Effect of Retinoic Acid on Murine Preosteoblastic MC3T3-E1 Cells

Hiroyuki NAGASAWA, Shu TAKAHASHI, Akira KOBAYASHI1, Hiroshi TAZAWA, Yohtalou TASHIMA1 and Kozo SATO

Department of Orthopedic Surgery and 1Department of Biochemistry, Akita University School of Medicine, 1–1–1 Hondo, Akita 010–8543, Japan
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Summary Retinoic acid (RA) plays an important role in bone metabolism in vivo through osteoclast activation and bone resorption. Retinoid X-activated receptor β (RXRβ) has been implicated in the genetic spinal defect of ossification of the posterior longitudinal ligament (OPLL). In this study, we examined the effects of 9-cis RA and all-trans RA (ATRA) on the proliferation, differentiation, and RXRβ expression of the murine preosteoblastic cell line MC3T3-E1. Both 9-cis RA and ATRA dose-dependently inhibited the increase in total soluble protein content at concentrations of 10 and 100 nM after 4 and 8 d co-culture with MC3T3-E1 cells. The inhibitory effect of 9-cis RA was slightly stronger than that of ATRA. Histone H4 mRNA expression was dose-dependently suppressed by both RAs on day 1. Alkaline phosphatase activity was increased by both RAs at 10 and 100 nM concentrations on day 4, with 9-cis RA-induced activity slightly stronger than that of ATRA. Osteopontin mRNA expression was increased by both RAs on day 1, but was suppressed on day 4. Bone Gla protein mRNA expression was inhibited by 10 and 100 nM 9-cis RA and by 100 nM ATRA on day 14. RXRβ mRNA expression was increased by 9-cis RA, an RXRβ ligand, in a dose-dependent manner. Our results suggested that while both RAs suppressed proliferation and stimulated the maturation of preosteoblastic MC3T3-E1 cells, 9-cis RA was slightly more potent than ATRA. It also appeared that RAs may contribute to the development of heterotopic ossification, including OPLL.

Key Words retinoic acid, osteoblast, differentiation, proliferation, retinoid X receptor

It is well known that retinoic acid (RA), an active metabolite of vitamin A, plays important roles in embryogenesis, cellular proliferation, and in the differentiation of many cell types including bone cells, such as osteoblasts, osteocytes, and osteoclasts. Many studies have reported the effects of RA on bone cell proliferation and differentiation (1, 2) and observed that retinoids, including RA, cause bone resorption by osteoclasts. This is based on both in vivo studies, including the finding that hypervitaminosis A leads to bone resorption (3), and in vitro studies, such as the demonstration of RA-induced osteoclastic bone-resorbing activity (4). However, retinoids are also thought to be involved in hyperostosis or the heterotopic ossification of ligaments, tendons, and joint capsules in humans (5, 6).

Ossification of the posterior longitudinal ligament (OPLL) of the spine, commonly occurring in the cervical spine, is the heterotopic ossification of the posterior longitudinal ligament that functions to stabilize the spine. Progressed and thickened OPLL compresses the spinal cord and can often lead to myelopathy and/or radiculopathy. While OPLL has been called "the Japanese disease" (7), being chiefly seen in Asians and especially in Japanese, clinical reports of OPLL have been received from throughout the world. Since 1975, a special study group organized by the Japanese Ministry of Health and Welfare has been charged to study the etiology, epidemiology, treatment, and quality of life issues associated with this disease. This group reported that spinal ligament cells derived from OPLL patients also contained mesenchymal osteoprogenitor cells that had differentiated into osteoblasts (8, 9). Moreover, Gazit et al. (10) reported that RA led to the differentiation of C3H10T1/2 cells (a mouse fibroblastic pluripotent mesenchymal stem cell line with the potential to differentiate in vitro into myoblasts, adipocytes, chondrocytes, and osteoblasts) into immature osteoblasts, but inhibited differentiation toward myoblasts and adipocytes. Thus, RA did not differentiate C3H10T1/2 stem cells into mature osteoblasts.

A link between vitamin A and OPLL or other skeletal changes in physiologically non-ossifying tissue has also been reported. Seawright et al. (11) reported that cats fed large amounts of vitamin A exhibited hyperostosis of the cervical spine along with high concentrations of vitamin A in plasma and the liver. Vitamin A and its metabolite RA used in the treatment of skin disorders such as psoriasis can also have the side effect of skeletal changes including OPLL (5, 12). Moreover, an OPLL patient who had not been treated with RA had high levels of serum retinol and retinol-binding protein (13). In vivo studies, retinyl palmitate administered to the abdominal cavity in rats caused skeletal changes that...
involved spinal ligaments (14) and hyperostosis of the vertebral column has been observed in rats orally administered RA (15).

One of the genetic factors in OPLL is the α2(XI) collagen gene (COL11A2) located within the human leukocyte antigen (HLA) region on chromosome 6p (16, 17). The COL11A2 gene is located near the retinoid X-activated receptor β (RXRβ) gene, one of the nuclear receptors for 9-cis RA, in both humans and mice. Indeed, Vandenberg et al. (18) described these two genes as having a “head-to-tail” arrangement. RXRs (RXRα, β, and γ) are known to form heterodimers with other nuclear hormone receptors such as vitamin D and thyroid hormone receptor, and regulate bone and mineral metabolism (19–21).

These findings suggest that RA and at least one RA nuclear receptor may be related to heterotopic ossifications, including OPLL, and that RA may differentiate not only mesenchymal stem cells to immature osteoblasts but also preosteoblasts to osteoblasts. Therefore, we decided to investigate the effect of RA on the proliferation, maturation, and RXRβ mRNA expression of the murine preosteoblastic cell line MC3T3-E1 using the ligand of RXRβ, 9-cis RA, and all-trans RA (ATRA). To assess proliferation, total soluble protein content and histone H4 mRNA expression were measured, and to detect differentiation, alkaline phosphatase activity and mRNA expression levels of osteopontin (OPN) and bone Gla protein (BGP) were determined. MC3T3-E1 cells, established from normal newborn murine calvaria (22), are immature osteoblasts able to differentiate only into osteoblasts. This cell line has been widely used to study the proliferation and differentiation of osteoblasts and has been shown to act as target cells for ATRA (1) and to express RXRβ mRNA (2).

MATERIALS AND METHODS

Cell culture. MC3T3-E1 cells, established from normal newborn mouse calvaria, were a gift from Dr. Kumegawa at the Riken Cell Bank (RCB 1126). Cells were transferred to 25-cm² tissue culture flasks to assay for total soluble protein content and alkaline phosphatase (ALP) activity, and to 75-cm² flasks for total RNA isolation and slot blot analysis. Cells were plated at a density of 1.2×10⁴ cells/cm² and cultured in α-modified Eagle’s medium (α-MEM; Gibco BRL, Life Technologies, Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Sanko Jyun-yaku Co. Ltd., Tokyo, Japan), 50 μg/mL L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries Co., Tokyo, Japan), 5 mM β-glycerophosphate (Wako Pure Chemical Industries Co.), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a fully humidified atmosphere containing 5% CO₂. After 48 h preincubation, various concentrations of 9-cis RA or ATRA (Sigma Chemical Co., St. Louis, MO, USA) were added to fresh α-MEM. Both RAs were dissolved in ethanol and used at less than 0.1% in medium. Experiments using RAs were conducted in a darkened room. Culture medium containing RAs was exchanged every 48 h.

Measurement of total soluble protein contents and alkaline phosphatase (ALP) activity. After 24 h, 48 h, 4 d or 8 d, cells were washed three times with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) (pH 7.4; 137 mM sodium chloride, 8.1 mM sodium hydrogen phosphate, 2.68 mM potassium chloride, 1.47 mM potassium dihydrogen phosphate) and scraped into 1 mL 25 mM Tris-buffered saline (TBS) (pH 7.4; 137 mM sodium chloride, 2.68 mM potassium chloride, 25 mM Tris) containing 0.1% Triton X-100. Cells were then sonicated for 10 min using a cell disruptor (Bioruptor UCD-200TM, Cosmo Bio, Tokyo, Japan) and centrifuged at 1,000 × g for 10 min. Supernatants were collected and assayed for total soluble protein content and ALP activity. Total soluble protein content was measured using the BCA protein assay kit (Pierce Co., Rockford, IL, USA) and ALP activity was determined using p-nitrophenyl phosphate as a substrate, according to previously described methods (23, 24).

Total RNA isolation and probe synthesis by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated at each time-point using an acid-guanidium-phenol-chloroform extraction method (ISOGEN; Wako Pure Chemical Industries, Ltd.), and quantitated spectrophotometrically.

Digoxigenin (DIG)-labeled DNA probes were synthesized by RT-PCR. Reverse transcription reactions were performed in 20 μL reaction volumes containing 5 μg

Table 1. Primers used in RT-PCR.

| Primer sequence | Product length (bp) | Location | Accession No. |
|-----------------|---------------------|----------|--------------|
| Histone H4      | forward 5’TGG AGA GGA AAG GTG GGA AA3’ reverse 5’TGG GTG CTC GGT GTA GGA GA3’ | 225 | 264–283 | V00753 |
| OPN             | forward 5’TGG ACA TCA GAG CCA AAT GT3’ reverse 5’TAC AGG AAC AAA AGC AA3’ | 355 | 822–841 | X14882 |
| BGP             | forward 5’TCT GAC AAA GCC TTC ATG TC3’ reverse 5’AAA TAG TAG TCA TAG GTG AGA TGG C3’ | 199 | 106–125 | U11542 |
| RXRβ            | forward 5’CCA GTC ATG ATG TCT TCC AT3’ reverse 5’TTC TCA CGA CAC GAG TAG GT3’ | 332 | 220–239 | M84818 |
| G3PDH           | forward 5’ACC ACA GTC CAT GCC ATC AC3’ reverse 5’TCC ACC ACC CTG TCC TAT3’ | 452 | 566–585 | M32599 |

BGP, RXRβ and G3PDH primers were designed based upon previous studies (25–27).
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total RNA, 50 mM Tris-HCl (pH 8.3), 75 mM potassium chloride, 3 mM magnesium chloride, 0.5 mM each dNTP, 0.09U random primers (Gibco BRL, Life Technologies, Inc.), 10 mM dithiothreitol, and 200 U SuperScript II reverse transcriptase (Gibco BRL, Life Technologies, Inc.). Resultant cDNA was used as templates for the PCR amplification of mouse histone H4, osteopontin (OPN), bone Gla protein (BGP), RXRβ and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Table 1). PCR was carried out in 100 μL reaction volumes using PCR DIG Labeling Mix (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and incubated in a GeneAmp PCR System 2400-R (Perkin-Elmer Co., NJ, USA) thermocycler. Reaction mixtures consisted of 1 μL of cDNA template, 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 200 μM each dATP and dCTP, 190 μM dGTP, 10 μM DIG-11-dUTP, 0.4 μM each forward and reverse primer, and 1 U TaKaRa Taq DNA polymerase (TaKaRa Inc., Shiga, Japan). PCR cycles used were 30 cycles of 94°C for 45s, 50–58°C (depending on primer set used) for 45s, and 72°C for 1 min. After 30 cycles, reactions were incubated at 72°C for 7 min, followed by cooling to 4°C. Annealing temperatures used were 54°C for histone H4, 50°C for OPN, 52°C for BGP, 55°C for RXRβ, and 58°C for G3PDH.

Slot blot analysis. Aliquots of 20 μg total RNA were dissolved in 10 μL H2O to which was added 20 μL formamide, 7 μL formaldehyde, and 2 μL 20×SSC (150 mM sodium chloride, 15 mM sodium citrate), and denatured by heating at 95°C for 15 min. After the addition of 2 volumes 20×SSC, samples were transferred onto nylon membranes (Boehringer Mannheim GmbH Biochemica) using a Slot Blot Filtration Manifold (PR 600, Hoefer, CA, USA) and washed in 10×SSC. After UV-cross-linking, membranes were prehybridized in standard prehybridization buffer (Boehringer Mannheim GmbH Biochemica) at 50°C for 1 h. Hybridization to DIG-labeled DNA probes (final concentration 200 ng/mL) was performed at 50°C for 16 h, after which membranes were washed (5 min×2) at room temperature and at 68°C (15 min×2) in a DIG wash and block buffer set (Boehringer Mannheim GmbH Biochemica) according to the manufacturer’s protocol. Membranes were then incubated with ALP-labeled anti-DIG antibody (1:10,000 dilution) and hybridized products visualized by CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2’-(5’-chlooro)tricyclo[3.3.1.1³,7]decan}-4-yl) phenyl phosphate) and exposure to X-ray film (Konica Co., Tokyo, Japan). Each mRNA level was normalized against G3PDH mRNA. The probes used did not produce non-specific bands when applied to Northern blot analysis.

Statistical analysis. Statistical significance (p<0.05) was determined by analysis of variance (ANOVA).

RESULTS

Total soluble protein content in MC3T3-E1 cells

As shown in Fig. 1, total soluble protein levels were not altered after 24 h regardless of treatment. However, both 9-cis RA and ATRA dose-dependently suppressed MC3T3-E1 cell proliferation. After 8 d of culture, both RAs at 1,000 nM concentration strongly suppressed the viability of MC3T3-E1 cells such that the cells could not reach confluency (data not shown). At 100 nM concentrations, the inhibitory effect of 9-cis RA was slightly stronger than that of ATRA on days 4 and 8.

Effect of 9-cis RA and ATRA on histone H4 mRNA expression

Histone H4 mRNA expression was measured on day 1 as an indicator of cell proliferation, based on previous reports (28–31) (Fig. 2). Both 9-cis RA and ATRA inhibited cell proliferation at concentrations higher

Fig. 1. Effect of RAs on total soluble protein content in MC3T3-E1 cells. Cells were inoculated into 25-cm² flasks and cultured alone or with 9-cis RA or ATRA for the indicated periods. 9-cis RA and ATRA were added at concentrations of 1, 10 and 100 nM. Cells were recovered in 1 mL TBS containing 0.1% Triton X-100, and assayed for protein concentration after sonication and centrifugation, as described in Materials and Methods. Results are expressed as mean±SE of three independent samples. * p<0.05, ** p<0.01, compared to controls.
than 10 nM. While 9-cis RA at 10 nM suppressed histone H4 mRNA expression, the same concentration of ATRA had no significant effect.

**ALP activity in MC3T3-E1 cells**

Neither RA at 1 nM concentration affected ALP activity relative to controls. Whereas 10 nM 9-cis RA enhanced ALP activity roughly 1.7-fold at day 2, the same concentration of ATRA did not increase ALP activity compared with controls throughout the experiment. The increased ALP activity enhanced by 10 nM 9-cis RA dropped to 1.2-fold of controls by day 8. At 100 nM, both 9-cis RA and ATRA maximally enhanced

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**Fig. 2. Effect of RAs on histone H4 mRNA expression in MC3T3-E1 cells.** Cells were inoculated into 75-cm² flasks and cultured for 24 h with the indicated amounts of 9-cis RA and ATRA. Total RNA (20 μg) was used for slot blot hybridization analysis as described in Materials and Methods. Histone H4 mRNA band densities were calibrated with reference to G3PDH-specific bands. Results are expressed as mean±SE of three independent samples. *p<0.05, **p<0.01, compared to controls.

**Fig. 3. Effect of RAs on MC3T3-E1 cell ALP activity.** Cells were inoculated into 25-cm² flasks and cultured without or with 1, 10 or 100 nM 9-cis RA or ATRA for indicated periods. Cells were recovered in 1 mL TBS containing 0.1% Triton X-100 and assayed for ALP activity after sonication and centrifugation as described in Materials and Methods. Results are expressed as mean±SE of three independent samples. *p<0.05, **p<0.01, compared to controls.
Fig. 5. Effect of RAs on BGP mRNA expression in MC3T3-E1 cells. Cells were inoculated into 75-cm² flasks and cultured for 14 d with the indicated amounts of 9-cis RA and ATRA. Total RNA (20 µg) was used for slot blot hybridization analysis as described in Materials and Methods. BGP band densities were calibrated with reference to G3PDH-specific bands. Results are expressed as mean±SE of three independent samples. * p<0.01 compared to controls.

ALP activity after 4 d of culture, but inhibited ALP activity to roughly 50% of control levels by day 8. **

Effect of 9-cis RA and ATRA on OPN mRNA expression
Both 9-cis RA and ATRA stimulated OPN mRNA expression after 1 d of culture (Fig. 4A), but inhibited expression by day 4 (Fig. 4B), in a dose-dependent manner. This significant increase and then decrease of OPN mRNA levels on days 1 and 4, respectively, was also observed when using 1 nM of the RAs. While no differences between the stimulatory effects of 9-cis RA and ATRA were observed on day 1, the inhibitory effect of 9-cis RA at day 4 was stronger than that of ATRA. **

Effect of 9-cis RA and ATRA on BGP mRNA expression
BGP, a non-collagenous protein found in bone tissue and expressed by mature osteoblasts, can be used as a differentiation marker of mature osteoblasts (28). The effects of both 9-cis RA and ATRA were assessed on day 14, by which time the cell line was considered to have undergone maturation. Both RAs showed inhibitory effects on BGP mRNA levels, with the effect of 9-cis RA stronger than that of ATRA at the same concentrations (Fig. 5). While 10 nM 9-cis RA inhibited BGP mRNA expression, 100 nM ATRA was required to show a similar effect. At 1,000 nM, both 9-cis RA and ATRA decreased total RNA levels to such an extent that slot blot analysis was no longer feasible as an assay system. **

Effect of 9-cis RA and ATRA on RXRβ mRNA expression
mRNA expression of the 9-cis RA receptor RXRβ was enhanced after treatment with 100 nM 9-cis RA. This effect appeared after 6 h culture (Fig. 6A), and was more marked on day 2 (Fig. 6B). While 10 and 100 nM 9-cis RA enhanced RXRβ mRNA expression, treatment of MC3T3-E1 cells with 1, 10, or 100 nM ATRA suppressed RXRβ mRNA expression.

DISCUSSION
9-cis RA exhibits a stronger inhibitory effect than ATRA on MC3T3-E1 cell proliferation
Total soluble protein and histone H4 mRNA expression were used as markers of proliferation in pre-osteoblastic MC3T3-E1 cells. While the effects of ATRA on osteoblast proliferation are well known, no reports have examined the effects of 9-cis RA. The effects of both RAs (9-cis RA and ATRA) on histone H4 gene expression have not been previously investigated, either. Total soluble protein content increases proportionally to increased cell number (32) and DNA content, and is widely used as a marker of proliferation in MC3T3-E1 cells. In this study, both RAs dose-dependently inhibited rising total soluble protein levels in immature osteoblast cultures with latency periods of more than 2 d. This result is supported by a previous observation that proliferating MC3T3-E1 cells exhibit a two-phase growth curve, with a rapid increase within the first 3 d of culture, and then a second rapid increase after 6 d (29).
At concentrations higher than 10 nm, both 9-cis RA and ATRA suppressed the rise in total soluble protein content, with 9-cis RA showing a significantly stronger effect than ATRA. In contrast to protein content, there was no significant difference between the two RAs in terms of histone H4 mRNA expression on day 1. These results confirmed previous studies that assayed osteoblastic MC3T3-E1 cell proliferation by direct cell counts (29), [3H]thymidine incorporation (29, 30), and DNA content (31).

9-cis RA has a stronger inducing effect on MC3T3-E1 cell differentiation and maturation than ATRA

ALP activity and mRNA expression increase during the early and middle stages of MC3T3-E1 cell differentiation, but decrease during late stages as cells become mature osteoblasts (28, 33). Our findings supported the early- and mid-phase effects of RA, with increased ALP activity more prominent after treatment with 9-cis RA than with ATRA. However, both RAs at 1,000 nm strongly suppressed MC3T3-E1 cell viability and inhibited ALP activity, which suggested that excessive RA levels were pharmacologically toxic.

OPN mRNA expression was significantly enhanced by both RAs at low concentrations (1 nm). On day 4, the RAs became inhibitory, with 9-cis RA being more potent than ATRA. Williams et al. (34) reported that 9-cis RA and ATRA had no effect on OPN mRNA expression in ROS 17/2.8 cells. ROS 17/2.8 cells are mature osteoblasts, and it was reported that OPN, a non-collagenous protein found in bone matrix and expressed in osteoblasts and kidney (35), appeared during the middle stages of differentiation (33). As MC3T3-E1 cells are pre-osteoblastic, it is possible that RA may affect OPN mRNA expression only in premature-stage osteoblastic cells.

ATRA induces BGP synthesis in primary cultured human osteoblasts (36), but does not induce or suppress BGP expression in mature rat osteoblasts (34, 37). In the present study, 10 and 100 nm 9-cis RA and 100 nm ATRA significantly suppressed BGP mRNA expression on day 14, at which point the cells were considered to have progressed to full maturity.

While MC3T3-E1 cells have been reported to express both RARs and RXRs (2), the regulatory mechanisms that underlie OPN and BGP expression by RAs in osteoblasts remain unclear. A vitamin D responsive element (VDRE) is present upstream of the mouse OPN and BGP genes (38, 39) and it has been suggested that 9-cis RA inhibits the RXR-enhanced binding of VDR-RXR heterodimers to VDRE within the OPN and BGP gene promoters (19, 20).

RAR and RXR regulatory mechanisms mediated by RA in MC3T3-E1 cells

Retinoic acid receptor family members (RARs and RXRs) mediate ligand-dependent transcription. While both 9-cis RA and ATRA bind to RARs, only 9-cis RA binds to RXRs (21, 40), although RXRs can still bind high concentrations of ATRA (1 and 10 nm) (21). The present study indicated that 9-cis RA at concentrations greater than 100 nm increased RXRβ mRNA levels in as little as 6 h after treatment. While the autoregulatory mechanism of RARβ gene expression by 9-cis RA is unclear, the mechanism of RARβ regulation by ATRA has been described. Sucov et al. (41) showed that the RARβ gene promoter contained a retinoic acid response element (RARE), and that the binding of ATRA to RARE induced RARβ gene expression. As RXRs heterodimerize with RARs, it is possible that ATRA controls RXR mRNA expression via RAR expression.

Differences between 9-cis RA and ATRA

Our study investigated the effects of 9-cis RA and ATRA on the proliferation, differentiation/maturation, and RARβ mRNA expression of preosteoblastic MC3T3-E1 cells, and found that 9-cis RA exerted a more potent effect on MC3T3-E1 cells than ATRA. It is already well known that the different RAs can have different functions. For instance, 9-cis RA induces cellular RA binding protein II more efficiently than ATRA in the MG-63 human osteosarcoma cell line (42) and produces more C-type natriuretic peptide, a local regulator of proliferation and differentiation in MC3T3-E1 cells (2). 9-cis RA plays important roles in proliferation, differentiation and RXR mediated ligand-dependent transcription. However, little is known of 9-cis RA absorption and metabolism in mammals. The physiological concentrations of RAs in plasma are approximately 10 nm. While ATRA and 13-cis RA exist in roughly equal quantities, 9-cis RA levels in human serum tend to be very low. Therefore, in our study we used 1, 10 and 100 nm as the working concentrations of the RA isomers. RA is synthesized from β-carotene by oxidation, although reports differ as to the isomerization of the all-trans isomer to the 9-cis isomer. Mertz et al. (43) and Romert et al. (44) reported that isomerization occurs when all-trans retinol and 9-cis retinol are oxidized to 9-cis RA by 9-cis retinol dehydrogenase. However, Murayama et al. (45) reported the possibility of 9-cis RA isomerization from ATRA, while Parker (46) described 9-cis RA formation directly from 9-cis β-carotene.

RA and heterotopic ossification

Several studies investigating the relationship between vitamin A and heterotopic ossification, including OPPL, reported that heterotopic ossification occurred after the administration of retinoids in human patients and in animal models (5, 11, 12). High concentrations of serum vitamin A in OPPL patients have also been reported (13). The RXRβ gene is located close to the collagen a2(XI) gene, and is considered a possible candidate gene for OPPL (16–18). The present study revealed the possibility that bone-resorbing agents 9-cis RA and ATRA had the potential to differentiate preosteoblastic MC3T3-E1 cells into mature osteoblasts. This hypothesis is supported by a study by Gazit et al. (10) that demonstrated that mesenchymal stem cells were differentiated into immature osteoblasts by ATRA. Thus, the strong cell-differentiating effects of RA may contribute to heterotopic ossification disease, including OPPL of the spine. Since the regulatory effects of 9-cis
RA on preosteoblastic MC3T3-E1 cells were more potent than those of ATRA, the regulatory mechanisms for heterotopic ossification and osteoblastic differentiation might be associated not only with ATRA, but also with 9-cis RA and RXR. Further studies to elucidate the regulatory mechanisms of heterotopic ossification mediated by RA are required.

In conclusion, the present study showed that 9-cis RA and ATRA inhibited the proliferation and enhanced the maturation of preosteoblastic MC3T3-E1 cells at an early differentiation stage, and suggested that RA may contribute to the development of heterotopic ossification, including OPLL.

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