin on control and osteoporotic MSCs analyzing whether the protective effect of leptin against osteoporosis could be expressed by inhibition of adipocyte differentiation. MSCs from control, and osteoporotic donors were subjected to adipogenic conditions, in the absence or in the presence of 62.5 nM leptin. The number of adipocytes, the content of PPAR $\gamma$  protein, and mRNA, and leptin mRNA were measured by flow cytometry, Western blot, and RT-PCR, respectively. Results indicate that control and osteoporotic MSCs differ in their adipogenic potential as shown by expression of active PPAR $\gamma$  protein. Leptin exerted an antiadipogenic effect only on control MSCs increasing the proportion of inactive phosphorylated PPAR $\gamma$  protein. Finally, results obtained during adipogenesis of osteoporotic cells suggest that this process is abnormal not only because of increased adipocyte number, but because of impaired leptin cells response. *J. Cell. Biochem.* 103: 1054–1065, 2008.

**Key words:** leptin; adipocytes; mesenchymal stem cells; osteoporosis; PPAR $\gamma$ ; adipogenesis

The bone marrow stroma contains mesenchymal stem cells (MSCs) that differentiate, among others, to the osteogenic, and adipogenic lineages. The clinical fact that a decrease in bone volume of age-related osteoporosis is accompanied by an increase in marrow adipose tissue [Meunier et al., 1971] also implies the possible reciprocal relationship that is postulated to exist between the two differentiation pathways.

Many experimental models have provided substantial evidence for a reciprocal relationship between these cell lineages [Meunier et al., 1971; Beresford et al., 1992; Jilka et al., 1996; Nuttall et al., 1998; Rodríguez et al., 1999, 2000; Rosen and Buxsein, 2006].

We have previously observed that MSCs obtained from control and osteoporotic women show differences in their capacity to differentiate into the osteogenic and adipogenic pathways [Rodríguez et al., 1999, 2000]. These observations, coupled with the proposal that osteoblastic, and adipocyte differentiation pathways are regulated jointly, points that marrow adipogenesis is of concern in some osteogenic disorders [Meunier et al., 1971; Nuttall et al., 1998; Rodríguez et al., 2000; Rosen and Buxsein, 2006].

The environment of intracellular and extracellular signals controls MSCs differentiation

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into osteoblast or adipocyte. Lineage commitment is determined through phenotype-specific transcription factors, such as osteoblast-specific Runx2/Cbfa 1, and adipocyte-specific peroxisome proliferators activated receptor gamma (PPAR $\gamma$ ) [Tontonoz et al., 1994; Ducy et al., 1997; Karsenty, 2001; Rosen and Spiegelman, 2001]. PPAR $\gamma$  is a key regulator of osteoblast, and adipocyte differentiation, and its activation play a pivotal role in selection of adipogenesis over osteoblastogenesis [Lecka-Czernik et al., 1999]. PPAR $\gamma$  is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily [Mangelsdorf and Evans, 1995; Kersten et al., 2000; Rosen and Spiegelman, 2001]. PPAR $\gamma$  is expressed early in the adipocyte differentiation program, and is activated by long-chain fatty acids, peroxisome proliferators, and the thiazolidinedione class of antidiabetic agents [Mangelsdorf and Evans, 1995; Kersten et al., 2000; Rosen and Spiegelman, 2001].

The transcriptional activity of PPAR $\gamma$  is positively regulated by specific lipophilic ligands [Rosen and Spiegelman, 2001] and negatively regulated by phosphorylation on a mitogen-activated protein kinase (MAPK) consensus site at Ser 112 [Hu et al., 1996; Reginato et al., 1998; Chan et al., 2001] as well as by activation of p38 MAPK $\alpha$  [Aouanadi et al., 2006]. Phosphorylation-induced inhibition of the transcriptional activity of this transcription factor provides a mechanism to switch off the response to the ligand. The post-translational modifications of PPAR $\gamma$  activity through phosphorylation appears to be the pathway by which several growth factors and cytokines affect the transcription of numerous genes involved in lipid metabolism [Diradourian et al., 2005].

Leptin has recently emerged as a potential candidate responsible for the protective effects of fat on bone tissue [Rosen and Bouxsein, 2006; Thomas et al., 2000]. Published data on leptin effects on bone metabolism are apparently contradictory [Thomas and Burguera, 2002]. The effects of leptin are thought to be largely mediated via the hypothalamus [Karsenty, 2001]; however, the observation that the leptin receptor (OB-R) is widely expressed throughout the body suggests that leptin also may operate directly in peripheral tissues [Tartaglia, 1997; Reseland et al., 2001; Holloway et al., 2002].

Leptin is able to stimulate, through MAPK, and signal transducers, and activators of trans-

cription (STAT) signals [Ahima and Osei, 2004; Catalano et al., 2004]. Leptin-activated OB-R regulates well-known insulin-targets, such as IRS-1, MAP kinase, ERK, Akt, AMP kinase, and PI3- kinase, raising the possibility that leptin pathways act in concert with insulin to control energy metabolism, and other cellular processes [Ahima and Osei, 2004]. The leptin signal is terminated by induction of suppressor of cytokine signaling-3 (SOCS-3), a member of the family of proteins which inhibits the JAK-STAT signaling cascade [Bjorbaek et al., 1999].

Several *in vitro* studies indicate that bone marrow stromal cells are responsive to leptin, which increases their proliferation, differentiation to osteoblastic lineage, and the number of mineralized nodules [Takahashi et al., 1997; Thomas et al., 1999; Reseland et al., 2001], but inhibits their differentiation to adipocytes [Thomas et al., 1999]. These observations suggest that leptin may participate in the regulation of bone mass, but the mechanism by which this occurs remains unclear. The expression of a functional leptin receptor has been demonstrated in precursor cells of the osteoblast lineage [Thomas et al., 1999; Kim et al., 2003] showing that they can be direct targets for leptin. In this regard, we have studied the direct action of leptin on MSCs, characterizing a high affinity OB-R in these cells [Hess et al., 2005]. We demonstrated that the binding capacity of OB-R in MSCs increased through osteogenic and adipogenic (AD) cell differentiation. Osteoporotic MSCs showed significant lower leptin binding capacity than control cells at early (2–3 days) cell differentiation. In addition, leptin exerted a similar and low stimulatory osteogenic effect on both control, and osteoporotic cells, while, it significantly inhibited adipocyte differentiation only in control cells. Leptin did not affect AD differentiation of osteoporotic cells [Hess et al., 2005]. Finally, we demonstrated that leptin increased aromatase activity of MSCs during osteogenic but not during adipogenic differentiation [Pino et al., 2006].

The aim of the present report was to further study the direct effect of leptin on control and osteoporotic MSCs analyzing whether the protective effect of leptin against osteoporosis could be expressed by inhibition of adipocyte differentiation, as proposed by Thomas et al. [1999] and Thomas and Burguera [2002]. Results indicate that control and osteoporotic

MSCs differ in their AD potential as shown by expression of active PPAR $\gamma$  protein. In addition, leptin exerted an antiadipogenic effect only on control MSCs by increasing the proportion of inactive phosphorylated PPAR $\gamma$  protein. Finally, osteoporotic MSCs had marked impaired leptin response, which was related with increased AD potential of cells.

## MATERIALS AND METHODS

### Subjects

Post-menopausal women aged 65–86 years old who required bone surgery because of bone fracture at the Trauma Section of Hospital S tero del R o, Santiago, Chile, were asked to volunteer as bone marrow donors. Bone marrow was obtained by iliac crest aspiration during surgical procedures [Rodr guez et al., 1999]; written informed consent was obtained from all subjects, and ethical approval was obtained from the Hospital S tero del R o and Instituto de Nutrici n y Tecnolog a de los Alimentos (INTA) ethics committees. Bone mineral density (BMD) was measured for each subject within 4 weeks after surgery using dual-energy X-ray absorptiometry (DXA) (LUNAR, Prodigy, General Electric Medical Systems, Madison, WI). Donors were classified as control or osteoporotic according to their BMD value; control donors had BMD higher than  $-2.5$  standard deviations (SD) of the mean BMD for young adults, and osteoporotic donors had BMD lower than  $-2.5$  SD, and hip fracture [Raisz, 1997]. Control and osteoporotic donors considered themselves healthy, except for fracture, and were not under glucocorticoid or estrogen replacement therapy. Demographic characteristics of donors are summarized in Table I.

**TABLE I. Demographic Characteristics of Donors**

Characteristic	Control	Osteoporotic	<i>P</i> values
N	10	6	
Age (years)	71.6 $\pm$ 3.7	73.2 $\pm$ 8.1	0.643
LS T score <sup>a</sup>	-0.2 $\pm$ 1.9	-4.0 $\pm$ 0.9	0.001
BMD (g/cm <sup>2</sup> ) <sup>b</sup>	1.2 $\pm$ 0.2	0.7 $\pm$ 0.1	0.001
BMI (kg/m <sup>2</sup> ) <sup>c</sup>	29.7 $\pm$ 5.2	25.5 $\pm$ 3.5	0.043

<sup>a</sup>LS, lumbar spine; T score, number of standard deviations by which an individual value differs from a young adult population.

<sup>b</sup>BMD, bone mineral density.

<sup>c</sup>BMI, body mass index.

### Reagents

Tissue culture media and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO), Gibco/BRL (Gaithersburg, MD) or US Biologicals (Swamscott, MA). Tissue culture plastic ware was obtained from Nunc (Naperville, IL). Recombinant human leptin (rh-leptin) was purchased from Chemicon International, Inc. (Termeclula, CA).

Antibodies: Monoclonal mouse anti-PPAR $\gamma$  was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal rabbit anti-phosphorylated PPAR $\gamma$  was from Upstate (Charlottesville, VA), monoclonal mouse anti-beta-actin was from Sigma, peroxidase conjugated goat anti-mouse and anti-rabbit were from Rockland (Gilbertsville, PA).

Taq Polymerase, dNTPs, primers, MgCl<sub>2</sub>, and PCR reaction buffer, were purchased from Invitrogen Corporation (Carlsbad, CA), reverse transcriptase M-MLV was from Promega (Madison, WI) and RNAWiz was from Ambion (Austin, TX).

SDS-PAGE and nitrocellulose membranes were acquired from Bio-Rad Laboratories (Hercules, CA). PVDF membranes and ECL kit were purchased from Perkin Elmer Life Sciences (Boston, MA).

### Cell Preparation and Culture Methods

MSCs were classified as control (c-MSCs) or osteoporotic (o-MSCs) according to whether they derived from control or osteoporotic donors. MSCs were isolated from bone marrow as previously described [Jaiswal et al., 1997]. Briefly, 10 ml of bone marrow aspirate was added to 20 ml of Dulbecco's Modified Eagle medium high glucose (D-MEM) containing 10% fetal bovine serum (FBS) (basal medium) and was centrifuged to pellet the cells, discarding the fat layer. Cells were resuspended in basal medium and fractionated on a 70% Percoll density gradient. The MSCs-enriched low-density fraction was collected, rinsed with basal medium, and plated at a density of  $1-2 \times 10^7$  nucleated cells/100 mm dish. Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. After 4 days in culture, non-adherent cells were removed and fresh culture medium was added. Basal medium was replaced by fresh medium twice weekly. When culture dishes became near confluence, cells were detached by mild treatment with trypsin (0.25%, 5 min,

37°C) and replated at 1/3 the original density to allow for continued passage. The experiments described here were performed after the fourth cell passage.

### Adipogenic Differentiation

MSCs obtained from control and osteoporotic donors ( $1 \times 10^5$  cells/35 mm dish) were maintained in culture in basal medium for at least 4 days before adding basal or AD medium during defined times. The adipogenic medium consisted of basal medium supplemented with 1  $\mu$ M dexamethasone, 10  $\mu$ g/ml insulin, 0.45 mM isobutyl-methyl-xanthine, and 0.1 mM indomethacin in the presence of freshly prepared leptin (62.5 nM, or 1  $\mu$ g/ml) or its vehicle (PBS). This was replaced by fresh adipogenic medium every 4 days.

The adipocyte number was determined after 14 days in culture. Cells were released from the culture dish with a mild trypsin treatment (0.25%, 5 min at 37°C) and counted in a hemocytometer. Cell concentration was adjusted to  $1 \times 10^6$  cells/ml and MSCs were tested for their adipocyte content by flow cytometric quantitation of lipid accumulation. Cells were placed in freshly diluted Nile Red (1 mg/ml) and analyzed by flow cytometry [Dennis et al., 1999] (FACS-Calibur, Becton Dickinson, Franklin, NJ).

### Immunofluorescence Staining

Cells were seeded on coverslips in 24 multiwell plates and cultured under basal or adipogenic conditions. After treatment, cells were washed two times with PBS, and fixed with a 3.7% formaldehyde solution in PBS, for 15 min at room temperature. Cells were afterwards permeabilized with a 0.2% Triton X-100 in 3.7% formaldehyde solution, for 15 min at room temperature. The fixed cells were re-hydrated with Tris buffer saline (TBS) (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris-base, pH 8.0), and incubated for 45 min in blocking solution (3% BSA in TBS) at room temperature. After this, cells were incubated with a specific primary mouse anti-PPAR $\gamma$  antibody, at 1:25 dilution in blocking solution during 45 min at 37°C and subsequently with the secondary antibody, FITC-conjugated rabbit anti-mouse IgG, at a 1:250 dilution in 3% BSA-TBS during 45 min at 37°C. Finally, the cells were rinsed in TBS, mounted in DABCO/mowiol and examined with an epifluorescence microscope (100 $\times$  objective, Nikon, Labophot-2, Japan). In the

controls, the first or the second antibodies were omitted.

### Western Blot

Cells cultured under basal or AD conditions were lysed as described by Lecka-Czernik et al. [1999]. Proteins (20  $\mu$ g) from cells lysated were denatured with sample buffer (2% SDS, 10% glycerol, 0.06 M Tris-HCl, 0.01% bromophenol blue, 20%  $\beta$ -mercaptoethanol, pH 6.8) during 5 min at 100°C. Proteins were separated by 8% SDS-PAGE. Then, samples were transferred to nitrocellulose or PVDF membranes, for 1 h at 100 V. Membranes were blocked with 3% BSA in 0.05% TBS-Tween, for 1 h at room temperature. Incubation with the corresponding primary antibody (anti-PPAR $\gamma$  at a 1:500 dilution, anti-phosphorylated PPAR $\gamma$  at a 1:3,000 dilution, and anti- $\beta$ -actin at a 1:5,000 dilution, in blocking solution) was performed during 16 h at 4°C. Membranes were washed three times with TBS-Tween 0.05%, and incubated with the corresponding peroxidase conjugated secondary antibody at a 1:5,000 dilution during 2 h at room temperature. Immunoreactive bands were visualized using the ECL kit.

Quantification was performed using Kodak Digital 1D software.  $\beta$ -Actin was used as load control.

### RNA Extraction and RT-PCR Analysis

Cells cultured for determined times were released from the culture dishes by a mild treatment with trypsin, cells were collected, and suspended with 1 ml of RNeasy. The extracted RNA was quantified in a spectrophotometer (MBE2000, Perkin Elmer) and stored at a -80°C. RNA integrity was visualized by electrophoresis under denaturing conditions.

The complementary cDNA was synthesized using reverse transcriptase M-MLV, through a 15 min cycle at 45°C, then at 4°C in ice for 5 min, and an extension of 45 min at 70°C. For PCR reactions, 300 ng of cDNA was amplified with 30  $\mu$ l of reaction mix 1 $\times$  PCR reaction buffer, 1.5 mM MgCl<sub>2</sub>, 6 pmol of each primer, 0.2 mM dNTPs, 22.3  $\mu$ l of nucleases-free water, and 2.5 U of Taq Polymerase. Table II shows the primer sequences used in this study, the melting temperature ( $T_m$ ), and the sizes of the PCR products. The PCR products were separated in 1.2% agarose gels.

**TABLE II. Primer Sequences for PCR Amplification**

Gen	Sequence	No. of cycles	T <sub>m</sub> (°C)	Size of PCR product
PPAR <sub>γ</sub>	5' CAGTGGGGATGCTCATAA 3' 5' CTTTTGGCATACTCTGTGAT 3'	35	54	422
Leptin	5' GTAGAGTTTGAAGGAGGTGA 3' 5' CTGGGAATTAGCCATTGTG 3'	35	57	349
aP2	5' AACCTTAGATGGGGGTGTCCTG 3' 5' TCGTGGAAGTGACGCCTTTC 3'	36	58	125
Adipsin	5' CAAGCAACAAAGTCCCGAGC 3' 5' CCTGCGTTCAAGTCATCCTC 3'	36	57	261
GPDH	5' ACCACAGTCCATGCCATCAC 3' 5' TCCACCACCCTGTTGCTGTA 3'	29	60	452

### Statistical Analysis

All measurements were performed in triplicate from at least three different samples. Values are expressed as mean ± SD. Two-sample Student's *t*-test was used to evaluate differences between samples and the respective controls; *P* < 0.05 was considered significant.

## RESULTS

### AD Differentiation of MSCs and Effect of Leptin

Control and o-MSCs showed dissimilar capacity for AD differentiation: o-MSCs developed twofold more adipocytes than c-MSCs (Table III). Since previous studies showed that MSCs developed responses to leptin, we tested the effect of 62.5 nM leptin on AD differentiation. This dose was selected considering previous observations [Hess et al., 2005; Pino et al., 2006]. We observed that the addition of leptin during AD treatment of cells decreased by 60% AD differentiation of c-MSCs, while it had no effect on o-MSCs. Therefore, in the presence of leptin AD differentiation is fivefold higher in osteoporotic than in c-MSCs. An initial contamination with adipocytes in both

**TABLE III. Effect of Leptin on Adipogenic Differentiation of MSCs**

Cells types	Adipocytes (%)	
	-Leptin	+Leptin
Control	11.5 ± 3.3	4.6 ± 0.8*
Osteoporotic	22.3 ± 6.5**	21.9 ± 5.7**

Cells (1 × 10<sup>5</sup>)/dish 35 mm obtained from control and osteoporotic donors were cultured in adipogenic medium in the absence or in the presence of leptin (62.5 nM). After 14 days in culture, adipocyte number was measured by flow cytometric analysis as described in Materials and Methods section.

\**P* < 0.05 as compared with -leptin value.

\*\**P* < 0.05 as compared with control values.

types of MSCs was discarded, since flow cytometric analysis, and adipogenic markers like adipsin, aP2, and leptin were negative at day 0 of AD differentiation (data not shown).

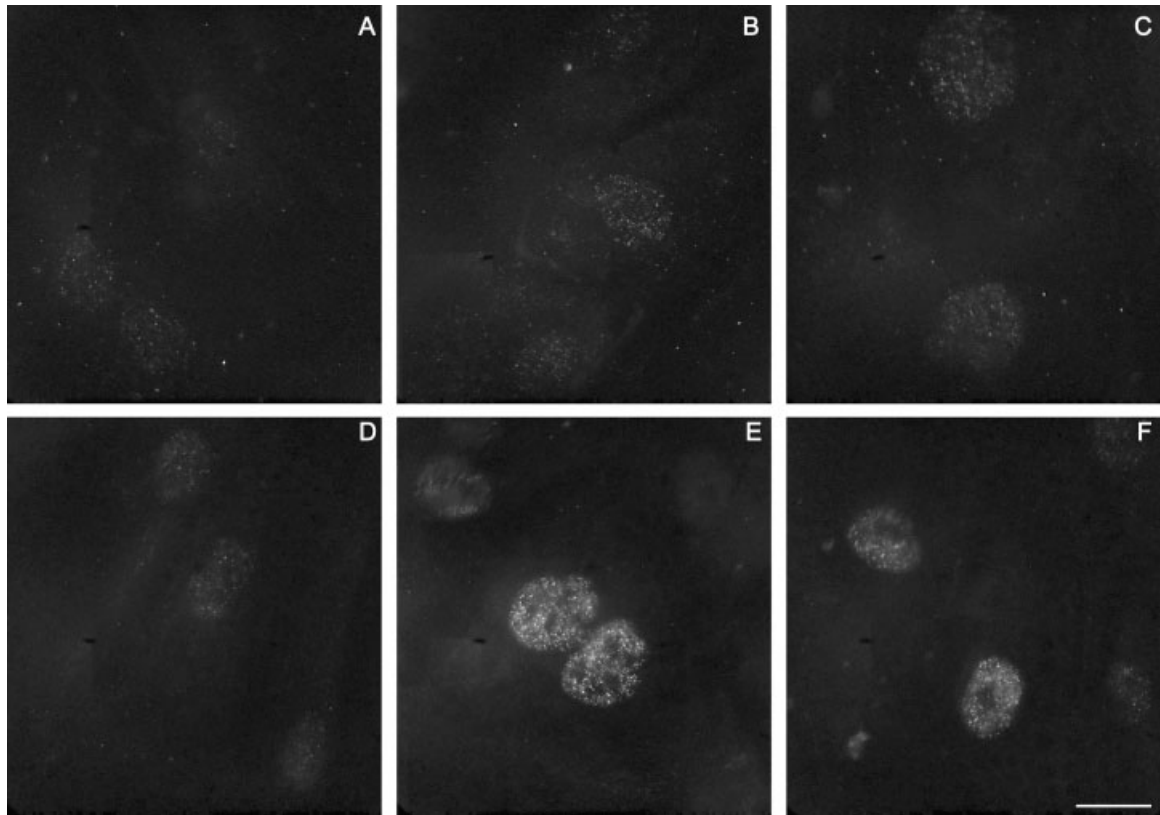
### MSCs Derived From Control and Osteoporotic Donors Have Different PPAR<sub>γ</sub> Activity

Immunocytochemistry for PPAR<sub>γ</sub> in MSCs showed in addition of an exclusive nuclear localization to the transcription factor, different fluorescence intensities in control, and o-MSCs. Low fluorescence for PPAR<sub>γ</sub>, both under basal, and adipogenic conditions was apparent in c-MSCs (Fig. 1A,B), while under these same experimental conditions a high fluorescent signal was developed by o-MSCs (Fig. 1D,E). Leptin addition during the adipogenic treatment of c-MSCs further decreased fluorescent signal for PPAR<sub>γ</sub>, but in o-MSCs no change in fluorescence intensity was observed after the addition of the hormone (Fig. 1C,F)

Western blots analysis allowed to further corroborate these observations (Fig. 2A). A significant difference in the amount of PPAR<sub>γ</sub> produced by control, and o-MSCs was detected by densitometric analysis in cells before or during AD differentiation, so that the mean PPAR<sub>γ</sub> protein level was sixfold higher in o-MSCs than in c-MSCs (Fig. 2B). During early AD differentiation PPAR<sub>γ</sub> level increased twofold in control cells, while o-MSCs maintained unchanged their high PPAR<sub>γ</sub> level until 15 days of AD differentiation.

### Leptin Affects Differently PPAR<sub>γ</sub> Protein Content in Control and Osteoporotic MSCs

Using Western analysis we appreciated that leptin added during AD differentiation affected differentially the immuno activity for PPAR<sub>γ</sub> in control and osteoporotic cells (Fig. 2A). Leptin significantly decreased the expression of PPAR<sub>γ</sub>



**Fig. 1.** Specific immunocytofluorescence for PPAR $\gamma$  during adipogenic (AD) differentiation of control (upper row) and osteoporotic (bottom row) human mesenchymal stem cells (MSCs). Cells were incubated in basal (A,D), adipogenic (B,E), or adipogenic plus 62.5 nM leptin (C,F) medium for 7 days. Cells were fixed and treated as specified in methods, using mouse anti PPAR $\gamma$  antibody as the primary antibody. Images were obtained from Nikon, Labophot-2 microscope at same exposure settings. The original magnification was 100 $\times$ . Bar = 10  $\mu$ m.

in control cells until day 5 of differentiation, the mean expression level of PPAR $\gamma$  decreased 60–70% in the presence of leptin, as compared with cells differentiating in the absence of leptin (Fig. 2C). In contrast, the presence of leptin during AD differentiation of o-MSCs did not affect the immunoactivity for PPAR $\gamma$  up to day 5 although a decrease of 30–40% of PPAR $\gamma$  immuno activity was observed only at day 7 of AD differentiation (Fig. 2D).

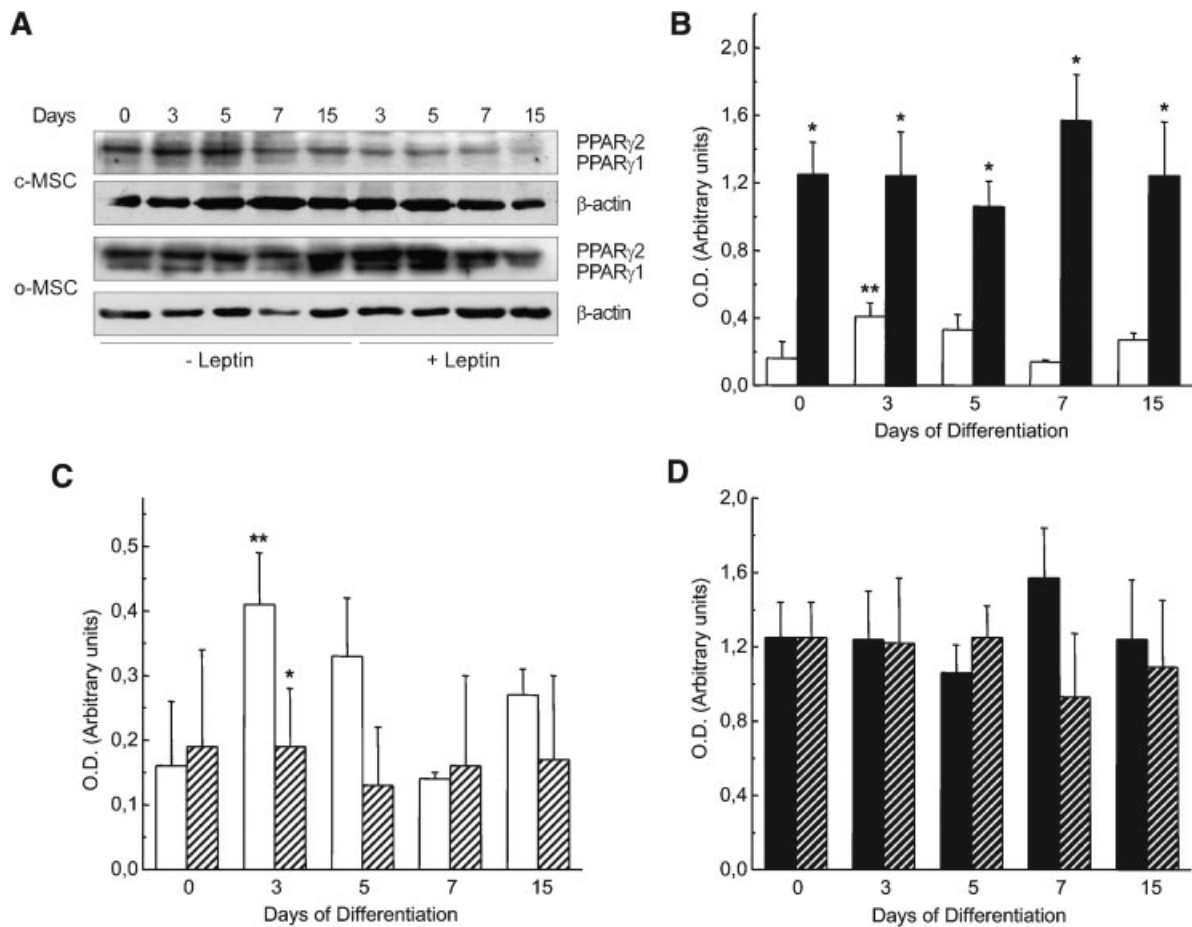
#### **Analysis of mRNA for PPAR $\gamma$ in Both Types of MSCs**

To analyze whether the difference in PPAR $\gamma$  among control and o-MSCs is also expressed at the mRNA for PPAR $\gamma$  level, the relative amount of PPAR $\gamma$  mRNA was measured by RT-PCR. As shown in Figure 3, the expression level of mRNA for PPAR $\gamma$  was similar in both cells types and remained almost constant through all period studied. Moreover, leptin did not modify the

levels of mRNA for PPAR $\gamma$  in both types of cells (Fig. 3)

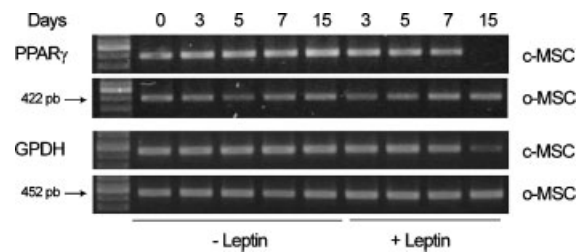
#### **Leptin Increased Phosphorylated PPAR $\gamma$ Level in Control MSCs**

Since PPAR $\gamma$  transcriptional activity is modified by phosphorylation at Ser 112, we analyzed by specific Western blots whether the level of this PPAR $\gamma$  differs among c-MSCs and o-MSCs (Fig. 4A). Before differentiation both control and o-MSCs had a similar level of phosphorylated PPAR $\gamma$  (Fig. 4B,C). Considering the high PPAR $\gamma$  content in o-MSCs (Fig. 2B), a much higher phosphorylated PPAR $\gamma$ /PPAR $\gamma$  ratio results in controls than in osteoporotic cells. During AD differentiation of control cells, phosphorylated PPAR $\gamma$  levels decreased by 50% up to day 7, recovering the basal level at the end of the differentiation period studied (Fig. 4B). However, when AD differentiation of these cells was developed in the presence of



**Fig. 2.** Effect of adipogenic differentiation with or without leptin on PPAR $\gamma$  protein level. Control (c-MSCs) and osteoporotic (o-MSCs) human mesenchymal stem cells were cultured under adipogenic conditions in the absence or presence of 62.5 nM leptin for specified time. **A:** Representative Western blots analysis of PPAR $\gamma$  proteins separated by SDS-PAGE. Histograms represent relative PPAR $\gamma$  level as detected by western blots and densitometric analysis in: **(B)** c-MSCs (white bars) and o-MSCs (black bars), \* $P < 0.05$  compared with respective value of

c-MSCs. \*\* $P < 0.05$  compared with day 0 value of AD differentiation; **(C)** c-MSC incubated in the absence (white bars) or in the presence of 62.5 nM leptin (dashed bars), \* $P < 0.05$  compared with value in the absence of leptin. \*\* $P < 0.05$  compared with value of day 0 of AD differentiation, and **(D)** o-MSC incubated in the absence (black bars) or in the presence of 62.5 nM leptin (dashed bars). All data were normalized by  $\beta$ -actin, expressed as optical density and represent the mean  $\pm$  SE of three different samples.

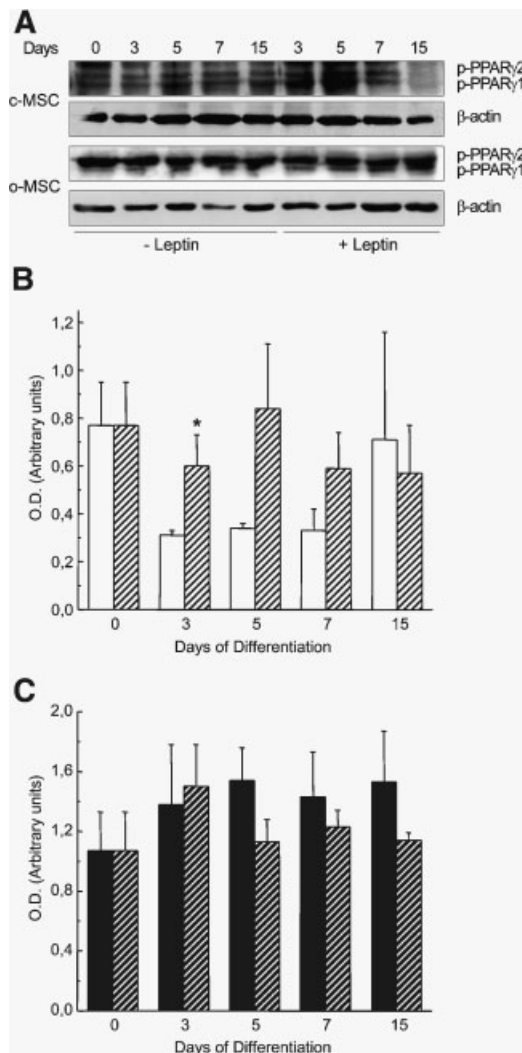


**Fig. 3.** Effect of leptin on PPAR $\gamma$  mRNA expression by control (c-MSCs) and osteoporotic (o-MSCs) human mesenchymal stem cells. Cells were cultured under adipogenic condition in the absence or in the presence of leptin 62.5 nM leptin, during specified time. Total RNA was extracted and subjected to RT-PCR analysis as described in Materials and Methods section.

leptin phosphorylated PPAR $\gamma$  levels were maintained at the basal levels throughout the period studied (Fig. 4B). On the other hand, no change in the level of phosphorylated PPAR $\gamma$  was observed in o-MSCs, maintaining their basal level during AD differentiation, either in the absence or the presence of leptin.

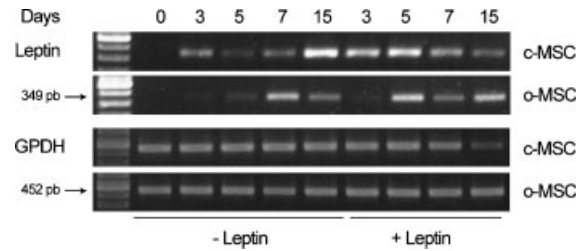
#### Leptin mRNA Expression by MSCs During Adipogenic Differentiation

A representative analysis by RT-PCR of the leptin gene expression during AD differentiation of both types of MSCs is shown in Figure 5. In the absence of exogenously added leptin, the expression of the hormone transcript in c-MSCs



**Fig. 4.** Effect of leptin on phosphorylated PPAR $\gamma$  (p-PPAR $\gamma$ ) level in control (c-MSCs) and osteoporotic (o-MSCs) human mesenchymal stem cells. Cells were cultured under adipogenic medium in the absence or presence of 62.5 nM leptin. **A:** Representative Western blots analysis for p-PPAR $\gamma$  separated by SDS-PAGE. Histograms represent relative p-PPAR $\gamma$  levels in c-MSC (**B**) and o-MSCs (**C**), incubated in the absence (white bars) or in the presence of 62.5 nM leptin (gray bars), as detected by Western blots and densitometric analysis. Data were normalized by  $\beta$ -actin, expressed as optical density and represent the mean  $\pm$  SE of three different samples. \* $P < 0.05$  compared with value in the absence of leptin. \*\* $P < 0.05$  compared with value of day 0 of AD differentiation.

changed from undetected in basal conditions to measurable levels from day 3 up to day 15 of AD differentiation. The addition of leptin during AD differentiation noticeably increased leptin transcript level during early differentiation (3–5 days), but afterwards the transcript level decreased. In contrast, o-MSCs showed



**Fig. 5.** Representative RT-PCR analysis of leptin mRNA expression by control (c-MSCs) and osteoporotic (o-MSCs) mesenchymal stem cells. Cells were cultured under adipogenic conditions in the absence or in the presence of 62.5 nM leptin during specified time. Total RNA was extracted and subjected to RT-PCR analysis as described in Materials and Methods section.

no leptin transcript before day 5 in the absence of exogenously added hormone, although the leptin transcript was detected from day 5 of AD differentiation in the presence of added leptin (Fig. 5).

## DISCUSSION

In the bone marrow, MSCs are common multipotential progenitors for osteoblasts, and adipocytes [Pittenger et al., 1999]. It has been proposed that the close relationship between these lineages underlies the reciprocal relationship between increased adipocytes and decreased bone formation in conditions of bone loss like age-related osteoporosis [Jilka et al., 1996; Verma et al., 2002], microgravity [Zayzafon et al., 2004] and diabetes [Forsen et al., 1999]. In this study, we observed that leptin significantly inhibited adipocyte differentiation only in control cells (60%). The effect of leptin on c-MSCs agrees with data obtained in immortalized human marrow stromal cell lines [Thomas et al., 1999]; however, results obtained during adipogenesis of osteoporotic cells suggest that this process is abnormal not only because of increased adipocyte number, but because of impaired leptin cells response.

We studied PPAR $\gamma$  protein, and mRNA as markers of the AD potential of control, and osteoporotic cells, since PPAR $\gamma$  appears to be a key component in the determination, and differentiation process [Rosen and Spiegelman, 2001]. The fact that at basal or along AD differentiation, control cells had much lower content of the transcription factor protein than osteoporotic cells explains at the molecular level the restricted adipogenesis observed in control cells, suggesting that in these cells an interplay



of cell signals restrict AD differentiation [Aouanadi et al., 2006]. Leptin could be one of such signals, since its activity limited cells AD potential in control cells by originating a further decrease in the level of PPAR $\gamma$  protein from day 3 to 15 of AD differentiation, accordingly with the reductive effect of the hormone on adipocyte number. In contrast, osteoporotic cells did not change their high PPAR $\gamma$  protein content through AD differentiation, nor as a response to leptin addition. Thus, in addition to differences in the level of PPAR $\gamma$  protein among control, and osteoporotic cells, these cells showed different capability for developing a leptin response.

Studies in human adipose tissue have found no difference in the mRNA levels of PPAR $\gamma$  among lean controls or obese or type 2 diabetic subjects, nor with aging [Auboeuf et al., 1997; Imbeault et al., 2001]. In this study, control and o-MSCs showed similar amount of PPAR $\gamma$  mRNA, either before or through AD differentiation, in the presence or the absence of leptin, suggesting that post-transcriptional regulation could explain the different PPAR $\gamma$  protein activity of both types of cells. Several factors that inhibit AD differentiation cause MAPK-mediated phosphorylation of PPAR $\gamma$  with subsequent loss of its transcriptional activity [Hu et al., 1996; Reginato et al., 1998; Chan et al., 2001]. Here we examined phosphorylated PPAR $\gamma$  protein on the MAPK consensus site Ser 112 as a measure of inactive PPAR $\gamma$  protein. A relationship among the AD potential and phosphorylated PPAR $\gamma$  level was observed only in control cells; accordingly with AD stimulation, the level of phosphorylated PPAR $\gamma$  decreased in these cells. Since both PPAR $\gamma$  mRNA and protein have a short half-life [Waite et al., 2001], even slight changes in the amount of active/inactive PPAR $\gamma$  protein could affect the AD potential of cells. Consequently, we found that leptin added to control cells during early adipogenesis significantly increased phosphorylated PPAR $\gamma$  protein, consistent with the protective effect of leptin on adipogenesis. In contrast, in osteoporotic cells the level of phosphorylated PPAR $\gamma$  did not change during AD differentiation either in the presence or absence of the adipokine. Therefore, it appears that o-MSCs have lost the MAPK-dependents activities that in control cells determine the appropriate active/inactive relationship of PPAR $\gamma$  protein.

Several observations suggest that leptin may be an important paracrine signaling molecule in the bone marrow microenvironment [Laharrague et al., 1998; Thomas et al., 1999; Reseland et al., 2001; Gordeladze et al., 2002; Hess et al., 2005]. Here we show that leptin restricts AD potential in c-MSCs, by increasing phosphorylated PPAR $\gamma$ ; in contrast, leptin action is hampered in osteoporotic MSCs during early adipogenesis, linking this failure with the increased AD potential of these cells. Apparently, this would be different to the response observed in vivo in the leptin-deficient *ob/ob* mouse [Hamrick et al., 2005; Wang et al., 2005], in which the defective leptin response would result from decreased leptin availability [Hamrick et al., 2005].

Diminished leptin action on MSCs could be the result of changes at the receptor or post-receptor levels. Previously, we showed an OB-R binding activity in these MSCs [Hess et al., 2005]. In the present study, we measured the activity of leptin gene by RT-PCR showing that osteoporotic MSCs had much lower expression of this gene than control cells. Leptin transcript in control cells was not detected under basal conditions, but its transcription was measurable from day 3 of AD differentiation. Moreover, after exogenously added leptin, its transcript level further increased in control cells. In contrast, despite increased AD capacity of osteoporotic MSCs, no leptin transcript was detected during differentiation of these cells in the absence of the hormone. Therefore, impaired leptin action on o-MSCs could result in part because of decreased leptin production. Abolished leptin transcription in these cells could result from the high activity of PPAR $\gamma$ , since experimental cells and human studies have concluded that high PPAR $\gamma$  activation inhibits leptin gene transcription [Kallen and Lazar, 1996; Sabatakos et al., 2000; Patel et al., 2003]. In addition, in human pre-adipocytes it has been observed that inhibition of different signaling pathways resulted in inhibition of leptin secretion, but not always inhibition of adipocyte differentiation [Patel et al., 2003].

Therefore, it could be proposed that during early adipogenesis of o-MSCs the diminished OB-R level [Hess et al., 2005] as well as low leptin production would determine sub-optimal cell leptin signaling. This proposition is supported by the observation that o-MSCs in the presence of exogenously added leptin increased

their production of the adipokine transcript from day 5 of AD differentiation, implying that these cells require high leptin doses to develop some cell response.

Insufficient leptin action has been recognized as underlying in clinical states like metabolic syndrome, obesity (induced either by diet or lack of leptin or its receptor), lipodystrophy, Cushing's syndrome, polycystic ovarian disease, and aging [Unger, 2003]. It appears exciting that a failure of leptin cell signaling may cooperate in the anomalous adipogenesis of o-MSCs. During development of osteoporosis several factors could play a part inducing cell impairment of leptin action, like increased SOC-3 induction by cytokines [Bjorbaek et al., 2000], increased endogenous or exogenous glucocorticoids [Mazziotti et al., 2006], decreased estrogen [Casabiell et al., 1998], and protein tyrosine phosphatase-1B [Cheng et al., 2002; Zabolotny et al., 2002]. On the other hand, the endogenous production of lipophylic ligands responsible for activating PPAR $\gamma$  transcription factors is rather unknown, both at physiological or pathological conditions.

From our observations it could be concluded that leptin signal malfunctioning in o-MSCs includes at least the following issues: (1) diminished OB-R binding at early AD differentiation [Hess et al., 2005]; (2) loss of the MAPK-dependents activities that determine the appropriate active/inactive relationship of PPAR $\gamma$  protein; and (3) poor leptin production by cells.

In summary, our results show different AD potential among control and osteoporotic cells that could be related to PPAR $\gamma$  protein activity. In addition, we demonstrated that leptin exerts an antiadipogenic effect on c-MSCs by increasing the proportion of inactive PPAR $\gamma$  protein. Osteoporotic MSCs characterized by impaired leptin cell response which result in increased AD potential of cells.

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