

# Simvastatin and Atorvastatin Enhance Gene Expression of Collagen Type 1 and Osteocalcin in Primary Human Osteoblasts and MG-63 Cultures

Silvia Ruiz-Gaspa,<sup>1</sup> Xavier Nogues,<sup>1,2,4\*</sup> Anna Enjuanes,<sup>1</sup> Joan C. Monllau,<sup>1,2</sup> Josep Blanch,<sup>1</sup> Ramon Carreras,<sup>2</sup> Leonardo Mellibovsky,<sup>1,4</sup> Daniel Grinberg,<sup>3</sup> Susana Balcells,<sup>3</sup> Adolfo Díez-Perez,<sup>1,2,4</sup> and Juan Pedro-Botet<sup>2,4</sup>

<sup>1</sup>URFOA, IMIM, Hospital del Mar, Barcelona, Spain

<sup>2</sup>Universitat Autònoma de Barcelona, Barcelona, Spain

<sup>3</sup>Department of Genetics, Universitat de Barcelona, Barcelona, Spain

<sup>4</sup>Department of Medicine, Hospital del Mar, Barcelona, Spain

**Abstract** To clarify the mechanism of the stimulatory effect of statins on bone formation, we have assessed the effect of simvastatin and atorvastatin on osteoblast activity by analysing cell proliferation, as well as collagen, osteocalcin, and bone morphogenetic protein-2 (*BMP2*) gene expression in primary human osteoblast (hOB) and MG-63 cell line cultures. Explants of bone from patients without any metabolic disease under orthopedic hip procedures were used to obtain hOB. Cell cultures were established, synchronized, and different concentrations of simvastatin or atorvastatin were added ( $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M,  $10^{-6}$  M) during the experiment. Cell proliferation was analyzed after 24 h. Collagen polypeptide  $\alpha 1$  type 1 (*COL1A1*) gene expression, osteocalcin, and *BMP2* expression levels were quantified by real-time PCR after 24 h incubation with statins. There was a statistically significant decrease in cell proliferation related to simvastatin or atorvastatin addition at all concentrations in primary hOB compared with those not treated. A significant increase in *COL1A1*, osteocalcin, and *BMP2* gene expression was detected when hOB cultures were treated with simvastatin or atorvastatin at different concentrations. Similar but less significant effects were found on MG-63 cells. After statin treatment we observed both an arrest of proliferation in hOB cells and an increase in collagen, osteocalcin, and *BMP2* gene expression, consistent with a stimulatory effect towards mature osteoblast differentiation. These findings support the bone-forming effect of statins, probably through the BMP2 pathway. *J. Cell. Biochem.* 101: 1430–1438, 2007. © 2007 Wiley-Liss, Inc.

**Key words:** cholesterol-lowering drugs; human osteoblast; collagen; calcium

Osteoporosis and atherosclerosis are widely prevalent conditions and induce serious effects on health, mainly in postmenopausal women and in the aging population. There is growing

evidence that osteoporosis and atherosclerosis are linked by biological associations [Hofbauer and Schoppet, 2004; Hamerman, 2005]. Although osteoporosis results from an imbalance between the bone-forming action of osteoblasts and the bone resorptive action of osteoclasts, resulting in an increased risk of fractures [NIH Consensus, 2001], the majority of currently available treatments, such as estrogens, bisphosphonates, and raloxifene, act through a decrease in osteoclastic bone resorption rather than enhancing bone formation [Hodgson et al., 2003]. However, a substantial number of fractures are still observed in patients on these medications, and in instances where advanced bone loss has already occurred. Therefore, these patients could greatly benefit from bone anabolic agents in order to restore

Grant sponsor: The Spanish Ministerio de Sanidad; Grant number: (FIS 01/1333); Grant sponsor: Spanish Atherosclerosis Society; Grant sponsor: Catalan Departament de Universitats, Recerca i Societat de la Informació; Grant number: (2005SGR 00762).

\*Correspondence to: Xavier Nogues, MD, PhD, URFOA, IMIM.UAB, Servei de Medicina Interna, Hospital del Mar, Passeig Marítim 5-29, E-08003 Barcelona, Spain.  
E-mail: xnogues@imas.imim.es

Received 20 June 2006; Accepted 7 December 2006

DOI 10.1002/jcb.21259

© 2007 Wiley-Liss, Inc.

bone mass, architecture, and mechanical properties. Thus, interest has turned, more recently, to treatments that stimulate osteoblast activity, such as parathyroid hormone (PTH) [Neer et al., 2001].

The recent discovery of bone anabolic effects of statins has spurred a great deal of interest among basic and clinical bone researchers. Both in vitro and animal data have suggested that statins stimulate bone morphogenetic protein-2 (BMP2) mediated osteoblast expression on bone cells, increase bone formation of calvaria mice when injected subcutaneously, and increase cancellous bone volume when administered orally to rats [Mundy et al., 1999]. Subsequent in vitro studies confirmed the stimulation of osteogenic differentiation and concurrent inhibition of adipogenic differentiation of bone marrow mesenchymal cells by different statins [Li et al., 2003; Song et al., 2003; Maeda et al., 2004]. However, further in vivo studies have yielded ambiguous results with respect to statin effects on bone metabolism in rodents [Maritz et al., 2001; Skoglund et al., 2002; Oxlund and Andreassen, 2004]. Additionally, statins directly affect osteoclasts through mechanisms that closely resemble the mode of action of nitrogen-containing bisphosphonates [Fisher et al., 1999] and it was demonstrated that inhibition of osteoclastic activity was inversely correlated with the magnitude of HMG-CoA reductase activity [Staal et al., 2003].

Many of the studies investigating the effects of statins on osteoblasts were performed in animal or human immortalized cell lines and only one has been done with primary human osteoblasts (hOB) [Viereck et al., 2005]. Inasmuch as studies that examine the influence of statins on the proliferation of bone cells are scarce, we performed the present study. We assessed the effect of simvastatin and atorvastatin, the two hypolipidemic drugs more commonly used in clinical practice, on osteoblast activity by analyzing cell proliferation, as well as collagen, osteocalcin, and *BMP2* gene expression in primary hOB and osteoblast-like MG-63 osteosarcoma cells.

## MATERIALS AND METHODS

### Experimental Design

The effect of simvastatin and atorvastatin on normal primary hOB from three healthy patients (69, 72, and 74 years old) and on the

human osteosarcoma cell line MG-63 was assessed. Simvastatin and atorvastatin were prepared at a concentration of 10 mM in ethanol and they were tested at concentrations that ranged from  $10^{-9}$  M to  $10^{-6}$  M. The parameters assessed included cytotoxicity, bromodeoxyuridine (BrdU) incorporation to cell DNA and Collagen polypeptide  $\alpha 1$  type 1 (*COL1A1*), osteocalcin, and *BMP2* gene expression. The effect of a 24 h incubation with simvastatin and atorvastatin was tested in triplicate after synchronization of cells, and was evaluated both with and without fetal calf serum (FCS). All experiments were repeated twice.

### Cell Culture

To obtain cultures of hOB, bone specimens were extracted from hip replacement procedures of three postmenopausal women without history of bone disease, metabolic or endocrine diseases, hormone-replacement therapy, or use of drugs that could affect bone mass. A standardized protocol was performed based on a method applied by Marie et al. [1989] with some modifications [Garcia-Moreno et al., 1998]. Briefly, the trabecular bone was separated and cut into small fragments extensively washed in phosphate-buffered solution (PBS) to remove non-adherent cells and finally placed on a Petri dish. The samples from the three donors were pooled and incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% FCS, pyruvate (1 mM), L-glutamine (2 mM), penicillin (56 U/ml), and streptomycin (56 U/ml), until confluence. After subculturing twice, cells were collected and frozen in liquid nitrogen.

MG-63 human osteosarcoma cells from ATCC (American Type Culture Collection, Rockville, MD) (<http://www.atcc.org>) were cultured in DMEM supplemented with 10% FCS. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### Osteoblast Characterization

#### Alkaline phosphatase histochemistry.

Cells were plated on 35 mm coverslips at a density of  $4 \times 10^5$  cells/ml in supplemented DMEM with 10% FCS,  $10^{-8}$  M 1-25(OH)<sub>2</sub>D<sub>3</sub> (vitamin D; Roche, Basel, Switzerland),  $10^{-8}$  M vitamin K, and 100 mg/ml ascorbic acid for 72 h. Alkaline phosphatase activity was assessed in

cells grown to confluence. The cultures were rinsed with PBS and fixed in cold 95% ethanol. The cells were incubated with a contrast solution (0.1 M Tris, pH = 10) containing 0.1% naphthyl phosphate (Sigma, Aldrich Quimica S.A., Madrid, Spain) with Fast Blue RR (Sigma), for 4 h at room temperature. A kidney tissue sample was used as a positive control.

**Qualitative osteocalcin PCR.** The PCR reaction was performed as described previously in a 50  $\mu$ l reaction mixture containing 5  $\mu$ l cDNA (1/10 dilution of RT mixture), 20 mM Tris-HCl, 50 mM MgCl<sub>2</sub>, 200  $\mu$ M of each deoxy-NTP, 0.3 mM of each sense and antisense specific primers (sense: 5'-TCA CAC TCC TCG CCC TAT T-3', exon 1 specific region, and antisense: 5'-CGA TGT GGT CAG CCA ACT-3' exon 4 specific region) and 0.03 U/ $\mu$ l Taq DNA polymerase (GibcoBRL, Grand Island, NY, USA), using an initial denaturation step at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 40 s, and polymerization at 72°C for 90 s, with a final elongation step at 72°C for 10 min. As an internal control,  $\beta$ -actin PCR was performed under the same conditions with the following specific primers: sense, 5'-TCA TGA AGT GTG ACG TTG ACA TCC GT-3', and antisense, 5'-CCT AGA AGC ATT TGC GGT GCA CGA TG -3'. The expected sizes of the PCR products of osteocalcin and  $\beta$ -actin were 246 bp and 285 bp, respectively.

#### Cell Viability Determination

The MTT test (Cell proliferation kit I (MTT); Roche Diagnostics, Mannheim, Germany) was used to measure cell viability. Osteoblast cells were plated at a density of  $2.0 \times 10^4$  cells/100  $\mu$ l/well in 96-well plates. After synchronization of the cells for 48 h in a medium containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich), the cells were incubated in DMEM for 24 h with or without FCS, and in the presence of the corresponding statin (simvastatin or atorvastatin) at concentrations ranging from  $10^{-6}$  M to  $10^{-9}$  M. Thereafter, 10  $\mu$ l of the MTT reagent were added and cells were incubated for 4 h at 37°C, prior to the addition of 100  $\mu$ l of the solubilizing reagent. After checking for complete dissolution of the purple crystals, the spectrophotometric absorbance at 550 nm and 600 nm was measured using a microplate (ELISA) reader. The MTT assay was performed in triplicate.

#### Cell Proliferation

Cell proliferation was assessed by the 5-bromo-2'-deoxy-uridine (BrdU) colorimetric assay. Osteoblast cells were plated at a density of  $2.0 \times 10^4$  cells/100  $\mu$ l/well in 96-well plates. After the synchronization of the cells for 48 h with medium containing 0.1% BSA, the cells were incubated in DMEM for 24 h with or without FCS, and in the absence or presence of the corresponding statin (simvastatin or atorvastatin), at concentrations ranging from  $10^{-6}$  M to  $10^{-9}$  M. Cell proliferation was determined using BrdU, which is incorporated in place of thymidine during the S-phase of the cell cycle. The BrdU Assay Kit (Cell Proliferation ELISA, BrdU (colorimetric) Roche Diagnostics) was used according to manufacturer's instructions. The reaction product was quantified by measuring the absorbance at 450 nm using a scanning multiwell spectrophotometer (ELISA reader).

#### Extraction of Total RNA and Reverse Transcription

Total RNA was prepared from cultured hOB by the guanidinium thiocyanate-phenol-chloroform method using Tri Reagent (Molecular Research Centre, Inc. Cincinnati, OH, USA) according to the manufacturer's protocol. The amount of RNA extracted from the cells was determined by spectrophotometry. Reverse transcription of RNA for first strand cDNA synthesis was performed using 900 ng of total RNA and 25 ng/ml oligo (dT)<sup>12-18</sup> primer (GibcoBRL, Piasley, UK) in a final volume of 6  $\mu$ l. The mix was incubated at 70°C for 10 min and immediately was frozen on ice. The extension reaction was performed at 42°C for 50 min after addition of reaction buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM of KCl, 3 mM of MgCl<sub>2</sub>, 10 mM of dithiothreitol, 1 mM of each dNTPs, and 100 U of the enzyme (*SuperScript™ II Rnase H<sup>-</sup> Reverse Transcriptase*, GibcoBRL), in a final volume of 10  $\mu$ l. Lastly, the reaction was inactivated at 70°C for 19 min and samples of cDNA were stored at -20°C.

#### Real-Time PCR

Real-time PCR amplification was performed with the ABI PRISM 7900 HT Sequence Detector (Applied Biosystems, Foster City, CA). Three Taqman Assays were used to quantify *COL1A1*, osteocalcin, and *BMP2* gene

expression (Assays-on-Demand Gene Expression Products, Applied Biosystems). Beta actin gene expression was used as an internal control.

Samples were assayed in triplicate in a 384-well optical plate with a final volume of 20  $\mu$ l each. All reactions consisted of 1  $\mu$ l cDNA, 10  $\mu$ l 2 $\times$  TaqMan Universal PCR Master Mix, 900 nM concentrations of each of the primers and 250 nM concentration of the fluorescent-labeled probe. The PCR mixture was incubated for 2 min at 50°C for AmpErase<sup>TM</sup> uracil-N-glycosylase-mediated decontamination, followed by 10 min at 95°C to activate AmpliTaq Gold DNA polymerase. Subsequently, a total of 50 cycles were performed; these consisted of a denaturation step for 15 sec at 95°C and a combined annealing-extension step for 1 min at 60°C. Data from the ABI Prism 7900 HT Sequence Detection instrument were analyzed with Sequence Detector software version 2.1 (SDS 2.1, Applied Biosystems), according to the comparative C<sub>T</sub> (threshold cycle) method.

#### Statistical Analysis

Analysis of variance was used to compare the means of quantitative variables among groups with independent data. For all analyses, a two-tailed nominal *P*-value less than 0.05 was considered statistically significant. Values were expressed as mean  $\pm$  SEM. Analyses were performed using the SPSS statistical package (12.0, Copyright (c) SPSS, Inc., 2004–2005).

## RESULTS

### Analysis of Statin Cytotoxicity on Normal hOB and MG-63 Cells

To confirm the osteoblast phenotype of the cells in culture, alkaline phosphatase activity and qualitative osteocalcin gene expression were assessed. The results of an MTT test performed on hOB and MG-63 cells after incubation with different simvastatin and atorvastatin concentrations (ranging from 10<sup>-6</sup> M to 10<sup>-9</sup> M) showed a percentage of cell viability above 85% and there were no differences with non-treated cells used as control.

### Effect of Simvastatin and Atorvastatin on Cell Proliferation

The BrdU incorporation was quantified to analyze the effect on cell proliferation of simvastatin and atorvastatin in hOB and

MG-63 cells. There was a statistically significant decrease in cell proliferation related to simvastatin addition at all concentrations in primary hOB compared with those not treated. This effect was observed both in the absence or presence of FCS (Fig. 1). In the case of atorvastatin, a significant decrease in cell proliferation was only observed at 10<sup>-7</sup> M and 10<sup>-6</sup> M in the absence of FCS and at 10<sup>-8</sup> M, 10<sup>-7</sup> M, and 10<sup>-6</sup> M when FCS was present (Fig. 1). Cell proliferation effect of simvastatin and atorvastatin on MG-63 cells was negligible (data not shown).

### Effect of Simvastatin and Atorvastatin on *COL1A1* Gene Expression

A significant increase in *COL1A1* gene expression was detected when hOB cultures were treated with simvastatin at 10<sup>-9</sup> M in the presence of FCS (161.79  $\pm$  7.7 AU (arbitrary units), *P* = 0.037), 10<sup>-8</sup> M (177.63  $\pm$  3.1 AU, *P* = 0.002), 10<sup>-7</sup> M (212.98  $\pm$  6.6 AU, *P* = 0.014), and

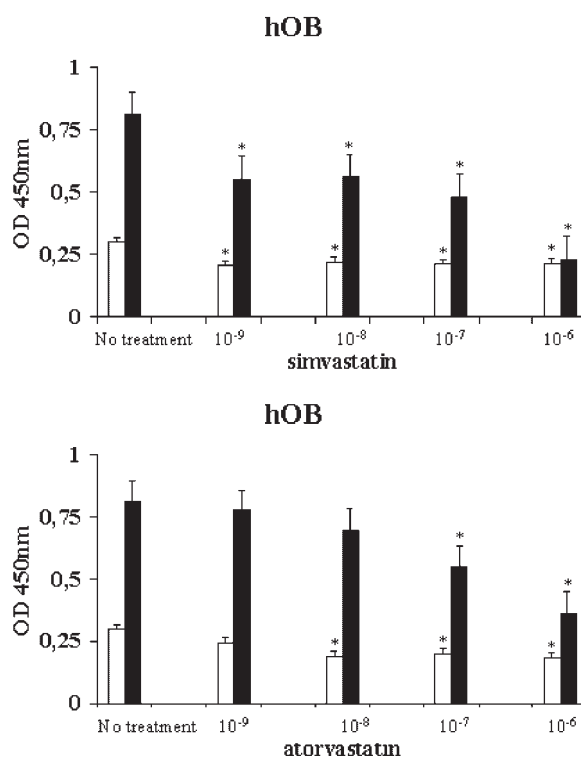


Fig. 1. Cell proliferation evaluated by DNA synthesis (BrdU incorporation) in a pool of human primary osteoblastic cells (hOB) treated 24 h with 10<sup>-9</sup> M, 10<sup>-8</sup> M, 10<sup>-7</sup> M, and 10<sup>-6</sup> M concentrations of simvastatin or atorvastatin compared with non-treated cells without FCS (white bars) and with FCS (solid bars). The data are mean ( $\pm$ SE) of values of quadruplicate determinations measured by optical density (OD 450 nm). \*Significant difference with respect to non-treated cells (*P* < 0.05).

$10^{-6}$  M ( $215.9 \pm 5.72$  AU,  $P = 0.009$ ) versus non-treated ( $74.36 \pm 2.70$  AU; Fig. 2a). A significant increase in *COL1A1* gene expression was found when hOB cultures were treated with atorvastatin and FCS at  $10^{-8}$  M ( $160.31 \pm 5.0$  AU,  $P = 0.020$ ),  $10^{-7}$  M ( $451.10 \pm 14.9$  AU,  $P = 0.003$ ), and  $10^{-6}$  M ( $212.88 \pm 5.37$  U,  $P = 0.012$ ; Fig. 2a). In the absence of FCS no statin effect on *COL1A1* gene expression was observed.

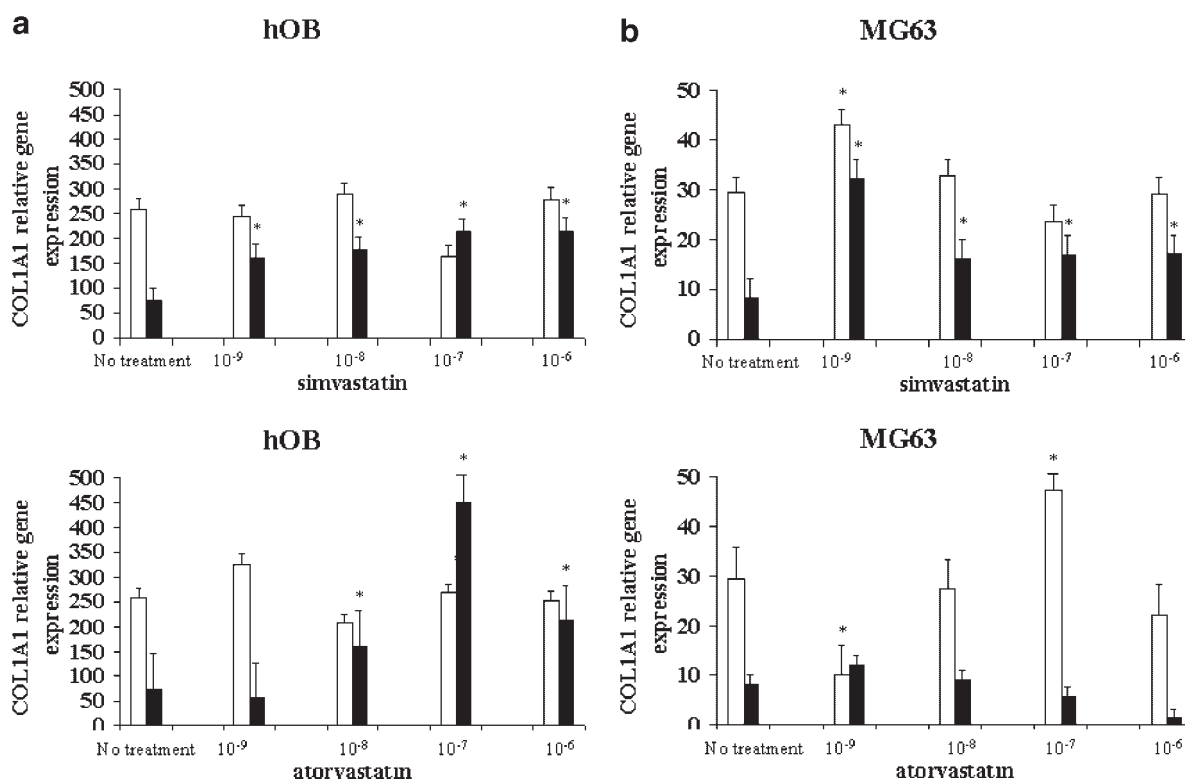
Regarding MG-63 cultures, a significant increase in *COL1A1* gene expression was detected when cells were treated with simvastatin at  $10^{-9}$  M and FCS ( $32.25 \pm 0.6$  AU,  $P = 0.025$ ),  $10^{-8}$  M ( $16.06 \pm 0.5$  AU,  $P = 0.01$ ),  $10^{-7}$  M ( $16.89 \pm 0.8$  AU,  $P = 0.049$ ), and  $10^{-6}$  M ( $17.10 \pm 0.4$  AU,  $P = 0.041$ ) versus non-treated (Fig. 2b). In the absence of FCS, a significant decrease in *COL1A1* gene expression was detected with atorvastatin at  $10^{-9}$  M ( $9.99 \pm 0.13$  AU,  $P = 0.05$ ), and a significant increase was found at  $10^{-7}$  M treatment ( $47.43 \pm 1.01$  AU,  $P = 0.05$ ) versus non-treated (Fig. 2b). No significant change in *COL1A1* gene expression was

detected at any concentration when cells were treated with atorvastatin with FCS (Fig. 2b).

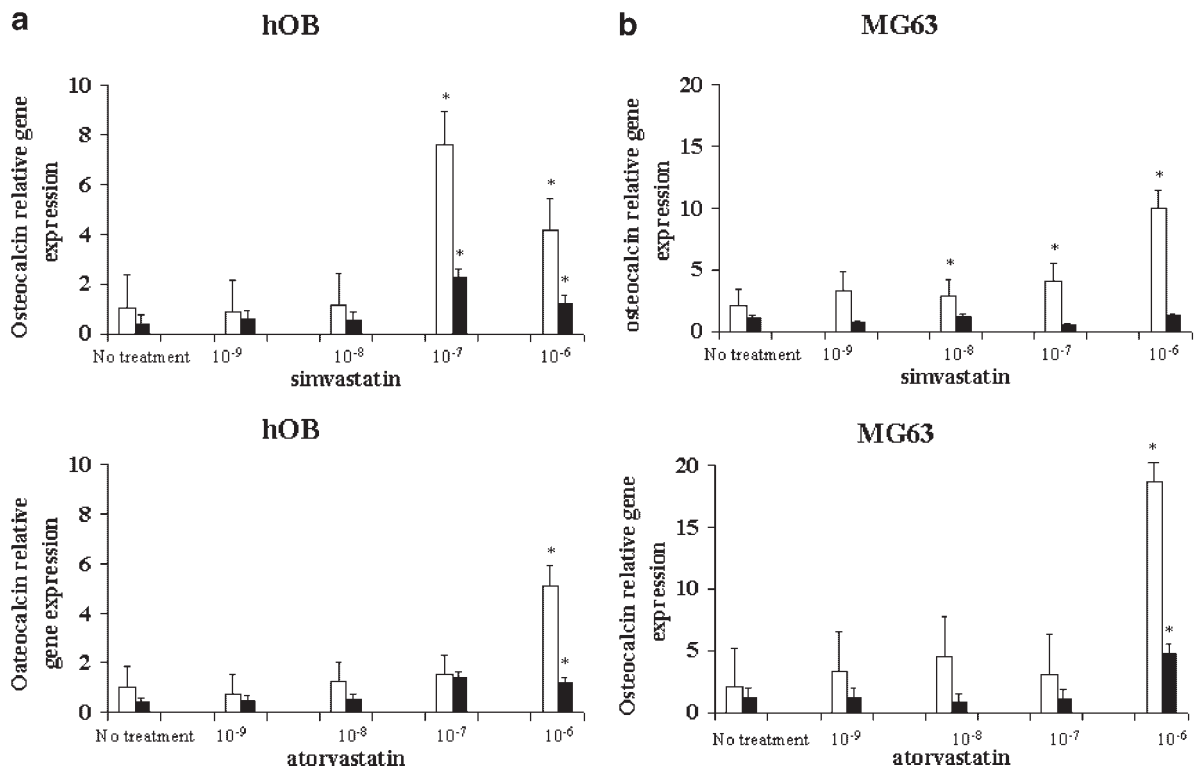
#### Effect of Simvastatin and Atorvastatin on Osteocalcin Gene Expression

A significant increase in osteocalcin gene expression was revealed when hOB cultures were treated with simvastatin at  $10^{-7}$  M ( $7.61 \pm 0.4$  AU,  $P = 0.03$ ) and  $10^{-6}$  M ( $4.14 \pm 0.1$  AU,  $P = 0.016$ ) concentrations without FCS and at  $10^{-7}$  M ( $2.27 \pm 0.1$  AU,  $P = 0.01$ ) and  $10^{-6}$  M ( $1.19 \pm 0.1$  AU,  $P = 0.022$ ) concentrations with FCS versus non-treated ( $1.04 \pm 0.09$  AU without FCS and  $0.41 \pm 0.05$  AU with FCS; Fig. 3a).

A significant increase in osteocalcin gene expression was identified when hOB cultures were treated with  $10^{-6}$  M atorvastatin without FCS ( $5.11 \pm 0.1$  AU,  $P = 0.032$ ) and with FCS ( $1.19 \pm 0.1$  AU,  $P = 0.008$ ) versus non-treated (Fig. 3a). A similar effect on osteocalcin gene expression was observed when MG-63 cells were treated with either simvastatin or atorvastatin (Fig. 3b).



**Fig. 2.** *COL1A1* relative gene expression in a pool of human primary osteoblastic cells (hOB) (a) and in MG-63 cells (b) treated with  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M, and  $10^{-6}$  M concentrations of simvastatin or atorvastatin compared with non-treated cells without FCS (white bars) and with FCS (solid bars). The data are mean ( $\pm$ SE) of values of triplicate determinations measured by real-time PCR. \*Significant difference with respect to non-treated cells ( $P < 0.05$ ).



**Fig. 3.** Osteocalcin relative gene expression in a pool of human primary osteoblastic cells (hOB) (a) and in MG-63 cells (b) treated with  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M, and  $10^{-6}$  M concentrations of simvastatin or atorvastatin compared with non-treated cells without FCS (white bars) and with FCS (solid bars). The data are mean ( $\pm$ SE) of values of triplicate determinations measured by real-time PCR. \*Significant difference with respect to non-treated cells ( $P < 0.05$ ).

#### Effect of Simvastatin and Atorvastatin on *BMP2* Gene Expression

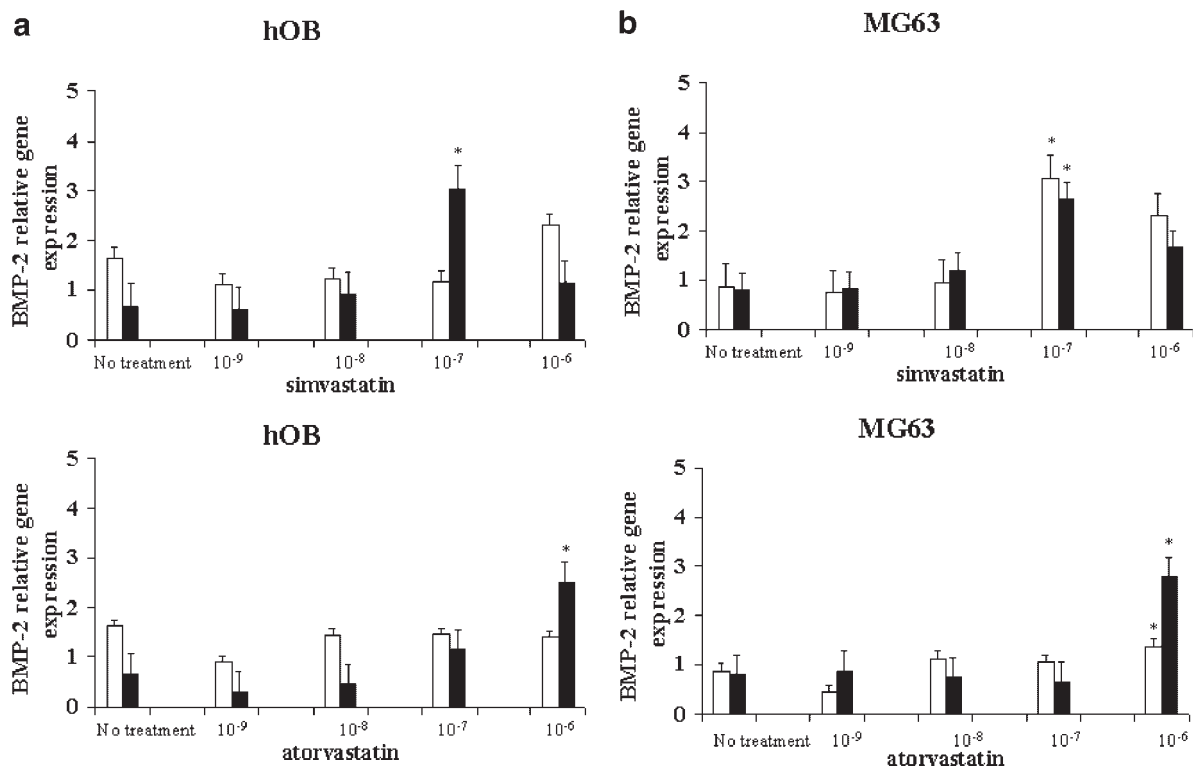
A significant increase of *BMP2* gene expression was observed in hOB cultures treated with simvastatin at  $10^{-7}$  M and FCS ( $3.05 \pm 0.1$  AU,  $P = 0.032$ ), versus those without ( $0.67 \pm 0.17$  AU; Fig. 4a). A significant increase in *BMP2* gene expression was found when hOB cultures were treated with atorvastatin at  $10^{-6}$  M and FCS ( $2.51 \pm 0.15$  AU,  $P = 0.016$ ) versus non-treated (Fig. 4a). A similar effect on *BMP2* gene expression was observed when MG-63 cells were treated with simvastatin or atorvastatin (Fig. 4b).

#### DISCUSSION

The results of the present study indicate that simvastatin and atorvastatin have a stimulatory effect on the expression of the osteoblast-specific genes *COL1A1*, osteocalcin, and *BMP2* in hOB and MG-63 cell cultures. Cell proliferation arrest after statin treatment is another important new finding that we observed.

Our results agree with previous reports on the osteoblast-differentiating effect of statins. The cell proliferation arrest may be explained by a stimulatory effect towards mature osteoblast, as shown by the increase of collagen and osteocalcin gene expression. In the stages of in vitro osteoblast differentiation, the initial phase consists on an active replication for several days. After confluence, cultures display a rapid increase in cell number. Collagen accumulation is minimal during this cell growth period but after this intermediate phase, cell replication decreases and alkaline phosphatase activity increases significantly [Quarles et al., 1992]. We started the experiment at this stage and here is when the addition of statins induced cell differentiation, as demonstrated by an increase in collagen gene expression. In this respect, osteocalcin was a good specific marker for the final phases of osteoblast maturation.

The intimate action mechanism of statins in inducing bone formation is not fully understood. The osteoblast-differentiating effect may be explained by a *BMP2* mediated action as



**Fig. 4.** BMP-2 relative gene expression in a pool of human primary osteoblastic cells (hOB) (a) and in MG-63 cells (b) treated with 10<sup>-9</sup> M, 10<sup>-8</sup> M, 10<sup>-7</sup> M and 10<sup>-6</sup> M concentrations of simvastatin or atorvastatin compared with non-treated cells without FCS and with FCS (solid bars). The data are mean ( $\pm$ SE) of values of triplicate determinations measured by real-time PCR. \*Significant difference with respect to non-treated cells ( $P < 0.05$ ).

described by Mundy et al. [1999]. Alternatively, in osteoblast-like MC3T3-E1 cells some studies have shown that simvastatin induces phosphorylation of mitogen-activated-protein (MAP) kinase through the heat shock protein 27 (HSP-27) [Wang et al., 2003]. Further evidence of a BMP2 involvement in the osteoblast-differentiating effect of statins has been provided by several groups [Sugiyama et al., 2000; Ohnaka et al., 2001; Maeda et al., 2004]. In particular, Ohnaka et al. [2001] found a significant increase in *BMP2* gene expression in hOB culture after addition of pitavastatin, a novel HMG-CoA reductase inhibitor, already available for clinical use in Japan [Mukhtar et al., 2005]. In the present study we found a significant increase in *BMP2* gene expression in primary hOB cultures and MG-63 cells treated with both simvastatin and atorvastatin, thus further supporting this mechanism.

Some strengths of the present study are worth mentioning. First, we used hOB cultures with a view to reflecting a close physiological

scenario. To date, almost all the in vitro and in vivo experiments have been performed in rat or murine experimental models. In our case, we used both hOB and the MG-63 osteosarcoma cell line in parallel. Interestingly, we observed a negligible effect of simvastatin and atorvastatin on MG-63 cell proliferation. This may be related with the particularly high growth rate of this human transformed cell line and illustrates a limitation of its use as an in vitro model for osteoblasts. In the gene expression analyses, the MG-63 cells behaved in a similar way as the hOBs although with more oscillations, notably in the case of *COL1A1* gene expression in response to atorvastatin.

Second, it is also interesting to emphasize that the statin doses, ranging between 10<sup>-6</sup> M and 10<sup>-9</sup> M, preserved cell viability as demonstrated by the MMT assay. Since a reduced cell viability using higher atorvastatin dosage (10<sup>-5</sup> M) has been previously described [Viereck et al., 2005], we have confirmed, and therefore used, cell viability with both statins at lower

concentrations. Although bone statin concentration after oral administration in humans is not known, we used the concentration reached in serum after clinical doses commonly used that correspond to 12.7–18.1 ng/ml or  $10^{-7}$ – $10^{-8}$  M.

Third, we have performed all the analyses in the absence or presence of 10% FCS. These two conditions may be viewed as extreme scenarios. In the first case, depletion of growth factors may lead to cell quiescence, while in the second, high proliferation is expected. We envisage that the physiological environment of osteoblasts in bone may be somewhere in between. Indeed, when no statins were added to the medium, the proliferation of cells on FCS was more than double that of cells without it. Upon statin addition, the antiproliferative effect was much more evident in cells growing on FCS than in cells without it probably because there was a much larger range of action for them. At the highest statin concentration, however, the proliferation of cells growing with or without FCS was very similar as if a limit had been reached. Regarding gene expression, differences in the effect of FCS on the studied genes were observed, which might be explained by the different requirements of these genes in terms of factors and differentiation stages.

One possible limitation of this study is that protein expression analysis has not been performed. However, real-time PCR accurately measures the mRNA levels of the gene and it is highly reliable in *in vitro* studies. Although posttranslational modifications may occur, the amount of mRNA may be considered a good predictor of protein levels. Therefore, it is a suitable first step towards later protein expression experiments. Another limitation is that only one time point was studied (24 h). Previous analyses using 24, 48, 72, and 96 h had shown that 24 h was enough to see the statin effect on cell proliferation. Thus, this time point was selected for the rest of analyses.

Clinically it is possible to study bone remodeling using bone biochemical markers. After treatment with simvastatin, some authors have found high levels of osteocalcin and bone alkaline phosphatase, indicating an increase in bone formation [Chan et al., 2001; Montagnani et al., 2003]. However, Berthold et al. [2004] have recently found that this effect is related to age, with a diverse effect of atorvastatin on bone turnover markers in young and old subjects,

suggesting a beneficial effect of statins only in patients older than 63 years.

The effects of statins on bone mineral density (BMD) and fracture risk are controversial. In a recent prospective study by Rejnmark et al. [2004a], after one year of treatment with 40 mg/day of simvastatin, changes in BMD at lumbar spine, total hip, femoral neck, and whole body in postmenopausal women were not statistically different from those observed in the placebo group. When this effect of statins was analyzed in large epidemiological studies it was associated with a fracture risk reduction even after adjustment by confounding factors [Rejnmark et al., 2004b]. Moreover, several meta-analyses performed have shown either non-clinically significant effect or only a marginal benefit [Bauer et al., 2004; Hatzigeorgiou and Jackson, 2005; Scranton et al., 2005]. The liver-specific nature of statins is one possible reason for the discrepancy between the results of the preclinical and clinical studies. Considering their high liver specificity and low oral bioavailability, distribution of statins to the bone microenvironment in optimal concentration is questionable. To unravel their exact mechanism and confirm beneficial action on bone, statins should reach the bone microenvironment at sufficient concentration.

For this reason cell culture systems provide a reliable way to demonstrate the concept of these actions. We consider that the experiment we performed is a well-established *in vitro* human model in which it will be possible to test new statins with a bone-targeted delivery. Our study confirms the bone-forming effect of statins on hOB cells and supports the goodness of a potential combined treatment of hyperlipidemia and osteoporosis.

#### ACKNOWLEDGMENTS

Robin Rycroft provided editorial assistance.

#### REFERENCES

- Bauer DC, Mundy GR, Jamal SA, Black DM, Cauley JA, Ensrud KE, van der Klift M, Pols HA. 2004. Use of statins and fracture: Results of 4 prospective studies and cumulative meta-analysis of observational studies and controlled trials. *Arch Intern Med* 164:146–152.
- Berthold HK, Unverdorben S, Zittermann A, Degenhardt R, Baumeister B, Unverdorben M, Krone W, Vetter H, Gouni-Berthold I. 2004. Age-dependent effects of atorvastatin on biochemical bone turnover markers: A



- randomized controlled trial in postmenopausal women. *Osteoporos Int* 15:459–467.
- Chan MH, Mak TW, Chiu RW, Chow CC, Chan IH, Lam CW. 2001. Simvastatin increases serum osteocalcin concentration in patients treated for hypercholesterolaemia. *J Clin Endocrinol Metab* 86:4556–4559.
- Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russell RG, Rodan GA, Reszka AA. 1999. Alendronate mechanism of action: Geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation *in vitro*. *Proc Natl Acad Sci USA* 96:133–138.
- Garcia-Moreno C, Serrano S, Nacher M, Farre M, Diez A, Marinoso ML, Carbonell J, Mellibovsky L, Nogues X, Ballester J, Aubia J. 1998. Effect of alendronate on cultured normal human osteoblasts. *Bone* 22:233–239.
- Hamerman D. 2005. Osteoporosis and atherosclerosis: Biological linkages and the emergence of dual-purpose therapies. *QJM* 98:467–484.
- Hatzigeorgiou C, Jackson JL. 2005. Hydroxymethylglutaryl-coenzyme A reductase inhibitors and osteoporosis: A meta-analysis. *Osteoporos Int* 16:990–998.
- Hodgson SF, Watts NB, Bilezikian JP, Clarke BL, Gray TK, Harris DW, Johnston CC Jr, Kleerekoper M, Lindsay R, Luckey MM, McClung MR, Nankin HR, Petak SM, Recker RR, Anderson RJ, Bergman DA, Bloomgarden ZT, Dickey RA, Palumbo PJ, Peters AL, Rettinger HI, Rodbard HW, Rubenstein HA. 2003. AACE Osteoporosis Task Force. American Association of Clinical Endocrinologists medical guidelines for clinical practice for the prevention and treatment of postmenopausal osteoporosis: 2001 edition, with selected updates for 2003. *Endocr Pract* 9:544–564.
- Hofbauer LC, Schoppet M. 2004. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. *JAMA* 292:490–495.
- Li X, Cui Q, Kao C, Wang GJ, Balian G. 2003. Lovastatin inhibits adipogenic and stimulates osteogenic differentiation by suppressing PPAR $\gamma$ 2 and increasing Cbfa1/Runx2 expression in bone marrow mesenchymal cell cultures. *Bone* 33:652–659.
- Maeda T, Matsunuma A, Kurahashi I, Yanagawa T, Yoshida H, Horiuchi N. 2004. Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. *J Cell Biochem* 92:458–471.
- Marie PJ, Lomri A, Sabbagh A, Basle M. 1989. Culture and behavior of osteoblastic cells isolated from normal trabecular bone surfaces. *In Vitro Cell Dev Biol* 25: 373–380.
- Maritz FJ, Conradie MM, Hulley PA, Gopal R, Hough S. 2001. Effect of statins on bone mineral density and bone histomorphometry in rodents. *Arterioscler Thromb Vasc Biol* 21:1636–1641.
- Montagnani A, Gonnelli S, Cepollaro C, Pacini S, Campagna MS, Franci MB, Lucani B, Gennari C. 2003. Effect of simvastatin treatment on bone mineral density and bone turnover in hypercholesterolemic postmenopausal women: A 1-year longitudinal study. *Bone* 32:427–433.
- Mukhtar RY, Reid J, Reckless JP. 2005. Pitavastatin. *Int J Clin Pract* 59:239–252.
- Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. 1999. Stimulation of bone formation *in vitro* and in rodents by statins. *Science* 286: 1946–1949.
- Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. 2001. Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 344:1434–1441.
- NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy. 2001. Osteoporosis prevention, diagnosis, and therapy. *JAMA* 285:785–795.
- Ohnaka K, Shimoda S, Nawata H, Shimokawa H, Kaibuchi K, Iwamoto Y, Takayanagi R. 2001. Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. *Biochem Biophys Res Commun* 287:337–342.
- Oxlund H, Andreassen TT. 2004. Simvastatin treatment partially prevents ovariectomy-induced bone loss while increasing cortical bone formation. *Bone* 34:609–618.
- Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. 1992. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: An *in vitro* model of osteoblast development. *J Bone Miner Res* 7:683–692.
- Rejnmark L, Buus NH, Vestergaard P, Heickendorff L, Andreassen F, Larsen ML, Mosekilde L. 2004a. Effects of simvastatin on bone turnover and BMD: A 1-year randomized controlled trial in postmenopausal osteopenic women. *J Bone Miner Res* 19:737–744.
- Rejnmark L, Olsen ML, Johnsen SP, Vestergaard P, Sorensen HT, Mosekilde L. 2004b. Hip fracture risk in statin users—A population-based Danish case-control study. *Osteoporos Int* 15:452–458.
- Scranton RE, Young M, Lawler E, Solomon D, Gagnon D, Gaziano JM. 2005. Statin use and fracture risk: Study of a US veterans population. *Arch Intern Med* 165:2007–2012.
- Skoglund B, Forslund C, Aspenberg P. 2002. Simvastatin improves fracture healing in mice. *J Bone Miner Res* 17:2004–2008.
- Song C, Guo Z, Ma Q, Chen Z, Liu Z, Jia H, Dang G. 2003. Simvastatin induces osteoblastic differentiation and inhibits adipocytic differentiation in mouse bone marrow stromal cells. *Biochem Biophys Res Commun* 308:458–462.
- Staal A, Frith JC, French MH, Swartz J, Gungor T, Harrity TW, Tamasi J, Rogers MJ, Feyen JH. 2003. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. *J Bone Miner Res* 18:88–96.
- Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. 2000. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. *Biochem Biophys Res Commun* 271:688–692.
- Viereck V, Grundker C, Blaschke S, Frosch KH, Schoppet M, Emons G, Hofbauer LC. 2005. Atorvastatin stimulates the production of osteoprotegerin by human osteoblasts. *J Cell Biochem* 96:1244–1253.
- Wang X, Tokuda H, Hatakeyama D, Hirade K, Niwa M, Ito H, Kato K, Kozawa O. 2003. Mechanism of simvastatin on induction of heat shock protein in osteoblasts. *Arch Biochem Biophys* 415:6–13.