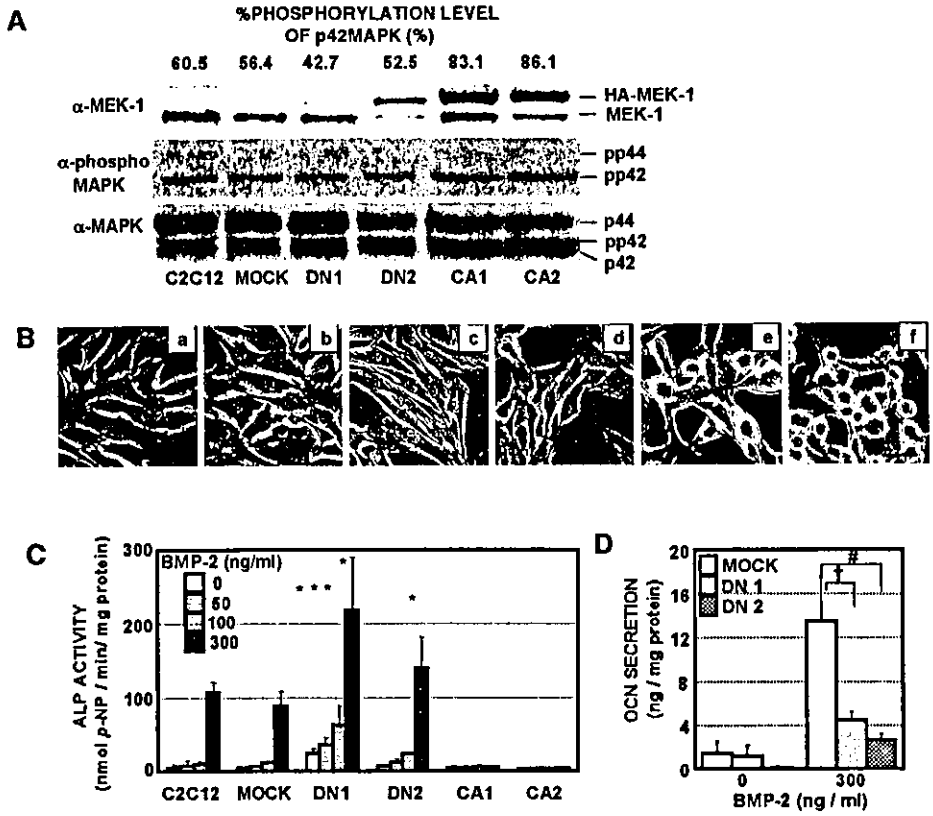


FIG. 3. The osteoblastic differentiation in constitutively active (CA) and dominant-negative (DN) MEK-1 stable transfectants. (A) Immunoblot analyses of expression of HA-tagged mutant MEK-1 and phosphorylation level of MAPK in parent C2C12 cells, mock, DN, and CA transfectants. (B) Parental C2C12 cells (a) and mock transfectant (b) were polygonal shaped. DN transfectant (c and d) spread in low cell density and showed a fibroblastic shape in high cell density. CA transfectants (e and f) showed a spindle shape and were rounded. Cells were cultured in the DMEM containing 10% FBS (bar = 50 $\mu$m). (C) DN transfectants showed higher ALP activity than mock one. Both CA1 and -2 transfectants represented little ALP activity even in the presence of 300 ng/ml of rhBMP-2. Cells were cultured for 3 days. (D) OCN secretion from DN transfectants was decreased significantly compared with the mock transfectant. Data were shown as mean ± SD of three independent experiments (*$p < 0.05$; †$p < 0.01$; #$p < 0.005$ compared with mock transfectant).

transfectants represented little ALP activity even in the presence of 300 ng/ml of rhBMP-2 (Fig. 3C).

Both mock and DN transfectants produced OCN into the culture media by stimulation with 300 ng/ml of rhBMP-2; OCN secretion from DN transfectants was decreased 33% in DN1 and 21% in DN2, as much as that of mock (Fig. 3D). CA transfectants did not secrete detectable OCN into the culture media (data not shown). These results were consistent with those using the MEK-1 inhibitor (Fig. 2).

### *Effects of the MEK-1 inhibitor on the osteoblastic differentiation of MC3T3-E1 preosteoblastic cells*

Next, we examined the effects of the MEK-1 inhibitor on MC3T3-E1 preosteoblastic cells.

The phosphorylation of MAPK was also reduced by PD98059 in MC3T3-E1 preosteoblastic cells in its dose-dependent fashion (79.5 ± 11.6% to 54.8 ± 6.99% at 0 to 100 $\mu$M; Fig. 4A).

ALP activity was increased by the treatment with PD98059 in the absence or presence of 50 ng/ml of rhBMP-2 (Fig. 4B). More than 10 $\mu$M of MEK-1 inhibitor significantly promoted ALP activity in MC3T3-E1 cells compared with that in the absence of MEK-1 inhibitor. The treatment with 25 $\mu$M of PD98059 increased ALP mRNA expression in the absence (data not shown) or presence of rhBMP-2 (Fig. 4D, left graph).

In contrast to the results using C2C12 cells, PD98059 enhanced OCN secretion from MC3T3-E1 cells in its dose-dependent fashion and peaked at 50 $\mu$M (0–50 $\mu$M; Fig. 4C). However, its secretion was reduced by the treatment with 100 $\mu$M of PD98059. This reduction might be caused by the cellular toxicity of this MEK-1 inhibitor. The increase of OCN mRNA expression was checked by RNA blot and OCN expression was consistent with its secretion between day 4 and 6 by the treatment with PD98059 (Fig. 4D, right graph and panels).

Calcium content of mineralized nodules was increased by the treatment with 25 $\mu$M of MEK-1 inhibitor. Twenty-five micromolars of PD98059 and 50 ng/ml of rhBMP-2 synergistically increased calcium content by 9.4-fold (Fig. 4 E).

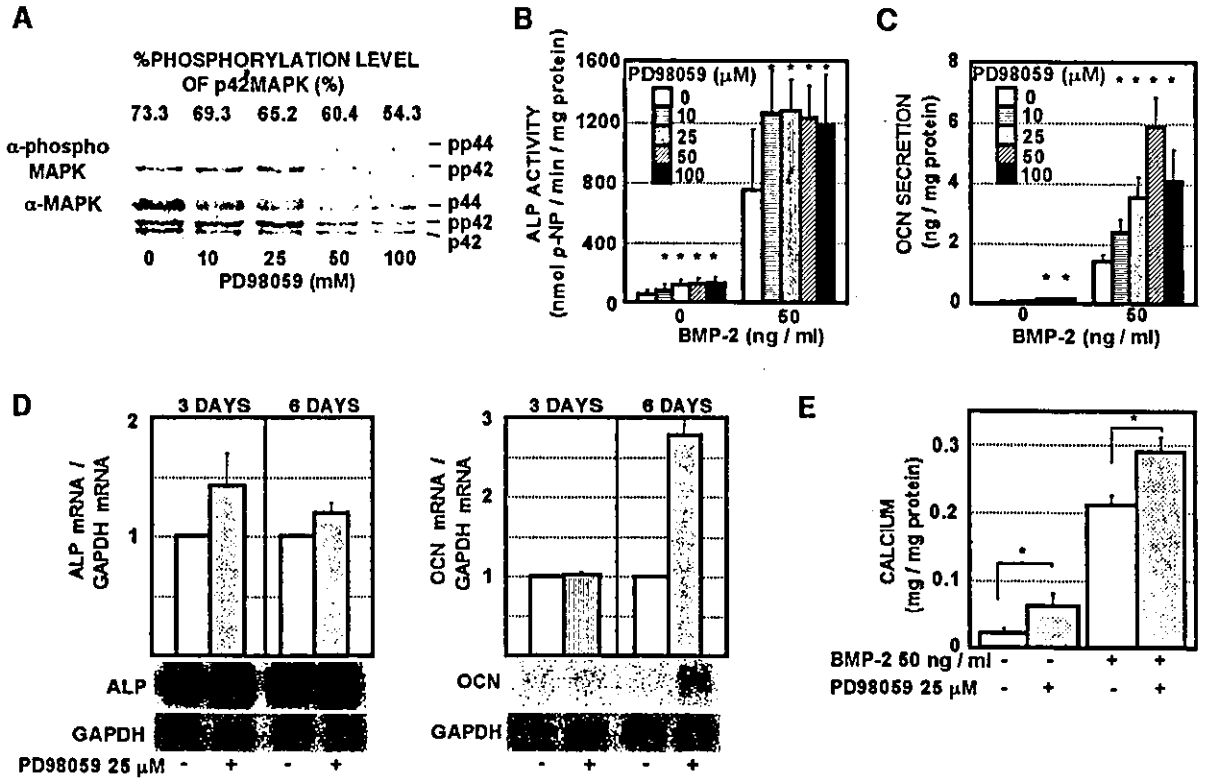


FIG. 4. Effects of the MEK-1 inhibitor on the osteoblastic differentiation in MC3T3-E1 preosteoblasts. (A) Phosphorylation level of MAPK was inhibited by PD98059 in a dose-dependent manner. (B) More than 10 μM of PD98059 promoted ALP activity in MC3T3-E1 cells in the absence or presence of 50 ng/ml of rhBMP-2. ALP activity was measured using *p*-nitrophenylphosphate as a substrate. Cells were cultured in α-MEM for 3 days. (C) OCN secretion between day 4 and 6 also was increased by treatment with the MEK-1 inhibitor in a dose-dependent fashion and peaked at 50 μM. Cells were cultured for 6 days. (D) mRNA levels of ALP and OCN stimulated with 50 ng/ml of rhBMP-2 were assayed by RNA blot analysis. Transcriptional level of OCN was increased after the culture for both 3 days and 6 days. The same amount (10 μg) of total RNA was applied to each lane. (E) MEK-1 inhibitor significantly promoted bone mineralization in the absence or presence of 50 ng/ml of rhBMP-2. Data are mean ± SD of three independent experiments (*$p < 0.05$ compared with the PD98059-untreated control).

## DISCUSSION

Murine C2C12 cells represented myogenic differentiation under low-serum (5%) condition.[25] It also has been reported that the same cells were converted from myogenic differentiation into osteoblastic differentiation by stimulation with rhBMP-2 in the low-serum (5%) culture media[25,26] and adipogenesis by thiazolidinediones or fatty acids.[27,28] Here, we found osteoblastic differentiation of these cells by stimulation with rhBMP-2 even in the presence of 10% FBS, which is usually used for maintaining these cells as undifferentiation status. Therefore, we considered these cells as pluripotent mesenchymal stem cells and a model for osteoblastic differentiation by stimulation with BMP-2. MC3T3-E1 cells are thought to be committed to osteoblastic lineage. They were used as mesenchymal cells that were more differentiated into osteoblasts than C2C12 cells.

Then, we screened the small molecule compounds, especially kinase inhibitors, to stimulate osteogenesis in vitro. ALP activity of rhBMP-treated C2C12 cells and MC3T3-E1 cells was used as a marker for our screening. We picked up an MEK-1 inhibitor as a candidate for this condition. Another candidate we found was a Rho kinase inhibitor, Y-27632 (Yoshikawa H, Yoshioka K, Itoh K, submitted, 2002). In contrast, SB203580 p38 MAPK inhibitor did not promote and even inhibit the osteoblastic differentiation. In this report, we focused on the MEK-1 inhibitor PD98059.

Our present data summarized the following four results. First, MAPK signaling pathway was involved in the initiation of the differentiation of mesenchymal cells. MEK-1 inhibition stimulated myogenic differentiation of C2C12 cells even in the presence of 10% FBS when the cells were not committed to osteoblastic lineage by BMP-2, while it promoted the osteoblastic differentiation of BMP-2–stimulated C2C12 cell and MC3T3-E1 cells when committed to osteoblastic lineage. In addition, both myogenic and osteoblastic differentiation of C2C12 cells were shut down by the expression of continuous activation of MEK-1 (Fig. 3C; data not shown). Bennet et al.[29] reported that early myogenic differentiation was inhibited by overexpression of MAPK phosphatase 1 in C2C12 cells and they suggested that inactivation of MAPK might be required for C2C12 myoblasts to initiate myogenesis. Our results were consistent with their report and the inactivation of MAPK might be required for these cells to initiate the osteoblastic differ-

entiation by BMP-2. MEK-1 inhibitor is thought to be an initiator or a promoter of the early stage differentiation of mesenchymal cells.

Second, investigating the osteoblastic differentiation of two cell lines in detail, ALP activity was increasing by the inhibition of MEK-1 activity in both C2C12 pluripotent mesenchymal cells treated with rhBMP-2 and MC3T3-E1 preosteoblastic cells with or without rhBMP-2 treatment. mRNA expression of type I collagen also was increasing by treatment with PD98059 (data not shown). Because ALP and type I collagen are used as early osteoblastic differentiation markers, these results indicated that MEK-1 inhibition promoted early osteoblastic differentiation in mesenchymal cells of both pluripotency and commitment into osteoblastic lineage. We speculate that two possibilities account for the reason for increase in ALP activity and type I collagen expression by MEK-1 inhibition. One idea is that the inhibition of MAPK signaling directly affects these expressions. The other is that MEK-1 inhibition promotes BMP production and subsequently induces these expressions. This issue should be focused on in future experiments.

Third, mineralization of ECM was promoted by the long-term inhibition of MAPK signaling pathway with MEK-1 inhibitor in both cell lines. It was reported that ALP cDNA–transfected cells promoted mineralized nodules.[30] Recently, Wennberg et al.[31] reported that osteoblasts derived from tissue nonspecific (TN) ALP knockout mice showed little mineralization of ECM and that mineralization was induced by adding soluble recombinant human TN ALP in these cells. They and other investigators suggested that ALP was involved in mineralization of ECM.[31,32] Taking our results and previous reports, the promotion of ALP activity might account for the effects of the MEK-1 inhibitor on the mineralization of ECM.

Fourth, OCN secretion and its transcriptional level are independent of MEK-1 inhibition and there is no correlation between its secretion and mineralization of ECM. OCN has been reported as a late osteoblastic differentiation marker.[2] However, bone formation was even increased in osteocalcin-deficient mice. In addition, bone mineralization and resorption were not impaired in those mice.[33] These reports suggested that OCN was a late osteoblastic marker but did not represent a degree of bone formation. In spite of the inconsistency of the effect of the MEK-1 inhibitor on OCN secretion and its transcript in between C2C12 and MC3T3-E1 cells, acceleration of mineralization of ECM in both cell lines is consistent with the results of OCN-deficient mice. Taking these facts, expression of OCN would not directly affect bone formation. Moreover, our data suggested that the difference between two cell lines caused the difference of OCN secretion by MEK inhibition. Xiao et al. reported that transcriptional factor Cbfa1, essential for the differentiation of osteoblasts, is phosphorylated by the activation of the MAPK pathway and regulated the activation of OCN gene promoter.[34] PD98059 did not affect Cbfa1 mRNA and protein expression level (data not shown). Further, Cbfa1 gene expression level was higher in MC3T3-E1 cells than in C2C12 cells, but OCN gene expression level was lower in the former than the latter (Figs. 2 and 4; data not shown). These data suggested that OCN gene expression was regulated not only by Cbfa1 but also by other signaling pathways affected directly or indirectly by MAPK signaling. Moreover, the same group recently reported that OCN gene expression was blocked by the short-term inhibition of the MAPK pathway by U0126 in confluent and differentiated MC3T3-E1 cells.[35] This report was partly inconsistent with our results. This difference might be due to the cellular background and/or the way of the application of the inhibitors.

Recently, Gallea et al. reported the positive effects of PD98059 on the osteogenic differentiation of C2C12 cells.[36] Our results were consistent with their report by means of ALP activity, OCN secretion, and these transcriptional levels. We found that the phosphorylation level of MAPK was reduced only slightly in the long-term inhibition of the MAPK pathway by high concentration of MEK-1 inhibitor because of the cellular compensatory mechanisms (Figs. 1A and 4A; data not shown). In addition to the experiments using the inhibitor, we could confirm those effects in C2C12 stable transfectants introducing constitutively active or dominant-negative MEK-1 cDNAs. Again, the change in MAPK phosphorylation was slight in these transfectants. Therefore, the cellular phenotype presented in this report represented the total (direct and indirect) effects of long-lasting inhibition of MAPK signaling. We also found that the MEK-1 inhibitor accelerated the mineralization of ECM, closely related to the bone formation in vivo. We also investigated that U0126[37] (Wako Pure Chemicals Industries, Ltd.) and U0124[37] (Calbiochem-Novabiochem Co., San Diego, CA, USA), another specific MEK-1 inhibitor and a negative control for U0126, showed similar effects on ALP activity and mineralization of ECM (data not shown). Our preliminary experiments, which were carried out to observe the effects of U0126 on in vivo ectopic bone formation by rhBMP-2 and type I collagen composite implanted submuscular fasciae in mice, showed enhanced ectopic bone formation to a certain degree (Higuchi C, Yoshioka K, Yoshikawa H, Itoh K, unpublished data, 2002).

Although the usefulness of the MEK-1 inhibitor for bone formation is clear, the precise underlying mechanism is not clear. The phosphorylation level of Smad5 or nuclear translocation of endogenous Smad5 was not influenced by the treatment with MEK-1 inhibitor (data not shown). Further studies should be required to solve the points.

This study suggested that a fine-tuning of the MAPK signaling pathway could promote mineralization of the ECM in parallel with up-regulation of ALP activity in BMP-2–induced pluripotent mesenchymal cells and preosteoblastic cells in vitro. MEK-1 inhibitors would be useful in vivo for the promotion of bone formation, for instance, delayed fracture healing or focal osteoporotic change, around fracture site.

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# A novel anti-rheumatic drug, T-614, stimulates osteoblastic differentiation in vitro and bone morphogenetic protein-2-induced bone formation in vivo

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**Abstract**

T-614 (*N*-[3-(formylamino)-4-oxo-6-phenoxy-4H-chromen-7-yl]methanesulfonamide), a newly developed anti-rheumatic drug under clinical trial, is an anti-inflammatory agent which has been reported to show the inhibitory effect of bone destruction in vivo arthritis model. We found that T-614 stimulated osteoblastic differentiation of stromal cell line (ST2) and preosteoblastic cell line (MC3T3-E1) in the presence or absence of recombinant human bone morphogenetic protein-2 (rhBMP-2). Calcium content of mineralized nodules was 14-fold elevated by the addition of T-614 in the presence of rhBMP-2 in ST2 but not MC3T3-E1. Oral administration of T-614 to mice also promoted rhBMP-2 induced bone formation in vivo. Northern blot analysis showed that transcriptional level of osterix, an essential transcription factor for osteoblastic differentiation, was 3-fold increased by T-614 with rhBMP-2 in ST2. Taken together, these results suggested that T-614 possessed anabolic effects on bone metabolism, besides suppressor of bone resorption, by increased expression of osterix.


*Keywords:* T-614; Osteoblast; Bone morphogenetic protein; Osterix; ST2; MC3T3-E1

Bone mass is always maintained through repeated cycle of destruction and rebuilding. This process, called bone remodeling, is regulated by both osteoclasts, which resorb old bones, and osteoblasts, forming new bones [1]. Osteoporosis, resulting from the imbalance of biological regulation, is one of the major health problems for not only elder people but also rheumatoid arthritis (RA) patients. Several drugs have been developed for the treatment of osteoporosis by inhibition of osteoclast [2,3] or by stimulation of osteoblast [4–6], however, none of the drugs showed the dual effects on both osteoclasts and osteoblasts. We carried out the screening of the several small molecules that stimulated alkaline phosphatase (ALP) activity in osteoblastic cells with the aim of clinical application for osteoporosis.

T-614, a member of the methanesulfonanilide class of anti-inflammatory agents, a newly developed anti-rheumatic drug, is undergoing phase III clinical trials in Japan. In vitro cell culture models, this drug significantly reduced the production of inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and IL-8 in human monocyte or human monocytic leukemia cell line [7,8], IL-1β, IL-6, IL-8, and monocyte chemoattractant protein 1 in synovial cells [9,10]. These biological effects of T-614 were considered due to the suppression of NF-κB activation [7,9]. In type II collagen induced and spontaneous arthritis models, this drug also suppressed the bone resorption and joint destruction [11]. Together, these reports suggested that T-614 inhibited the osteoclast activity. However, the precise mechanism of this drug as anti-osteoporotic activity is still ambiguous.

In the present study, we examined the effect of T-614 on osteoblastic differentiation using murine bone mar-

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row stromal cell line ST2 and preosteoblastic cell line MC3T3-E1 cultured with or without rhBMP-2, since these cell lines have been established as the undifferentiated osteoblastic cells [12,13]. Using a mouse model, we also studied its effect on rhBMP-2 induced bone formation in vivo. The present results suggested that T-614 had the dual effects for the osteoporotic patients by promoting bone formation and inhibiting bone resorption.

## Materials and methods

*Materials.* T-614 and its metabolites (T-614M1: *N*-(3-amino-4-oxo-6-phenoxy-4H-chromen-7-yl)methanesulfonamide, T-614M2: *N*-{7-[(methylsulfonyl)amino]-4-oxo-6-phenoxy-4H-chromen-3-yl}acetamide, and T-614M4: *N*-[3-(formylamino)-6-(4-hydroxyphenoxy)-4-oxo-4H-chromen-7-yl]methanesulfonamide) were chemically synthesized in Toyama Chemical (Toyama, Japan). Recombinant human BMP-2 was provided from Genetics Institute (Cambridge, MA) and Yamanouchi pharmaceutical (Tokyo, Japan).

*Cell culture.* Murine bone marrow stromal cell line ST2 and preosteoblastic cell line MC3T3-E1 were purchased from RIKEN CELL BANK (Tsukuba, Japan). ST2 cells were cultured in RPMI medium 1640 (RPMI 1640; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Equitech-bio, Kerrville, TX) and MC3T3-E1 cells were maintained in α-minimum essential medium (α-MEM; Invitrogen) with 10% FBS at 37°C in a humidified 5% $CO_2$ incubator. For each assay, ST2 cells were plated on type I collagen-coated plate (Sumitomo Bakelite, Tokyo, Japan) at $1.5 \times 10^4$ cells/cm$^2$ and MC3T3-E1 cells on non-coated plate at $2.5 \times 10^4$ cells/cm$^2$. At 24 h after being plated, the medium was changed to α-MEM supplemented with 10% FBS containing various concentrations of T-614 or its metabolites with or without rhBMP-2.

*Alkaline phosphatase staining and activity.* Both cell lines were treated with rhBMP-2 and/or T-614 compounds for 3 days. For ALP staining, cells were fixed for 15 min with 3.7% formaldehyde at room temperature after washing with phosphate-buffered saline (PBS). Then, the cells were incubated with the mixture of nitroblue tetrazolium (NBT; Promega, Madison, WI) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Promega) in ALP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) for 1 h in the dark at room temperature. To measure ALP activity, cells were washed twice with PBS and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) following its protocol. ALP activity was assayed using *p*-nitrophenylphosphate as a substrate by Alkaline Phospha Test Wako (Wako Pure Chemicals Industries, Osaka, Japan) and the protein content was measured using bicinchoninic acid (BCA) protein assay kit (Pierce).

*Osteocalcin secretion.* The amount of osteocalcin (OCN) secreted into the culture medium from day-4 to -6 was determined by radioimmunoassay using mouse osteocalcin immunoradiometric assay (IRMA) Kit (Immutopics, San Clemente, CA). OCN secretion was normalized to the total cellular protein content.

Alizarin Red S staining and calcium content in mineralized nodules. ST2 and MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS and 10 mM β-glycerophosphate (Sigma) in the absence or presence of 50 ng/ml rhBMP-2 and 5 μg/ml T-614 for 30 days. The medium was replaced every 3 days. For deposited calcium staining to detect mineralized nodules, the cells were washed with deionized water after being fixed for 15 min with 3.7% formaldehyde at room temperature and stained with Alizarin Red S (Sigma, St. Louis, MO) at pH 6.3. To measure calcium content of the nodules, 500 μl of 1 N HCl was added to each well to decalcify mineralized nodules after fixation of the cells. After 24 h, calcium content in the supernatant was determined using the *o*-cresolphthalein complexon color development method by Calcium Test WAKO (Wako Pure Chemicals Industries). Duplicated wells were used to determine the protein content.

*In vivo rhBMP-2 induced bone formation.* Collagen composites containing rhBMP-2 were prepared according to the previous report [14]. In brief, 3 μg rhBMP-2 solution was blotted into a porous collagen disk (6 mm diameter with 1 mm thickness) purchased from Helistat, Integra Life Sciences (Plainsboro, NJ) and freeze-dried. Fourteen male ICR mice, 6 weeks old, were purchased from Nippon SLC (Shizuoka, Japan). Under general anesthesia with diethyl ether, one composite was implanted into the left dorsal muscle pouch of each mouse. The mice were divided into two groups (seven mice for each group). To each group, 0 or 10 mg/kg/day of T-614, suspended in 0.5% (w/v) methylcellulose (Wako Pure Chemicals Industries) solution, was given orally twice a day for 17 days. On day 17, composites were collected and then radiographed with a soft X-ray apparatus. The ossicles were fixed with 3.7% formaldehyde and decalcified with 5 ml of 0.6 N HCl, dehydrated, and embedded in paraffin. Sagittal sections of 4 μm thickness were cut on a microtome and subjected to hematoxylin and eosin staining. The calcium content of the decalcified HCl solution was measured as above.

*Northern blot analysis.* Total RNA was extracted from ST2 and MC3T3-E1 cells using TRIZOL reagent (Invitrogen, Carlsbad, CA). Ten micrograms of total RNA was electrophoresed in 1% agarose–formaldehyde gels and transferred onto Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Tokyo, Japan). Twenty-five nanograms of the probes was radiolabeled with [α-$^{32}$P]dCTP using Rediprime II DNA Labelling System (Amersham Pharmacia Biotech, Tokyo, Japan). In order to make probes, mouse osteocalcin cDNA was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) with sense (5′-GAACAGACAAGTCCCACAC-3′) and antisense (5′-CCTCATCTGAACTTTATTTTGGAGC-3′) primers (GenBank Accession No. X04142), for mouse osterix (OSX), with sense (5′-GGCAGTCACTAAGATCCCCA-3′) and antisense (5′-CCC AGACTCCATGGCTTTAC-3′) primers (GenBank Accession No. NM130458). For Cbfa1/Runx2 probe, a 0.61 kb-*PstI*/*Hin*dIII fragment was used (GenBank Accession No. XM128663) [15]. All probe sequences were confirmed by 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The membranes were prehybridized, hybridized using Rapid-Hyb Buffer (Amersham Pharmacia Biotech) with radioactive probes, and then washed with 2× SSC containing 0.1% SDS or 0.2× SSC containing 0.1% SDS at room temperature or 65°C. The hybridized blots were exposed to an imaging plate at room temperature and relative levels of messenger RNA (mRNA) were estimated by a laser scanning densitometer (Fuji FLA 2000; Fuji, Tokyo, Japan).

*Statistic analysis.* Data are expressed as means ± SD in all figures. Statistic significance was analyzed by Student's *t* test. $P < 0.05$ value was considered statistically significant.

## Results

### *Effects of T-614 on ALP staining and activity in ST2 and MC3T3-E1 cells*

Alkaline phosphatase, an early phase marker of osteoblastic differentiation, was assayed in ST2 and MC3T3-E1 cultured for 3 days in the presence or absence of rhBMP-2 or T-614. T-614 (0–30 μg/ml) and rhBMP-2 (0–50 ng/ml) synergistically increased both staining and activity of ALP in ST2 in a dose-dependent fashion (Figs. 1A and B, left panels). In MC3T3-E1, T-614 also increased ALP staining and activity with the

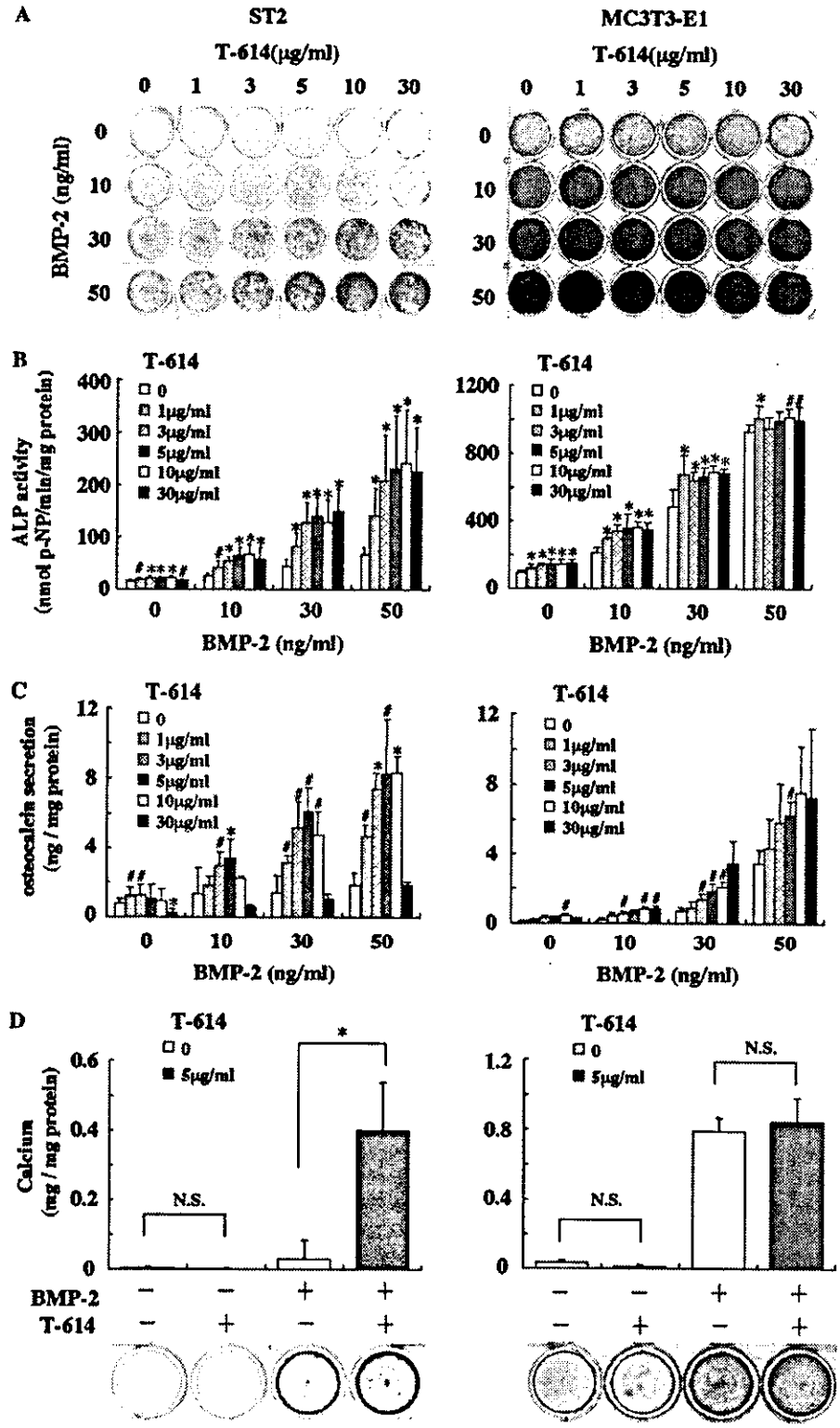



Fig. 1. ALP staining (A) of ST2 (left panel), MC3T3-E1 (right panel) and ALP activity (B) in ST2 (left panel) and MC3T3-E1 (right panel). The data are expressed as means ± SD of six determinations. Osteocalcin secretion (C) from ST2 (left panel) and MC3T3-E1 (right panel) was analyzed with radioimmunoassay. The data are expressed as means ± SD of three determinations. Calcium contents of mineralized nodules (D, top panels) in ST2 (left panel) and MC3T3-E1 (right panel) were analyzed. Alizarin Red S staining was performed (D, lower panels) in ST2 (left panel) and MC3T3-E1 (right panel). #$P < 0.05$ and *$P < 0.01$ (compared with vehicle control).

lower concentration (≤ 30 ng/ml) of rhBMP-2, but little increased effect was seen at 50 ng/ml rhBMP-2. Further, the stimulatory effect of this drug for MC3T3-E1 was less than that for ST2 (Figs. 1A and B, right panels).

*Effects of T-614 on osteocalcin secretion ST2 and MC3T3-E1 cells*

Osteocalcin is the marker of terminally differentiated osteoblast. T-614 increased osteocalcin secretion in both cell lines in its dose-dependent fashion except 30 μg/ml T-614 in ST2 (Fig. 1C).

*Effects of T-614 on bone nodule formation in ST2 and MC3T3-E1 cells*

In the presence of 50 ng/ml rhBMP-2, the calcium content of ST2 cultured with T-614 was 14-fold increased as compared to that cultured without T-614 (Fig. 1D, left panel). In the absence of rhBMP-2, mineralized nodules were less detected. By contrast, in MC3T3-E1, T-614 did not affect the calcium content in the presence or absence of rhBMP-2 (Fig. 1D, right panel).

*Effects of T-614 on rhBMP-2 induced bone formation in vivo*

Newly formed ectopic ossicles induced by rhBMP-2 were photographed (Fig. 2A) and radiographed (Fig. 2B). The calcium contents of newly formed ossicles of T-614 group were 1.7-fold increased compared to those of control group (Fig. 2C). Histological analysis showed normal bone containing fully matured bone marrow formation in each group. There were no apparent histological differences between two groups (Fig. 2D).

*Effects of T-614 on the expression of Cbfa1/Runx2, osterix, and osteocalcin mRNA in ST2 and MC3T3-E1 cells*

In order to speculate the effect of T-614 on osteoblastic differentiation, we examined the expression level of two transcription factors, Cbfa1/Runx2 and osterix, both playing essential roles in osteoblast differentiation [15–17]. Cbfa1/Runx2 was expressed in both cell lines and not stimulated by T-614 (Fig. 3A, top panels). On the other hand, we found that the expression level of osterix transcript in ST2 cells was approximately 3-fold increased when cultured with 10 μg/ml T-614 and 50 ng/ml rhBMP-2 for 3–6 days (Fig. 3A, middle left panel). In contrast, in MC3T3-E1, the expression of osterix was consistently detected even in the absence of rhBMP-2 (Fig. 3A, middle right panel). The transcriptional level of osteocalcin was also increased by the treatment with 10 μg/ml T614 and 50 ng/ml rhBMP-2 for 3–6 days (Fig.

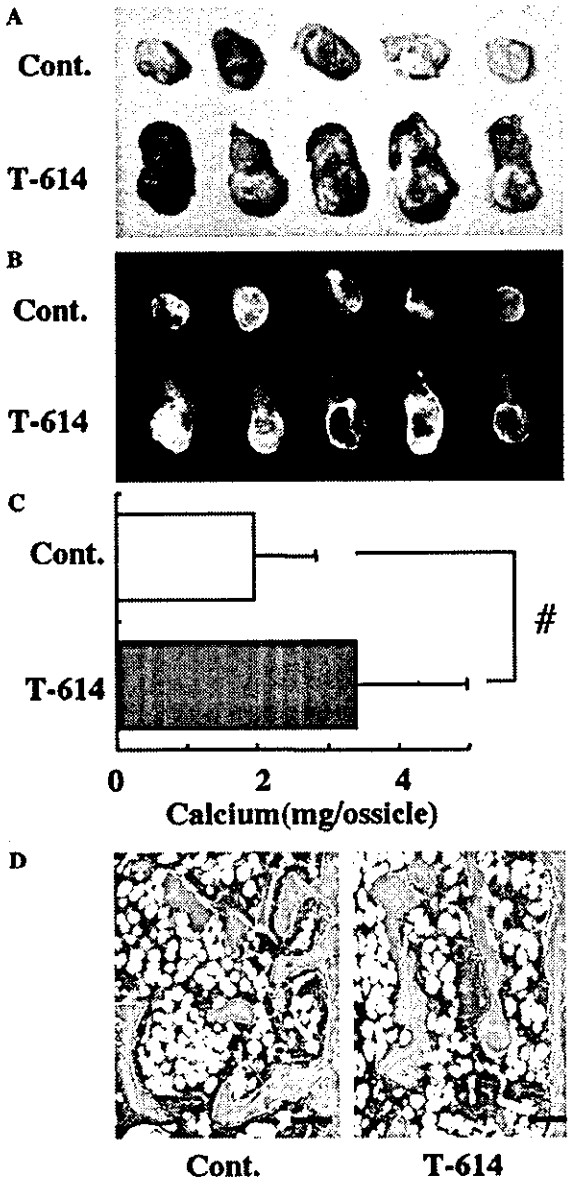



Fig. 2. Photograph (A) and soft X-ray radiograph (B) of rhBMP-2 induced newly formed bones at 17 days after implantation. Calcium contents of newly formed bones were measured (C). Histological appearance of newly formed bones stained with hematoxylin and eosin (D). Cont.: control group. T-614: T-614 10 mg/kg/day group. #$P < 0.05$ (compared with vehicle control). Bar: 100 μm.

3A, lower panels, lanes 6 and 8). We examined that T-614 might be involved in the early signal transduction pathway of BMP-2 in osteoblasts. Smad 5 is one of the intracellular signal transducers of BMP signaling [18]. The phosphorylation levels of Smad 5 were increased at 10, 20, and 30 min after treatment with 50 ng/ml rhBMP-2. Ten μg/ml T-614 did not affect this increase in the level of phosphorylation (data not shown). We also examined the expression levels of Id1, which was a direct target of BMP signaling [19]. The expressions of Id1 transcripts were stimulated after 1 hour treatment with

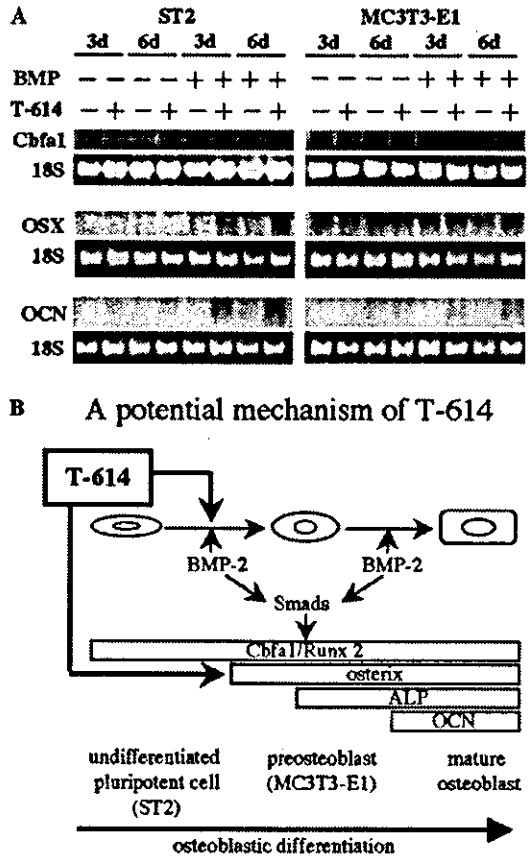
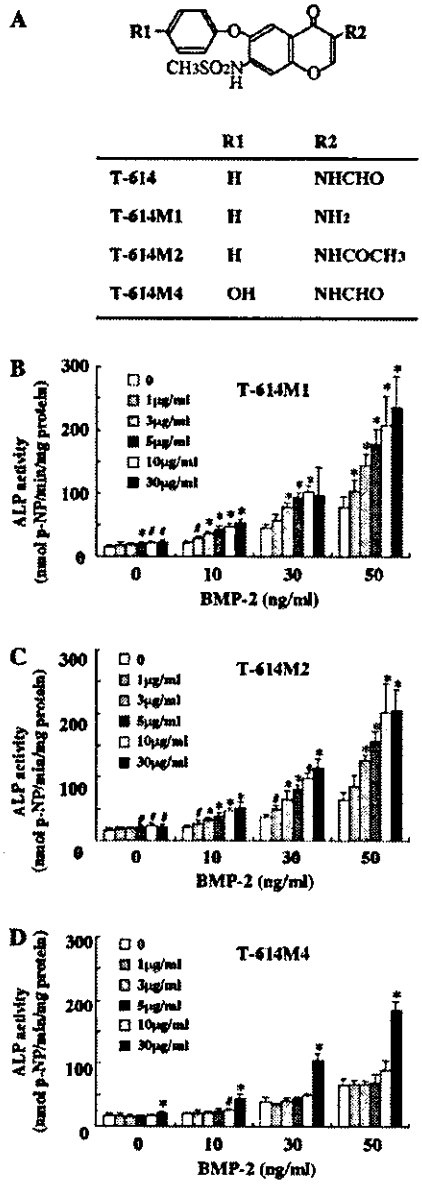



Fig. 3. The expression of Cbfa1/Runx2 (top panel), osterix (middle panel), and osteocalcin (lower panel) transcripts in ST2 (left panel) and MC3T3-E1 (right panel). Cells were treated with vehicle or 10 μg/ml T-614 in the absence or 50 ng/ml rhBMP-2 for 3–6 days (A). A potential mechanism of T-614 on osteoblastic differentiation. T-614 affected neither the phosphorylation level of Smad 5 nor the expression of Cbfa1/Runx2. T-614 stimulated the expression of osterix (B).

50 ng/ml rhBMP-2 and gradually decreased. They were also stimulated to the same extent even in the presence of 10 μg/ml T-614 (data not shown). Taken together, we speculated that T-614 was not involved in the early signal transduction pathway of BMP-2 in ST2 cells. We summarized a potential mechanism of the anabolic effect of T-614 (Fig. 3B).

*Effects of T-614 metabolites on ALP activity in ST2 cells*

In order to estimate the structure–activity relationship of T-614, we compare ALP activity in ST2 using several T-614 metabolites (Fig. 4A). Although T-614M1 and T-614M2 had little effect on TNF-α production compared to T-614 in human monocyte cell line THP-1 cell ($IC_{50}$: T-614; 17 μg/ml, T-614M1; >30 μg/ml, and T-614M2; >30 μg/ml, unpublished data), these two compounds stimulated ALP activity to a similar degree as T-614 itself (Figs. 4B and C). Further, even T-614M4 has been shown as an inactive compound for TNF-α




| | R1 | R2 |
|---|---|---|
| T-614 | H | NHCHO |
| T-614M1 | H | $NH_2$ |
| T-614M2 | H | $NHCOCH_3$ |
| T-614M4 | OH | NHCHO |

Fig. 4. Chemical structures of T-614 and its metabolites (T-614M1, M2, and M4) (A) and the effects of the T-614 metabolites on ALP activity of ST2 cells (B: T-614M1, C: M2, D: M4). The data are expressed as means ± SD of five determinations. #$P < 0.05$ and *$P < 0.01$ (compared with vehicle control).

production in THP-1 cell, this metabolite stimulated ALP activity at 30 μg/ml (Fig. 4D).

## Discussion

In this study, we have found that T-614 and its metabolites stimulated osteoblastic differentiation in ST2 and MC3T3-E1. In addition, T-614 also stimulated rhBMP-2 induced bone formation in vivo. The possible mechanism accounting for these effects is increased

expression of osterix by T-614. Osterix has been reported to play a pivotal role in osteoblast differentiation and bone formation [16]. In MC3T3-E1, the expression level of osterix was consistent even in the absence of rhBMP-2 (Fig. 3A, right panel). In ST2, by contrast, osterix was scarcely detected in the absence of rhBMP-2, T-614 and rhBMP-2 about 3-fold stimulated the expression of osterix (Fig. 3A, left panel). Cbfa1/Runx2 [15,17], the other essential transcription factor for osteoblastic differentiation, was expressed consistently in both cell lines. It is reported that osterix was located in the downstream of Cbfa1/Runx2 in the transduction pathway of BMP-2 [16]. In addition, it is believed that ST2 is an undifferentiated pluripotent bone marrow stromal cell line, whereas MC3T3-E1 is a more differentiated preosteoblastic cell line. Taken together, we speculated the potential mechanism of anabolic effect of T-614 in Fig. 3B. T-614 promoted osteoblastic differentiation via the stimulation of osterix expression. Therefore, the different expression levels of osterix might account for the difference in the effects of T-614 on the osteoblastic differentiation for ST2 and MC3T3-E1. Whether this stimulatory effect of T-614 on osterix expression is direct or indirect should be addressed in future.

T-614 is a member of the methanesulfonanilide class of anti-inflammatory drug, which was developed for the treatment of patients suffering from RA. Since osteoporotic changes, besides joint destruction, are often recognized in RA patients, the drug which suppresses bone resorption and promotes bone formation will bring great advantages to those patients. T-614 has been reported to reduce the production of inflammatory cytokines by inhibition of NF-κB activation [7,9]. Knockout mice study revealed that NF-κB played an essential role in osteoclastogenesis, but did not affect osteoblastic differentiation [20,21]. In addition, salicylate, the other molecule of NF-κB inhibitor [22,23], did not promote ALP activity in our experiments (data not shown). Further, inactive metabolite of T-614 for cytokine production, T-614M4, still showed the stimulatory effect for osteoblastic differentiation (Fig. 4D). Taken together, these evidence suggested that the other mechanism, besides NF-κB inactivation, could exist for the promotion of osteoblastic differentiation by T-614.

T-614 also has been reported to inhibit both the activity and expression of cyclooxygenase (COX)-2 in cultured fibroblast [24]. However, NS-398, other COX-2 inhibitor, inhibited ALP activity in osteoblast [25]. These reports suggested that the stimulatory effect of osteoblastic differentiation by T-614 was not due to the inhibition of COX-2. Recently, COX-2 was indispensable for *de nove* bone formation, such as fracture healing [26,27]. Therefore, the application of COX-2 inhibitor for fractured patients may not be recommended for fear of non-union. Besides major bone fracture, in the postmenopausal women, they often suffer from pain by minor trabecular bone fracture due to osteoporosis. For those elder people, T-614 might have more benefit for decreasing the adverse effect. The effective concentration of T-614 in vitro study (1–10 μg/ml) and in vivo study (10 mg/kg/day) are comparable to the plasma concentration of T-614 estimated in the clinical trial (unpublished data).

In this report, we found that a novel anti-rheumatic drug, T-614, promoted osteoblastic differentiation in ST2, MC3T3-E1 cells in vitro and rhBMP-2 induced bone formation in vivo. The possible mechanism of these effects was stimulation of osterix expression. Therefore T-614 has an anabolic property as well as an inhibitory effect of bone resorption. The osteogenic activity of this compound under clinical setting should be addressed in future.

## Acknowledgments

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トピックス

# 軟骨形成におけるBMPの役割

*Role of BMPs during cartilage development*

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## はじめに

Bone morphogenetic protein (BMP) は当初，皮下に植えると異所性に内軟骨性骨化を誘導する分泌蛋白として同定された[1]．そして遺伝子クローニングによってBMPは多くのメンバーからなるファミリーを構成することが明らかにされた[2]．そのアミノ酸配列の類似性から，BMPはいくつかのサブファミリーに分類され，growth and differentiation factor (GDF) もその1つに含まれる (表1)．その後の研究により，BMPは発生過程において骨の形成のみならず，種々の臓器や組織の形成や分化や，胎児の軸決定にも深く関与していることが明らかにされてきた[3]．実際，BMP2やBMP4を欠失したノックアウトマウスでは発生初期に中胚葉の誘導に失敗して致死となり[4,5]，BMP-7を欠失したノックアウトマウスでは骨格，腎臓，眼に異常が認められている[6]．このようにBMPの機能はネーミングの範疇を超えて多岐にわたるが，やはり発見のきっかけとなった骨形成能に注目が集まり，骨折治療や骨粗鬆症における骨質の改善を目指して臨床応用が試みられている．

表1　BMP family members

| | BMP subfamily |
|---|---|
| BMP-2<br>BMP-4 | BMP-2/4 |
| Osteogenin (BMP-3)<br>GDF-10 | BMP-3 |
| BMP-5<br>Vgr-1 (BMP-6)<br>OP-1 (BMP-7)<br>OP-2 (BMP-8)<br>OP-3 (BMP-8B) | OP-1/BMP-7 |
| BMP-9<br>BMP-10<br>GDF-11 (BMP-11) | |
| CDMP-1 (GDF-5)<br>CDMP-2 (GDF-6)<br>CDMP-3 (GDF-7)<br>BMP-15 | CDMP (GDF) |

Key words ▶
軟骨 (cartilage)
骨形成因子 (bone morphogenetic protein)
Noggin

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ところで，身体の骨格は，まず軟骨で形成され，次いで骨に置換される．この内軟骨性骨化の過程は，発生の初期において未分化間

葉系細胞(間充織細胞)が，将来軟骨ができあがる場所で凝集することから始まる．骨格の各コンポーネントに相当する個々の細胞凝集塊は，位置に固有な増殖と分化を行い，おのおのの形をもった軟骨原基(cartilage primordia)が形成される．軟骨原基はこの段階ですでにその形が将来の骨の形を表わしているため，骨格の鋳型(template)と呼ばれる．さらに発生の進行とともに，この軟骨の鋳型の中心部では軟骨細胞が増殖そして分化・肥大する．肥大した細胞の周囲のマトリックスは石灰化が進行し，やがて血管や骨芽細胞が侵入して軟骨は骨および骨髄へと置換される．この結果軟骨は長管骨の両端に残って分化を続け，骨端軟骨となる．骨端軟骨と骨幹の境界では成長軟骨板が形成され，ここでは軟骨細胞は柱状に配列し，骨幹に向かって静止軟骨，増殖軟骨，肥大軟骨の3層構造をとるようになる．さらに成長が進むにつれ，骨端の中心部にも骨が形成され，その内面側の成長軟骨板は成長の終了とともに消失するが，表面側の軟骨は永久に存在し，関節を構成体する．このような関節形成を含めた骨格形成において軟骨は非常に重要な役割を果たしており，その増殖・分化は種々の液性因子のシグナルによってコントロールされている．BMPはその1つであり，BMPには骨に劣らず，軟骨を作る強力な作用がある．本稿では軟骨形成におけるBMPシグナルの役割を概説する．

## Ⅰ．BMPの生体における機能の研究方法

BMPの骨格形成における機能を知るために，まずその発現パターンが調べられた．発生過程において，軟骨原基周囲の軟骨膜には*Bmp2*, *Bmp4*, *Bmp7*そして*Gdf5*が発現することが，*in situ* hybridization法によって明らかにされた[7,8]．そしてそれらのレセプターであるBMP receptors type IA，type IB，type IIが軟骨原基内の軟骨細胞に発現していることから[9]，BMPが軟骨形成に重要な役割を果たしていることが推測された．また，BMPシグナルの調節には，BMPに結合してレセプターとの相互作用を障害し，シグナルの伝達をブロックするアンタゴニストが存在する．Nogginはその1つで[10]，軟骨原基内の軟骨細胞に強く発現する[11]．ゆえに軟骨形成はNogginの活性によっても制御されていると考えられた．

次に，これらBMPが実際には生体においてどのような機能を果たしているのかを調べるために，人為的にBMPシグナルを活性化あるいは不活化した動物が作成された．その表現型の解析を行い，正常との違いの原因をBMPシグナルの強弱に帰することにより，BMPの機能を推測したのである．

## Ⅱ．BMPシグナルの不活化と軟骨形成

まず，BMPシグナルを不活化したノックアウトマウスが作製された．しかし軟骨形成に関しては，*Bmp2*, *Bmp4*は着床後の発生初期の胎児に発現するために，胎生9日頃に致死となり，軟骨が発生を始める12.5日目以降の解析が不可能であった[4,5]．BMP receptors type IAやtype IIのノックアウトマウスも，中胚葉の形成不全で9日頃に致死となり，BMPシグナルの伝達が障害されたためと考えられた．一方，*Bmp5*, *Bmp6*, *Bmp7*そして*Gdf5*のノックアウトマウスはホモ接合体でも生き残り，骨格の一部に形成不全を示した[6,12~14]．BMP receptors type IBのノックアウトマウスもほぼ四肢の骨格に限局する異常

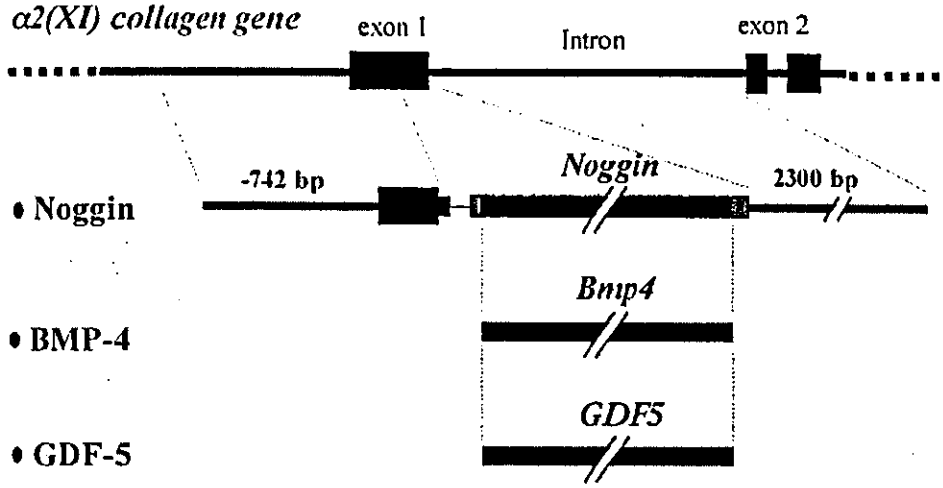


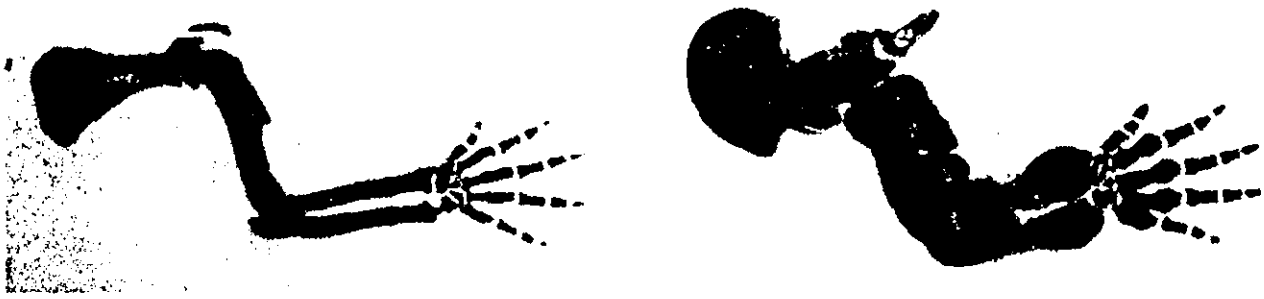

図1 Noggin, BMP4, GDF5トランスジェニックマウスの作製に用いたDNAコンストラクト
おのおののcDNAに，XI型コラーゲンα2鎖遺伝子のプロモーター／エンハンサー配列を結合して作出した．このプロモーター／エンハンサー配列はトランスジェニックマウスにおいて，結合した遺伝子を極めて軟骨特異的に発現させる[20]．

を呈した．これらの比較的軽微な表現型の異常は，同じ組織に発現する他のBMPファミリーメンバーやレセプターが，欠失したBMPやレセプターの機能を代償したためと推測されている．この考えは*Bmp5*と*Bmp7*の両方を欠失したダブルノックアウトマウスが発生初期に致死となることから証明された[15]．

そこで，ファミリーメンバー間の代償を排除し，BMPの軟骨形成における役割を知るために，アンタゴニストであるNogginを強制発現させて複数のBMPシグナルをブロックすることが試みられた．しかも，生体すべてのBMPシグナルを不活化すると*Bmp2*, *Bmp4*ノックアウトマウスの例から発生初期に致死となるので，限られた組織でNogginを発現させた．その1つはchickの肢にNogginをコードしたレトロウイルスを感染させ，肢の発生を観察したものである[16,17]．この肢では骨格，筋，腱，脂肪，皮膚などすべての組織に感染程度に応じてNogginが過剰発現し，結果的に軟骨発生が起こらなかった．もう1つの方法としてわれわれは，軟骨特異的プロモーターを用いて(図1) Nogginを軟骨に過剰発現させたトランスジェニックマウスを作製した[18]．このマウスでは軟骨がほとんど形成されず，またtype X collagenを発現する分化した肥大軟骨細胞が認められなかった．これらのことから，軟骨形成にはBMPが決定的に必須であり，その機能は他の液性因子，例えばヘッジホグやFGFなどによって代償できないと考えられた．軟骨の無形成は軟骨マトリックス遺伝子の転写因子であるSox9をノックアウトしたES細胞でも認められる．Nogginを過剰発現したchick肢軟骨やトランスジェニック軟骨でSox9の発現が著しく低下していたことから，BMPシグナルの不活化がSox9の発現低下を通して軟骨の無形成をもたらしたと推測される．

## Ⅲ．BMPシグナルの活性化と軟骨形成

BMPシグナルを軟骨で活性化したモデル動物としてNogginノックアウトマウスが1998年に報告された[11]．Nogginはかなり軟



正常マウス　　BMP-4トランスジェニックマウス

図2　BMP4トランスジェニックマウスの骨格

胎生16.5日の正常およびBMP4トランスジェニックマウスの前肢骨格を，軟骨をアルシャンブルーで，骨をアリザリンレッドで染めたもの．BMP4トランスジェニックマウスの軟骨原基は正常マウスに比べて，増大している．関節の形成は保たれている．

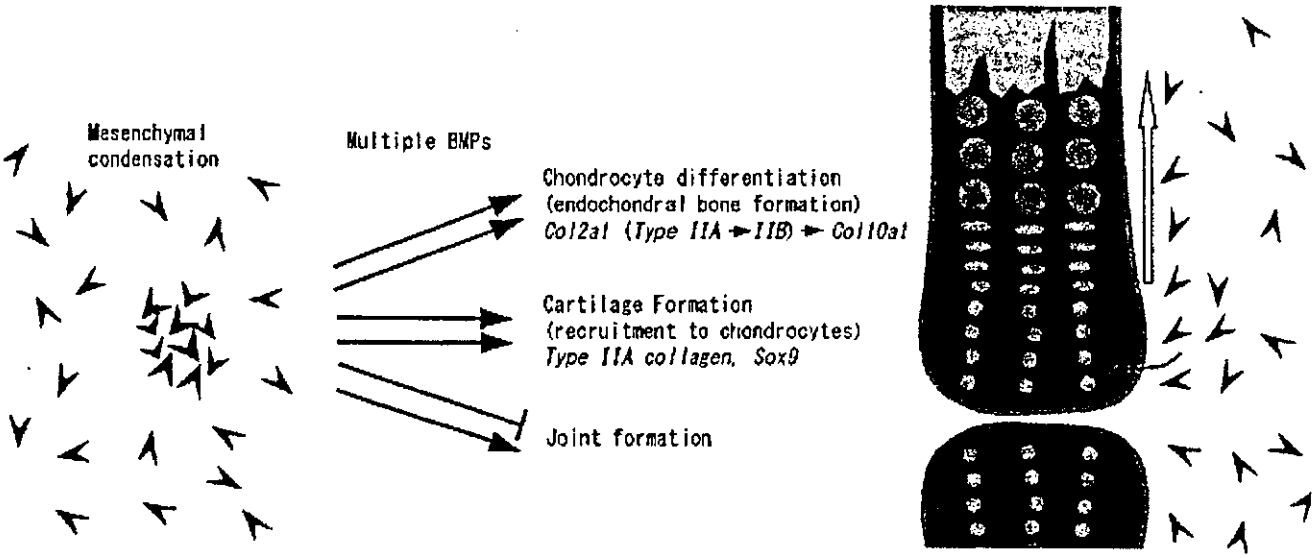



図3　軟骨の形成・分化および関節の形成過程における各BMPの役割

軟骨周辺には種々のBMPが発現している．BMP4とGDF5には未分化間葉系細胞を軟骨細胞にリクルートして軟骨原基を増大させるとともに，軟骨分化を亢進させるという共通の作用がある．一方関節形成に関しては，GDF5にはBMP4に比べて，強い関節形成の抑制作用が認められる．

骨特異的に発現しているので，Nogginを欠失したこのマウスは，軟骨で相対的にBMPの活性が上昇し，軟骨原基が大きくなった．これはNogginトランスジェニックマウスの表現型と反対である．またこのノックアウトマウスには関節が存在せず，BMPシグナルと関節形成に深い関与があることが示された．NogginはBMP2, BMP4, BMP7そしてGDF5と結合するので，このノックアウトマウスではこれらすべてのBMPのシグナルが軟骨で活性化されていると考えられる．軟骨を含め1つの組織の同じ場所で複数のBMPメンバーが発現することが見いだされているが[7,8]，その理由や，各BMPに特有の機能があるかどうかについては興味がもたれているところである．われわれは軟骨特的プロモーターを用いて(図1)BMP4とGDF5を軟骨に強制発現させたトランスジェニックマウスを作製し[18,19]，比較した．BMP4トランスジェニックマウス，GDF5トランスジェニックマ

ウスともに，軟骨原基が増大し(図2)，またtype K collagenを発現する分化した肥大軟骨細胞が数多く認められた．一方相違点としては，GDF5トランスジェニックマウスでは関節が形成されなかったのに対し，BMP4トランスジェニックマウスでは関節が形成されることが多かった．すなわち，BMP4とGDF5は，軟骨形成，分化では同様の機能を果たすが，関節形成に関しては異なる役割を担っていると考えられる(図3)．Nogginノックアウトマウスの表現型は軟骨形成に関してはBMP4とGDF5の両方の作用が相加あるいは相乗的に働き，関節形成に関してはGDF5の役割が優勢に現れたと考えられる．分子レベルにおいては，BMP4とGDF5の作用の相違点についてはレセプター以下のシグナル伝達に差があるということであろうから，今後この伝達経路の解明が待たれるところである．

## おわりに

BMPシグナルをブロックすると軟骨ができないことから，BMPが軟骨形成に必須の蛋白であることは疑いない．一方，変形性関節症の変性軟骨の一部の軟骨細胞ではBMPの発現が強く認められ，この部では軟骨の修復あるいは再生機転が働いている可能性がある．発生過程に軟骨原基として体を支持し，生後は関節の構成体として，荷重や運動に耐える器官として軟骨の機能を保つため，BMPシグナルによる軟骨形成のコントロールは，非常に重要であると考えられる．このコントロールメカニズムを理解し，さらに操作することが可能になれば，変形性関節症などの軟骨変性疾患における軟骨修復へ向けて臨床応用できる日が来るかもしれない．

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平成13年度脊柱靱帯骨化症に関する調査研究班第1回班会議

# 3) BMPの応答制御機構の解析

A study on the regulation mechanism of BMP responsiveness

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key words

骨形成因子（BMP）
異所性骨化
細胞骨格
Rho キナーゼ
カルポニン

**脊柱靱帯骨化症をはじめとする異所性骨化では，間葉系細胞の骨形成因子（BMP）に対する応答性の亢進が推測されている．BMPの応答性は，細胞骨格関連遺伝子その他の種々のシグナル伝達系を介して制御を受けている．**

## はじめに

骨形成因子（bone morphogenetic protein：BMP）は，*in vivo* で異所性骨形成を誘導する生物活性を有するため，脊柱靱帯骨化症をはじめとする，異所性骨化症の成因に深く関与することが示唆される．BMPの作用機序から異所性骨化を考える場合，次の3つの病態が推測される．①BMPの全身的な過剰発現：fibrodysplasia ossificans progressiva[1]，②BMPの局所的な過剰発現：化骨性筋炎，アキレス腱骨化症，類骨骨腫[2]など，③BMPに対する応答性の亢進（全身的/局所的）．なかでも，③のBMPの応答制御機構については，いまだ不明な点が多く，脊柱靱帯骨化症の成因を解明するための新しいアプローチであると考えられる．本稿では，細胞骨格関連遺伝子とBMPによる骨形成との関連に注目し解析を進めたので，その知見を概説する．

## BMPの生物活性

骨組織は，再生修復能に優れていることから，古くから骨組織中には，骨再生（骨形成）を促進する活性物質（osteogenin）の存在が推測されていた．1965年，Urist は0.6N 塩酸処理した脱灰骨基質に骨誘導物質が存在することを発見した[3]．1988年，Wozney らは，ウシ脱灰骨基質よりBMPを精製し，BMP-1-7の遺伝子のクローニングに成功した[4]．BMPを筋肉内に移植すると，内軟骨性骨形成過程を経て，移植後3週には，移植片全体が，骨組織に置換される（図1）．骨梁間には，造血骨髄が形成され，一つのorganとしての骨の形態を呈する（図2）．この異所性骨形成過程は，一連の生物学的連鎖反応からなり，この過程は，胎生期の軟骨・骨の発生過程を再現するものである．BMPは初期の未分化間葉系細胞の増殖と集合（chemotaxis）や軟骨細胞/骨芽細胞への分化を担っていると考えられる．その後の反応は，*in vivo* の環境下で，自動的に進行すると考えられる．rhBMP-2を用いた場合は，その純度が高いためか，軟骨細胞の出現が少なく，骨芽細胞の出現が早期にみられる．BMPは異所性骨形成のみならず，骨折治癒過程の初期[5]や，骨

3) BMP の応答制御機構の解析

図1 rhBMP-2(5μg)を含有するアテロコラーゲン(3mg)をマウス背部筋膜下に移植後3週の異所性骨形成

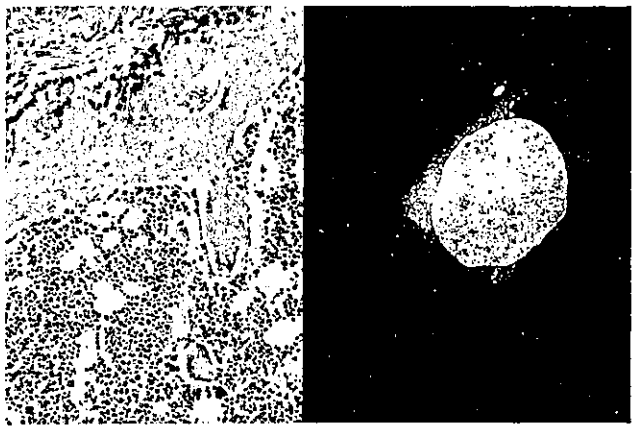

図2 rhBMP-2による異所骨の組織像(左)および軟X線像(右)

腫瘍の骨膜反応[6]においても発現が確認されており，骨形成を担う，最も重要な分化・増殖因子であると考えられている．現在，リコンビナント・ヒトBMP-2の臨床治験がなされており，その臨床応用が期待されている．

## BMP の活性制御機構の解析

BMP の活性制御機構の解析方法としては，① *in vitro* における，間葉系細胞から骨芽細胞への分化誘導系，② *in vivo* における異所性骨誘導系が用いられている．BMP の *in vivo* における生物活性の制御は，酸，TNF-α[7]，エストロゲン，血管新生阻害薬(TNP-470)[8]，ホスホジエステラーゼ阻害薬[9]などにより行われていることが報告されている．一方，*in vitro* における BMP による未分化間葉系細胞から骨芽細胞への分化は，Smad その他の種々のシグナル伝達系により制御されていることが推測されている[10]．骨に対する力学的負荷，機械的刺激が骨形成に重要であることは，従来から提唱されてきたが，その機構はいまだ不明であった．細胞骨格(cytoskeleton)は，力学的負荷により，著しく変化を受ける細胞構成体であり，これを制御する Rho-Rho キナーゼ，カルポニンなどの細胞骨格関連遺伝子(□．)と BMP による骨形成との関連に注目し解析を行った．

### 1. カルポニンによる制御

アクチン結合蛋白であるカルポニンは Rho キナーゼの基質の一つであり[11]，ストレスファイバーの重合作用を有する[12]．カルポニン遺伝子のホモ型欠失マウス(−/−)の胎生13.0〜14.5日の骨格組織を比較すると，ホモ型欠失マウスで，胎生期の観察から，長管骨の骨化中心の出現および頭蓋骨の膜性骨化がより早期に観察された．5〜8週齢マウスでは，大腿骨の横径成長の亢進を認め，骨皮質，骨膜の肥厚および BMP-4 mRNA の過剰発現を伴う骨膜骨芽細胞の活性化を認めた[13]．血清アルカリフォスファターゼ(ALP)活性は，ホモ型欠失マウスで有意に高値を示した(野生型マウス：277±9 IU/L，ホモ型欠失マウス：406±17 IU/L，mean ± S.E., n =31，P <0.001)．また，