Journal of Cellular Biochemistry 92:458–471 (2004)

# Induction of Osteoblast Differentiation Indices by Statins in MC3T3-E1 Cells

**Toyonobu Maeda,[1] Ayako Matsunuma,[1] Izuru Kurahashi,[2] Toru Yanagawa,[3] Hiroshi Yoshida,[3] and Noboru Horiuchi[1]***

[1]Department of Biochemistry, School of Dentistry, Ohu University, Koriyama 963-8611, Japan
[2]Department of Oral Surgery, School of Dentistry, Ohu University, Koriyama 963-8611, Japan
[3]Department of Oral Maxillofacial Surgery, Institute of Clinical Medicine, University of Tsukuba, Tsukuba 305-8575, Japan

**Abstract** Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes conversion of HMG-CoA to mevalonate, a rate-limiting step in cholesterol synthesis. The present study was undertaken to understand the events of osteoblast differentiation induced by statins. Simvastatin at $10^{-7}$ M markedly increased mRNA expression for bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), alkaline phosphatase, type I collagen, bone sialoprotein, and osteocalcin (OCN) in nontransformed osteoblastic cells (MC3T3-E1), while suppressing gene expression for collagenase-1, and collagenase-3. Extracellular accumulation of proteins such as VEGF, OCN, collagenase-digestive proteins, and noncollagenous proteins was increased in the cells treated with $10^{-7}$ M simvastatin, or $10^{-8}$ M cerivastatin. In the culture of MC3T3-E1 cells, statins stimulated mineralization; pretreating MC3T3-E1 cells with mevalonate, or geranylgeranyl pyrophosphate (a mevalonate metabolite) abolished statin-induced mineralization. Statins stimulate osteoblast differentiation in vitro, and may hold promise drugs for the treatment of osteoporosis in the future. J. Cell. Biochem. 92: 458–471, 2004. 

**Key words:** statins; osteoblast differentiation; mineralization; vascular endothelial growth factor; bone sialoprotein

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase to block conversion of HMG-CoA to mevalonate, a rate-limiting step in cholesterol synthesis [Goldstein and Brown, 1990]. These drugs, including simvastatin and atorvastatin, thus are potent inhibitors of cholesterol biosynthesis that are widely prescribed to lower cholesterol in hyperlipidemic patients at risk for cardiovascular disease [Hamelin and Turgeon, 1998; Maron et al., 2000]. By inhibiting the initial part of the cholesterol synthesis pathway, statins decrease availability of several important lipid intermediate compounds including isoprenoids such as geranylgeranyl pyrophosphate (GGPP); these are attached as posttranslational modification to certain proteins, such as small G proteins including Ras and Ras-like proteins (Rho, Rap, Rab, and Ral) [Casey and Seabra, 1996].

Osteoblasts, which arise from mesenchymal stem cell precursors, undergo differentiation in response to a number of factors including bone morphogenetic proteins (BMPs), transforming growth factor (TGF), insulin-like growth factor I (IGF-I), vascular endothelial growth factor (VEGF), and glucocorticoids [McCarthy et al., 1989; Noda and Camilliere, 1989; Celeste et al., 1990; Midy and Plouet, 1994; Hughes et al., 1995; Goad et al., 1996; Gerber et al., 1999; Spelsberg et al., 1999]. In addition, several different molecules are associated with deposition and maintenance of mineralized skeletal elements. Once matrix synthesis begins in osteoblast culture models such as primary osteoblast cultures, the cells differentiate as genes encoding osteoblastic markers such as alkaline phosphatase (ALP), type I collagen (Col I), and osteocalcin (OCN) are activated. Finally, osteoblasts become embedded in the extracellular matrix

Grant sponsor: The Ministry of Education, Science, Sports, and Culture of Japan.

*Correspondence to: Noboru Horiuchi, DDS, PhD, Department of Biochemistry, Ohu University School of Dentistry, Koriyama 963-8611, Japan.
E-mail: fwga4746@mb.infoweb.ne.jp

Received 11 December 2003; Accepted 16 January 2004
DOI 10.1002/jcb.20074

consisting mainly of collagen fibrils, and matrix mineralization begins as mineral deposit extend along and within collagen fibrils [Lian et al., 2003].

Mundy et al. [1999] first reported that statins stimulated in vivo bone formation in rodents and increased new bone volume in cultures from mouse calvaria. Recently, we showed that statins stimulate expression of bone anabolic factors such as VEGF and BMP-2 [Maeda et al., 2003], and promote osteoblast differentiation and mineralization in MC3T3-E1 cells derived from new bone mouse calvaria [Maeda et al., 2001]. However, little is known about effects of statins on regulation of osteoblast function. The present study was undertaken to investigate changes in marker expression corresponding to stages of osteoblast differentiation in statin-treated MC3T3-E1 cells. We found that statins initially enhance BMP-2 expression and then induce synthesis of differentiation markers characteristic late of osteoblast stages. Furthermore, we showed for the first time that simvastatin increased abundance of mRNA encoding bone sialoprotein (BSP) at the middle stage of differentiation and decreased mRNA encoding collagenase-3, i.e., matrix metalloproteinase (MMP)-13 at the late stage.

## MATERIALS AND METHODS

### Cell Culture

MC3T3-E1 cells (a clonal preosteoblastic cell line derived from newborn mouse calvaria) were grown in α-MEM (ICN Pharmaceuticals, Aurora, OH) supplemented with 10% fetal bovine serum (FBS) as previously described [Maeda et al., 2001; Maeda et al., 2003]. Three days after plating, cells reached confluence and subsequently were cultured for 4–24 days in differentiation medium consisting of 90% α-MEM containing a 50 μg/ml concentration of the phosphate ester of ascorbic acid (Wako Chemicals, Osaka, Japan), 10 mM β-glycerophosphate (Wako Chemicals), and 10% FBS. Simultaneously, cells were exposed to statins to be tested such as simvastatin at $10^{-7}$ M (Calbiochem, San Diego, CA), atorvastatin at $10^{-6}$ M (Yamanouchi Pharmaceutical, Tokyo, Japan), or cerivastatin $10^{-8}$ M (Takeda Pharmaceutical, Osaka, Japan) for the time periods indicated. In studies of inhibitors of statin-induced mineralization, MC3T3-E1 cells were treated with 1 mM mevalonate (Sigma, St. Louis, MO) or 20 μM GGPP (Sigma) in the absence or presence of a statin [simvastatin ($10^{-6}$ M), cerivastatin ($10^{-7}$ M), or atorvastatin ($10^{-5}$ M)] for 16 days.

### Northern Blot and RT-PCR Analyses of Gene Expression

For Northern analysis we isolated total RNA from MC3T3-E1 cells. The following cDNA probes were used to detect transcripts in Northern blots: a 433-bp fragment of murine TGF-β (TGF-β1 isoform), a 409-bp fragment of murine VEGF, a 654-bp fragment of murine Col-I, a 284-bp fragment of murine OCN, a 799-bp fragment of murine OPN, a 598-bp fragment of murine collagenase-1 (MMP-1), and a 626-bp fragment of murine collagenase-3 (MMP-13). Total RNA was extracted from cells using guanidine thiocyanate. Total RNA was fractionated on 1.2% agarose gels containing formaldehyde and transferred to a nylon membrane (Hybond-XL; Amersham Biosciences, Buckinghamshire, UK). The mouse cDNA probes used to hybridize with mRNAs on the membranes were labeled with [α-$^{32}$P]deoxy-CTP (specific activity, 110 TBq/mmol; ICN) using a Megaprime DNA labeling kit (Amersham). All hybridizations were performed under high-stringency conditions as previously described [Maeda et al., 2003]. Membrane signal visualized with an Molecular Imager FX (Bio-Rad, Hercules, CA) was analyzed quantitatively using Quantity One 4.1.1 (Bio-Rad) image analysis software. RT-PCR were performed using a Gene Amp RNA PCR kit (Applied Biosystems, Foster City, CA). One microgram of total RNA was reverse-transcribed at 42°C for 1 h to synthesize cDNA. Amplification reactions were performed using the following primers and protocols: BMP-2: forward, 5′-CCAAGACACAGTTCCCTACA-3′; reverse, 5′-CACGGCTTCTAGTTGATGGA-3′ (annealing at 57°C, 22 cycles, 562-bp product); BMP-4: forward, 5′-AGTTTGATACCTGAGACCGG-3′; reverse, 5′-ATTTCTGCTGGGGGCTTCAT-3′ (annealing at 57°C, 22 cycles, 600-bp product); Runx2/Cbfa1: forward, 5′-GGTGGTCCGCGATGATCTC-3′; reverse, 5′-GAGGGCACAAGTTCTATCTGGA-3′ (annealing at 57°C, 24 cycles, 941-bp product); and BSP: forward, 5′-CTGTAGCACCATTCCACACT-3′; reverse, 5′-ATGGCCTGTGCTTTCTCGAT-3′ (annealing at 57°C, 22 cycles, 1,055-bp product). Amounts of these transcripts were calculated relative to the amount of cyclophilin mRNA

present. Signal intensity was quantified with the Molecular Imager FX.

### Synthesis of Collagen and Noncollagen Protein

Bone matrix protein synthesis was analyzed by incorporation of [$^3$H]-proline into proteins digestable and not digestable with bacterial collagense [McCarthy et al., 1989]. Confluent MC3T3-E1 cells in 24-well plates were cultured in differentiation medium containing test substances for the periods indicated. We added [2,3,4,5-$^3$H]-proline (specific activity, 4.8 TBq/mmol; Moravak Biochemicals, Brea, CA) at 5 μCi/well containing 0.5 ml of medium for the last 4 h of culture. After cells were harvested, extracted with 0.9 ml of 0.004 N NaOH, and sonicated, 0.1 ml of 1 M HEPES was added. Extracts from cells and extracellular matrix were incubated with 0.7 ml of vehicle or bacterial collagenase (Type II, Sigma) at 37°C for 3 h. The solution was added with 50 μl of 10% trichloroacetic acid, and centrifuged at 12,000 rpm for 5 min. Radioactivities in supernatant and precipitates corresponded to collagenase-digested proteins (CDP) and noncollagenous proteins (NCPs), respectively.

### Determination of VEGF and OCN Proteins

MC3T3-E1 cells were treated with vehicle or test substances such as simvastatin and cerivastatin for the periods indicated. Conditioned media from the last 24 h of culture were stored at −80°C until assay. VEGF concentrations were measured by a mouse VEGF quantitative sandwich enzyme immunoassay (R&D Systems, Minneapolis, MN). This assay measures primarily the 165-amino acid isoform of VEGF, the main soluble isoform. Fifty microliters of conditioned medium was used for the assay. OCN was measured with a mouse osteocalcin IRMA kit (Immutopics, San Clemente, CA).

### Measurement of ALP Activity

Cultures in 24-well plates were rinsed in PBS. Cells were sonicated in 0.1 M Tris buffer (pH 7.2) containing 0.1% Triton X-100. ALP activity was quantitated in cell lysate using an ALP B-test kit (Wako Chemicals). Aliquots of cell lysate were subjected to protein assay using Bradford's method [Maeda et al., 2001].

### Assay of Mineralized Matrix Formation

Cells in 24-well plates were cultured in differentiation medium after confluence for the periods indicated. After removing the medium, cells were washed twice with PBS. The extent of mineralized matrix in the plates was determined by Alizarin Red S (AR-S) staining [Maeda et al., 2003]. Briefly, cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS, and stained with 40 mM AR-S at pH 4.2 for 10 min at room temperature. Next, cell preparations were washed five times with deionized water and incubated in PBS for 15 min to eliminate nonspecific staining. The stained matrix was photographed using a 35-mm camera. AR-S staining was released from the cell matrix by incubation in 10% (w/v) cetylpyridinium chloride for 15 min. The amount of dye released was quantified by spectrophotometry at 562 nm.

### Statistical Analysis

All values are presented as the mean ± SEM for four measurements. Differences between treated and untreated groups were assessed by Student's *t*-test. Multiple comparisons were evaluated by one-way analysis of variance, followed by Scheffe's *F*-test. Statistical analysis was performed with the StatView 4.02 software package (Abacus Concepts, Inc., Berkeley, CA). A $P$ value less than 0.05 was considered to indicate statistical significance.

## RESULTS

### Effect of Statins on Expression of Bone Anabolic Factors

The time course of the effect of statins on BMP-2 expression was first examined in MC3T3-E1 cells. Simvastatin at $10^{-7}$ M significantly increased BMP-2 mRNA synthesis at 4 and 8 days of culture (Fig. 1A). Treatment of the cells with simvastatin at $10^{-7}$ M did not affect BMP-4 mRNA abundance during the entire culture period (Fig. 1B). During osteoblast differentiation, BMP-2 increases mRNA expression of Runx2/Cbfa1, a master transcription factor in the osteoblast lineage; accordingly, gene expression for Runx2 was examined using RT-PCR (Fig. 1C). Runx2 mRNA expression was maintained at a steady level between 0 and 16 days of culture, and then gradually decreased until the end of culture at day 24. Simvastatin at $10^{-7}$ M did not affect Runx2 mRNA expression in MC3T3-E1 cells during the entire culture period. We next tested the effect of statins on expression of bone growth factors

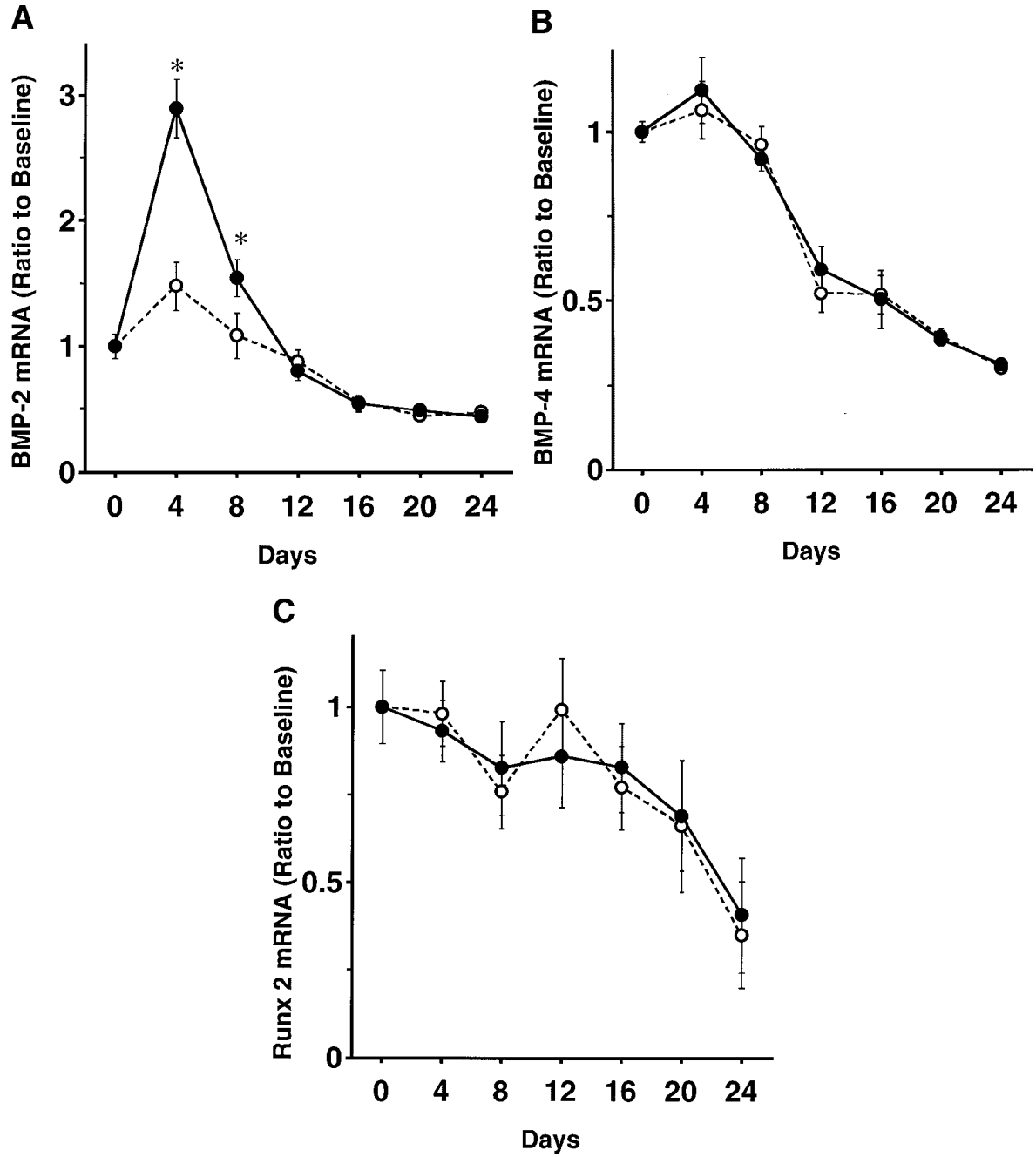


**Fig. 1.** Time course of the effect of simvastatin on BMP-2, BMP-4, and Runx2 mRNA expression in MC3T3-E1 cells. Cells were treated with vehicle (○) or $10^{-7}$ M simvastatin (●) for the time periods indicated. Total RNA was subjected to RT-PCR. PCR products encoding BMP-2, BMP-4, and Runx2 were measured by Southern blot analysis. Amounts are stated relative to expression of cyclophilin mRNA. **A**: BMP-2 mRNA expression. **B**: BMP-4 mRNA expression. **C**: Runx2 mRNA expression. Data represent the mean ± SEM for 4-wells. *, $P < 0.01$ compared to vehicle-treated control at each time point.

synthesized by MC3T3-E1 cells at a later stage of culture. TGF-β mRNA abundance increased in a time-dependent manner, but was not influenced by treatment with simvastatin (Fig. 2A). VEGF mRNA concentrations became markedly elevated at 16–24 days of culture, when $10^{-7}$ M simvastatin significantly enhanced this mRNA expression (Fig. 2B). Significant increases in VEGF protein secretion were observed at 16 and 20 days of treatment with $10^{-7}$ M simvastatin or $10^{-8}$ M cerivastatin; subsequently, secretion decreased until the end of culture (Fig. 2C).

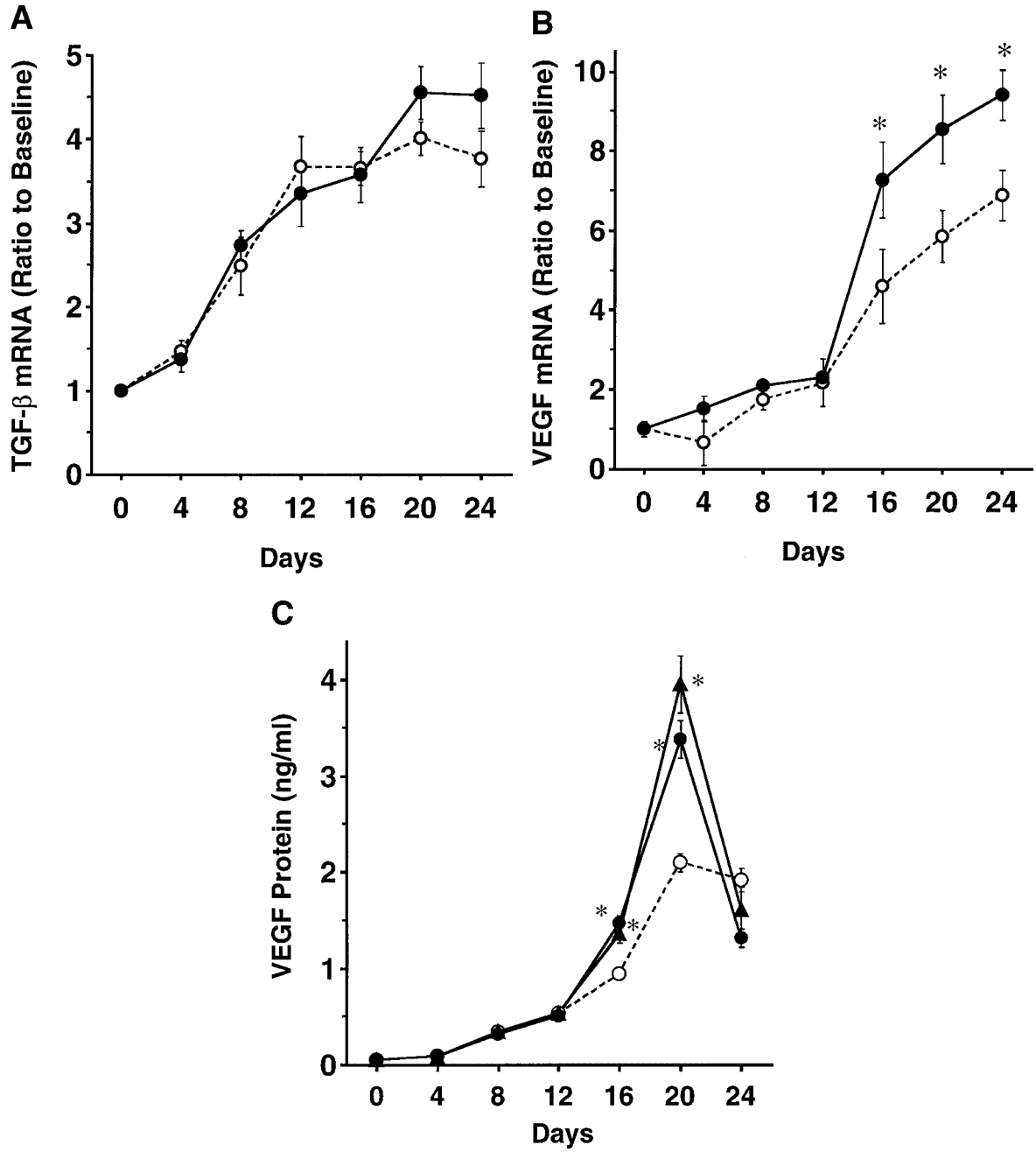


**Fig. 2.** Time course of the effect of simvastatin on TGF-β and VEGF mRNA expression and VEGF protein secretion in MC3T3-E1 cells. Cells were treated with vehicle (○), $10^{-7}$ M simvastatin (●), or $10^{-8}$ M cerivastatin (▲) for the time periods indicated. Amounts of TGF-β and VEGF mRNA were determined by Northern blot analysis and are stated relative to expression of cyclophilin mRNA. **A**: TGF-β mRNA expression. **B**: VEGF mRNA expression. **C**: VEGF protein secretion. Conditioned media for a 24-h period were collected, and VEGF concentrations were measured by a mouse VEGF assay kit. Data represent the mean ± SEM for 4-wells. *, $P < 0.01$ compared to vehicle-treated control at each time point.

### Effect of Statins on ALP Expression

We examined expression of ALP, an enzyme serving as a marker of osteoblast differentiation, in MC3T3-E1 cells treated with simvastatin or cerivastatin (Fig. 3). ALP transcript concentration increased during 8 and 16 days of culture, and simvastatin at $10^{-7}$ M or cerivastatin at $10^{-8}$ M significantly stimulated mRNA expression at days 8 and 12 of culture (Fig. 3A). ALP activity increased time-dependently, peaking at 12 days of culture and then gradually

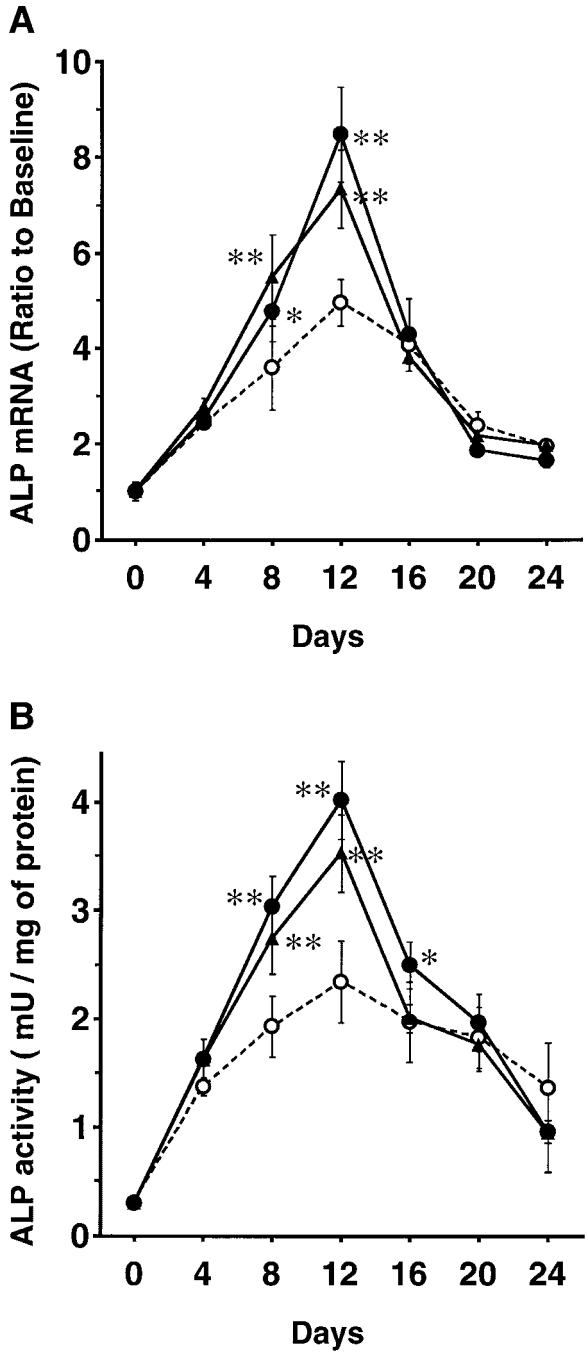


**Fig. 3.** Time course of the stimulation of ALP expression by statins in MC3T3-E1 cells. Cells were treated with vehicle (O), $10^{-7}$ M simvastatin (●), or $10^{-8}$ M cerivastatin (▲) for the time periods indicated. **A**: ALP mRNA expression. Total RNA was extracted and ALP mRNA abundance corrected for cyclophilin mRNA expression was determined by Northern blot analysis. **B**: ALP activity. Samples from whole-cell extracts were assayed using an ALP kit. Data represent the mean ± SEM for 4-wells. *, $P < 0.05$ and **, $P < 0.01$ compared to vehicle-treated control at each time point.

decreasing. Simvastatin ($10^{-7}$ M) and cerivastatin ($10^{-8}$ M) significantly enhanced ALP activity at days 8, 12, and 16 and at days 8 and 12, respectively (Fig. 3B).

### Effect of Statins on Collagen Expression

Type I collagen is a major extracellular matrix protein in bone. MC3T3-E1 osteoblastic cells abundantly express collagen. The effect of simvastatin on Col-I gene expression was determined during osteoblast maturation. Northern blot analysis showed that Col-I mRNA expression was elevated during day 8–24 of culture and was significantly increased during 8–20 days of culture by $10^{-7}$ M simvastatin as compared to vehicle treatment cells (Fig. 4A). MMP-1 (collagenase-1) mRNA abundance increased time-dependently, while simvastatin at $10^{-7}$ M significantly suppressed this mRNA expression during 16–24 days of culture, when mRNA in control cells showed a marked increase (Fig. 4B). Since osteoblasts produce additional types of interstitial collagenase, changes in mRNA encoding MMP-13 (collagenase-3) also were monitored over 24 days of culture. MMP-13 mRNA expression was greatly increased during 12–20 days of culture, when simvastatin at $10^{-7}$ M significantly suppressed this increased mRNA expression (Fig. 4C). We assessed synthesis of collagen-like proteins (CDP; Fig. 5) by labeling proteins with tritiated proline for 3 h, collecting the matrix, and digesting matrix proteins with bacterial collagenase. Amounts of CDP increased time-dependently until the end of culture (day 24). Simvastatin at $10^{-7}$ M and cerivastatin at $10^{-8}$ M significantly increased amounts of CDP during 4–24 days of culture. Thus, statins markedly elevate accumulation of collagen-like proteins throughout the culture period.

### Effect of Statins on NCP Expression

Because expression of NCPs such as BSP, OCN, and OPN changes during maturation of osteoblasts, we examined the effect of statins on their expression by MC3T3-E1 cells. BSP mRNA increased during 8–20 days of culture, and $10^{-7}$ M simvastatin markedly enhanced this mRNA expression at 16 and 20 days of culture (Fig. 6A). When changes in OCN mRNA and protein concentrations were monitored over time in the absence and presence of statins, OCN mRNA concentration increased time-dependently, peaking at 20 days of culture and

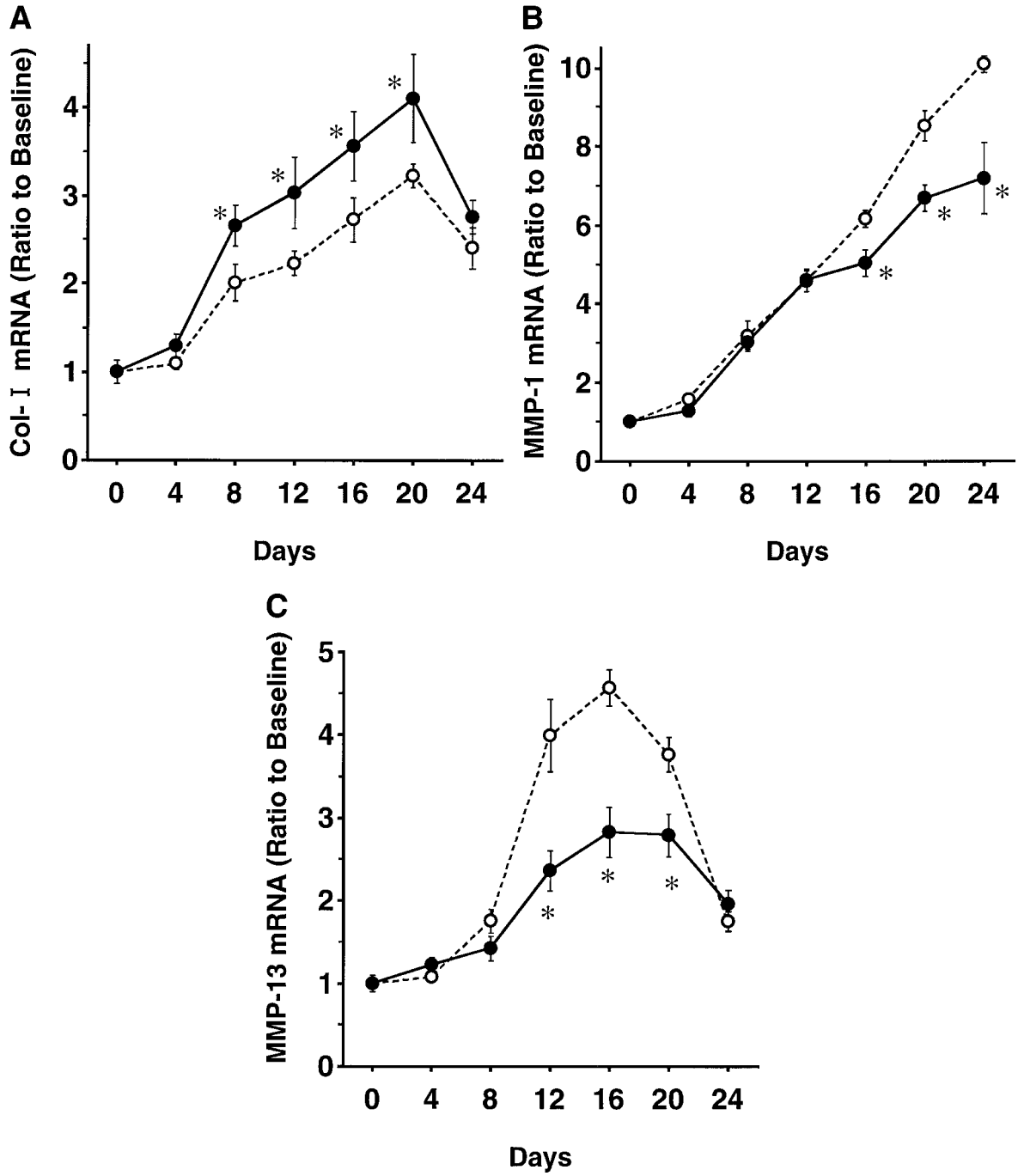


**Fig. 4.** Time course of the effect of simvastatin on mRNA expression for type I collagen and collagenases in MC3T3-E1 cells. Cells were treated with vehicle (○) or $10^{-7}$ M simvastatin (●) for the time periods indicated. Amounts of mRNA were determined by Northern blot analysis and are stated relative to cyclophilin mRNA expression. **A**: Type I collagen (Col-I) mRNA expression. **B**: MMP-1 (collagenase-1) mRNA expression. **C**: MMP-13 (collagenase-3) mRNA expression. Data represent the mean ± SEM for 4-wells. *, $P < 0.01$ compared to vehicle-treated control at each time point.

then decreasing markedly at day 24. A significant further increase in OCN mRNA occurred at 16 and 20 days of culture in simvastatin-treated cells (Fig. 6B). OCN protein accumulation also increased time-dependently until the end of culture (day 24). Cells treated with simvastatin ($10^{-7}$ M) and cerivastatin ($10^{-8}$ M) showed augmented time-dependent increases in OCN protein concentration, with the maximum effect at days 20 and 16, respectively. Overall, these statins significantly elevated OCN concentrations during 12–20 days of culture (Fig. 6C).

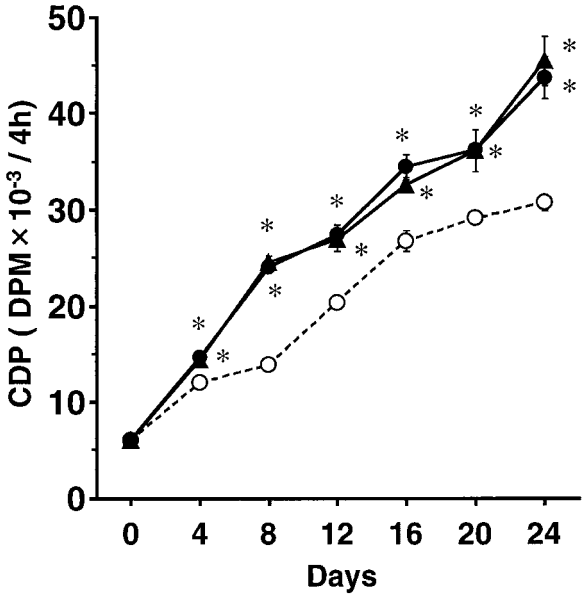


**Fig. 5.** Time course of the effect of statins on collagenase-digested proteins (CDP) synthesis in MC3T3-E1 cells. Cells were treated with vehicle (○), $10^{-7}$ M simvastatin (●), or $10^{-8}$ M cerivastatin (▲) for the time periods indicated. Proteins produced by the cells were labeled with tritiated proline during a 3-h incubation, collected and digested with bacterial collagenase. Amounts of protein sensitive to the enzyme are indicated along the ordinate. Data represent the mean ± SEM for 4-wells. *, $P < 0.01$ compared to vehicle-treated control at each time point.

OPN mRNA expression increased near the end of culture (days 20 and 24), but was not affected by treatment with simvastatin during the entire period of culture (Fig. 6D). Synthesis of NCPs increased time-dependently, and simvastatin at $10^{-7}$ M and cerivastatin at $10^{-8}$ M significantly enhanced increases of these proteins synthesis during the entire culture period (Fig. 7).

### Effect of Statins on Mineralized Nodule Formation

We finally tested the effect of statins on osteoblast differentiation as evidenced by mineralization (Fig. 8). MC3T3-E1 cells were cultured after confluence for the time periods indicated in the absence and presence of statins (simvastatin at $10^{-7}$ M, cerivastatin at $10^{-8}$ M, and atorvastatin at $10^{-6}$ M). Mineralization by these cells was significantly increased with exposure to these statins during 12–24 days of culture (Fig. 8A). Statin-stimulated mineralization by MC3T3-E1 cells was blunted by addition of substances related to cholesterol synthesis such as mevalonate and GGPP (Fig. 8B).

## DISCUSSION

We demonstrated that statins potently induced mRNAs encoding osteoblast differentiation markers such as BMP-2, ALP, type I collagen, BSP, and OCN in MC3T3-E1 cells. This is the first demonstration that a relatively low concentration of simvastatin ($10^{-7}$ M) enhances BSP mRNA expression and suppresses gene expression of MMP-13 (collagenase-3) in osteoblastic cells in vitro. These data support the impression that statins increase synthesis and secretion of a wide spectrum of factors and matrix proteins related to osteoblast differentiation and ultimately stimulate mineralization via an inhibition of the cholesterol synthesis pathway in MC3T3-E1.

The present study was undertaken to evaluate statin-induced changes in expression of osteoblast differentiation markers using a non-transformed preosteoblast cell line (MC3T3-E1) that maintains much of the tightly linked control between proliferation and differentiation usually seen only in primary culture cells [Franceschi and Iyer, 1992; Quarles et al., 1992]. After confluence, the cells deposit a collagenous extracellular matrix accompanied by activation of specific genes associated with the osteoblastic phenotype, such as ALP, OCN, and BSP, and finally form mineralized nodules (Fig. 9). Thus, MC3T3-E1 cells are suitable for detailed analysis of the order of osteoblast differentiation.

Since osteoblasts produce several cytokines and growth factors including members of the TGF-β superfamily and VEGF [Harada et al., 1994; Harris et al., 1994a; Maeda et al., 2003], the effect of statins differentiating MC3T3-E1 cells was assessed in terms of expression of BMP-2, BMP-4, TGF-β, and VEGF. Simvastatin clearly enhanced BMP-2 but not BMP-4 mRNA expression at an early stage of differentiation, which is consistent with findings of previous studies [Mundy et al., 1999; Sugiyama et al., 2000; Maeda et al., 2001].

Runx2/Cbfa1 is a master transcription factor for the osteoblast lineage; mice deficient in Runx2 show complete lack of bone [Komori et al., 1997; Otto et al., 1997]. MC3T3-E1 cells constitutively expressed Runx2 mRNA, especially in the early and middle stages of differentiation, as previously reported by Banerjee et al. [2001]. Although BMP-2 increased expression of Runx2 mRNA in osteoblastic cells,

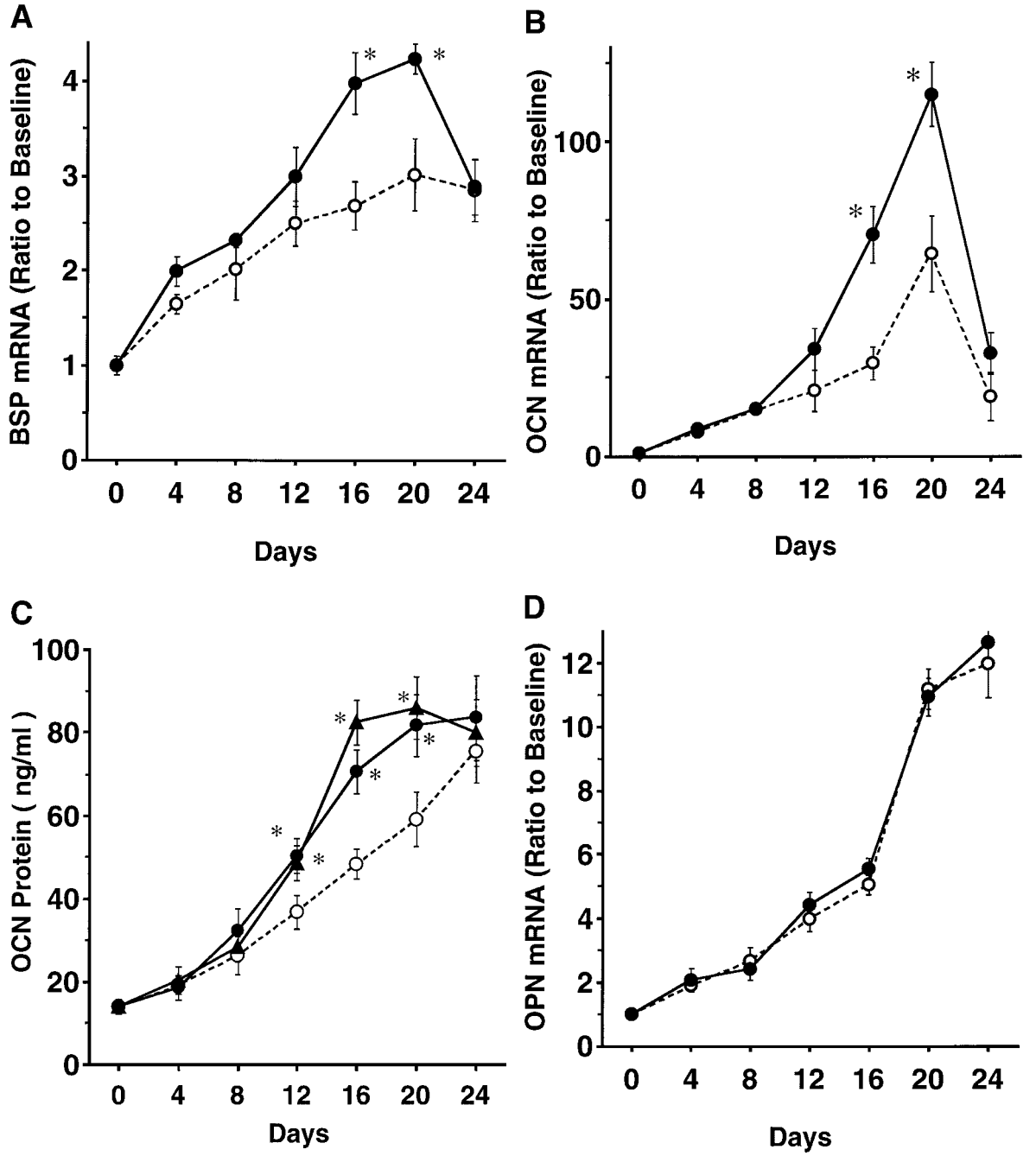


**Fig. 6.** Time course of the effects of statins on noncollagenous protein (NCP) expression in MC3T3-E1 cells. Cells were treated with vehicle (○), $10^{-7}$ M simvastatin (●), or $10^{-8}$ M cerivastatin (▲) for the time periods indicated. Abundance of mRNA corrected for cyclophilin mRNA was determined. **A**: BSP mRNA expression measured by RT-PCR. **B**: OCN mRNA expression measured by Northern blotting. **C**: OCN protein accumulation measured by a mouse OCN RIA kit. **D**: OPN mRNA expression measured by Northern blotting. Data represent the mean ± SEM for 4-wells. *, $P < 0.01$ compared to vehicle-treated control at each time point.

treatment with simvastatin did not affect Runx2 mRNA abundance. Shui et al. [2003] showed that Runx2 mRNA and protein were expressed constitutively, and that actively differentiating osteoblasts showed a significant increase in Runx2 DNA binding activity accompanied by increased phosphorylation of the transcription factor. We, therefore, suspect that increase in expression of BMP-2 mRNA and protein in response to statins up-regulate activity

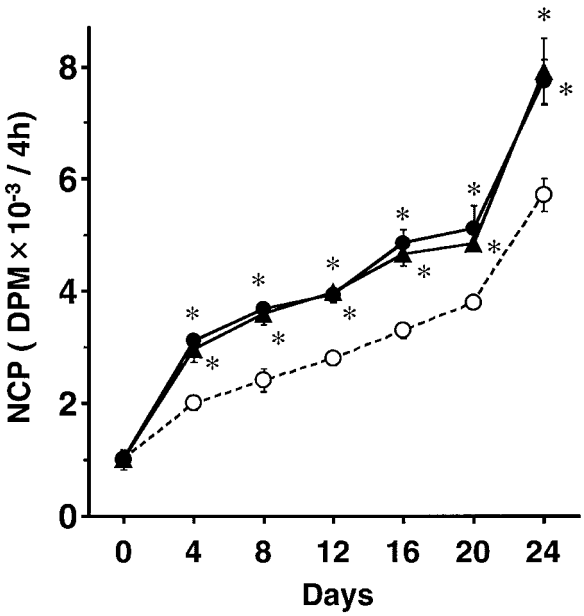


**Fig. 7.** Time course of the effect of statins on NCP synthesis in MC3T3-E1 cells. Cells were treated with vehicle (○), $10^{-7}$ M simvastatin (●), or $10^{-8}$ M cerivastatin (▲) for the time periods indicated. Proteins produced by the cells were labeled with tritiated proline over 3 h of incubation, collected, and digested with bacterial collagenase. Amounts of protein resistant to the enzyme are expressed along the ordinate. Data represent the mean ± SEM for 4-wells. *, $P < 0.01$ compared to vehicle-treated control at each time point.

of Runx2, promoting osteoblast differentiation. Alternatively, the degree of increase of BMP-2 in response to simvastatin may have been insufficient for stimulating Runx2 gene expression.

A growth factor abundant in bone matrix, TGF-β, caused a clear increase in new bone formation after injection in rodents [Noda and Camilliere, 1989]. Contrastingly, however, TGF-β inhibited differentiation in primary cultures of osteoblasts [Harris et al., 1994b]. Moreover, TGF-β represses Runx2 transcription via activation of Smad 3, inhibiting osteoblast differentiation [Alliston et al., 2001]. Based on these in vitro effects of TGF-β, increased TGF-β mRNA expression would not have been a likely consequence of simvastatin exposure in MC3T3-E1 cells.

During the past decade, investigation of VEGF has focused largely on regulation of skeletal growth. Midy and Plouet [1994] reported that VEGF was a much more potent inducer of osteoblast differentiation on a molar basis than BMP-2; in another study, inactivation of the *VEGF* gene was shown to inhibit endochondral bone formation via inhibition of angiogenesis [Gerber et al., 1999]. Recently, we demonstrated that short-term treatment with statins stimulated gene expression and protein synthesis for VEGF in MC3T3-E1 cells [Maeda et al., 2003]. In the present study, we assessed whether simvastatin could enhance VEGF mRNA and protein expression under conditions of long-term culture for up to 24 days. We found that VEGF mRNA and protein were significantly increased in the later stages of culture, suggesting involvement of VEGF in promoting differentiation of osteoblasts during mineralization (Fig. 9). Furthermore, our previous investigation demonstrated that interference with VEGF signaling pathway by SU1498, a VEGF receptor 2 (Flk-1) kinase inhibitor, suppressed mineralization by simvastatin-treated cells cultured

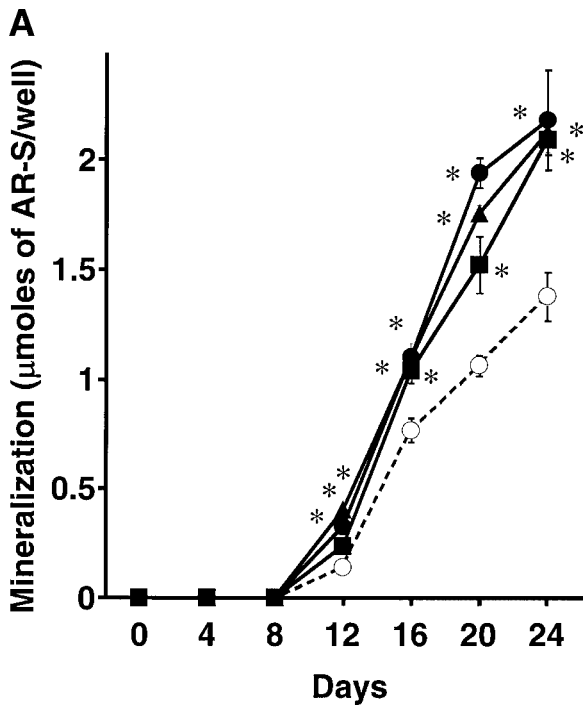


**Fig. 8.** Effect of statins on mineralization by osteoblastic cells. **A**: Time course of mineralization by simvastatin at $10^{-7}$ M (●), cerivastatin at $10^{-8}$ M (▲), and atorvastatin at $10^{-6}$ M (■) in MC3T3-E1 cells. Cells were treated with vehicle (○) or statins for the time periods indicated. AR-S staining was performed for demonstration of mineralized nodule formation. **B**: Effects of mevalonate and GGPP on statin-induced mineralization in MC3T3-E1 cells. Cells were cultured in the medium containing vehicle (V), 1 mM mevalonate (M), or 20 μM GGPP (G) with vehicle (Veh), $10^{-6}$ M simvastatin (Sim), $10^{-7}$ M cerivastatin (Cer), or $10^{-5}$ M atorvastatin (Ato) for 16 days. Mineralized nodules stained with AR-S were photographed (**upper panel**); then AR-S concentrations were measured (**lower panel**). Data represent the mean ± SEM for four determinations. *, $P < 0.01$ compared to vehicle control.

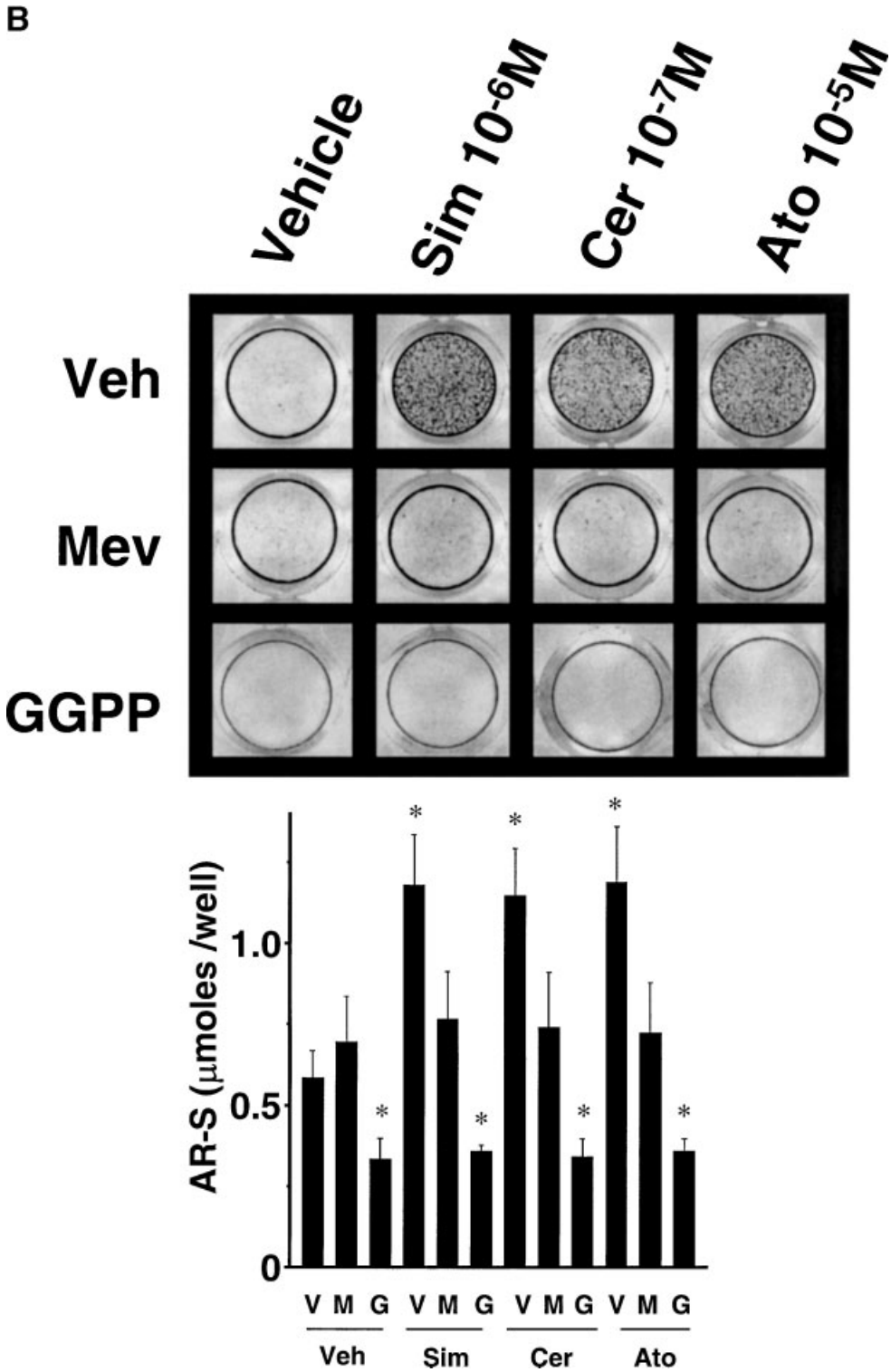


**Fig. 8.** (*Continued*)

for a long period after confluence. These findings suggest that enhanced VEGF production by osteoblasts is involved importantly in osteoblast differentiation and mineralization response to statins [Maeda et al., 2003], especially at the later stage of maturation. In the end of culture (day 24), extracellular matrix was highly mineralized and the number of cells were

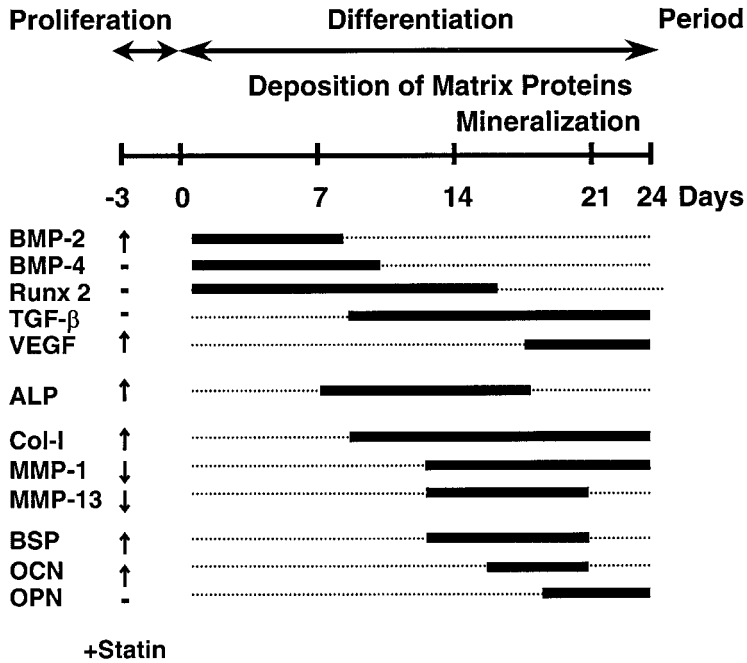


**Fig. 9.** Temporal expression pattern of markers typical of osteoblast differentiation in MC3T3-E1 cells. Cell proliferation ceases at confluence; then, osteoblast differentiation occurs. Finally, the cells form mineralized nodules. Solid bars indicate periods of elevated expression of genes during culture. Effects of statins on gene expression are shown as: ↑, increase; ↓, decrease; −, no change.

markedly reduced, because amounts of DNA and total RNA decreased in a well. Although production of VEGF per cell may be restored at high levels, accumulated amounts of the growth factor were reduced in a well. Furthermore, cultured osteoblasts in highly mineralized milieu, or osteocytes, would not secrete substantial amounts of bioactive proteins such as VEGF which were saturated in the microenvironment. Most of VEGF protein may be broken down in the cells. Based on these reasons, we explained the results that VEGF accumulation in the medium were markedly reduced despite of increased expression of the mRNA per statin-treated cell at day 24.

Up-regulation of ALP, an enzyme studied as a marker of osteoblast differentiation, occurs at the middle stage of differentiation [Aubin et al., 1995a]. Both simvastatin and cerivastatin clearly increased ALP gene expression and enzyme activity in this middle period, which is important for initiation of mineralization. Therefore, statins stimulate osteoblastic activity at least in part by enhancing synthesis of ALP.

We next focused on determining responses of MC3T3-E1 osteoblastic cells to statin treatment in terms of extracellular matrix protein expression. Like fibroblasts, osteoblasts abundantly synthesize and secrete Col-I, a major bone matrix constituent and extracellular macromolecule in osteoblast cultures. Statins significantly increased mRNA expression and collagen-like proteins (CDP) as determined by bacterial collagenase in MC3T3-E1 cells. Furthermore, gene expression of tissue collagenases such as MMP-1 and MMP-13 was strongly suppressed by treatment with simvastatin, especially in the later stage of culture. MMP-13 mRNA expression was markedly inhibited by simvastatin during culture from days 12–20, while the cells were actively expressing Col-I mRNA and forming mineralized nodules. Thus, our data support findings that statins augment collagen accumulation in the extracellular space by both an increase in production and a decrease in degradation; the end result is enhancement of mineralization.

Increased gene expression for NCPs during maturation of MC3T3-E1 cells occurred sequentially: first BSP, followed by OCN, and OPN (Fig. 9). BSP, a major protein produced and secreted by osteoblasts, regulates mineralization by direct interaction with cell-surface integrin receptors [Oldberg et al., 1988] and by initiating nucleation of hydroxyapatite, the bone mineral [Hunter and Goldberg, 1993].

Inactivation of the *BSP* gene in mice results in bone abnormalities, supporting a role for BSP in the mineralization process [Aubin et al., 1995b]. Based on these studies, increased production of BSP is important for initiation of osteoblast differentiation in culture. The present study showed that simvastatin increased BSP mRNA expression during the mineralizing phase; the increase in BSP expression induced by simvastatin is associated with enhanced mineralized nodule formation by osteoblasts.

OCN is a late marker of osteoblast differentiation that is related closely to osteoblast maturation [Franceschi and Iyer, 1992; Aubin et al., 1995a]. We clearly demonstrated increased OCN mRNA expression and protein secretion in response to statins at a late stage of MC3T3-E1 cell culture, consistent with the results of Ohnaka et al. [2001]. Since OCN may be important for maximizing calcification at a late stage of culture, statins would be likely to elevate OCN gene expression and protein synthesis by MC3T3-E1 cells. OPN is another noncollagenous bone matrix protein secreted by osteoblasts. Since greatly increased OPN gene expression was observed at very late stage such as 20 and 24 days of the culture, OPN is regarded as the last in a chronologic sequence of markers of osteoblast differentiation. Furthermore, OPN expression is regulated by hormones and cytokines, and inhibits mineral crystal growth in vivo and in vitro [Hunter et al., 1996; Boskey et al., 2002]. In our results, simvastatin did not affect OPN mRNA expression, suggesting that statin exposure did not affect secretion of this mineralization-inhibiting protein by older osteoblasts and osteocytes.

MC3T3-E1 osteoblastic cells showed mineralized nodule formation during the second half of the culture period, when statins including simvastatin, cerivastatin, and atorvastatin significantly enhanced mineralization beyond the extent seen in vehicle-treated controls; this confirmed our previous observation [Maeda et al., 2001, 2003]. Statins sequentially stimulate expression of differentiation markers (BMP-2, ALP, Col-I, BSP, VEGF, and OCN) by osteoblasts, leading to enhanced formation of mineralized nodules. Many studies document that statins trigger multiple cellular events through their action as HMG-CoA reductase inhibitors [Goldstein and Brown, 1990; Lacoste et al., 1995; Hamelin and Turgeon, 1998; Kureishi et al., 2000; Maron et al., 2000]. By modulating the initial part of the cholesterol synthesis pathway, statins decrease concentrations of many important compounds including mevalonate and GGPP. The present study showed involvement of the mevalonate pathway and isoprenoids in inhibiting mineralized nodule formation; either mevalonate, the direct product of HMG-CoA reductase, or GGPP, an isoprenoid, opposed stimulation of mineralization by statins in MC3T3-E1 cells. Geranylgeranylated proteins including small G proteins (Ras) and Ras-like proteins may be involved in upregulation of accelerated mineralization by osteoblasts [Ohnaka et al., 2001; Maeda et al., 2003]. Our results indicate that statins stimulate osteoblast differentiation and matrix mineralization in vitro, which suggests possible clinical applicability of statins to treatment of osteoporosis.

Statins stimulate BMP-2 expression, which promotes osteoblast differentiation at early and middle stages of the culture. Furthermore, it is likely that VEGF stimulated by statins enhances the differentiation mineralization at late stage of the culture. Further studies will identify other critical factors induced by statins on promotion of osteoblast differentiation.

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