# Menaquinone-7 regulates gene expression in osteoblastic MC3T3E1 cells

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**Abstract.** Previous study has shown that the vitamin $K_2$ analog menaquinone-7 (MK-7) induces expression of the osteoblast-specific genes osteocalcin, osteoprotegerin, receptor activator of NFκB, and its ligand. Since MK-7 may also regulate osteoblast cell function, we examined the expression of osteoblast genes regulated by MK-7 administration. Differences between gene expression in control and MK-7-administered MC3T3E1 cells were analyzed using the suppression subtractive hybridization method. After 24 h of MK-7 administration, genes upregulated by MK-7 included *tenascin C* and *BMP2*. Genes downregulated by MK-7 administration included *biglycan* and *butyrophilin*. Real-time PCR showed a marked increase in *tenascin C*. When the protein level was examined using Western blot analysis, tenascin C was higher in MK-7-administered cells than in control cells. These results indicated that MK-7 affected the cellular function of osteoblastic MC3T3E1 cells. Considering BMP2 mRNA expression was higher in MK-7-administered cells than in control cells, the effect of MK-7 administration on the signal transduction system was examined. Western blot analysis showed that cells administered MK-7 displayed a higher phosphorylated Smad1 level than control cells. Because MC3T3E1 cells have a nuclear binding receptor for MK-7, this result might indicate an indirect effect of MK-7 through BMP2 production.

## Introduction

Bone modeling and remodeling are essential for development, maturation, maintenance, and repair of bones. Osteoblasts and osteoclasts are the main cells responsible for bone remodeling. Osteoblasts are differentiated from precursor cells by their response to bone morphogenetic protein (BMP) (1). MC3T3E1 cells, derived from neonatal mouse calvariae, are well-established as an *in vitro* model for osteoblast differentiation (2). When BMP2 binds to its specific receptor on the cell surface, the downstream molecules Smad1, 5, and 8 are phosphorylated (1). The phosphorylated Smads form a complex with Smad4. This complex is subsequently transported into the nucleus where it exerts gene regulation either directly or indirectly. Thus, the BMP2 signal produces osteoblast-specific proteins such as osteocalcin.

Attention has been focused on vitamin $K_2$, which has 14 isomers, in bone metabolism. Menaquinone-4 (MK-4) or menatetrenone, a vitamin $K_2$ analogue, is considered to stimulate osteoblastogenesis and inhibit osteoclastogenesis in human bone marrow cell culture (3). Although MK-4 produces the most potently enhanced mineralization *in vitro* of all vitamin $K_2$ analogues (4), a lower concentration is observed in various foods (5). Rather than MK-4, fermented foods contain significant amounts of menaquinone-7 (MK-7) (5). In epidemiological studies, the continuous intake of MK-7 from fermented soybeans (natto) contributes to the maintenance of bone health (6).

Menaquinones are essential for the γ-carboxylation of osteocalcin, a calcified tissue protein containing γ-carboxyglutamic acid, which is synthesized in osteoblasts (7). A previous study showed that MK-7 induced the production of osteocalcin, osteoprotegerin, receptor activator of NFκB (RANK), and its ligand (RANKL) in osteoblastic MC3T3E1 cells (9). These findings suggest that vitamin K plays a significant role in bone homeostasis. Recently, a novel mechanism by which vitamin $K_2$ functions in transcriptional regulation in osteoblasts was identified. Both vitamin $K_2$ and the known steroid and xenobiotic receptor (SXR) ligand rifanpicin upregulated expression of the prototypical SXR target gene *CYP3A4* and bone markers such as alkaline phosphatase (ALP) and matrix Gla protein (MGP) (8). These findings indicated that MK-7 might directly affect osteoblastic function through SXR.

Suppression subtractive hybridization (SSH) is a useful method to compare gene expression in two biological

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situations. This method is a useful tool to identify broad-spectrum changes in gene expression in tissues in response to a given stimulus (9). We performed SSH analysis to identify differences between the gene expression of MK-7-treated osteoblastic MC3T3E1 cells and control cells. Several genes were identified, 6 of which were upregulated and 3 of which were downregulated by MK-7. In addition, the expression of specific proteins was investigated.

## Materials and methods

*Reagents*. Fetal bovine serum (FBS) and α-modification of Eagle's minimal essential medium (α-MEM) were obtained from Dai-Nippon Seiyaku (Osaka, Japan). Menaquinone-7 (vitamin $K_2$; MK-7) highly purified from fermented soybeans was generously supplied by Eisai Pharmaceutical Co. (Tokyo, Japan) and dissolved in ethanol. Anti-tenascin C antibody, anti-Smad1 antibody, and anti-phosphosmad1 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Other chemicals were reagent grade from Nakalai Tesque (Osaka, Japan). Plastic cell culture dishes were purchased from Becton Dickinson (Lincoln Park, NJ, USA). Other materials were commercial products of the highest grade available.

*MC3T3E1 cell culture*. Osteoblastic MC3T3E1 cells derived from the calvaria of a newborn C57BL/6 mouse were obtained from the RIKEN cell bank, and used in the 3rd-5th passages. The cells were maintained in α-MEM containing 10% (v/v) FBS. All cells were plated at a density of $2x10^5$ cells in 35-mm culture dishes at 37˚C in a humidified atmosphere containing 5% $CO_2$. When cells reached confluency, $10^{-5}$ M MK-7 was added to the medium. After 24 h of incubation with MK-7, RNA was prepared.

*Preparation of total RNA*. Twenty-four hours after the administration of MK-7, total RNA was extracted using the AGPC method (Isogen, Wako Chemicals, Osaka, Japan). Briefly, the cells were lysed in Isogen solution and homogenized, and 0.2 volume of chloroform was added. Phase separation was achieved by centrifugation at 12,000 x g for 15 min at 4˚C. The aqueous phase was transferred to fresh RNase-free tubes, and the RNA was precipitated with an equal volume of isopropanol at 4˚C. The RNA pellet was washed once with 75% ethanol and re-suspended in RNase-free water.

*Construction and analysis of subtractive cDNA libraries*. For the construction of a subtractive cDNA library, total RNA from either control cells or MC3T3E1 cells treated with MK-7 for 24 h were reverse transcribed using a Super SMART PCR cDNA synthesis kit (Clontech, CA, USA). Transcribed cDNAs were then used for suppression subtractive hybridizations, which were performed using a PCR-Select cDNA subtraction kit according to the manufacturer's instructions (Clontech). Finally, the resulting subtracted cDNA was ligated into an Advantage PCR cloning kit (Clontech) to make a subtracted cDNA library. After construction of the library, bacterial colonies were randomly selected, and cloned cDNA sequences were determined using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, CA, USA) with either an M13 forward (5'-CGCCAGGGT TTTCCCAGTCACGAC-3') or M13 reverse (5'-CACA GGAAACAGCTATGACCATG-3') primer.

*Dot blot analysis*. Dot blot hybridization was performed to confirm that the subtraction products were from the tester. The PCR products in the analysis of positive clones were prepared for dot blot hybridization. Because the cDNA of

Table I. Primer sets used in real-time PCR.

| Genes | Primer sets | | Size (bp) |
|---|---|---|---|
| | Forward | Reverse | |
| *Arrestin 3* | CACTCCTGGCTGCCAACTGT | GGTGTCTTCATGCTTGAGTTTGC | 54 |
| *Biglycan* | CCTTCCGCTGCGTTACTGA | GCAACCACTGCCTCTACTTCTTATAA | 51 |
| *BMP 2* | CCAAAATCCCTAAGGCATGCT | TTCATTTTCATCTAGGTACAACATGGA | 73 |
| *Butyrophilin* | AAGTGCTGGAAACATAGCCACAT | CCTGAGCGTTGCTGAATCAA | 58 |
| *Receptor (calcitonin) activity modifying protein 2* | GGAGAGCAGAGCAGCGTAGAG | CGATTTCCTCGGGCTGAGT | 68 |
| *Deafness, autosomal dominant 5 homolog* | CATCCCTTTGCGGTGTTACC | AGGATTTTCTGCAGGACACAGAA | 51 |
| *Eukaryotic translation elongation factor 1 α 1* | CCGGCCACCTGATCTACAA | GCAGCCTCCTTCTCAAACTTTTC | 70 |
| *TAF12 RNA polymerase II, TATA box binding protein-associated factor* | TCTAGAGCGCCAGTGGAACA | TGCTTTTTTGTAGGGTCGGATT | 70 |
| *Tenascin C* | GGACTTACGGGTGTCTGAAACC | TGAGGCGGTAACGATCAAACT | 86 |
| *Upregulated during skeletal muscle growth 5* | AATTGTGTCCTGGCCACATATG | CTTTCACAGCTGGAGTTTTCTTAGG | 85 |

Table II. Gene expression regulated by MK-7 using SSH.

| Genes | GenBank no. | SSH results |
|---|---|---|
| *Arrestin 3* | NM_133205 | upregulated |
| *Biglycan* | BC019502 | downregulated |
| *Butyrophilin* | BC031459 | downregulated |
| *Receptor (calcitonin) activity modifying protein 2* | NM_019444 | upregulated |
| *Deafness, autosomal dominant 5 homolog* | AK089459 | upregulated |
| *Eukaryotic translation elongation factor 1 α 1* | NG_002347 | upregulated |
| *TAF12 RNA polymerase II, TATA box binding protein-associated factor* | BC019668 | upregulated |
| *Tenascin C* | NM_011607 | upregulated |
| *Upregulated during skeletal muscle growth 5* | BC024355 | downregulated |

SSH, suppression subtractive hybridization.

MC3T3E1 cells was used as the probe, the hybridized membrane was further visualized using an ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech Inc., NJ, USA).

*BLAST with GenBank*. The resulting partial sequences were compared with sequences in GenBank using the NCBI BLAST program (10).

*Quantification of various molecules using real-time PCR*. First-strand cDNA was synthesized from 5 μg of total RNA using a commercial First Strand cDNA synthesis kit (Fermentas Ltd, Vilnius, Lithuania) according to the manufacturer's instructions. Real-time PCR was performed in an ABI PRISM-7700 sequence detection system (Applied Biosystems) using SYBR-Green as a double-strand DNA-specific binding dye. Thermocycling was performed in a final volume of 20 μl containing 1 μl of cDNA sample, 5 pmol of each primer, and 10 μl of SYBR-Green kit. The quantification of each target gene and GAPDH mRNA was performed in separate wells using the gene-specific primer sets described in Table I. Primer sets were designed using Primer Express (Applied Biosystems). The cycle conditions for the sequence detection system were determined by using an experimental plate as a source for collecting well factors according to the operating instructions of Applied Biosystems, followed by a denaturing step at 95˚C for 10 min and 50 cycles at 95˚C for 15 sec and 60˚C for 1 min. Each reaction was run in duplicate or triplicate, and fluorescence data were collected at the end of the extension step in every cycle. Real-time PCR products were run on 1.2% agarose gel and were confirmed as a single band.

In separate experiments, the identities of the amplified PCR products were confirmed by direct sequencing using an automatic DNA sequencer (Applied Biosystems, CT, USA).

*Western blot analysis*. To detect the expression of specific proteins, Western blot analysis was carried out. All antibodies including horseradish peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat IgG secondary antibodies were purchased from Santa Cruz Biotechnology, Inc., Germany. Briefly, whole cell lysates (10 μg) derived from MC3T3E1 cells cultured with $10^{-5}$ or $10^{-6}$ M MK-7 were applied to a 10% acrylamide gel, electrophoresed, and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). After blocking, the membranes were reacted with individual antibodies. All blots were analyzed using the FAS-II UV-image analyzer (Toyobo Co. Ltd., Tokyo, Japan), and the densities of the bands were quantitated using Quantity One™ version 2.5 (PDI Inc., NY, USA).

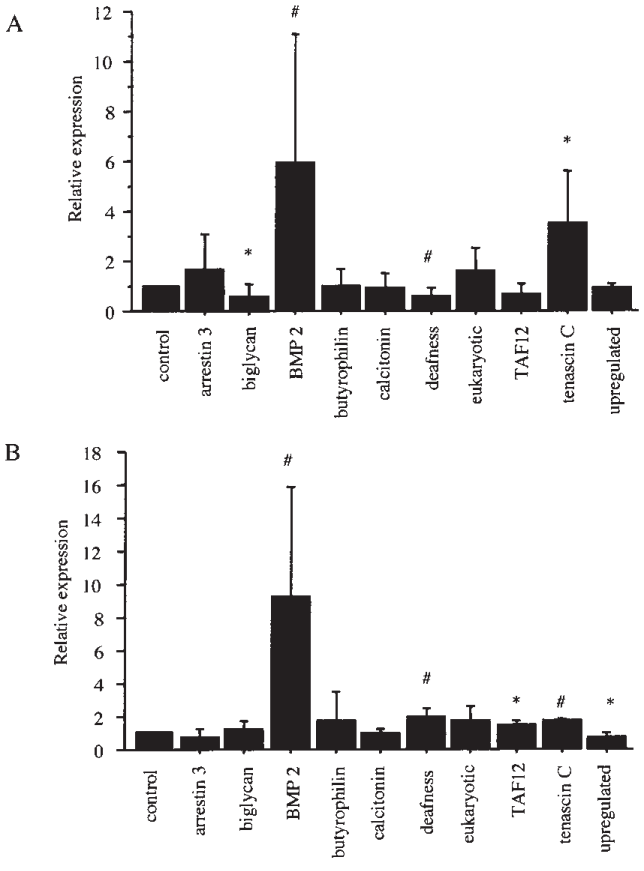


Figure 1. Gene expression due to MK-7 administration after (A) 1 h and (B) 6 h using real-time PCR. The expression levels were normalized to the value of GAPDH mRNA and represent relative expression. #p<0.10, *p<0.05 (Mann-Whitney test).

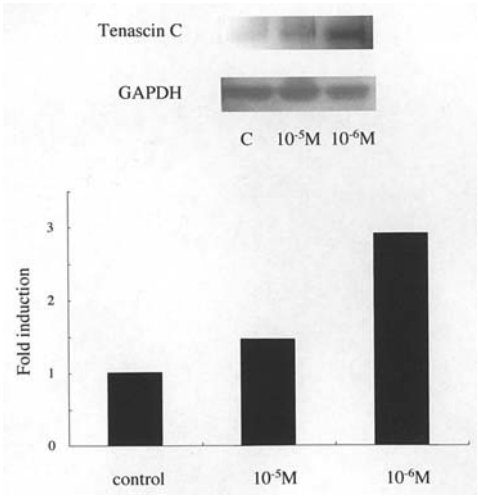


Figure 2. Effect of MK-7 on tenascin C expression by Western blot analysis. The expression levels were normalized to the value of GAPDH and represent relative expression.

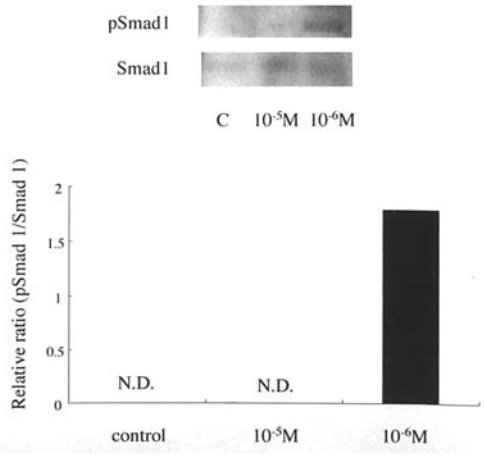


Figure 4. Effect of MK-7 on phosphorylation of Smad1 by Western blot analysis. The expression levels were normalized to the value of Smad1 and represent relative expression.

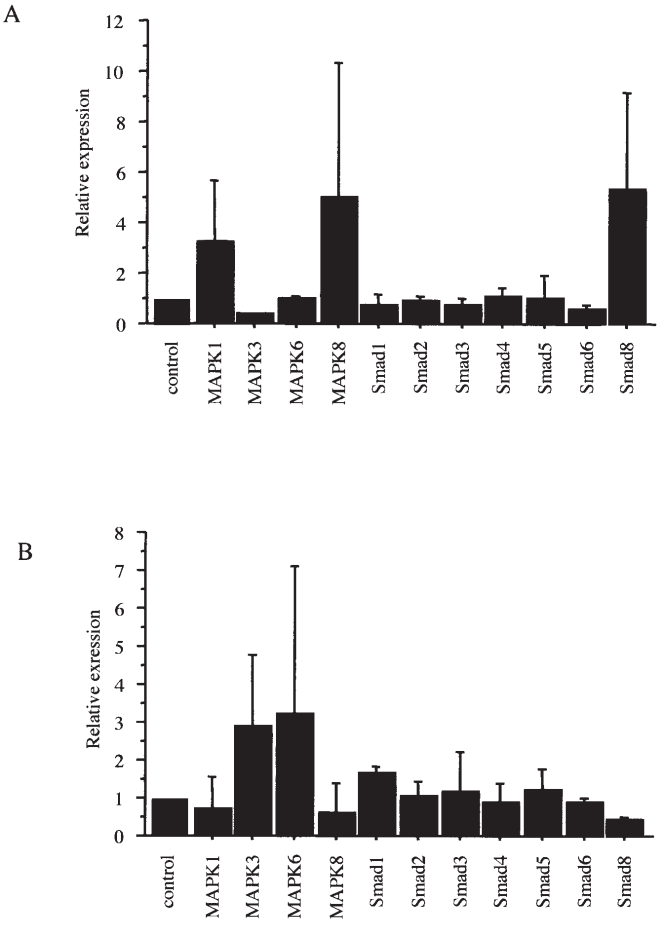


Figure 3. Effects of MK-7 on signal transduction system by real-time PCR. Gene expression due to MK-7 administration after (A) 1 h and (B) 6 h. The expression levels were normalized to the value of GAPDH mRNA and represent relative expression. No statistical differences were observed using the Mann-Whitney test.

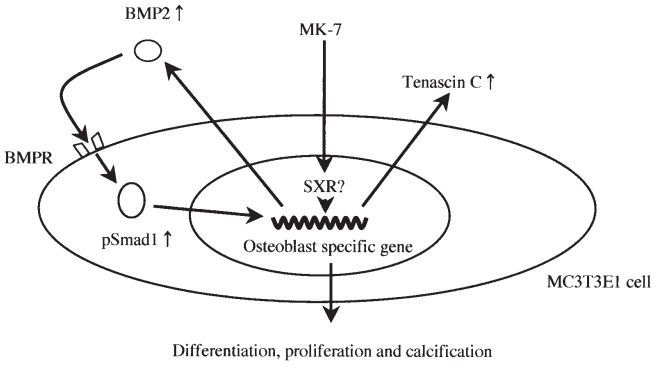


Figure 5. Effect of MK-7 on osteoblastic MC3T3E1 cells.

*Statistical analysis*. The gene expression results are shown as means ± SD. In order to compare the differences between control and various gene expression, the Mann-Whitney test was employed. All statistical analyses were performed using StatView (v. 5.0; SAS Corp., CA, USA).

## Results

*MK-7-induced gene expression using SSH*. We employed the suppression subtractive hybridization (SSH) method to quantify the differences between control and MK-7-treated MC3T3E1 cell cDNAs. Ninety-three genes were obtained by SSH. Dot blot analysis was further performed in order to confirm the differences in gene expression (data not shown). A total of 11 clones were randomly selected from the library and their cDNA inserts were partially sequenced and compared with GenBank sequences by using the BLAST algorithm. We selected 9 genes of interest (Table II), 6 of which were upregulated by MK-7 administration and 3 of which were downregulated.

*Confirmation of differential gene expression using real-time PCR*. To confirm the differential expression of the candidate genes detected by SSH, we performed real-time PCR using specific primers as described in Materials and methods. As shown in Fig. 1, the relative expression of *tenascin C* 1 h after MK-7 administration was more than three times higher than that of the control. The expression of *BMP2* also showed a

marked increase relative to the control, even though no statistical difference was observed. On the other hand, the relative expression of *biglycan* was ~one-half that of the control. After 6 h of MK-7 administration, the *TAF12* gene was ~2 times that of the control, and the *upregulated during skeletal muscle growth 5* gene was ~one-half of the control. Genes for *BMP2*, *deafness*, and *tenascin C* had tendencies to increase compared to the control. These results indicated that MK-7 might induce tenascin C expression.

*MK-7 administration correlated with tenascin C expression using Western blot analysis*. We next examined whether MK-7 administration to MC3T3E1 cells correlated with tenascin C expression. When MC3T3E1 cells were cultured with $10^{-5}$ M MK-7, tenascin C expression was ~1.5 times higher than that of the control (Fig. 2). Moreover, when $10^{-6}$ M MK-7 was used, tenascin C expression was ~three times higher than that of the control. This result indicated that MK-7 is correlated with MC3T3E1 cell function.

*Effects of MK-7 administration on signal transduction system*. Because the expression of the BMP2 gene was upregulated by MK-7 administration, as shown in Fig. 1, upregulation of BMP2 might affect the intracellular signal transduction system in MC3T3E1 cells. Administration of MK-7 did not affect the gene expression of MAPKs and Smads either at 1 h (Fig. 3A) or 6 h (Fig. 3B), according to real-time PCR.

In order to clarify whether MK-7 was related to phosphorylation of intracellular molecules, the expression level of phosphorylated Smad1 was investigated using Western blot analysis (Fig. 4). After administration of $10^{-6}$ M MK-7, phosphorylated Smad1 was detected. On the contrary, phosphorylated Smad1 was undetected in either control or cells cultured with $10^{-5}$ M MK-7.

## Discussion

The differential expression between genes of menaquinone-7 (MK-7)-treated cells and control cells helps us understand the molecular basis of the MK-7 pathway. MK-7 is vitamin $K_2$ with seven isoprene units, and is essential for the γ-carboxylation of osteocalcin (11). Among the 14 isomers of vitamin $K_2$, MK-4 most potently enhanced mineralization *in vitro* (4). When experimental diets containing MK-7 alone were fed to ovariectomized rats, both MK-4 and MK-7 accumulated in the femoral bone and significantly increased the Ca concentration in the femur (12). Soybeans fermented by *Bacillus natto* (Natto), a traditional Japanese food, contain >100 times more $VK_2$ (mainly MK-7) than various cheeses (5) and are recognized to be a healthy food. In addition to γ-carboxylation, MK-7 may affect the cellular function of osteoblasts (13). In the present study, we clarified the gene expression of MK-7 administration using SSH.

Although we found nine genes that showed a significant or marked change 24 h after MK-7 administration using SSH, real-time PCR showed that only tenascin C expression was significantly higher than in the control. Western blot analysis supported this result. Since the steroid and xenobiotic receptor (SXR) exists in the nucleus and mediates vitamin $K_2$-activated transcription of extracellular matrix-related genes (8), expression of tenascin C may be associated with this pathway (Fig. 5). Five tenascin proteins (tenascin-C, -R, -W, -X, and -Y) have been found, among which tenascin cytotactin (tenascin C) plays a major role in development, tissue remodeling, and cell adhesion activity (14). As tenascin C promotes osteoblast differentiation (15), its presence may be important in bone remodeling (16). Tenascin C is secreted by osteoblasts but absent from mineralized bone (15). Although tenascin C inhibits cell adhesion to fibronectin by the suppression of focal adhesion kinase (17), endogenous tenascin C plays an important role in maintaining the morphology, state of differentiation, and proliferation of osteoblasts (15). Thus, MK-7 administration may contribute to the differentiation of osteoblastic MC3T3E1 cells. Since transforming growth factor-ß (TGF-ß) regulates the expression of tenascin C (18), mRNA expression of bone morphogenetic protein 2 (BMP2), which belongs to the TGF-ß superfamily, was further investigated. Real-time PCR results showed a marked increase of BMP2 mRNA expression at both 1 h and 6 h after administration of MK-7. Injection of BMP2 locally over the surface of calvariae of mice induces periosteal bone formation on the surface of the calvariae without a prior cartilage phase (19). BMP2 interacts with the fibroblast growth factor 4 and sonic hedgehog, inhibits limb bud expression, and induces the formation of chondrocyte and osteoblast precursors (20,21). When BMP2 binds to its specific receptors (BMPR-I and -II), it activates the intracellular molecules Smad1, 5 and 8 (1). Phosphorylated Smad1, 5 and 8 proteins form a complex with Smad4 and then are translocated into the nucleus where they interact with other transcription factors. In the present study, phosphorylated Smad1 was observed when cells were cultured with $10^{-6}$ M MK-7. As MK-7 administration may activate the BMP2 pathway, this pathway might cause the upregulation of tenascin C expression. The BMP2 pathway showed an autocrine-like effect when MK-7 was administered to MC3T3E1 cells (Fig. 5).

In conclusion, MK-7 activated the BMP2-Smad1 pathway through production of BMP2. Moreover, tenascin C expression might be involved in this pathway. Although the biological effect of MK-7 is weaker than that of MK-4, MK-7 alone may affect cellular function and induce bone mineralization. We can absorb MK-7 from food, so that MK-7 may contribute to bone health. Further studies are necessary in order to clarify the relationship between MK-7 administration and its nuclear receptor SXR and downstream molecules that act as transcription factors.

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