Role of Osteoglycin in the Linkage between Muscle and Bone*

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Background: The interaction between muscle tissues and bone metabolism has recently been noted.

Results: Osteoglycin is produced in myoblastic cells and enhances bone formation parameters in osteoblasts.

Conclusion: Osteoglycin may be a crucial humoral bone anabolic factor that is produced by muscle tissues.

Significance: Osteoglycin may be the first potential humoral bone anabolic factor produced from muscle cells.

The interaction between muscle tissues and bone metabolism is incompletely understood. We hypothesized that there might be some humoral factors that are produced in muscle tissues and exhibit bone anabolic activity. We, therefore, performed comparative DNA microarray analysis between mouse myoblastic C2C12 cells transfected with either stable empty vector or ALK2 (R206H), the mutation that constitutively activates the bone morphogenetic protein (BMP) receptor, to search for muscle-derived bone anabolic factors. Twenty-five genes whose expression was decreased to <1/4, were identified; these included osteoglycin (OGN). Stable overexpression of OGN significantly decreased the levels of Runx2 and Osterix mRNA compared with those in cells transfected with vector alone in MC3T3-E1 cells. On the other hand, it significantly enhanced the levels of ALP, Col1, and osteocalcin (OCN) mRNA as well as alkaline phosphatase (ALP), type I collagen (Col1), and osteoglycin (OGN). Stable overexpression of OGN significantly decreased the levels of Runx2 and Osterix mRNA compared with those in cells transfected with vector alone in MC3T3-E1 cells. In conclusion, this study suggests that OGN may be a crucial humoral bone anabolic factor that is produced by muscle tissues.

Progress has recently been made in investigations of the relationships between bone metabolism and organ systems other than bone. For example, the linkages between bone and cardiovascular systems, nervous systems, adipose tissues, and pancreas have been noted. Previous studies indicate that muscle mass is closely related to high bone mass and a decrease in fracture risk in postmenopausal women (1–3). From these findings, we hypothesized that there might be some interactions between muscle tissues and bone metabolism.

Bone morphogenetic proteins (BMPs)2 are known to be important in fracture healing but have been disappointing in human trials (4). However, a recent study showed that fractures that are covered with relatively intact muscle improve more rapidly than fractures associated with more severe damage (5, 6). In that study muscle flaps applied to autogenous bone grafts improved healing, but coverage with skin did not. Moreover, proinflammatory cytokines, in particular tumor necrosis factor-α (TNF-α), at the site of fracture induced the differentiation of stromal cells present in muscle into osteoprogenitor cells and promoted bone fracture healing (7). In that study muscle-derived mesenchymal cells were more effective as the source of cells that differentiate into osteoblastic cells than bone marrow-derived mesenchymal cells. These findings suggest that muscle tissues play some important physiological and pathological roles through certain interactions between muscle tissues and bone metabolism. Muscle tissues can produce local growth factors, which have anabolic effects in bone tissues. For example, IGF-binding protein 5 (IGFBP5) is secreted from muscle tissues (8). We, therefore, hypothesized that there might be some humoral factors that are produced in muscle tissues and affect bone in an anabolic fashion, although there have been no reports about the relationships between the factors secreted from muscle tissues and bone metabolism.

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2 The abbreviations used are: BMP, bone morphogenetic protein; ALK2, activin-like kinase 2; OCN, osteocalcin; OGN, osteoglycin; ALP, alkaline phosphatase; Col1, type I collagen; α-MEM, α-minimal essential medium.
Fibrodysplasia ossificans progressiva is a rare autosomal dominant disorder of skeletal malformations, which are characterized by postnatal progressive heterotopic ossification in soft tissues, especially skeletal muscle, and acute heterotopic ossification induced by muscle injury, such as accidental trauma or surgical operations (9–11). A mutation causing constitutive activation (617G—A; R206H) in a BMP type 1 receptor, the activin receptor type 1 (ACVR1)/activin-like kinase 2 (ALK2), is found in patients with the classic form of fibrodysplasia ossificans progressiva (10). This disease might provide some clue to link muscle tissues to bone.

In this study we performed comparative DNA microarray analysis between stable empty vector- and ALK2(R206H)-transfected mouse myoblastic C2C12 cells to find novel factors that are produced in muscle tissues and exhibit bone anabolic activity. Osteoglycin (OGN) was identified as one of 25 genes whose expressions were decreased to <1/4 by ALK2 (R206H) overexpression in these cells. Our data suggest that OGN may be an important factor produced by muscle-derived cells and secreted into blood that exhibits bone anabolic effects.

**EXPERIMENTAL PROCEDURES**

**Materials**—MC3T3-E1 cells were provided by Dr. H. Kodama (Ohu Dental College, Koriyama, Japan). Mouse calvarial osteoblasts from 2–4-day-old ICR mice were obtained from Primary Cell Co., Ltd., Sapporo, Japan. Human (h) recombinant BMP-2, anti-β-actin, PD98059, SB203580, curcumin, and recombinant human TGF-β1 were obtained from Sigma. Anti-alkaline phosphatase (ALP), anti-β-catenin, anti-phosphorylated Smad1/5/8, anti-Smad1, anti-Smad5, anti-TGF-β1, anti-OGN antibodies, OGN siRNA(m), and control siRNA were from Santa Cruz Biotechnology. Anti-extracellular signal-regulated kinase (ERK1/2) and anti-phospho ERK1/2 antibodies were from Cell Signaling Technology. Anti-ALK2 (R206H) V5-tagged construct was as previously described (11). SB431542 was from Tocris Cookson Ltd. (Bristol, UK). Anti-Col1 antibody and human recombinant OGN were from Calbiochem and ADIPO BIOSCIENCE Inc. (Santa Clara, CA).

The coding region of mouse OGN was amplified by reverse transcription PCR to total RNA from C2C12 cells using 5'-GCTGAAATGGAGACTGTGCACTCTA-3' as the forward primer and 5'-GGTTAGAAGATGACCCATGGAATTA-3' as the reverse primer. The cDNA was TA-cloned into the pCR2.1 vector (Invitrogen) according to the manufacturer’s specifications. The OGN cDNA insert was cloned into the mammalian expression vector, pcDNA3.1(−) (Invitrogen). All constructs were verified by restriction enzyme analysis and nucleotide sequencing.

**Cell Culture**—Mouse osteoblastic MC3T3-E1 and mouse calvarial osteoblastic cells were cultured in α-MEM (containing 50 μg/ml ascorbic acid) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). Mouse myoblastic C2C12 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) as previously described (12). Six hours later, the cells were supplied with fresh α-MEM or DMEM containing 10% FBS. Forty-eight hours later the transiently transfected cells were used for experiments. To generate stably transfected MC3T3-E1 or C2C12 cells, after incubation in α-MEM or DMEM containing 10% FBS for 48 h, the cells were passaged, and clones were selected in α-MEM or DMEM supplemented with GaNF (0.3 mg/ml or 0.7 mg/ml, respectively; Invitrogen) and 10% FBS. Twenty-four clones were selected after 3 weeks of culture in GaNF. Several clones were selected after Western blotting with anti-OGN antibody. At least three independent clones for each stable transfection were characterized to rule out the possibility of clonal variation. Empty vector-transfected cell clones were used as control.

**Protein Extraction and Western Blot Analysis**—Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture (Roche Applied Science), 1% Triton X-100, and 1 mM sodium orthovanadate. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride. Blots were blocked with 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20 containing 3% dried milk powder. The membranes were immunoblotted with each primary antibody. The antigen-antibody complexes were visualized using the appropriate secondary antibodies (Sigma) and an enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham Biosciences). The results depicted in each figure are representative of at least three independent cell preparations. Each experiment was repeated three times.

**RNA Extraction and Real-time PCR**—Total RNA was prepared from cells using TRizol reagent. cDNA was synthesized using the Superscript II First-Standard Synthesis System for RT-PCR (Invitrogen). Specific mRNA was quantified by real-time PCR using a 7500 Real-time PCR system (Applied Biosystems, Rotkreuz, Switzerland) with SYBR Premix Ex TaqTM II (Perfect Real Time) kits (TaKaRa) according to the manufacturer’s standard protocol. The mRNA value for each gene was normalized relative to the mouse GAPDH mRNA levels in RNA samples. Primer sequences (forward and reverse) were as follows: GAPDH, 5'-GTGTACATGTGGTTCCCAGATAGGAGCCTC-3' and 5'-AGTGAAGTTGTGATATTCTGTGCTG-3'; OGN, 5'-GTCTTTTGTGCCATGGAT-3' and 5'-GAAGCTGCAACAGCACAAT-3'; osteocalcin (OCN), 5'-CTGAGTTCTGCAACAGGCTTTCA-3' and 5'-GCCGGAAGCTGTCTCATACCTTT-3'; Runx2, 5'-AATGGCCTCCTGTTGTAGAAG-3' and 5'-GTCGCCGCCCCCAACAATCT-3'; Osterix, 5'-AGCCACAGGACTGGAAAGACGC-3' and 5'-GGCCTTATGCTAGGACTGAGC-3'.

**Microarray Analysis**—Total RNA was extracted from C2C12 cells stably transfected with empty vector or ALK2 (R206H)
using TRIzol reagent. Total RNA was purified using RNeasy MiniElute Clean Up kit (Qiagen, Tokyo, Japan) to yield an A$_{260/280}$ > 1.90. Double-stranded cDNA was synthesized using a T7-oligo(dT) primer with the 3’IVT Express Kit (Affymetrix Inc., Santa Clara, CA). Hybridization samples were prepared and processed according to the GeneChip Expression Analysis Technical Manual. The Affymetrix Murine Genome 430 2.0 set was used to compare gene expression. Data were analyzed using the GeneChip Operating Software Version 1.4 (Affymetrix 690036) according to the GeneChip Expression Analysis Fundamentals. Data were analyzed using the GeneChip Operating Software Version 1.4 (Affymetrix Inc., Santa Clara, CA). Hybridization samples were prepared and processed according to the GeneChip Expression Analysis Technical Manual. The Affymetrix gene chip, we identified 25 genes whose expression was decreased to 1/4 in the experimental versus the control group (Table 1). The levels of OGN mRNA were significantly suppressed in the stable ALK2 R206H-transfected C2C12 cells compared with those in the empty vector-transfected cells and was further reduced by BMP-2 treatment (Fig. 1A). The levels of OGN

### Table 1: Gene transcripts down-regulated in stable ALK-2(R206H)-transfected C2C12 cells versus vector-alone transfected cells

| Gene name | GenBank accession no. | Ratio |
|-----------|-----------------------|-------|
| B8S004S5N13Rik | BM942851 | -6.3 |
| Trim44 | NM_020267 | -4.8 |
| S110039M20Rik | AW494150 | -4.6 |
| Fndc1 | AK003938 | -4.5 |
| Hif2 | AV909098 | -4.5 |
| SUSD5 | AV226398 | -4.5 |
| S210468N07Rik | AK013390 | -4.5 |
| Dorx1 | AV321547 | -4.3 |
| Auts2 | BB429147 | -4.0 |
| C1r | BB558917 | -3.6 |
| Follistatin-like 1 | BI452772 | -3.3 |
| Ddx11 | AF031524 | -3.0 |
| Pros1 | Z25469 | -2.8 |
| Nov | X96585 | -2.5 |
| Osteoglycin | BB542051 | -2.5 |
| Carp2 | NM_007792 | -2.5 |
| Mospd2 | BB45345 | -2.4 |
| 2610301H18Rik | AK019956 | -2.4 |
| IL-33 | NM_133775 | -2.2 |
| LOC672215///Ntn1 | NM_008744 | -2.1 |
| Rnf157 | BB261882 | -2.1 |
| Sand9l | BB145092 | -2.1 |
| Tslp | NM_021367 | -2.1 |
| Hhip | BB088162 | -2.0 |

**FIGURE 1.** The levels of OGN in myoblasts stably transfected with empty vector (V) and ALK2 (R206H). A, total RNA was extracted from C2C12 cells stably transfected with V or ALK2 (R206H) cultured in the presence or absence of 300 ng/ml BMP-2 for 48 h, and then real-time RT-PCR was performed. Data are expressed relative to the GAPDH mRNA value. *, p < 0.01 relative to V-transfected cells. **, p < 0.01 relative to ALK2 R206H-transfected C2C12 cells without BMP-2. B, total protein from C2C12 cells stably transfected with V or ALK2 (R206H) (#6, #8) was extracted, and Western blot analysis was performed with anti-OGN and β-actin antibodies.
protein were suppressed in the stable ALK2 (R206H)-transfected C2C12 cells compared with those in the empty vector-transfected cells (Fig. 1).

**OGN Expression during Osteoblast and Myotube Differentiation**—MC3T3-E1 cells in culture undergo osteoblastic differentiation with mineralization starting after 2–3 weeks. The levels of OGN mRNA increased for up to 3 weeks in MC3T3-E1 cells (Fig. 2A). Myoblastic C2C12 cells differentiated into osteoblastic cells when stimulated with BMP-2, and the levels of OGN mRNA increased with time (Fig. 2B). OGN levels did not change in the absence of BMP-2 (data not shown). C2C12 cells differentiated into myotube cells when stimulated with horse serum, and the levels of OGN mRNA increased with the myotube differentiation (Fig. 2C).

**Effect of OGN on Osteoblast Phenotype and Mineralization**—We examined whether OGN would affect osteoblast phenotype
in MC3T3-E1 cells. As shown in Fig. 3A, stable overexpression of OGN significantly suppressed the levels of Runx2 and Osterix mRNA compared with those of cells transfected only with vector in MC3T3-E1 cells. On the other hand, it significantly enhanced the levels of ALP, Col1, and OCN mRNA. Moreover, stable OGN overexpression enhanced the levels of Col1 and β-catenin protein in MC3T3-E1 cells (Fig. 3B). The optimal concentration of recombinant OGN protein in MC3T3-E1 cells was 10 μg/ml in our preliminary study (data not shown). As shown in Fig. 3C, recombinant OGN protein
enhanced the levels of Col1, ALP, and OCN mRNA levels, although it significantly suppressed the levels of Runx2 and Osterix mRNA in MC3T3-E1 cells. Moreover, OGN enhanced the levels of Col1 and β-catenin protein in these cells (Fig. 3D). These results were consistent with the effects of OGN overexpression on osteoblast markers. Next, we examined the effects of OGN overexpression on mineralization in MC3T3-E1 cells. Stable OGN overexpression induced mineralization compared with empty vector-transfected cells in the presence of ascorbic acid and β-glycerophosphate. These findings were confirmed in quantitation analysis of Alizarin Red (Fig. 3E). Although a reduction in endogenous OGN levels by siRNA enhanced the levels of Runx2 and Osterix mRNA, it suppressed the levels of Col1, ALP, and OCN mRNA in MC3T3-E1 cells (Fig. 3F). A reduction in endogenous OGN level by siRNA suppressed the levels of Col1 and β-catenin protein in MC3T3-E1 cells (Fig. 3G). Moreover, we examined the effects of a reduction in endogenous OGN level by siRNA on the osteoblast phenotypes in mouse primary calvarial osteoblast cultures. As shown in Fig. 3H, a reduction in endogenous OGN levels by siRNA enhanced the level of Runx2 mRNA, and it suppressed the levels of Col1, ALP, and OCN mRNA in mouse primary osteoblasts. It suppressed the levels of Col1 and β-catenin protein in mouse primary osteoblasts (Fig. 3I).

Effects of OGN on BMP-2-induced Differentiation of Myoblasts into Osteoblasts—We examined the effects of OGN on BMP-2-induced differentiation of myoblasts into osteoblasts. Transient OGN overexpression significantly suppressed the levels of Runx2, Osterix, Col1, ALP, and OCN mRNA induced by BMP-2 in C2C12 cells (Fig. 4A). As shown in Figs. 4, B and C, transient OGN overexpression significantly antagonized BMP-2 and Smad5-induced transcriptional activity in C2C12 cells, although it did not affect the phosphorylation of Smad1/5/8. Moreover, OGN was not coimmunoprecipitated with Smad1 and Smad5 in these cells (data not shown), suggesting that OGN does not interact with Smad1 and Smad5 physically.

Role of Myoblastic Cell-derived OGN in Osteoblasts—We obtained the conditioned medium from OGN-overexpressed or -suppressed mouse myoblastic C2C12 cells. The levels of OGN in conditioned medium were shown in Fig. 5A. The levels of OGN were elevated and decreased in the conditioned medium from C2C12 cells, in which OGN was overexpressed by OGN transient transfection and decreased by siRNA transfection, respectively. Conditioned medium from transient OGN-overexpressing C2C12 cells decreased the levels of Runx2 and Osterix mRNA, and it enhanced the levels of Col1, ALP, and OCN mRNA in MC3T3-E1 cells compared with conditioned medium from empty vector-transfected C2C12 cells (Fig. 5B). Conditioned medium from stable OGN-overexpressing C2C12 cells enhanced the levels of Col1 and β-catenin protein in MC3T3-E1 cells (Fig. 5C). Moreover, although conditioned medium from endogenous C2C12 cells with OGN suppression by siRNA increased the levels of Runx2 and Osterix mRNA, it decreased the levels of Col1, ALP, and OCN mRNA in MC3T3-E1 cells compared with conditioned medium from control siRNA-transfected C2C12 cells (Fig. 5D). Moreover, it decreased the levels of Col1 and β-catenin protein in these cells (Fig. 5E). In addition, we examined the effects of conditioned medium from OGN-overexpressed or -suppressed C2C12 cells on the osteoblast phenotypes in mouse primary calvarial osteoblast cultures. Conditioned medium from transiently OGN-overexpressing mouse myoblastic C2C12 cells decreased the levels of Runx2 mRNA, and it enhanced the levels of Col1, ALP, and OCN mRNA in mouse primary osteoblasts (Fig. 5F). Moreover, conditioned medium from endogenous C2C12 cells with OGN suppression by siRNA increased the levels of Runx2 mRNA and decreased the levels of Col1, ALP, and OCN mRNA in mouse primary osteoblasts (Fig. 5G). Similar data were obtained from the experiments using the conditioned medium from stably OGN-overexpressing, horse serum-treated C2C12 cells (the putative myotube cells) (data not shown). Moreover, in our preliminary study the OGN protein was detected in human serum by Western blot analysis (data not shown).

Role of TGF-β in Enhancement of Col1 Expression by OGN—Because a previous study indicated that OGN induces bone formation in conjunction with TGF-β (15, 16), we examined the relationships between OGN and TGF-β in the enhancement of Col1 expression by OGN in osteoblasts. We employed 3TP-Lux, a Smad3/4-responsive luciferase reporter, to examine the effects of OGN on TGF-β-induced transcriptional activity in osteoblasts. As shown in Fig. 6A, OGN significantly increased Smad3/4-responsive transcriptional activity in MC3T3-E1 cells, and it additionally enhanced TGF-β-induced transcriptional activity. SB431542, an inhibitor of endogenous TGF-β signaling (ALK5 inhibitor), did not affect OGN-induced transcriptional activity, although it antagonized TGF-β effects (Fig. 6A). OGN significantly enhanced TGF-β-induced Col1 mRNA levels in MC3T3-E1 cells, and SB431542 did not affect OGN-induced Col1 mRNA levels, although it antagonized...
TGF-β effects (Fig. 6B). Next we examined whether OGN would affect the expression of TGF-β in osteoblasts. As shown in Fig. 6, C and D, transient OGN overexpression or endogenous OGN suppression by siRNA did not affect the levels of TGF-β mRNA and protein compared with those in each control group in MC3T3-E1 cells. Moreover, OGN did not affect TGF-β-induced phosphorylation of Smad2/3 protein (Fig. 6E). Previous studies suggest that MAP kinases are related to Smad-independent signaling of TGF-β (13, 40). We, therefore, performed the additional experiments to examine the involvement of MAP kinases in OGN-induced Col1 mRNA levels using several MAP kinase inhibitors in MC3T3-E1 cells. PD98059, SB203580, and curcumin are inhibitors of ERK1/2, p38 MAP kinase, and c-Jun N-terminal kinase (JNK), respectively. As shown in Fig. 6F, inhibitors of p38 MAP kinase or JNK did not affect Col1 mRNA levels induced by OGN. However, OGN enhanced the levels of phosphorylated ERK1/2, and an inhibitor of ERK1/2 significantly antagonized the levels of Col1 mRNA enhanced by OGN in MC3T3-E1 cells (Fig. 6, F and G), suggesting that OGN seemed to enhance the levels of Col1 mRNA partly through ERK1/2 in osteoblasts.

**DISCUSSION**

Several lines of evidence suggest certain interactions between muscle tissues and bone metabolism. In this study we aimed to find some humoral factors that are produced in muscle tissues and exhibit bone anabolic activity. We hypothesized that the signal suppressed by the conversion of muscle tissues into bone might give us a clue to find out muscle-derived bone anabolic factors because those factors could be predominantly expressed in muscle tissues, compared with their expressions in...
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bone, and their systemic effects through blood could be more important than their local effects in muscle tissues. We, therefore, performed comparative DNA microarray analysis between stable empty vector- and ALK2(R206H)-transfected mouse myoblastic cells, as fibroblasts ossificans progressiva and its molecular pathogenesis could be a clue to help identify some muscle-derived humoral bone anabolic factors. Then we selected several factors that exhibited decreased expression levels upon ALK2(R206H) expression. Twenty-five genes showed expression that decreased to $<1/4$ in ALK2 (R206H)-transfected C2C12 cells compared with that in empty vector-transfected cells. Among them were several bone-related factors, such as decorin, follistatin, Nov, interleukin 33 (IL-33) and OGN. Decorin is a well characterized, secreted small leucine-rich proteoglycan in bone, which binds to collagen and several bone matrix proteins (17). In a previous microarray analysis of C2C12 cells with and without BMP-2 treatment, several members of the leucine-rich-repeat family of proteoglycans, such as decorin, byglycan, osteomodulin, fibromodulin, and OGN, were down-regulated by BMP-2 treatment (18). Our preliminary study in MC3T3-E1 and C2C12 cells suggested that follistatin, Nov, and IL-33 do not enhance the osteoblast phenotype (data not shown). We, therefore, investigated the function of OGN because several studies have suggested its potential as a bone anabolic factor. OGN is the seventh member of the small leucine-rich proteoglycans that was originally called osteoinductive factor. The small leucine-rich proteoglycans have core proteins ranging in size from 25 to 62 kDa and share a common protein structure that is composed of $6–11$ repeats of leucine-rich regions. OGN belongs to class III of the small leucine-rich proteoglycans, and it was initially isolated from bovine bone as an inducer of matrix mineralization (19). OGN cDNA is highly expressed in mouse hypertrophic chondrocytes as well as cochlea tissues (20, 21). OGN down-regulation was found to be correlated with arteriogenesis in the adventitia of rabbit collateral arteries (22). High expression of OGN inhibits metastatic behavior and decreases gelatinase activity of murine hepatocarcinoma Hca-F cells (23, 24). In this study the levels of OGN were elevated during osteoblast differentiation, which was consistent with a previous study (25). Moreover, some comparative microarray studies revealed that OGN is one of the mechanosensitive genes that mediate an anabolic response of mechanical loading in mice and in osteoblasts using a rotating wall vessel and a random positioning machine (26, 27). These findings suggest that OGN might play an important role in bone formation by osteoblasts at the well differentiated stage. In our data the levels of OGN were decreased by activation of ALK2 signaling, which pathologically converts muscle tissues to bone in fibroblasts ossificans progressiva, and the levels of OGN increased when myoblastic cells differentiated into myotubes upon horse serum treatment. Moreover, Chan et al. (25) recently reported that OGN was included in differentially up-regulated secretome components during skeletal myogenesis in C2C12 cells in a recent study. These findings suggest that OGN might play some important role in muscle. In this study stable OGN overexpression enhanced the levels of ALP, Col1, $\beta$-catenin, and OCN as well as mineralization in MC3T3-E1 cells, although it decreased the levels of Runx2 and Osterix. Moreover, endogenous reduction of OGN levels by siRNA decreased the levels of ALP, Col1, $\beta$-catenin, and OCN in these cells, although it elevated the levels of Runx2 and Osterix in these cells. In addition, OGN overexpression antagonized BMP-2-induced differentiation of myoblastic cells into osteoblasts. A previous report showed that the osteoblast number increases at an early differentiation stage, but the osteoblast functions were impaired in osteoblast-specific Runx2-overexpressing transgenic mice (28), suggesting that Runx2 negatively acts on osteoblast differentiation of the osteoblastic cells at a later differentiation stage, whereas it induces osteoblast differentiation in immature osteoblasts at an earlier differentiation stage. From these findings we can speculate that OGN is a factor that suppresses osteoblastic differentiation of premature osteoblasts and enhances osteoblast phenotype and mineralization in well differentiated osteoblasts.

There are some humoral factors that are produced in non-endocrine organs and affect bone. Adiponectin is one of the adipokines that is produced in adipose tissue and regulates energy homeostasis and insulin sensitivity. Several studies indicate that adiponectin increases osteoclast apoptosis and decreases proliferation of osteoclast precursor cells (29, 30). Leptin, a circulating hormone produced by adipose tissue, is a multifunctional hormone that plays important roles in body weight homeostasis, neuroendocrine function, fertility, immune function, and angiogenesis (31). A recent study showed

FIGURE 5. Effects of conditioned medium from OGN-overexpressed or -suppressed myoblastic cells on osteoblast phenotype. A, conditioned medium was obtained from the cultures of C2C12 cells transiently transfected with empty vector (V) or OGN, or the cultures of C2C12 cells transfected with control siRNA- or OGN siRNA for 24 h. The conditioned medium was analyzed with Western blot analysis for anti-OGN and $\beta$-actin antibodies. B, conditioned medium was obtained from the cultures of C2C12 cells transiently transfected with empty vector (V) or OGN for 24 h. Wild-type MC3T3-E1 cells were cultured with $20\%$ of each conditioned medium for 24 h. Total RNA was extracted from these cells, and real-time RT-PCR for Runx2, Osterix, Col1, ALP, OCN, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. *, $p<0.01$ relative to V-transfected groups. C, wild-type MC3T3-E1 cells were cultured with $20\%$ of each conditioned medium for 48 h. Total protein from these cells was extracted, and Western blot analysis for anti-Col1, $\beta$-catenin, and $\beta$-actin antibodies was performed. D, conditioned medium was obtained from the cultures of control siRNA- or OGN siRNA-transfected C2C12 cells for 24 h. Wild-type MC3T3-E1 cells were cultured with $20\%$ of each conditioned medium for 24 h. Total RNA was extracted from these cells, and real-time RT-PCR for Runx2, Osterix, Col1, ALP, OCN, or GAPDH was performed. E, wild-type MC3T3-E1 cells were cultured with $20\%$ of each conditioned medium for 48 h. Total protein from these cells was extracted, and Western blot analysis for anti-Col1 and $\beta$-catenin or $\beta$-actin antibodies was performed. F, conditioned medium was obtained from the cultures of transient V- or OGN-transfected C2C12 cells for 24 h. Mouse calvarial osteoblasts were cultured with $20\%$ of each conditioned medium for 24 h. Total RNA was extracted from these cells, and real-time RT-PCR for Runx2, Col1, ALP, OCN, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. *, $p<0.01$ relative to V-transfected groups. G, conditioned medium was obtained from the cultures of control siRNA- or OGN siRNA-transfected C2C12 cells cultured for 24 h. Mouse calvarial osteoblasts were cultured with $20\%$ of each conditioned medium for 24 h. Total RNA was extracted from these cells, and real-time RT-PCR for Runx2, Col1, ALP, OCN, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. *, $p<0.01$ relative to control siRNA-transfected groups. **, $p<0.05$ relative to control siRNA-transfected groups.
that the role of leptin receptor signaling is as a negative modulator of bone mechanosensitivity (32). Moreover, leptin affects bone in a catabolic fashion through the central nervous system (33). On the other hand, IL-6, fibroblast growth factor 21, IL-8, and IL-15 are myokines that are produced by muscle tissues and affect other organs. IL-6 produced by type 1 and 2 muscle fibers increases hepatic glucose production during exercise and lipolysis in adipose tissues (34, 35). Fibroblast growth factor 21, produced from skeletal muscle, is an important endogenous regulator for systemic glucose and lipid metabolism (36, 37). IL-8, produced by contracting myofibers, exerts its effect locally and plays a role in exercise-induced angiogenesis (38). IL-15 is produced in human skeletal muscle in vitro and in vivo, and it takes part in reducing adipose tissue mass (39). In this study conditioned medium from OGN-overexpressed and OGN-suppressed myoblastic cells enhanced and decreased the levels of...
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ALP, Col1, and β-catenin in mouse osteoblastic cells, respectively. These effects were parallel to the effects of OGN overexpression and repression in osteoblasts. Moreover, OGN protein is detected in human serum and culture supernatant of mouse myoblastic cells. These findings indicate that OGN, produced in muscle, exhibits bone anabolic activity in osteoblasts. However, we cannot completely rule out the possibility that the conditioned media would contain changes besides just the enhanced production and secretion of OGN.

TGF-β is produced by osteoblasts and released from the bone matrix and regulates bone metabolism in various ways (40). TGF-β binds to its type I membrane receptor promoting dimerization with the type II receptor, stimulating its Ser/Thr kinase activity, and causing phosphorylation of Smad2 and Smad3 (p-Smad2/3); the activated Smads associate with Smad4 and translocate into the nucleus (41, 42). Previous studies indicate that Smad3 is an important molecule for osteoblastic bone formation (13, 43–45). Several bioactive proteins, such as p53, basic fibroblast growth factor, interferon-formation (13, 43–45). Moreover, OGN did not affect the levels of TGF-β as well as the phosphorylation of Smad2/3 induced by TGF-β. These findings suggest that OGN enhances TGF-β-induced collagen expression through its interaction with the TGF-β pathway downstream rather than the phosphorylation of Smad2/3 by TGF-β. This interaction between OGN and the TGF-β pathway might play some role in the bone anabolic activity by OGN. OGN plays some role in collagen fibrillogenesis in many tissues, such as eye and skin (50, 51). OGN is one of the three major keratan sulfate-containing proteoglycans in the cornea, and its glycosaminoglycan side chains seem to affect fibril diameter, interfibrillar spacing, and normal tissue hydration (52). In an OGN-deficient mouse, the diameter of the corneal collagen fibrils was significantly increased by transmission electron microscopy and reduced tensile strength of the skin was observed (51, 53). These data suggest that OGN might determine elasticity and tensile strength. Moreover, OGN might play a role in regulating the collagen structures of the auditory system and in hair cell development (20). Although further studies are necessary to clarify the details of the relationship between OGN and collagen in bone, OGN may enhance bone strength through alterations of the structure and quality of collagen matrix, other bone matrix proteins, and proteoglycans.

Exercise therapy and an increase in muscle mass are considered to be very effective for an increase in bone mineral density and a reduction in fracture risk in osteoporotic patients. However, therapy to improve these factors is clinically very difficult as the physical activity of osteoporotic patients is usually disturbed. Humoral bone anabolic factors, produced in muscle tissues, may be important as target molecules for the treatment and prevention of osteoporosis as well as bone metabolic index-related muscle and exercise therapy. Because OGN may be one of the humoral bone anabolic factors produced from muscle, a further clinical study using patient samples and an in vivo study using muscle-specific OGN-deleted or -transgenic mice are necessary.

In conclusion, OGN was selected as a molecule that is down-regulated by ALK2 (R206H)-transfected myoblastic cells. The level of OGN as well as the effects of the conditioned medium from OGN-modulated myoblastic cells was positively correlated with osteoblast phenotype and mineralization in osteoblastic cells, although it seemed to reduce osteoblast differentiation in osteoblasts at the early differentiation stage and myoblasts. OGN may be a crucial humoral bone anabolic factor that is produced in muscle.

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