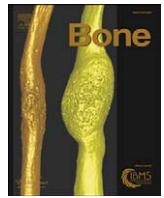




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## HO-1 expression increases mesenchymal stem cell-derived osteoblasts but decreases adipocyte lineage<sup>☆</sup>

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## ABSTRACT

Human bone marrow mesenchymal stem cells (MSC) are pleiotropic cells that differentiate to either adipocytes or osteoblasts as a result of cross-talk by specific signaling pathways including heme oxygenase (HO)-1/-2 expression. We examined the effect of inducers of HO-1 expression and inhibitors of HO activity on MSC differentiation to the osteoblast and adipocyte lineage. HO-1 expression is increased during osteoblast stem cell development but remains elevated at 25 days. The increase in HO-1 levels precedes an increase in alkaline phosphatase (AP) activity and an increase in BMP, osteonectin and RUNX-2 mRNA. Induction of HO-1 by osteogenic growth peptide (OGP) was associated with an increase in BMP-2 and osteonectin. Exposure of MSC to high glucose levels decreased osteocalcin and osteogenic protein expression, which was reversed by upregulation of the OGP-mediated increase in HO-1 expression. The glucose-mediated decrease in HO-1 resulted in decreased levels of pAMPK, pAKT and the eNOS signaling pathway and was reversed by OGP. In contrast, MSC-derived adipocytes were increased by glucose. HO-1 siRNA decreased HO-1 expression but increased adipocyte stem cell differentiation and the adipogenesis marker, PPAR $\gamma$ . Thus, upregulation of HO-1 expression shifts the balance of MSC differentiation in favor of the osteoblast lineage. In contrast, a decrease in HO-1 or exposure to glucose drives the MSC towards adipogenesis. Thus, targeting HO-1 expression is a portal to increased osteoblast stem cell differentiation and to the attenuation of osteoporosis by the promotion of bone formation.

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## Introduction

Human bone marrow-derived mesenchymal stem cells give rise to osteoblastic and adipogenic lineages. The shift from osteoblastic to adipocyte lineage is a result of cross-talk and the various factors that drive MSC to the adipocyte lineage inhibiting osteoblast formation [1]. Diabetes affects dynamic bone formation in both humans and animals leading to osteopenia and osteoporosis [2,3] and increased adipogenesis [4–6]. Bone mineral density and other biochemical markers of

bone turnover are adversely affected in individuals with diabetes [7]. Reduction of bone mass, occurring with increased frequency in individuals with diabetes mellitus, has been attributed to poor glycemic control, but the pathogenic mechanisms remain unknown. High concentrations of glucose (hyperglycemia) in diabetics exacerbate this complication [7–9]. Osteoblasts secrete growth factors including platelet-derived growth factor, insulin like growth factors and bone morphogenetic proteins [10] that are stored in the bone matrix. Whether these factors are affected by diabetes remains to be seen. Furthermore, the molecular mechanism underlying osteoblast stem cell differentiation under hyperglycemic conditions has not been fully elucidated.

In contrast to diabetes, recent studies have shown that several growth factors including osteogenic growth peptide (OGP) enhance differentiation of mesenchymal stem cells to osteoblasts [11] and that endothelial growth factor (EGF) and OGP enhance osteoblast cell proliferation by regulating several signaling pathways. Osteogenic growth peptide is a naturally occurring tetradecapeptide that is both

*Abbreviations:* HO-1, heme oxygenase-1; AP, alkaline phosphatase; VEGF, vascular endothelial growth factor; OGP, osteogenic growth peptide; NO, nitric oxide; CO, carbon monoxide; MSC, mesenchymal stem cell; ROS, reactive oxygen species; EGF, endothelial growth factor.

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an anabolic agent and a hematopoietic stimulator [12]. OGP increases osteoblast proliferation, alkaline phosphatase (AP) activity and matrix synthesis and mineralization. It prevents glucocorticoid-induced apoptosis and the subsequent bone remodeling alterations that are associated with steroids [13].

Heme oxygenase-1 (HO-1) plays a major role during bone marrow stem cell differentiation [14–16]. Heme oxygenase which exists in two forms, HO-1 (inducible) and HO-2 (constitutive), catalyzes the rate-limiting step in heme degradation, resulting in the formation of carbon monoxide (CO), iron and biliverdin, the latter is subsequently reduced to bilirubin by biliverdin reductase. Additionally, both CO and nitric oxide (NO) protect against tumor necrosis factor-induced apoptosis in osteoblasts [17]. In addition, during fracture repair, activation of HIF-1 and its target genes, VEGF and HO-1, regulate osteoblastogenesis and bone reabsorption [18], suggesting a role of HO-1 in bone metabolism. Finally, several lines of evidence suggest that HO-1 plays an important physiological role in obesity and diabetes. Increased HO-1 expression decreases adipogenesis in obese animals [4,5,19]. However, the role of HO-1 expression in mesenchymal stem cell (MSC) development and differentiation into either osteoblasts or adipocyte stem cells is poorly understood. HO-1 expression and its role in diabetes and other pathologies is a burgeoning area of research [20]. HO-1 is a target gene for the prevention of diabetes and obesity. Increased HO-1 expression resulted in higher adiponectin levels and improved insulin sensitivity [19].

The goal of the present study was to determine whether HO-1 and HO-2 expression shifts the balance of MSC-mediated lineage expression towards osteoblast or adipocyte differentiation. MSC are multipotent cells that have the potential to proliferate and differentiate into a variety of cell types characteristic of bone, skeletal and cardiac muscle, adipose tissue and neural cells [21–24] and are used as a model system to study the effect of HO-1 expression on osteoblast and adipocyte differentiation. We report here that inducers of HO-1 expression such as OGP affect osteoblast differentiation via increased levels of HO-1, pAKT and eNOS. We demonstrate that osteoblast differentiation is positively regulated by HO-1 expression, which was associated with a reduction of reactive oxygen species (ROS), thereby permitting the restoration of osteoblast markers, specifically, induction of OPG and osteocalcin, while HO-1 expression negatively regulates adipocyte stem cell differentiation.

## Materials and methods

### Cell culture from BM and differentiation into osteoblasts

Frozen bone marrow mononuclear cells were purchased from Allcells (Allcells, Emeryville, CA). After thawing the cells, mononuclear cells were resuspended in an  $\alpha$ -minimal essential medium ( $\alpha$ -MEM, Invitrogen, Carlsbad CA) supplemented with 20% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA). The cells were plated at a density of  $1\text{--}5 \times 10^6$  cells per 100 cm<sup>2</sup> dish. The cultures were maintained at 37 °C in a 5% CO<sub>2</sub> incubator, and the medium was changed after 48 h and every 3–4 days thereafter. When the MSCs were confluent, the cells were recovered by the addition of 0.25% trypsin/EDTA (Life Technologies, Frederick, MD). Mesenchymal stem cells (MSCs passages 2–3) were plated in a 60-cm<sup>2</sup> dish at a density of  $1\text{--}2 \times 10^4$  and cultured in  $\alpha$ -MEM with 10% FBS for 7 days at 37 °C. HO-1 silencing was achieved by infecting cells 2 days after seeding with a commercially available lentiviral siRNA for HO-1, at a viral total of 4–6 transduction units per milliliter; as a control, a siRNA scrambled mixture was used. siRNA HO-1 was purchased from Sigma-Aldrich, St. Louis, MO.

Beginning at day 7, cultures were switched to differentiation medium in phenol red-free BGJ<sub>b</sub> (Fitton-Jackson modification) and supplemented with 5% FBS containing 50  $\mu$ g/ml ascorbic acid (for

appropriate collagen and extracellular matrix production). From day 14 forward, 3 mM  $\beta$ -glycerophosphate (for appropriate mineralization) was added to the differentiation media. For some studies, cultures were grown entirely in phenol red-free Ham's F-12 with 5% FBS without the addition of differentiation medium.

### HO-1 siRNA transfection

Cells were treated with three different predesigned siRNAs of HO-1 gene (SASI\_Hs01\_00035068, SASI\_Hs01\_00035065 and SASI\_Hs01\_00035067 from Sigma-Aldrich, St. Louis, MO). According to the manufacturer's protocol, Osteogenic media containing siRNA using N-TER (Sigma-Aldrich, St. Louis, MO) was replaced every 48 h. Briefly, nanoparticle solution was incubated with 10 nM siRNA. After 20 min, cells were treated with siRNA solution, with or without OGP during osteogenesis for 21 days.

### DNA content and fragmentation

Osteoblasts were plated (4000 cells/96 wells) in two plates, for cellular density using CyQuant™ kit (Molecular Probes, Eugene, OR) and for the fragmentation DNA assay using Apostain Elisa Kit (Alexis Biochemicals, Inc., Plymouth Meeting, PA) according to the manufacturer's protocol. Cultures were treated with both Osteogenic media and OGP for 21 days.

### Alkaline phosphatase activity

Cells were plated in 6-well plates. Cell layers were washed twice with ice-cold phosphate-buffered saline (PBS), then harvested in 1 ml 50 mM Tris-HCl (pH 7.6), sonicated twice on ice and then centrifuged at 4 °C for 15 min at 1000 $\times$ g. The supernatants were stored at –20 °C until analysis for alkaline phosphatase activity was conducted, using *p*-nitrophenylphosphate as substrate. Absorbance was read at 405 nm using a microplate reader. Alkaline phosphatase activity was expressed as nmol *p*-nitrophenol released/min per  $\mu$ g DNA. All analyses were replicated 6 times. Each experiment was repeated two or three times.

### Measurement of DNA content and bone mineralization

Osteoblasts were plated in 6-well plates. Cell layers were washed twice with ice-cold PBS, harvested in 50 mM Tris-HCl (pH 7.6), sonicated and then centrifuged for 15 min at 1000 $\times$ g. The supernatants were analyzed for DNA content by measuring fluorescence at 458 nm spectrophotometrically, using purified calf thymus as a DNA standard [25]. All analyses were performed in six replicates. Bone mineralization was determined using Alizarin Red S (Sigma-Aldrich, St. Louis, MO) staining and phase-contrast microscopy 21 days after treatment. Cell were incubated with 2% alizarin red at pH 4.2 for 10 min and subsequently washed with distilled water. Subcultured cells were examined by phase-contrast microscopy at 21 days to determine cell morphology and to verify the presence of mineralized nodules.

### Immunoblot analysis

Osteoblasts or adipocytes were incubated with stimulants in T75 flasks for 24 h. They were then washed with PBS and trypsinized (0.05% trypsin w/v with 0.02% EDTA). The pellets were lysed in buffer (Tris-Cl 50 mM, EDTA 10 mM, Triton X-100 1% v/v, PMSF 1%, pepstatin A 0.05 mM and leupeptin 0.2 mM) and, after mixing with sample loading buffer (Tris-Cl 50 mM, SDS 10% w/v, glycerol 10% v/v, 2-mercaptoethanol 10% v/v and bromophenol blue 0.04%) at a ratio of 4:1, were boiled for 5 min. Samples (10  $\mu$ g protein) were loaded onto 12% gels and subjected to electrophoresis (150 V, 80 min). The separated proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA; 1 h, 200 mA per gel). After transfer, the blots

were incubated overnight with 5% nonfat milk in TBS followed by incubation with 1:1000 dilution of the primary antibody for 3 h. Polyclonal rabbit antibodies directed against the human HO-1, AMPK, pAMPK, peNOS and HO-2 were obtained from Stressgen Biotechnologies (Victoria, BC). After washing with TTBS, the blots were incubated for 2 h with secondary antibody (1:5000) and conjugated with alkaline phosphatase. Finally, the blots were developed using a premixed solution containing 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.48 mM nitro blue tetrazolium (NBT) in buffer (Tris-HCl 10 mM, NaCl 100 mM, MgCl<sub>2</sub> 59.3 μM, pH 9.5). The blots were scanned and the optical density of the bands was measured using Scion (New York, NY) Image software.

#### Human bone marrow-derived adipocyte mesenchymal stem cells

Frozen bone marrow mononuclear cells were purchased from Allcells (Allcells, Emeryville, CA). After thawing, the cells were resuspended in an  $\alpha$ -minimal essential medium ( $\alpha$ -MEM, Invitrogen, Carlsbad CA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad CA) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad CA). The cells were plated at a density of  $1-5 \times 10^6$  cells per 100 cm<sup>2</sup> dish. The cultures were maintained at 37 °C in a 5% CO<sub>2</sub> incubator, and the medium was changed after 48 h and every 3–4 days thereafter. When the MSCs were confluent, the cells were recovered by the addition of 0.25% trypsin/EDTA (Life Technologies, Frederick, MD). MSCs (passages 2–3) were plated in a 60-cm<sup>2</sup> dish at a density of  $1-2 \times 10^4$  and cultured in  $\alpha$ -MEM with 10% FBS for 7 days. The medium was replaced with adipogenic medium, and the cells were cultured for an additional 21 days. The adipogenic medium consisted of complete culture medium supplemented with OM DMEM-high glucose, 10% (v/v) FBS, 10 μg/ml insulin, 0.5 mM dexamethasone (Sigma-Aldrich, St. Louis, MO), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO) and 0.1 mM indomethacin (Sigma-Aldrich, St. Louis, MO).

#### Oil Red O staining

For Oil Red O staining, 0.5% Oil Red O solution (Sigma-Aldrich, St. Louis, MO) was used. Briefly, adipocytes were fixed in 1% formaldehyde, washed in Oil red O for 20 min, rinsed with 85% propylene glycol (Sigma-Aldrich, St. Louis, MO) for 3 min, washed in distilled water and mounted with aqueous mounting medium, [26].

#### Heme oxygenase activity assay

HO activity was assayed in homogenates of osteoblasts. Cell homogenates were incubated with 50 μM heme, 2 mg/ml rat liver cytosol (as a source of biliverdin reductase), 1 mM MgCl<sub>2</sub>, 3 units glucose-6-phosphatase dehydrogenase, 1 mM glucose-6-phosphate and 2 mM NADP<sup>+</sup> in 0.5 ml 0.1 M potassium phosphate buffer, pH 7.4, for 30 min at 37 °C. The reaction was terminated by placing the tubes on ice and bilirubin was then extracted with chloroform. The amount of bilirubin generated was determined using a dual-beam scanning spectrophotometer (Perkin-Elmer, Norwalk, CT, Lambda 17 UV/VIS) and is defined as the difference between 464 and 530 nm (extinction coefficient: 40 mM<sup>-1</sup> cm<sup>-1</sup> for bilirubin). The results were expressed as nmol of bilirubin/5 × 10<sup>6</sup> cells/h. Tin mesoporphyrin (SnMP), purchased from Frontier Science, Logan UT, was used to inhibit heme oxygenase activity [27]. Cobalt protoporphyrin (CoPP) was purchased from Frontier Science, Logan, UT, to induce HO-1 expression and HO activity [28].

#### mRNA isolation and real-time PCR quantification

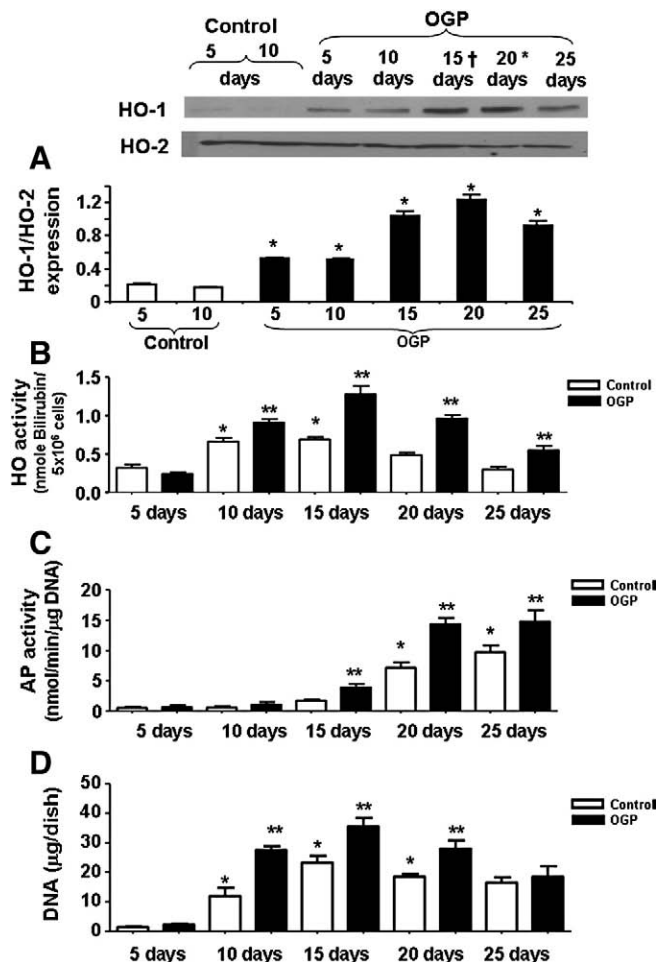
Total RNA was isolated using Trizol<sup>®</sup> (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was

synthesized with Roche reverse transcription reagents. Total RNA (1 μg) was analyzed by real-time PCR.

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the TaqMan gene expression assay on an ABI Prism 7900 sequence analyzer according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA). Each reaction was run in triplicate. The comparative threshold cycle (C<sub>T</sub>) method was used to calculate the amplification fold as specified by the manufacturer. A value of 10 ng of reverse-transcribed RNA samples was amplified by using the TaqMan Universal PCR Master Mix and TaqMan gene expression assays (ID Hs01055564\_m1 for human BMP-2, ID Hs00231692\_m1 for RUNX2, ID HS99999901\_s1 for 18S as an endogenous control; Applied Biosystems).

#### ELISA assay for osteocalcin and osteoprotegerin

By using a specific ELISA test, according to the manufacturer's recommendation, we evaluated the levels of osteocalcin (BioSource International, Inc., Camarillo, CA, USA) and OPG (osteoprotegerin) in the culture supernatant (BioVendor Laboratory Medicine, Modrice, Czech Republic).



**Fig. 1.** (A) Effect of OGP (Upper panel) and protein levels in osteoblasts as a function of time during the periods of proliferation and differentiation. Western blot analyses of HO proteins in osteoblast homogenates were performed using antibodies against human HO-1 and HO-2 proteins. Blots shown are representative of 3 separate experiments. (B) Effect of OGP on HO activity in isolated cell homogenates of cells harvested after 5, 10, 15, 20 and 25 days of growth. HO activity in osteoblasts was measured as described in the Materials and methods section. Results are the mean ± SE; n = 3; \*p < 0.05 compared to 5 days OGP treated, effect of OGP on AP activity (C) and DNA accumulation (D). AP activity and DNA content were measured as described in the Materials and methods section. Data presented are the result of 3 separate experiments. \*p < 0.001.

### Statistical analysis

Data are presented as mean  $\pm$  standard error (SE) for the number of experiments. Statistical significance ( $p < 0.05$ ) was determined by the Fisher method of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by single factor analysis of variance (ANOVA) for multiple groups or unpaired *t*-test for two groups.

### Results

#### Effect of OGP on HO-1 protein levels and HO activity

The basal levels of HO-1 expression increased during MSC osteoblast-culture growth and peaked at 20 days following treatment with OGP. HO activity followed a similar pattern but with a peak at day 15 ( $p < 0.05$ ) and then gradually declining (Fig. 1B). HO-2 protein levels did not change during osteoblast proliferation in absence of OGP (days 5 and 10) or in presence of OGP (Figs. 1A and B). To examine whether HO-1 protein expression was associated with a corresponding increase in osteoblast proliferation and differentiation, the temporal sequence of differentiation was also determined. Alkaline phosphatase (AP) activity gradually increased, reaching a maximum at day 20, where it plateaued. OGP treatment significantly ( $p < 0.05$ ) increased AP levels above control (Fig. 1C). A temporal increase in DNA ( $\mu\text{g}/\text{plate}$ ) followed a pattern similar to that of HO-1 protein. As seen in Fig. 1D, DNA accumulation in control increased to reach a maximum at day 15 and declined to low levels at 25 days. OGP treatment resulted in significantly ( $p < 0.05$ ) increased levels of DNA at days 10, 15 and 20 over control.

#### Effect of OGP on differentiation of mesenchymal stem cells into osteoblasts

PCR quantification of undifferentiated MSCs and of cells at day 21 after culture in differentiation medium confirmed that the cells were positive for early mature osteoblastic markers such as BMP-2, RUNX-2, osteonectin and osteocalcin (Fig. 2A). The effect of OGP real-time PCR quantification of osteonectin mRNA indicated a time-dependent increase reaching a maximum at day 15 followed by a decline at day 21. BMP-2 and RUNX-2 exhibited a similar time-dependent increase that peaked at day 21 of OGP exposure (Fig. 2B).

#### Effect of OGP on eNOS, peNOS and pAKT

To investigate the possibility that differential activation of eNOS, peNOS and the anti-apoptotic signaling molecule pAKT could account for the increase in osteoblast cell proliferation and differentiation, we assessed the levels of these proteins after 10 days of culture in the presence of OGP. As seen in Fig. 3, OGP treatment resulted in a significant ( $p < 0.05$ ) increase in the amount of HO-1 protein. OGP supplementation also resulted in a significant increase in the anti-apoptotic protein pAKT. Upregulation of HO activity by OGP also resulted in a significant increase in eNOS and peNOS. It should be noted that the significant increase in pAKT occurred without any change in AKT ( $p < 0.05$ ; Fig. 3).

#### Effect of OGP on HO-1 expression and pAMPK in the presence of siRNA

We tested the hypothesis that OGP increases signaling molecules in osteoblasts via an increase in HO-1. MSCs were transduced with HO-1 siRNA to test whether this treatment could inhibit HO-1 mRNA

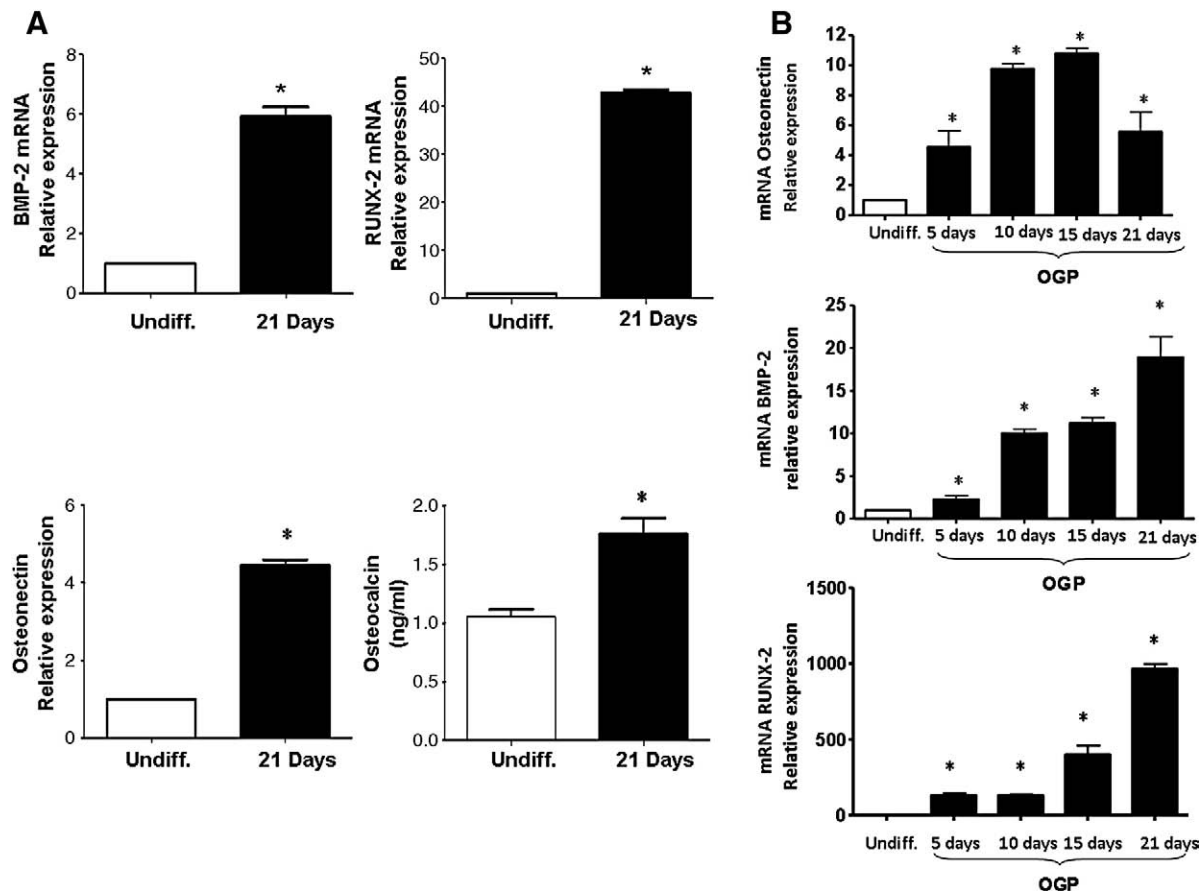
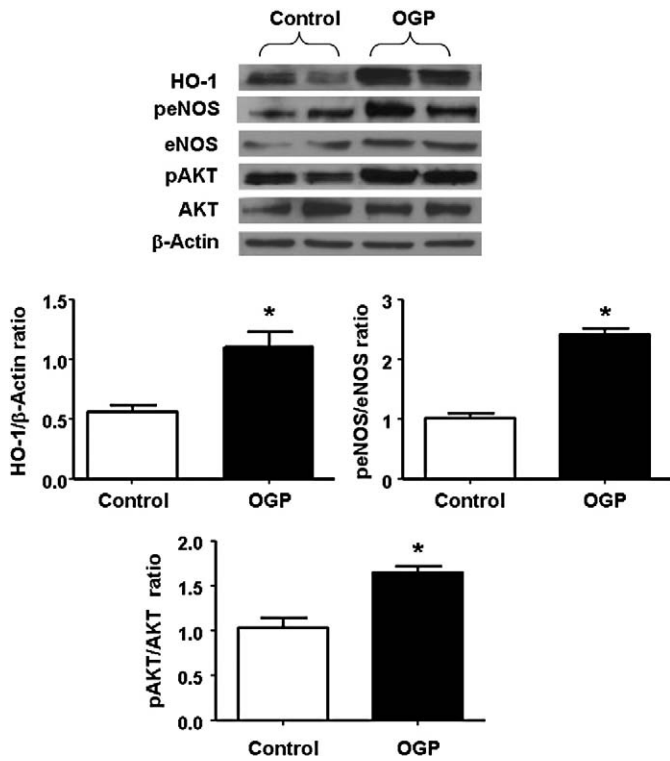


Fig. 2. (A) Expression of osteoblastic markers in differentiated MSCs by PCR. qRT-PCR revealed a marked increase in BMP-2, RUNX2, osteocalcin and osteonectin at 21 days of osteoblastic differentiation. Bars represent the mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$  vs. undifferentiated cells. (B) Expression of mRNA of osteonectin, BMP-2 and RUNX-2 with time of exposure to OGP (10–6 M). The results are of 3 independent experiments. \* $p < 0.05$  vs. undifferentiated cells.



**Fig. 3.** Western blot and densitometry analysis of effect of OGP on HO-1 expression, peNOS and eNOS, pAKT and AKT proteins measured after 21 days of osteoblast differentiation. We performed quantitative densitometry evaluation of peNOS, eNOS, pAKT and AKT in the cells. \* $p < 0.05$  control vs. OGP. Each bar represents mean  $\pm$  SE of 3 experiments.

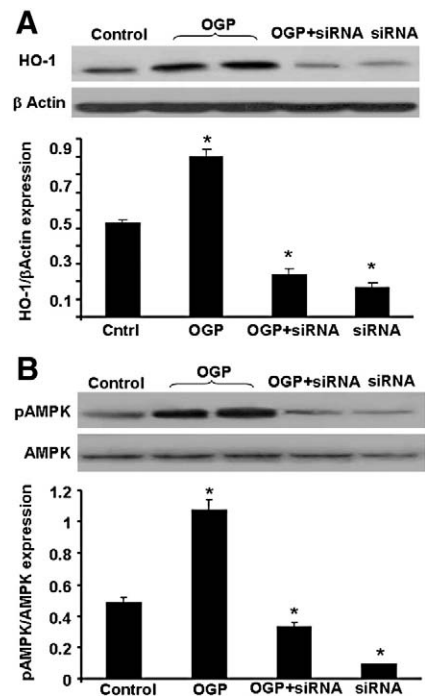
and the signaling molecule pAMPK expression. The results presented in Fig. 4A strongly support the hypothesis that OGP increased both HO-1 and pAMPK expression in osteoblasts. HO-1 siRNA added to osteoblast cells dramatically reduced both HO-1 expression (Fig. 4A) and pAMPK levels (Fig. 4B).

#### Effect of high glucose levels on OPG and osteocalcin levels during osteoblastic differentiation: role of OGP

We verified whether suppression of HO-1 as a result of hyperglycemia (glucose 20 mM) can affect other gene expression. OPG expression (Fig. 5A) and osteocalcin secretion (Fig. 5B) during differentiation were significantly ( $p < 0.05$ ) decreased when MSCs were exposed to glucose (20 mM) compared to untreated cells. In addition, OGP treatment in the presence of glucose resulted in an increase in osteocalcin levels after secretion was reduced by a high glucose concentration. Similarly, glucose exposure showed a significant ( $p < 0.05$ ) reduction in osteonectin secretion compared to untreated control cells (data not shown). This is the first demonstration that OGP can protect osteoblasts from hyperglycemia by increasing the levels of OPG and osteocalcin (Figs. 5A and B) during osteoblast cell growth and differentiation. Inhibition of HO activity by SnMP treatment in the presence of glucose abrogated OGP-mediated increases in both OPG and osteocalcin (Figs. 5A and B).

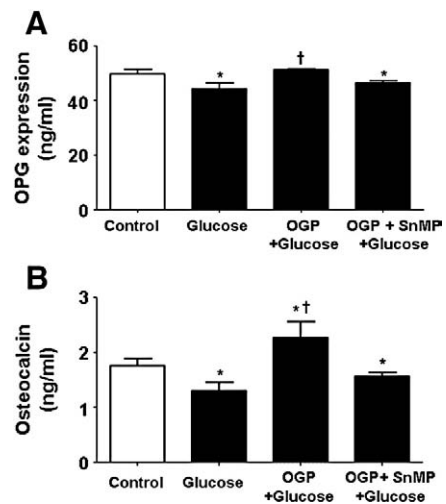
#### Effect of OGP and glucose on HO-1, eNOS, peNOS, pAMPK and pAKT expression

The induction of diabetes by culturing cells in glucose resulted in a significant decrease in HO-1 protein levels Fig. 6A. This decrease was partially restored by culturing the cells in the presence of OGP. Similarly, eNOS and pAMPK phosphorylation were decreased by culturing osteoblasts in glucose (Fig. 6A). OGP treatment had no effect

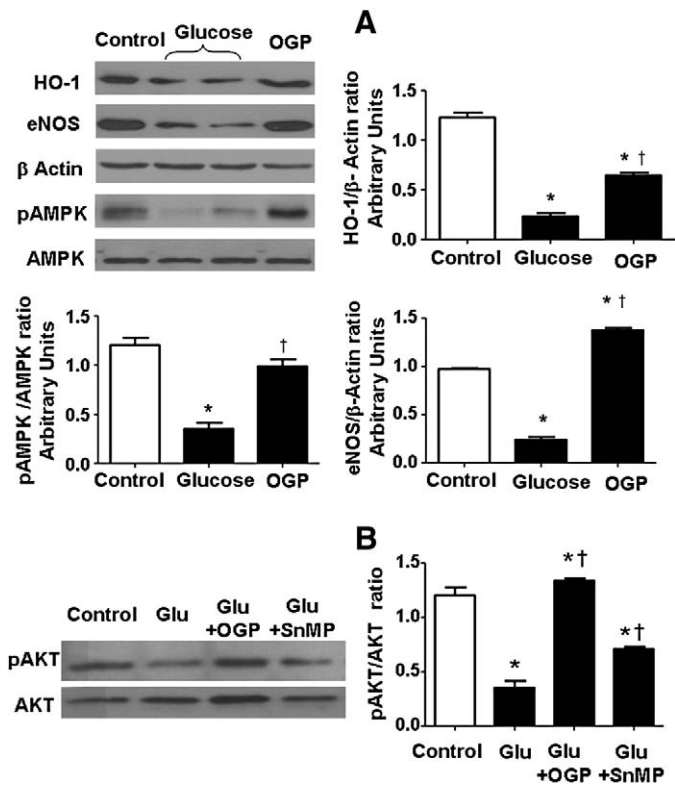


**Fig. 4.** (A) Western blot analysis of HO in osteoblasts homogenates treated with OGP ( $10^{-6}$  M) for 10 days. In the presence and absence of siRNA HO-1, immunoblots were performed using antibodies against human HO-1 protein. Blots shown are representative of 3 separate experiments, \* $p < 0.05$ . (B) Effect of siRNA HO-1 on OGP-mediated pAMPK activation. \* $p < 0.05$  and \*\* $p < 0.01$ , compared to OGP,  $n = 4$ .

on AMPK expression. However, there was a significant ( $p < 0.05$ ) increase in the expression of pAMPK and eNOS (Fig. 6A). As seen in Fig. 6B, pAKT phosphorylation was decreased by culturing osteoblasts in glucose and there was no effect on AKT. The presence of OGP reversed the effect of glucose and increased activation of pAKT. The changes in protein expression of pAKT (Fig. 6B) mirrored those seen with HO-1 protein expression (Fig. 6A). Similarly, MSCs cultured with SnMP, an inhibitor of HO-1/-2 activity [29], and glucose displayed a significant  $p < 0.05$  decrease in pAKT (Fig. 6B) compared to MSC-cultured with OGP and glucose.



**Fig. 5.** (A and B) Effect of glucose (30 mM), OGP + glucose and SnMP + glucose + OGP on osteoblast marker expression. ELISA shows an increase of OPG (A) and osteocalcin (B). Control bars represent the mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$  vs. undifferentiated cells <sup>†</sup> $p < 0.05$  compared to glucose. Addition of SnMP caused a statistically significant reduction in the expression of both,  $p < 0.05$ .



**Fig. 6.** (A) Lysates of osteoblasts cultured in the presence of a high glucose concentration (30 mM) and OGP were assayed. Representative Western blots are shown for HO-1, eNOS, pAMPK, AMPK and  $\beta$ -actin proteins. Bars represent the mean  $\pm$  SEM of four independent experiments. (B) pAKT and AKT levels were measured in presence of vehicle solution, glucose (30 mM) and glucose + OGP in combination with SnMP. Both CoPP and OGP increased pAKT levels. \* $p < 0.05$  vs. differentiated cells (control); † $p < 0.05$  vs. glucose.

#### Effect of HO-1 induction on osteogenesis, adipogenesis and PPAR $\gamma$ levels

We used a potent inducer of HO-1, CoPP, to assess the differential effect of HO-1 on osteogenesis and adipogenesis. CoPP treatment resulted in an increase in bone mineralization when measured at day 21 (Fig. 7A). The increase was significant ( $p < 0.01$ ) when compared to vehicle. In contrast, the reverse was seen on adipogenesis with a significant ( $p < 0.05$ ) decline apparent at day 21 (Fig. 7B). In agreement with the effect of HO-1 on adipogenesis (Fig. 7C), the decline in HO-1 expression at day 10 of adipogenic differentiation ( $p < 0.05$ ) was accompanied by a significant ( $p < 0.01$ ) increase in the levels of PPAR $\gamma$  (Fig. 7C). In addition, HO-1 siRNA increased the area of lipid droplets, i.e., adipogenesis (Fig. 8).

#### Discussion

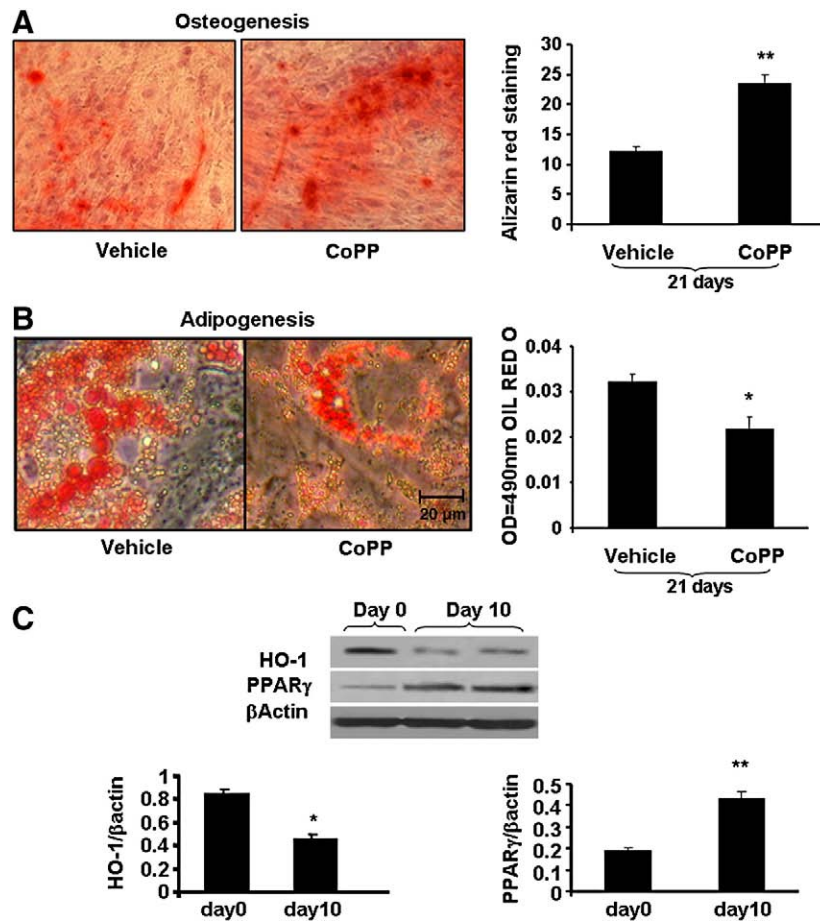
In the present study we show, for the first time, that the upregulation of HO-1 increases MSC-mediated osteoblasts with concomitant reduction in adipocytes. The OGP-mediated increase in HO-1 levels increases osteoblast proliferation and differentiation and is associated with an increase in osteoblast function, via an increase in AKT. A significant increase in OGP-mediated cell proliferation was observed while, in contrast, both SnMP, a competitive inhibitor of HO activity, and HO-1 siRNA reversed the OGP-mediated effect, suggesting that the effect of OGP was dependent on an increase in both HO-1 expression and HO activity. To further explore the functional expression of HO-1 in human MSC-mediated osteoblast lineages, we measured AP, DNA accumulation and bone mineralization. The increase in HO-1 resulted in an increase in the rate of AP and DNA accumulation and mineralization as a function of time when

compared to untreated osteoblasts. In addition, osteoblasts cultured in the presence of an inhibitor of HO-1, as in cells exposed to high glucose, exhibited a decrease in the levels of BMP-2, osteonectin, pAMPK and eNOS. However, upregulation of HO-1 by OGP in cultured osteoblasts rescued the hyperglycemia-mediated decrease in BMP-2, HO-1, eNOS and pAMPK. Previous studies have shown that eNOS was expressed in osteoblasts and that a deficiency of this enzyme resulted in a significant reduction in bone formation in mice [30]. Thus, the OGP-mediated increase in HO-1 and eNOS can be regarded as a pivotal step in bone metabolism through an ability to modulate osteoblast function. eNOS and NO are stimulators of the levels of BMP-2 and increase differentiation of osteoblasts [31,32].

More recently, we reported that HO-1 overexpression in animal models of both type 1 and type 2 diabetes attenuates vascular dysfunction via an increase in pAMPK and AKT and a decrease in oxidative stress [4,28,33]. Diabetes affects the integrity and functionality of bone tissue [34–36] possibly through increased adiposity [37]. Patients with diabetes frequently show either low bone mass (osteopenia) or increased bone mineral density with an increased risk of fracture and an impairment in bone healing [38], presumably due to stimulation of osteoblast apoptosis [39], recently reviewed [40].

Finally, the present data demonstrate a differential effect of HO-1 on MSC-mediated adipocyte stem cells. We investigated the effect of HO-1 expression on differentiation. A clear induction of adipogenic transformation was observed upon exposure of MSC to glucose. The capacity of high glucose to activate adipogenic differentiation has been described in isolated adipocytes [19] and was shown to be dependent on suppression of HO-1. In agreement with these results, glucose increased adipogenesis and this was associated with the suppression of HO-1 protein levels. Glucose has been shown to suppress HO-1 promoter and HO-1 levels [41,42]. High glucose suppressed HO-1 expression in cell lines [42–44] as well as in animal models [19,45,46]. In fact, supplementation of high glucose, 20 mM, increased adipogenesis which was further increased in the presence of HO-1 siRNA (Fig. 8). The identification that inhibition of HO-1 expression increased the MSC shift towards adipocytes has at least two important conceptual implications. Firstly, high glucose has an adipogenic potential; secondly, a direct link exists between the suppression of HO-1 and the increase in adipogenesis and metabolic syndrome. Upregulation of HO-1 was involved in detecting and decoding a variety of stress conditions including hyperglycemia and angiotensin II-mediated stress [4]. We recently showed that HO-1 recruits the EC-SOD to act as an anti-oxidant and to dissipate H<sub>2</sub>O<sub>2</sub> [45] and in triggering an increase in adiponectin and the signaling pathway pAMPK–pAKT [47]. Additionally, Fig. 7 shows that HO-1 expression decreased during differentiation, while PPAR $\gamma$  levels increased. PPAR $\gamma$  is commonly referred to as the master regulator of adipogenesis [48,49]. Ectopic expression and activation of PPAR $\gamma$  are sufficient to induce adipocyte differentiation. Given the role of HO-1 expression in preventing obesity [15], it is possible that the differential role of HO-1 in adipocyte and osteoblast lineages might represent a strategy to curb adiposity and increase osteogenesis.

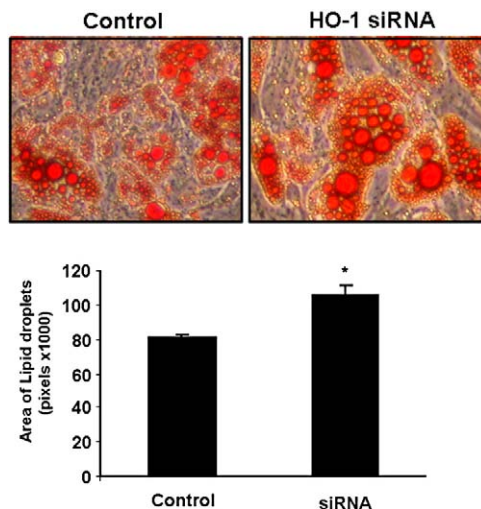
The results of the present study show that increased HO-1 expression and HO activity are essential for MSC growth to the osteoblast lineage and is consistent with the role of HO-1 in hematopoietic stem cell differentiation [14,15,50] in which HO-1 regulates stem cell differentiation to a number of lineages [14–16]. The HO-1/HO-2 system participates in the regulation of cell differentiation in osteoblasts and adipocytes in a cell-specific but very different manner. Although, the basal levels of HO-1 protein and HO activity are needed for osteoblast cell growth, an increase in the basal level of HO-1 resulted in the enhancement of osteoblast differentiation. Induction of HO-1 is essential for the resultant increase in pAKT, pAMPK, peNOS levels and NO bioavailability [5,28,47]. An increase in NO may be necessary for OGP-mediated osteoblastic activity [51]; upregulation of NO was



**Fig. 7.** Samples were stained as described in the [Materials and methods](#) section to determine (A) bone mineralization. CoPP (2.0  $\mu$ M) was added once every 5 days during culture media change for 21 days. These pictures are representative of 3 repeated experiments at 16 days,  $**p < 0.01$  and  $*p < 0.05$  compared to control. (B) Adipogenesis MSCs were treated with 2.0  $\mu$ M CoPP for 14 days and lipid droplets were stained with Oil red O then measured adipogenesis on 490 nm wave length. Levels of significance:  $*p < 0.05$  control vs. CoPP; and (C) effect of HO-1 expression on PPAR $\gamma$  expression,  $*p < 0.05$ ,  $**p < 0.02$ ,  $n = 4$ .

shown to play a positive role in bone formation [30–32]. Although the mechanism by which HO-1 overexpression decreased adipocyte differentiation but increased osteoblast differentiation is still unclear, it is apparent that HO activity and its products, bilirubin, CO and iron, play a different role in cell proliferation. More recently, it was shown that the elevation of HO-1-derived CO in endothelial cells enhanced

endothelial cell proliferation [52,53]. In contrast, increased HO-1 levels caused a decrease in vascular smooth cells [4]. The effect of HO-1 expression on osteoblasts and adipocytes is mirrored by the effect of HO-1 on endothelial cells and vascular smooth muscle cells. In fact, adipocyte stem cells from both obese rats and mice have low levels of HO-1 protein and HO activity, which may reflect an increase in adiposity [4,19]. Upregulation of HO-1 in obesity decreases adiposity and increases adiponectin [4]. Thus, the site-specific delivery of HO-1 to adipocytes may play a regulatory role in the prevention of adipocyte differentiation in a variety of vascular diseases including the metabolic syndrome [4]. Our results provide direct evidence that HO-1 gene expression has a differential effect on osteoblast and adipocyte cell proliferation and differentiation. Thus, by manipulating the expression of HO-1, it will be possible to attenuate the hyperglycemia-mediated inhibition of osteoblast differentiation, while simultaneously inhibiting adipocyte differentiation and thereby offering potential targets in the management of the metabolic syndrome.



**Fig. 8.** The effect of HO-1 siRNA on adipogenesis. Lipid droplets area was determined by Oil red O staining after 21 days.  $*p < 0.05$  control vs. HO-1 siRNA.

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