

Osteoadherin serves roles in the regulation of apoptosis and growth in MC3T3-E1 osteoblast cells

ERI HAMAYA^{1,2}, TOSHIKI FUJISAWA¹ and MASATO TAMURA²

Departments of ¹Dental Anesthesiology and ²Biochemistry and Molecular Biology, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University, Sapporo, Hokkaido 060-8586, Japan

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Abstract. Small leucine-rich proteoglycans (SLRPs) are a class of proteoglycans that are characterized by small protein cores and structures of leucine-rich repeats. SLRPs are expressed in most extracellular matrices and share numerous biological functions that are associated with binding of collagens and cell surface receptors. Osteoadherin (also termed osteomodulin) is encoded by the *Omd* gene and is a keratan sulfate proteoglycan of the class II subfamily of SLRPs. Osteoadherin is highly expressed in mineralized tissues, including bone and dentin; however, its precise roles remain unknown. The present study determined the *Omd* expression levels and investigated the effects of over- and under-expression of osteoadherin in osteoblastic cells. *Omd* mRNA expression increased with osteoblast differentiation in MC3T3-E1 cells. In C2C12 cells, *Omd* mRNA expression was induced by bone morphogenetic protein (BMP)2. Reporter assays similarly demonstrated activation of the *Omd* gene promoter following co-transfection with Smad1 and Smad4, which are intracellular signaling molecules of the BMP2 signaling pathway. Overexpression of *Omd* increased the viability and decreased caspase 3/7 activity in MC3T3-E1 cells. By contrast, following transfection with small interfering RNA for *Omd*, viable cell numbers were decreased and caspase 3/7 activity was increased. Furthermore, overexpression of *Omd* reduced the expression of CCN family 2 in these cells. These results demonstrate that *Omd* expression is regulated during osteoblast differentiation, and that the protein product osteoadherin serves roles in the apoptosis and growth of osteoblast cells.

Introduction

Extracellular matrices (ECMs) provide cell microenvironments with several secreted molecules, such as collagens, glycoproteins, glycosaminoglycans (GAGs) and proteoglycans. Proteoglycans regulate cell behaviors by binding GAG chains or through direct protein-protein interactions via the core protein (1). The structure of small leucine-rich proteoglycans (SLRPs) consists of a small protein core with various GAG side chains, and SLRPs are grouped into five classes based on common structural properties (1,2). Osteoadherin, also termed osteomodulin (the *Omd* gene product), was originally isolated from guanidine extracts of bovine bone, and is a 47-kDa keratan sulfate proteoglycan of the class II subfamily of SLRPs (2,3). Several SLRPs are ECM components; however, osteoadherin is the only member that is restricted to mineralized tissues, such as bones and teeth (4-6). In bone tissues, *Omd* is expressed by fetal and adult osteoblasts, and its product osteoadherin can reach up to 400 µg/g wet weight in bovine bone (3).

As with numerous SLRPs, osteoadherin binds a number of ECM components; in particular, it binds fibrillar collagens to stabilize fibrillar tissue frameworks (7,8). Leucine-rich repeat (LRR) motifs comprise 6-10 repeats of ~20-29 amino acids with conserved leucine spacing, and are folded into structures with one β-sheet and one α-helix. LRRs participate in a wide range of biological processes, and interact directly with collagen, heparin-binding proteins and cell surface receptors to block ligand binding (9). Osteoadherin contains six closely spaced tyrosine sulfate residues in its N-terminal region and two in its C-terminal region (9,10). Tyrosine sulfate domains bind heparin-binding proteins, such as basic growth factor-2 and thrombospondin I (10). Osteoadherin also contains large numbers of acidic amino acid residues in its N-terminal region (3), which may bind to hydroxyapatite in calcified tissues and basic cluster motifs of heparin-binding proteins and growth factors (9,11). SLRPs also carry GAG chains that are covalently attached to the core protein, and can modulate biological functions depending on the nature of GAG chains. Through these interactions, SLRPs are involved in the initial triggering of multiple cellular responses in various tissues (2). However, it is unclear whether osteoadherin in bone tissues serves roles in the regulation of osteoblast growth, apoptosis, and differentiation.

Correspondence to: Professor Masato Tamura, Department of Biochemistry and Molecular Biology, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University, North 13, West 7, Sapporo, Hokkaido 060-8586, Japan
E-mail: mtamura@den.hokudai.ac.jp

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Bone morphogenetic proteins (BMPs) regulate differentiation and proliferation in various cell types, including osteoblasts (12). BMP2 reportedly triggers osteoblast differentiation and upregulates several genes that encode osteoblast phenotype-related proteins *in vitro* (13). In the intracellular BMP2 signaling pathway, Smad1 is phosphorylated directly by type I receptors for BMP2 and is transported into the nucleus in complexes with Smad4 (14). This complex interacts with the regulatory elements of target genes and regulates their expression. To date, little is understood of how *Omd* expression is regulated in response to BMP2 during osteoblast differentiation.

Apoptosis can be initiated by various stimuli and is directed by a family of cysteine proteases known as caspases. At the molecular level, caspase activation plays a central role in the execution of apoptosis. To the best of our knowledge, so far, 14 mammalian caspases have been identified, three of which (caspase-3, -6 and -7) are the effector caspases that coordinate the execution phase of apoptosis by degrading multiple substrates, including the structural and regulatory proteins in the cell nucleus, cytoplasm and cytoskeleton (15).

The present study determined the *Omd* expression levels during osteoblast differentiation and investigated the effects of overexpression and knockdown of osteoadherin in osteoblast cells. Overexpression of *Omd* increased the numbers of viable cells and decreased the activity of caspase 3/7 in MC3T3-E1 cells. By contrast, knockdown of *Omd* decreased viable cell numbers and increased caspase 3/7 activity in these cells. *Omd* overexpression also reduced the expression of CCN family 2 (CCN2). Thus, it can be concluded that osteoadherin regulates apoptosis and growth in osteoblast cells.

Materials and methods

Reagents. Recombinant human BMP2 was kindly supplied by Astellas Pharma Ltd. Dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich; Merck KGaA.

Cell cultures. MC3T3-E1 mouse osteoblast cells and C2C12 myoblast cells were obtained from RIKEN BioResource Center and were cultured in α -minimal essential medium (α -MEM; Sigma-Aldrich; Merck KGaA) containing 100 μ g/ml kanamycin (Meiji Seika Kaisha, Ltd.) and 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) at 37°C under a humidified atmosphere containing 5% CO₂, as described previously (16). MC3T3-E1 cells were cultured and differentiated in α -MEM containing 10% FBS, 10 mM β -glycerophosphate (Tokyo Chemical Industry Co., Ltd.) and 50 μ g/ml ascorbic acid (Wako Pure Chemical Industries, Ltd.) for 1-3 weeks. Pre-adipocyte 3T3-L1 cells were purchased from DS Pharma Biomedical Co., Ltd. and grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS at 37°C until confluence. At 2 days after confluence (day 0), differentiation was induced in adipocyte cultures by adding 500 μ M IBMX, 10 μ g/ml insulin (Cell Science and Technology Institute, Inc.) and 1 μ M dexamethasone to the basal medium. On day 3, media were replaced with adipogenic medium comprising DMEM supplemented with 10% FBS and 10 μ g/ml insulin, which was changed every

2 days thereafter until analysis. C2C12 cells were seeded into culture dishes, and then cultured in media containing 50 or 200 ng/ml of BMP2 for 6-24 h. Cells of the mouse stromal cell line ST2 (RIKEN BioResource Center) were obtained and cultured as described previously (17). Similarly, bEnd.3 mouse vascular endothelial cells (American Type Culture Collection) (18) were cultured in DMEM supplemented with 10% FBS as described previously (19) and culture media were changed every 3-4 days.

Reverse transcription (RT)-semi-quantitative PCR (qPCR). Total RNA was extracted from cells using ISOGEN (Nippon Gene Co., Ltd.) and complementary DNA was synthesized with the Omniscript RT kit (Qiagen GmbH) using an oligo(dT)₁₅ primer (1 μ M) at 37°C for 30 min. semi-qPCR analyses were performed with Taq DNA polymerase (Qiagen GmbH) as described previously (20). The specific primer sequences for each gene are listed in Table I. Each reaction consisted of an initial denaturation at 94°C for 3 min followed by three-step cycling: Denaturation at 94°C for 30 sec, annealing at a temperature optimized for each primer pair for 30 sec, and extension at 72°C for 40 sec. After the requisite number of cycles (25-35 cycles), reactions underwent a final extension at 72°C for 5 min. To accommodate the differences in RNA quantities, expression levels were normalized to those of the housekeeping gene GAPDH. Amplification products were separated using electrophoresis on 2% agarose gels and visualized by SYBR Green staining followed by UV light illumination. The relative intensity of the gel bands was measured using ImageJ software (version 1.52a; National Institutes of Health), and results were normalized to the GAPDH mRNA level.

RT-qPCR. Total RNA was extracted from cells using ISOGEN (Nippon Gene Co., Ltd.) and complementary DNA was synthesized with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 37°C for 60 min. RT-qPCR analyses were performed using the assay-on-demand TaqMan probes (Mm00449589_m1 for *Omd*, Mm00650798g1 for *Npy1r*, Mm00475834_m1 for *Alp*, Mm03413826_mH for *Bglap*, Mm01192932_g1 for *Ccn2*, and Mm99999915_g1 for *GAPDH*; Applied Biosystems; Thermo Fisher Scientific, Inc.) and the StepOne® real time PCR system, according to the manufacturer's protocol, as described previously (21). The qPCR thermocycling conditions included an initial denaturation of 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative gene expression levels were quantified using the comparative 2^{- $\Delta\Delta C_q$} method (22) using *GAPDH* expression as an endogenous control.

Western blot analysis. MC3T3-E1 cells were transfected according to the experimental conditions and then collected at specific time points, washed in ice-cold PBS, and suspended in CellLytic-M Mammalian cell lysis/extraction reagent (Sigma-Aldrich; Merck KGaA) plus a protease inhibitor (Complete mini; Roche Diagnostics). Whole-cell extracts were quantified using a Protein assay reagent (Bio-Rad Laboratories, Inc.) and ~30 μ g protein/lane was separated using 10% SDS-PAGE followed by transfer to a polyvinylidene fluoride membrane (EMD Millipore). Blocking was performed

Table I. Primers used for reverse transcription-semi-quantitative PCR.

Specificity	Forward sequence (5'-3')	Reverse sequence (5'-3')	Predicted size, bp
<i>Omd</i>	ATGGTGTATTTCGCTAAACTTTCAA	AAGATGATTATAGCAGAGGTCAAG	194
<i>Bgn</i>	TGCCACCGCCATTGTTCCATC	GTTTGTGGAGGGACTGGCTAGA	486
<i>Dcn</i>	ATGGCAGTCTGGCCAATGTT	GCCGTCTGAGGGTTACTTGT	308
<i>Fmod</i>	CATGATCTGCACCCCTTCTT	AATGCAGAGGAAGCCAGGTC	300
<i>Lum</i>	CCTGAGGAATAACCAAATCGAC	AGACCAGCAGGCAGCTTGCTCA	393
<i>GAPDH</i>	TCCACCACCCTGTTGCTGTA	ACCACAGTCCATGCCATCAC	452

Omd, osteoadherin; *Dcn*, decorin; *Bgn*, biglycan; *Fmod*, fibromodulin; *Lum*, lumican.

with 0.3% skim milk (BD Biosciences) dissolved in TBS with 0.05% Tween-20 (TBST) for 1 h at room temperature. The membrane was probed with antibodies against osteoadherin (rabbit polyclonal anti-human polyclonal antibody; 1:2,000; cat. no. NBP2-19626; Novus Biologicals, LLC) or β -actin (rabbit polyclonal antibody; 1:2,000; cat. no. 20536-1-AP, ProteinTech Group, Inc.) for 1 h at room temperature. The membrane was subsequently washed three times and incubated with anti-mouse horseradish peroxidase-linked antibody (1:20,000; cat. no. NA934; GE Healthcare Life Sciences) in TBST for 1 h at room temperature. Following three additional washes, chemiluminescence detection was performed with ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) and a LAS 1000 image analyzer, according to the manufacturer's instructions.

Reporter constructs and assays of luciferase activity. Luciferase reporter plasmids for the murine *Omd* promoter were generated as follows. The 1774-bp *Omd* promoter fragment (-1,609 to +165) (chromosome 13; MGI: 1350918) was isolated from mouse genomic DNA using PCR and was subcloned into the pGL4.12 vector (Promega Corporation) to generate the luciferase reporter plasmid pOmd-luc. The nucleotide sequences of promoter regions were verified by sequencing. The Smad expression plasmids encoding wild-type Smad4 and constitutively active Smad1 (DVD) were provided by Dr Katagiri (Saitama Medical University, Saitama, Japan) (23). For the reporter assay, C2C12 cells were plated 24 h prior to transfection at a density of 1×10^5 cells/well (24-well plate) and cultured in α -MEM supplemented with 10% FBS. Transfection of plasmid DNA into cells was performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase reporter assays were performed as described previously (20). Briefly, cells were lysed with a PicaGene cell culture lysis reagent Luc (Wako Pure Chemical Industries, Ltd.; cat. no. 300-04351) and firefly luciferase activities were measured using a Mini Lumat LB 9506 luminometer (Berthold Technologies). Luciferase activity was normalized by comparison with β -galactosidase activity and fold-changes in the luciferase activities of transfected cells were calculated on the basis of those in control MC3T3-E1 cells that were transfected with pcDNA3.

Transfection of small interfering RNAs (siRNAs). MC3T3-E1 cells were transfected with 2.5 nM Silencer Select

pre-designed siRNAs for osteoadherin (siOmd; Thermo Fisher Scientific Inc.; cat. nos. s77492 and s77494), the Y1 receptor (siY1; Thermo Fisher Scientific, Inc.; cat. no. s70765) or Silencer negative control siRNA no. 1 (siCont; Thermo Fisher Scientific, Inc.; cat. no. 4390843) using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (16). Total RNA was extracted after 2 or 6 days for the determination of mRNA level. Total protein was extracted after 2 days for western blot analysis.

Generation of plasmid construct and overexpression of *Omd*. *Omd* overexpression plasmid was generated as follows and was subsequently designated pOmd. Total RNA was extracted from MC3T3-E1 cells using ISOGEN (Nippon Gene Co., Ltd.). Mouse *Omd* complementary DNA was reverse-transcribed from RNA using Omniscript RT kit (Qiagen), and then amplified using primers that flank the mouse *Omd* open reading frame (forward, 5'-CCAGCCCGAGGACAAGAAAA-3' and reverse, 5'-GGCTTTATGGAGGCATAAATGTGT-3') and PrimeSTAR Max DNA polymerase (Clontech Laboratories, Inc.), according to the manufacturer's instructions. PCR products were electrophoresed on 1% agarose gels and were purified and subcloned into *Kpn*I and *Xba*I restriction sites of the pcDNA3 vector (Invitrogen; Thermo Fisher Scientific, Inc.) using In-Fusion HD cloning kit (Clontech Laboratories, Inc.; cat. no. 639648), according to the manufacturer's protocol. Individual clones of transformed *Escherichia coli* were isolated from agar plates and nucleotide sequences of each plasmid were confirmed by DNA sequencing. MC3T3-E1 cells were transfected in 24-well plates with 0.1 μ g pOmd or empty vector (pcDNA3) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Alkaline phosphatase (ALP) staining analyses. MC3T3-E1 cells were plated onto 24-well plates at a density of 1.7×10^4 /ml. Following 24 h, cells were transfected in 24-well plates with 0.1 μ g pOmd or pcDNA3 and were cultured in α -MEM for 14 days. ALP staining was performed as previously described (20). Briefly, cells were rinsed in PBS, fixed in 10% formalin at room temperature for 30 min, rinsed again in PBS, and then incubated at room temperature for 1 h in 300 μ l aliquots of solution containing 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate, 0.3 mg/ml nitro-blue tetrazolium (Wako Pure Chemical Industries, Ltd.), 0.1 M Tris-HCl

(pH 9.0), 0.01 N NaOH and 0.05 mM MgCl₂. Images were captured using an iPhone7 smartphone (Apple Corporation). The same settings were applied to all experimental samples.

Quantitation of cell viability. Cell counting kit-8 (Dojindo Molecular Technologies, Inc.; cat no. CK04-01) were used to evaluate cell viability. In these experiments, 10- μ l aliquots of the WST-8 substrate [5-mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] were added to each well. After incubation for 1 h at 37°C, optical densities were measured at a wavelength of 450 nm using a microplate absorbance reader. Data are expressed as fold changes of transfected samples relative to cell numbers in samples transfected with control siRNA or pcDNA3.

Measurements of caspase 3/7 activity. The enzyme activities of caspase 3/7 were determined using a caspase colorimetric assay (Caspase-Glo 3/7 assay system; Promega Corporation) as described previously (21). Briefly, for each reaction, cells were lysed and incubated with luminogenic substrates containing the Asp-Glu-Val-Asp sequence, which is cleaved by activated caspase 3/7. Following incubation at room temperature for 1 h, luminescence was quantified using a Mini Lumat LB 9506 luminometer (Berthold Technologies). Fold changes in caspase 3/7 activity in transfected cells were calculated on the basis of those in control siRNA or pcDNA3 transfected MC3T3-E1 cells.

Statistical analysis. Data are expressed as the mean \pm standard deviation of three to six independent experiments. Comparison of two groups was performed with a Student's t-test using Microsoft Excel version 2016 (Microsoft Corporation). Comparisons of multiple groups were performed using one-way analysis of variance using Microsoft Excel version 2016 (Microsoft Corporation) followed by Dunnett's multiple comparison test using software provided by Osaka University, Osaka, Japan (<http://www.gen-info.osaka-u.ac.jp/MEPHAS/dunnett-e.html>). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SLRP mRNA expression levels in cultured cells. To evaluate the roles of osteoadherin in mesenchymal cells, *Omd* mRNA expression levels were determined in various mesenchymal cell cultures and compared with those of other SLRPs, including biglycan, decorin, fibromodulin, and lumican, using RT-semi-qPCR analyses. *Omd* mRNA was expressed in MC3T3-E1 cells (osteoblasts) but not in C2C12 myoblasts, adipocyte differentiated 3T3-L1 cells, ST2 bone marrow stromal cells or bEnd.3 endothelial cells (Fig. 1A). By contrast, biglycan (*Bgn*), decorin (*Dcn*) and fibromodulin (*Fmod*) mRNA expression was detected in MC3T3-E1, C2C12 and 3T3-L1 cells. Meanwhile, lumican (*Lum*) mRNA was expressed only in MC3T3-E1 and 3T3-L1 cells. Analysis of the *Omd* and *Bgn* mRNA/*GAPDH* mRNA ratio in various mesenchymal cell cultures is presented in Fig. 1B. These data indicate that *Omd* expression varies during osteoblast differentiation. To determine whether *Omd* expression reflects the stages of osteoblast differentiation, MC3T3-E1 cells were cultured in

differentiation medium for 1-3 weeks and expression levels of *Omd* mRNA were determined using RT-qPCR. *Omd* mRNA expression was significantly increased from 1 week and increased further over the culture period of 3 weeks in differentiating osteoblast cells (Fig. 1C). Protein levels were also assayed using western blot analysis and were identified to be increased during *in vitro* osteoblastic differentiation in MC3T3-E1 cells (Fig. 1D).

BMP2 induces *Omd* expression in C2C12 cells and regulates *Omd* promoter activity via *Smad* signaling. To further investigate the roles of osteoadherin in osteoblast differentiation, experiments were performed with C2C12 cells, which are well-characterized model cells that differentiate into myotubes or osteoblasts by the presence of BMP2 (13). In C2C12 cells, *Omd* mRNA expression was not detected using RT-semi-qPCR analyses (Fig. 1A). However, in subsequent RT-qPCR analyses, *Omd* mRNA expression was increased >10-fold after treatment with 50 ng/ml BMP2. A higher BMP2 concentration increased the expression of *Omd* mRNA to >30-fold at 200 ng/ml (Fig. 2A). In time course analyses of BMP2-induced *Omd* mRNA expression, *Omd* mRNA expression increased after 6 h, and a significant time-dependent increase in mRNA level was observed at 24 h (Fig. 2B). These findings indicate that BMP2 induces *Omd* expression in these cells.

To elucidate the mechanisms by which BMP2 signaling activates *Omd* transcription, the present study cloned an ~1.7 kb-pair mouse genomic DNA fragment corresponding to the 5'-flanking promoter region of the mouse *Omd* gene. To determine responsiveness to BMP2 signaling, this *Omd* promoter region was ligated into a luciferase reporter expression vector (pOmd-luc), and luciferase activity was observed in BMP2-treated C2C12 cells (Fig. 2C). Transient transfection of these cells with pOmd-luc and co-transfection with Smad1 (DVD) and Smad4 expression plasmids resulted in a significant increase in luciferase activity (Fig. 2D). However, co-transfection with Smad1 (DVD) and Smad4 expression plasmids did not alter pOmd-luc activity in MC3T3-E1 cells (data not shown).

Knockdown of Y1 receptor upregulates *Omd* mRNA expression in MC3T3-E1 cells. Several studies have reported that neuropeptide Y and its Y1 receptor are directly involved in osteoblast regulation (24-26). Previously, we demonstrated that knockdown of the Y1 receptor using siRNA promotes osteoblast differentiation (16). Therefore, the present study examined the effects of Y1 receptor inhibition on *Omd* mRNA expression. Following transfection of MC3T3-E1 cells with siRNA for the Y1 receptor, Y1 receptor (*Npy1r*) mRNA expression level was decreased to undetectable levels, confirming that the siRNA was effective at silencing endogenous Y1 receptor expression (Fig. 3A). Under these conditions, *Omd* mRNA expression was significantly increased (Fig. 3B), indicating that *Omd* expression is Y1 receptor dependent and that the NPY signaling pathway regulates *Omd* expression in MC3T3-E1 cells.

Effects of overexpression or knockdown of *Omd* on viability and apoptosis in MC3T3-E1 cells. To determine the biological

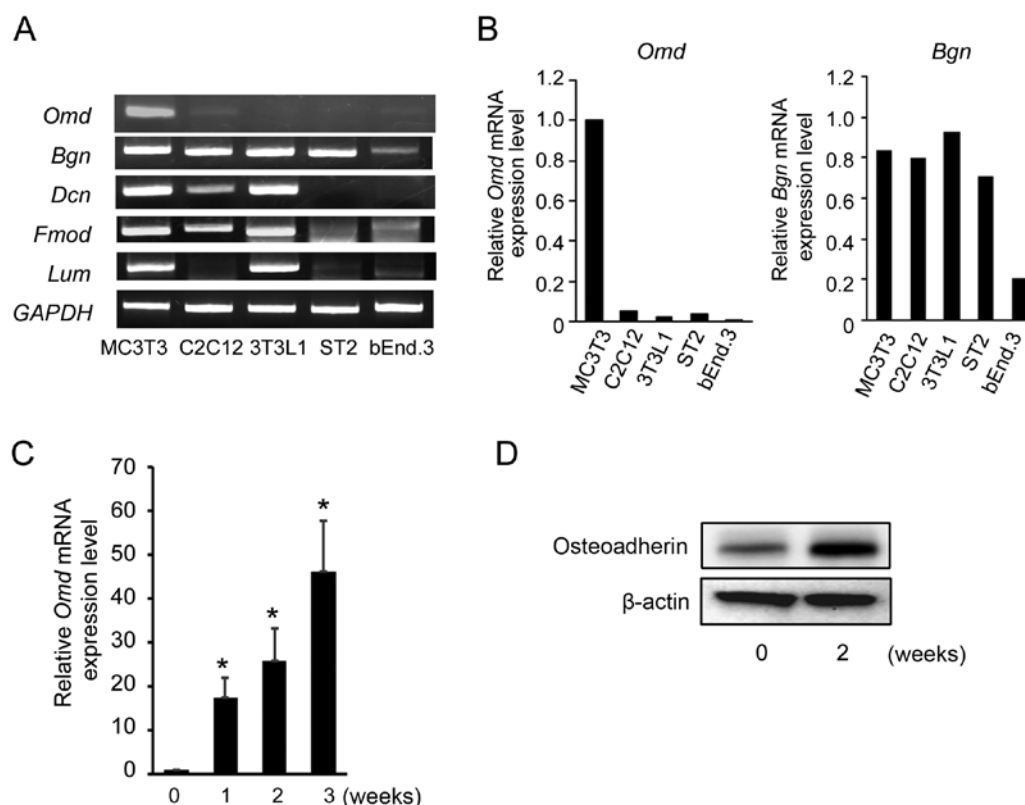


Figure 1. SLRP mRNA expression levels under various culture conditions. (A) Total RNA was extracted from MC3T3-E1, 3T3-L1, C2C12, ST2 and bEnd.3 cells, and mRNA expression levels of the SLRPs *Omd*, *Dcn*, *Bgn*, *Fmod* and *Lum* were determined using RT-semi-qPCR analyses. Equal loading of cDNA samples was confirmed by amplification of *GAPDH* cDNA. The presented data are representative of three experiments with similar results. (B) mRNA expression levels of *Omd* and *Bgn* normalized to *GAPDH*. (C) Confluent MC3T3-E1 cells were incubated in differentiation media for 1-3 weeks. Total RNA was extracted from cells and *Omd* mRNA expression was determined using RT-qPCR. mRNA expression levels were normalized to those of *GAPDH*, and are presented as fold changes relative to the expression levels in control cells. (D) Confluent MC3T3-E1 cells were incubated in differentiation media for 2 weeks. Total protein was extracted from MC3T3-E1 cells and the levels of *Omd* protein were determined by western blot analysis. β -actin was used as an endogenous control. Data are presented as the mean \pm standard deviation of separate experiments performed in triplicate. * $P < 0.05$ vs. week 0. SLRP, small leucine-rich proteoglycan; *Omd*, osteoadherin; *Dcn*, decorin; *Bgn*, biglycan; *Fmod*, fibromodulin; *Lum*, lumican; RT, reverse transcription; qPCR, quantitative PCR; cDNA, complementary DNA.

relevance of *Omd* regulation in osteoblast differentiation, *Omd* was significantly overexpressed in MC3T3-E1 cells by transfecting cells with the p*Omd* expression plasmid (Fig. 4A). Western blot analysis demonstrated that osteoadherin protein was overexpressed in p*Omd*-transfected MC3T3-E1 cells (Fig. 4B). Viability was significantly increased in these cells compared with those transfected with control plasmid (Fig. 4C). Caspase 3/7 activity, which is associated with apoptosis (15), was also significantly decreased following transfection with p*Omd* (Fig. 4D), which further indicates that *Omd* is involved in the regulation of apoptosis in MC3T3-E1 cells.

In further experiments, the effects of *Omd*-knockdown was examined using RNA interference in MC3T3-E1 cells. Following transfection with *Omd* siRNA, *Omd* mRNA expression was significantly decreased (Fig. 4E). As presented in Fig. 4F, cells transfected with *Omd* siRNA exhibited a reduction in osteoadherin expression compared with cells transfected with siCont. Viable cell numbers were decreased compared with those in control siRNA-transfected cells, although statistical significance between control siRNA- and *Omd* siRNA-transfected cells was not confirmed (Fig. 4G). By contrast, caspase 3/7 activity was significantly increased following exposure to *Omd* siRNA (Fig. 4H). These results

indicate that cell survival/death is *Omd*-dependent and that osteoadherin may regulate cell growth and apoptosis in osteoblast cells.

Effects of *Omd* overexpression on the mRNA expression of specific osteoblast differentiation genes in MC3T3-E1 cells. To investigate the effects of osteoadherin in osteoblast differentiation, the present study examined the effects on genes with characterized roles in osteoblast differentiation. Following transfection with *Omd* siRNA, *Alp* mRNA expression level and ALP activities were not affected in MC3T3-E1 cells (data not shown). Although *Alp* and *Bglap* mRNA expression levels were not significantly altered by *Omd* overexpression, *Ccn2* expression was significantly reduced (Fig. 5A). However, as ALP activity was not affected by *Omd* overexpression on day 14 (Fig. 5B), it can be concluded that osteoblast-related gene expression is not dependent on *Omd* in MC3T3-E1 cells.

Discussion

SLRP family members are secreted into the ECM after synthesis by the cells of connective tissues. These proteoglycans have core proteins with LRRs and are important

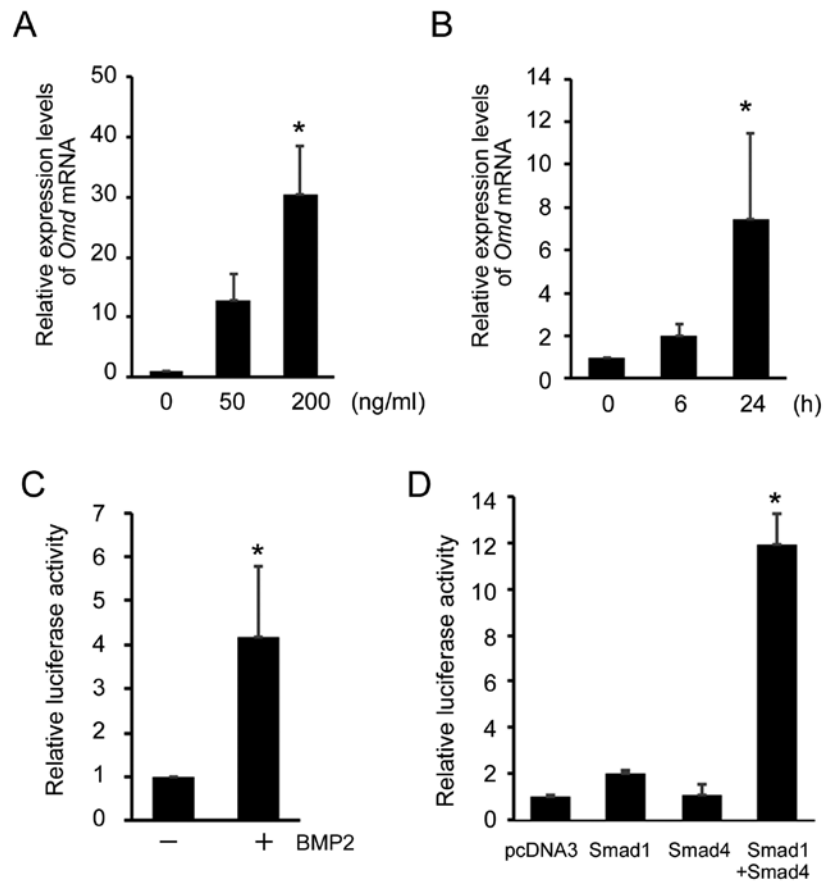


Figure 2. BMP2 signaling induces *Omd* expression and *Omd* promoter activity in C2C12 cells. (A) C2C12 cells were seeded into culture dishes at 1.5×10^6 cells/cm². On day 1 of culture, cells were cultured in media containing indicated concentrations of BMP2 or vehicle (0), and total RNA was extracted from cells after 2 days. * $P < 0.05$ vs. 0 ng/ml. (B) C2C12 cells were seeded into 100-mm cell culture dishes and, after 24 h, were cultured in fresh media containing 200 ng/ml BMP2 for the indicated times. *Omd* mRNA expression was determined using reverse transcription-quantitative PCR. * $P < 0.05$ vs. 0 h. (C) C2C12 cells were transiently transfected with the pOmd-luc reporter plasmid in 24-well plates. Subsequently, 200 ng/ml BMP2 (+) or vehicle (-) was added and cells were cultured for a further 24 h prior to determinations of luciferase activity. (D) C2C12 cells were transiently co-transfected in 24-well plates with the reporter plasmid pOmd-luc and expression constructs for Smad1 or Smad4, or an empty expression plasmid (pcDNA3). Luciferase activities were determined after 48 h. Normalized luciferase activities are presented as relative ratios. * $P < 0.05$ vs. pcDNA3. Data are presented as the mean \pm standard deviation from separate experiments performed in triplicate. BMP, bone morphogenetic protein; *Omd*, osteoadherin.

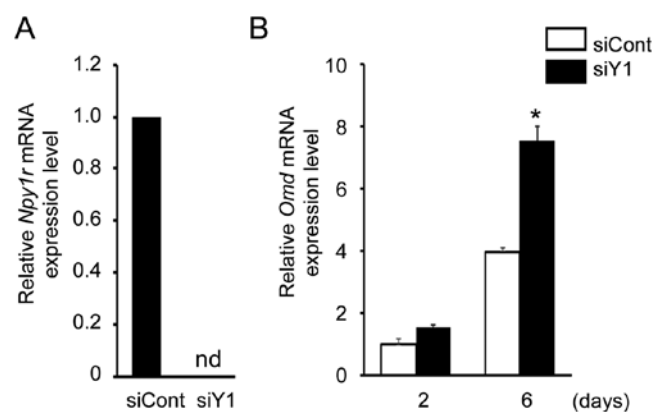


Figure 3. Knockdown of the Y1 receptor upregulates *Omd* mRNA expression in MC3T3-E1 cells. (A) MC3T3-E1 cells were plated at 1.7×10^4 cells/cm² and were cultured for 24 h. Cells were then transfected with siY1 or siCont (both at 5 nM) and were incubated again for 2–6 days. Total RNA was extracted, and (A) after 2 days *Npy1r*, and (B) after 2 and 6 days *Omd* mRNA expression levels were determined using reverse transcription-quantitative PCR analyses. Expression levels were normalized to those of *GAPDH* and fold changes were calculated relative to the control. Data are presented as the mean \pm standard deviation of separate experiments performed in triplicate. * $P < 0.05$. siY1, Y1 receptor small interfering RNA; siCont, scramble small interfering RNA; *Omd*, osteoadherin; *Npy1r*, Y1 receptor.

structural components of the ECM. Accordingly, numerous SLRPs have been reported to bind multiple ECM constituents, particularly fibrillar collagens, and thereby stabilize

tissue frameworks (7,8). SLRPs include five classes of structurally related proteoglycans. Among them, osteoadherin, fibromodulin, keratocan and lumican are class II SLRPs (2),

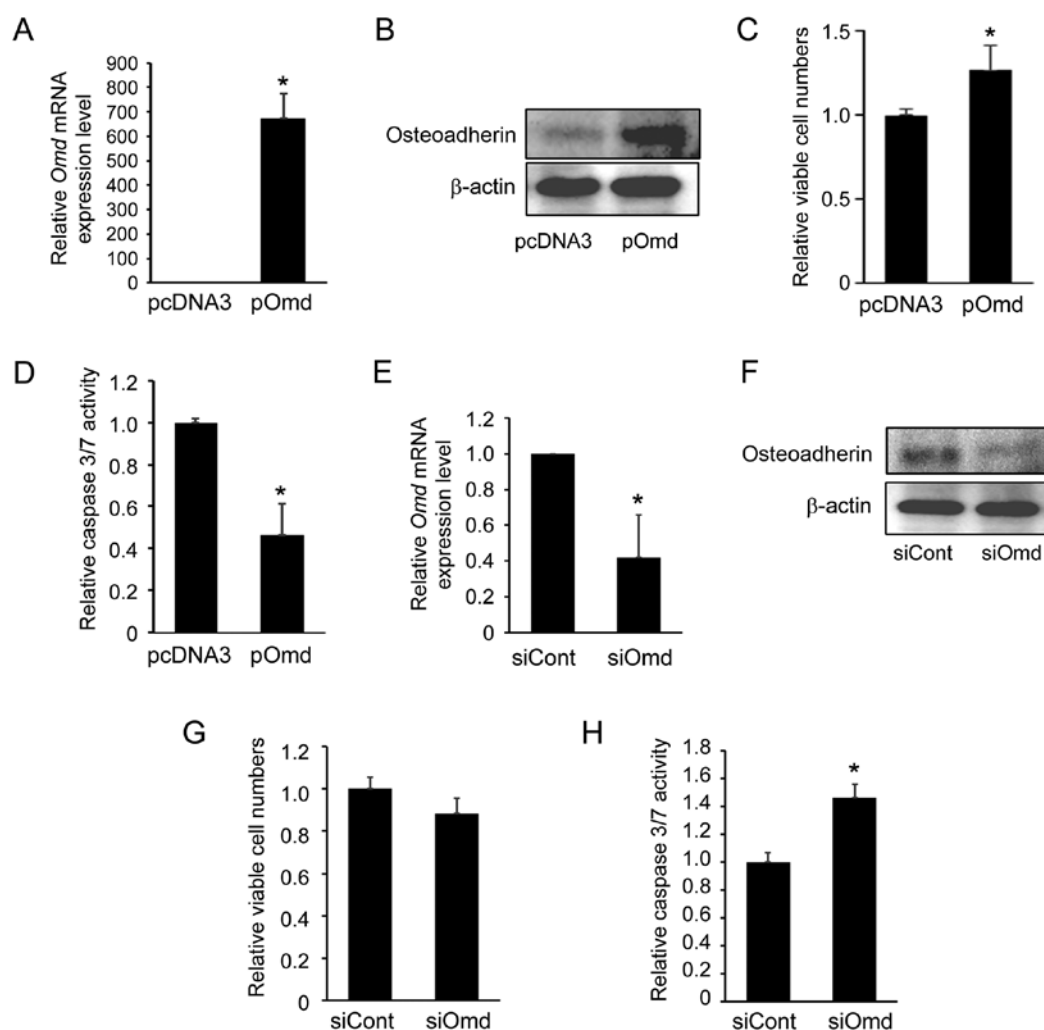


Figure 4. Effects of overexpression and knockdown of *Omd* on cell proliferation and apoptosis in MC3T3-E1 cells. MC3T3-E1 cells were plated at 1.0×10^5 cells/cm². Following culture for 24 h, cells were transfected with pOmd or pcDNA3. (A) Total RNA was extracted after 2 days and *Omd* mRNA expression levels were determined using RT-qPCR. (B) Total protein was extracted from MC3T3-E1 cells and the levels of osteoadherin protein were determined by western blot analysis. (C) Viable cell numbers were quantified. (D) The activity of caspase 3/7 was measured. (E) MC3T3-E1 cells were plated at 1.7×10^4 cells/cm². Following culture for 24 h, cells were transfected with siOmd or siCont (both at 5 nM). Total RNA was extracted after 1 day and *Omd* mRNA expression levels were determined using RT-qPCR. (F) Total protein was extracted from MC3T3-E1 cells and the levels of osteoadherin protein were determined by western blot analysis. (G) Viable cell numbers were quantified. (H) The activity of caspase 3/7 was measured. (A and E) Expression levels were normalized to those of *GAPDH* and are presented as fold changes relative to the control. (B and F) β -actin was used as an endogenous control. Fold changes in viable cell numbers and activities were calculated on the basis of those in control cells (those transfected with siCont or pcDNA3). Data are presented as the mean \pm standard deviation of separate experiments performed in triplicate. * $P < 0.05$ vs. pcDNA3 or siCont. Omd, osteoadherin; siOmd, *Omd* small interfering RNA; siCont, scramble small interfering RNA; pOmd, *Omd* overexpression plasmid; RT-qPCR, reverse transcription-quantitative PCR.

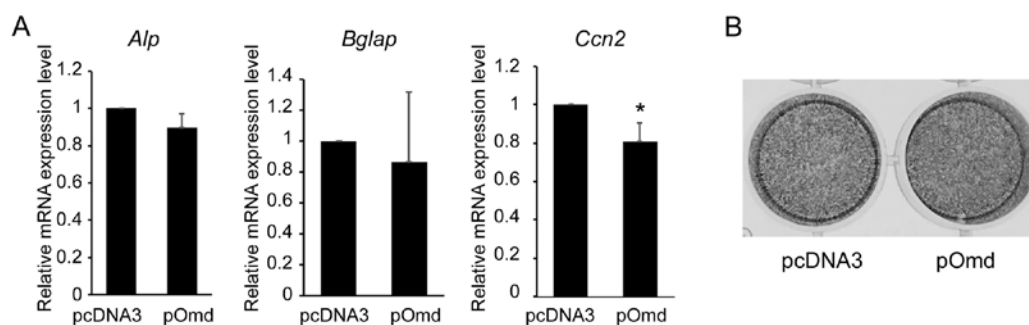


Figure 5. Effects of *Omd* overexpression on the mRNA expression levels of genes related to osteoblast differentiation in MC3T3-E1 cells. MC3T3-E1 cells were plated at 1.7×10^4 cells/cm² and were cultured. After 24 h, cells were transfected with pOmd or pcDNA3 plasmids and were incubated. (A) Total RNA was then extracted after 2 days and *Alp*, *Bglap* and *Ccn2* mRNA expression levels were determined using reverse transcription-quantitative PCR. Fold changes in mRNA expression levels were calculated relative to expression levels in control cells transfected with pcDNA3. Data are presented as the mean \pm standard deviation from separate experiments performed in triplicate. * $P < 0.05$ vs. pcDNA3. (B) After transfection with pOmd or pcDNA3, cells were cultured for 14 days and ALP activities were investigated using staining analyses. *Bglap*, osteocalcin; *Alp*, alkaline phosphatase; *Ccn2*, CCN family 2; *Omd*, osteoadherin; pOmd, *Omd* overexpression plasmid.

and osteoadherin has highly restricted expression in cells of mineralized tissues (4,6). In the present study, BMP2 enhanced *Omd* expression in C2C12 cells. Y1R inhibition also enhanced *Omd* expression, suggesting specific functions of osteoadherin in bone homeostasis and osteoblast differentiation. The class I SLPRs biglycan and decorin are also expressed in osteoblasts and are present in bone ECM (2,27), and their affinity for bone apatite has been demonstrated by chromatographic purification with hydroxyapatite columns (28). These observations suggest significant roles of these SLPRs in the regulation of mineralization (28). Osteoadherin also interacts with calcium phosphate mineral (29), suggesting that osteoadherin contributes to biomineralization and the control of mineral crystal nucleation, growth, and maturation.

Although few previous reports show regulation of *Omd* expression by growth factors and signaling molecules, Rehn *et al* (30) reported that transforming growth factor- β (TGF- β) down-regulates *Omd* expression in osteoblasts. The present study demonstrated that BMP2 enhances *Omd* gene expression and activates the *Omd* gene promoter. Upon activation by BMP2, Smad1/Smad4 complexes regulate the transcription of various target genes, and among these *Id1*, *Tlx-2* and *Mix.2* reportedly respond to BMP signaling via the Smad-binding motifs on their promoters (23,31,32). Several DNA binding motifs for Smads, including GTCT, have been identified (33). The present analyses indicate the presence of transcriptional machinery that is sensitive to BMP2 signaling and regulates transcription through interactions with the *Omd* promoter.

SLRPs have multiple complex roles. Class II SLRPs contain charged KS chains in their LRRs and N termini with multiple sulfated tyrosine residues that contribute to the anionic properties of these proteoglycans. The GAG KS is reportedly present in several tissues, including bone, cartilage, reproductive and neural tissues (1,2). KS binds the growth factors fibroblast growth factor 2, TGF- β and sonic hedgehog with high affinity (34,35), suggesting roles of KS proteoglycans in the regulation of growth factor activities and morphogen gradient formation. Decorin has been characterized as a proteoglycan with roles in the control of cell growth (2), as indicated by binding and inhibition of TGF- β . Biglycan also interacts with TGF- β and modulates its activities (2). In addition, decorin was reported to interact with epidermal growth factor receptor and vascular endothelial growth factor receptor, and consequently regulate the cell cycle (2,9,36). The KS of osteoadherin interacts with fibronectin to promote osteoblast attachment *in vitro* (3). Furthermore, the tyrosine sulfate-rich domains of osteoadherin have been shown to bind motifs of basic clusters in a variety of heparin-binding proteins, including some with bioactive properties (10). These studies suggest that osteoadherin binds various growth factors, growth factor receptors and matrix proteins that regulate apoptosis and cell growth via cell cycle- and proliferation-related mechanisms.

CCN2, which is widely considered a connective tissue growth factor, is a cysteine-rich ECM protein that acts as an anabolic growth factor to regulate cell functions (37,38). *Ccn2* mRNA expression also induces apoptosis in human aortic smooth muscle cells (39), breast cancer cells (40) and mouse osteocytes (41) by down-regulating anti-apoptotic genes. In accordance with these observations, *Omd* overexpression

reduced *Ccn2* expression in MC3T3-E1 cells in the present study, suggesting an apoptotic mechanism involving CCN2 that is regulated by osteoadherin expression in osteoblasts.

In conclusion, the present study, to the best of our knowledge, is the first to show that osteoadherin regulates apoptosis and proliferation in osteoblast cells, and thus established a molecular relationship between osteoblast-derived SLRP osteoadherin and caspase 3/7 activity in osteoblasts. However, the mechanism of regulation on caspase 3/7 activity by osteoadherin remains unclear. Further investigations are required to identify molecular mechanisms of osteoadherin and associated signaling molecules.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

EH and MT conceived and designed the experiments. EH performed the experiments. EH and TF analyzed the data. EH and MT wrote the manuscript. All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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