J Bone Miner Metab (2013) 31:53–63
DOI 10.1007/s00774-012-0385-x

ORIGINAL ARTICLE

Retinoic acid regulates commitment of undifferentiated mesenchymal stem cells into osteoblasts and adipocytes

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Received: 29 December 2011 / Accepted: 21 August 2012

Abstract Evidence indicates that the balance between osteoblastogenesis and adipogenesis of mesenchymal stem cells (MSCs) is regulated by several hormones, growth factors, and their downstream signaling cascades. Previous studies suggest that retinoic acid (RA) plays a role in osteoblastogenesis and adipogenesis. However, it is unknown whether RA regulates commitment of MSCs into osteoblasts and adipocytes. In this study, we investigated the role of RA in differentiation of MSCs using the C3H10T1/2 cell line. RA stimulated activity and expression of alkaline phosphatase (ALP) and upregulated activity of the ALP gene promoter. The effects of RA were further enhanced by bone morphogenetic protein 2 (BMP2) and resultant Smad signaling. Furthermore, overexpression of Runx2 and Msx2, critical transcription factors for bone formation and BMP2-dependent osteoblastogenesis, enhanced RA-dependent ALP activity. In view of these findings, RA likely stimulates osteoblast differentiation through the BMP2–Smad–Runx2/Msx2 pathway. In contrast, RA markedly inhibited BMP2-induced adipocyte differentiation, suppressing expression of peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein (C/EBP)α and C/EBPδ, and inhibiting adipogenic function of C/EBPβ, C/EBPδ, and PPARγ. In conclusion, our data suggest that RA regulates commitment of MSCs into osteoblasts and adipocytes by controlling transcriptional regulators.

Keywords Retinoic acid · Osteoblast · Adipocyte · Bone morphogenetic protein

Introduction

Pluripotent mesenchymal stem cells (MSCs) can differentiate into osteoblasts, adipocytes, chondrocytes, and myoblasts in response to various hormones, growth factors, and cytokines [1–3]. Recent studies suggest that regulation of the differentiation of MSCs into osteoblasts and adipocytes is tightly controlled and required for maintenance of appropriate bone mass [4]. In metabolic bone diseases such as osteoporosis, adipocyte numbers often increase in association with decreased bone volume, consequently resulting in production of fatty marrow [5]. Bone morphogenetic protein (BMP) is a powerful cytokine involved in promoting differentiation of MSCs into osteoblasts in vitro and inducing bone formation in vivo [6–8]. More than 15 members of the BMP family have been identified, and BMP2 has been shown to strongly induce osteogenesis in vitro as well as in vivo [9]. Smad1, -4, and -5 are known to mediate intracellular signaling and consequently stimulate osteogenic actions of BMP2 [10]. After BMP2-receptor binding, Smad1 and -5 are phosphorylated and bind to Smad4 before translocation into the nuclei [10]. In the nuclei, Smad1, -4, and -5 regulate expression and function

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of transcription factors essential for bone formation, including Runx2, Msx2, and Osterix [11–15]. Similarly, adipogenesis is regulated by transcription factors CCAAT/enhancer-binding protein (C/EBP) family members and peroxisome proliferator-activated receptor-γ (PPARγ) [16, 17]. Overexpression of C/EBPβ in MSCs induces peroxisome PPARγ and adipocyte differentiation in cooperation with C/EBPδ [16].

Several cytokines and transcription factors are reported to be involved in regulating the balance between osteoblast and adipocyte differentiation. BMP2 stimulates both osteoblastogenesis and adipogenesis [18]. Canonical Wnt family members, including Wnt3a, stimulate osteoblast differentiation through the β-catenin/LEF signaling pathway and inhibit adipocyte differentiation by suppressing PPARγ and C/EBPα mRNA expression [19–21]. A recent study indicated that Wnt5a, a member of the noncanonical Wnt family, increases osteoblast differentiation with induction of Runx2; however, Wnt5a suppresses adipocyte differentiation and PPARγ function by regulating histone methyltransferase [22]. The inflammatory cytokine tumor necrosis factor-α (TNFα) prevents adipogenesis by inhibiting PPARγ function [23, 24] and inhibits bone formation and osteoblastogenesis through suppression of Runx2 [25]. The homeobox gene, Msx2, stimulates osteogenesis [26–29] but suppresses adipogenesis [28, 29]. In addition, an isoform of C/EBPβ, LIP, which lacks a transcriptional activation domain, inhibits adipocyte differentiation but stimulates osteoblast differentiation [30]. Furthermore, TAZ has been identified as a transcriptional modulator of osteoblastogenesis and adipogenesis [31]. Despite these findings, the regulatory mechanism for commitment of MSCs into either the osteoblast or adipocyte lineage remains to be elucidated.

Retinoic acid (RA) has been reported to play roles in osteoblast differentiation [32] and conversion of pre-adipocytes into osteoblasts [33]. In addition, RA has been shown to stimulate osteoblast differentiation in cooperation with ascorbic acid [34]. Interestingly, opposite effects of RA and glucocorticoid on osteoblast differentiation have been shown [35]. Similarly, RA and glucocorticoid showed different effects on expression of osteoblast gene markers [36]. On the other hand, RA has been reported to suppress adipogenesis and C/EBPβ function [37]. These findings suggest that RA might be involved in commitment of pluripotent MSCs. To date, however, the role of RA in MSC differentiation and the associated molecular mechanisms remain elusive.

To understand the functional role of RA in MSC differentiation, we studied the relationship between RA and transcription factors that are critical for osteoblastogenesis and adipogenesis. We found that RA stimulates osteoblast differentiation through interaction with the BMP2–Smad–Runx2/Msx2 signaling pathway. In contrast, RA markedly inhibits adipogenesis by suppressing C/EBP family members and PPARγ. These findings provide new insight into the understanding of the molecular mechanisms regulating MSC differentiation into osteoblasts and adipocytes.

Materials and methods

Cells and reagents

The 293, C3H10T1/2, and C2C12 cells were purchased from Riken Cell Bank (Tsukuba, Japan) and cultured in modified α-minimum Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS). RD-C2 cells isolated from the calvariae of Runx2-deficient mice were also purchased from Riken Cell Bank. 3T3-F442A cells, a kind gift from Dr. Minoru Morikawa (Chugai Pharmaceutical), were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % FBS. All trans-RA, Troglitazone, and insulin were purchased from Sigma-Aldrich. BMP2 was generated and used as previously described [29, 30]. Anti-PPARγ and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Constructs and transfection

CCAAT/enhancer-binding protein β and C/EBPδ cDNA were a kind gift from Dr. Shizuo Akira (Osaka University). Runx2 cDNA was a gift from Dr. Yoshiaki Ito (Kyoto University). PPARγ expression vector and aP2 promoter luciferase constructs were a kind gift from Dr. Bruce M. Spiegelman (Dana Faber Cancer Institute) [38]. Luciferase construct containing tandem repeats of BMP-responsible Smad-binding element (SBE) were provided by Dr. Takeshi Imamura (Cancer Institute). Alkaline phosphatase (ALP) luciferase reporter constructs (−1,313 and −724 bp) were used as previously described [39]. An ALP luciferase reporter construct lacking a putative RAR-binding element (RARE) was generated by digestion with *Bst*EII, which is present in the element, followed by treatment with Mung Bean Nuclease (New England Biolabs) for 30 min. Deletion of the element was confirmed by DNA sequence analysis. All expression vectors were purified using a High Speed Midi Kit (Qiagen, Valencia, CA, USA). Transfection was performed using Fugene6 (Roche, Indianapolis, IN, USA) or Superfection (Qiagen) according to the manufacturers' protocol.

Generation of adenovirus

Recombinant adenovirus carrying Smad1, Smad4, Runx2, Msx2, Osterix, C/EBPβ, C/EBPδ, constitutively active

BMP type 1a receptor, ALK3 mutant (Q233D), and PPARγ were constructed by homologous recombination between the expression cosmid cassette (pAxCAwt) and the parental virus genome in 293 cells using an adenovirus construction kit (Takara), as previously described [29, 30, 40, 41]. Titers of adenovirus were determined by a modified point assay [40]. Infection of C3H10T1/2, C2C12, and 3T3-F442A cells was performed by incubation with adenovirus at a multiplicity of infection of 50.

Western blotting

Cells were washed four times with ice-cold phosphate-buffered saline (PBS) and solubilized in lysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM ethyleneglycoltetraacetic acid (EGTA), 1.5 mM $MgCl_2$, 10 % glycerol, 1 % Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.2 mM sodium orthovanadate). Lysates were centrifuged at 16,000 *g* for 20 min at 4 °C. Samples were treated with sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and reacted with anti-PPARγ antibody before reaction with horseradish peroxidase-conjugated protein A or G (Jackson Laboratory, Bar Harbor, ME, USA). Luminescence signals were amplified using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposed using X-ray film (Kodak, New York, NY, USA).

Luciferase assay

Cells transfected with firefly luciferase reporter construct and TK-Renilla luciferase expression vector were lysed and cultured for 48 h. Lysates were measured using a luminometer (Promega, Madison, WI, USA). Transfection efficiency was normalized by determining the activity of *Renilla* luciferase. Data represent fold differences compared with control group.

Measurement of ALP activity

Cells were washed three times with PBS and lysed by freeze-thawing twice in 0.05 % Triton X-100 solution. Lysates were incubated with substrate solution (1 mM sodium *p*-nitrophenylphosphate hexahydrate, 10 mM $MgCl_2$, 2.5 mM 2-aminomethyl-2-methyl-1 propanol) for 1 h at 37 °C, and ALP activity of the lysates was measured on a micro plate reader (BioRad, Hercules, CA, USA) at 405-nm absorbance using *p*-nitrophenol (1 µmol/ml) as standard. ALP activity was normalized against protein content using the Bradford method.

Staining of ALP activity in the cells

Cells were washed twice with PBS, fixed in 3.7 % neutral formalin solution (Wako Pure Chemical Industries), and incubated with 100 mM Tris–HCl buffer (pH 9.5) containing 330 ng/ml nitro blue tetrazolium (Sigma), 165 ng/ml bromochloro-indolyl phosphate (Sigma), 100 mM NaCl, and 5 mM $MgCl_2$ at 37 °C.

Oil red O staining

Cells were washed twice with PBS and fixed with 10 % formalin-PBS. The cells were washed with 60 % isopropyl alcohol solution and stained with 0.5 % oil red O solution for 20 min. The area of oil red O-positive cell staining was measured using ImagePro Plus software (Palmetron).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells using the RNeasy Kit (Qiagen). Total RNA was denatured at 70 °C for 10 min and reverse-transcribed using Oligo-dT primer and reverse transcriptase (Invitrogen) to synthesize cDNA. To determine expression levels of ALP, Osterix, bone sialoprotein (Bsp), and osteocalcin mRNA, cDNA samples were analyzed by real-time PCR using an ABI PRISM 7300 unit (Applied Biosystems) and specific TaqMan probes as follows: mouse Osterix (sense primer, 5′-AGCGACCACTTGAGCAAACAT-3′; antisense primer, 5′-GCGGCTGATTGGCTTCTTCT-3′; probe, 5′-CCCGACGCTGCGACCCTCC-3′); mouse osteocalcin (sense primer, 5′-GCAATAAGGTAGTGAACAGACTCC-3′; antisense primer, 5′-GTTTGTAGGCGGTCTTCAAGC-3′; probe, 5′-TGGAGCCTCAGTCCCCAGCCCA-3′); mouse ALP (sense primer, 5′-ATCTTTGGTCTGGCTCCCATG-3′; antisense primer, 5′-TTTCCCGTTCACCGTCCAC-3′; probe, 5′-TGAGCGACACGGACAAGAAGCCCTT-3′); mouse Bsp (sense primer, 5′-AAGCAGCACCGTTGAGTATGG-3′; antisense primer, 5′-CCTTGTAGTAGCTGTATTCGTCCTC-3′; probe, 5′-CGGTAAGTGTCGCCACGAGGCTCCC-3′); Msx2 (sense primer, 5′-CCATATACGGCGCATCCTACC-3′; anti-sense primer, 5′-CAACCGGCGTGGCATAGAG-3′; probe, 5′-AGACCTGTGCTCCCCATCCCGCC-3′); C/EBPα (sense primer, 5′-AGACGCAACAGAAGGTGCTG-3′; anti-sense primer, 5′-GTGTCCAGTTCACGGCTCAG-3′; probe, 5′-AGTTGACCAGTGACAATGACCGCCTGC-3′); C/EBPβ (sense primer, 5′-ACGACTTCCTCTCCGACCTC-3′; anti-sense primer, 5′-CGAGGCTCACGTAACCGTAG-3′; probe, 5′-CGCCAAGCCGAGCAAGAAGCCGG-3′); C/EBPδ (sense primer, 5′-AGACGCAACAGAAGGTGCTG-3′; anti-sense primer, 5′-GTGTCCAGTTCACGGCTCAG-3′; probe, 5′-AGTTGACCAGTGACAATGACCGCCTGC-3′). Expression levels of each sample were normalized by β-actin mRNA expression.

Knockdown of Msx2

Cells were transfected with Stealth small interfering RNA (siRNA) (Invitrogen) and Mission siRNA (Sigma) using Lipofectamine 2000 according to the manufacturer's protocol. Msx2 siRNA (siMsx2; 5′-CCGCCAGAAACAGUA CCUGUCCAUA-3′) was used for knockdown of Msx2. Control siRNA was purchased from Invitrogen.

Statistical analysis

All data were analyzed by a paired *t* test, Dunnett's test, or Tukey's test. Values are given as mean ± standard deviation.

Results

To determine whether RA is involved in regulating the commitment of MSCs into osteoblasts and adipocytes, we investigated the effect of RA on the C3H10T1/2 pluripotent MSC cell line, which differentiates into both osteoblasts and adipocytes. In agreement with the results of a previous study [42], we found that BMP2 stimulates osteoblast and adipocyte differentiation of C3H10T1/2 cells (Fig. 1a). RA also increased the activity of ALP, a well-known osteoblast marker, in C3H10T1/2 cells (Fig. 1a). In the presence of BMP2, RA was found to further enhance ALP activity (Fig. 1a). In contrast, RA was found to strongly inhibit BMP2-induced adipocyte differentiation (Fig. 1a). Similar effects were observed in the pre-adipocyte cell line, 3T3-F442A (Fig. 1b). Based on these findings, RA appears to be one of the important regulators for commitment of MSCs into osteoblasts and adipocytes.

To understand more precisely the role of RA in osteoblastogenesis, we examined its effect on expression of several osteoblast markers using real-time PCR analysis. Treatment with RA stimulated ALP expression in C3H10T1/2 cells (Fig. 2a). In addition, RA significantly enhanced ALP expression in the presence of BMP2 (Fig. 2a). Although RA showed significant increase in Bsp expression, RA had very little effect on expression of Osterix and osteocalcin in both the absence and presence of BMP2 (Fig. 2b, c, d). These results suggest that RA specifically controls ALP expression, but not the other investigated markers, during MSC osteoblast differentiation, even in the presence of BMP2.

It has been reported that a RARE is present in the ALP gene promoter region (Fig. 3a) [39]. RA was found to increase ALP gene promoter activity when using the −1,313-bp ALP gene promoter luciferase construct containing a RARE (Fig. 3b, c). In contrast, RA failed to increase luciferase activity of the −724-bp ALP gene promoter construct (Fig. 3b), shown previously in SaOS-2 osteosarcoma cells [39]. In addition, RA did not increase luciferase activity of the −1,313-bp Del ALP gene promoter construct lacking a RARE in the −1,313-bp ALP gene promoter construct (Fig. 3a, c). These data indicate that RARE is also critical for ALP gene promoter activity in undifferentiated MSCs. Investigation into the relationship between RA and BMP2 in regulating the ALP gene promoter identified that RA and BMP2 cooperatively increased ALP gene promoter activity (Fig. 3b). Consistently, a constitutively active mutant BMP receptor (ALK3QD) increased the −1,313-bp ALP gene promoter luciferase activity and enhanced the activity in the presence of RA (Fig. 3c). However, the cooperative effect between RA and BMP2 signaling was not observed in the −724-bp ALP gene promoter (Fig. 3b) and −1,313-bp ALP Del gene promoter (Fig. 3c). To understand further the cooperative effects of RA and BMP2 signaling on regulating ALP gene expression, we examined the relationship between RA and BMP2 signaling. As shown in Fig. 3d, ALK3QD markedly stimulated Smad signaling, determined using a luciferase construct containing SBE. Interestingly, RA enhanced the effect of the mutant BMP receptor on Smad activity, although RA itself had no effect on SBE-luciferase activity (Fig. 3d). In addition, RA enhanced ALP activity induced by overexpression of Smad1 and 4 (Fig. 3e). RA and Smad1/4 consistently and synergistically stimulated ALP gene promoter activity (Fig. 3f). These findings suggest that RA stimulates osteoblast differentiation through functional interaction with Smad1 and -4.

To understand further the relationship between RA and BMP2 signaling in regulating ALP gene expression, we examined the effect of RA on BMP2-regulated osteoblastogenic transcription factors, including Runx2, Msx2, and Osterix [3, 26, 27, 43–46]. As shown previously [12, 40], overexpression of Runx2 increased ALP activity and RA further enhanced Runx2-induced ALP activity (Fig. 4a). RA also modestly increased ALP activity in the presence of Msx2 (Fig. 4b). In contrast, RA did not clearly increase the ALP activity when Osterix was overexpressed (Fig. 4c). These results suggest that RA stimulates osteoblast differentiation in association with Runx2 and Msx2, but not with Osterix. To support this, RA treatment or overexpression of Smad1/4 stimulated ALP activity in Runx2-deficient RD-C2 cells in which Msx2 remains functional [41] (Fig. 4d). In addition, overexpression of Smad1/4 further increased ALP activity in the presence of RA (Fig. 4d). To examine further the role of Msx2 and RA in the regulation of ALP gene expression, we performed knockdown experiments of Msx2 in Runx2-deficient RD-C2 cells. Knockdown of Msx2 using siRNA clearly

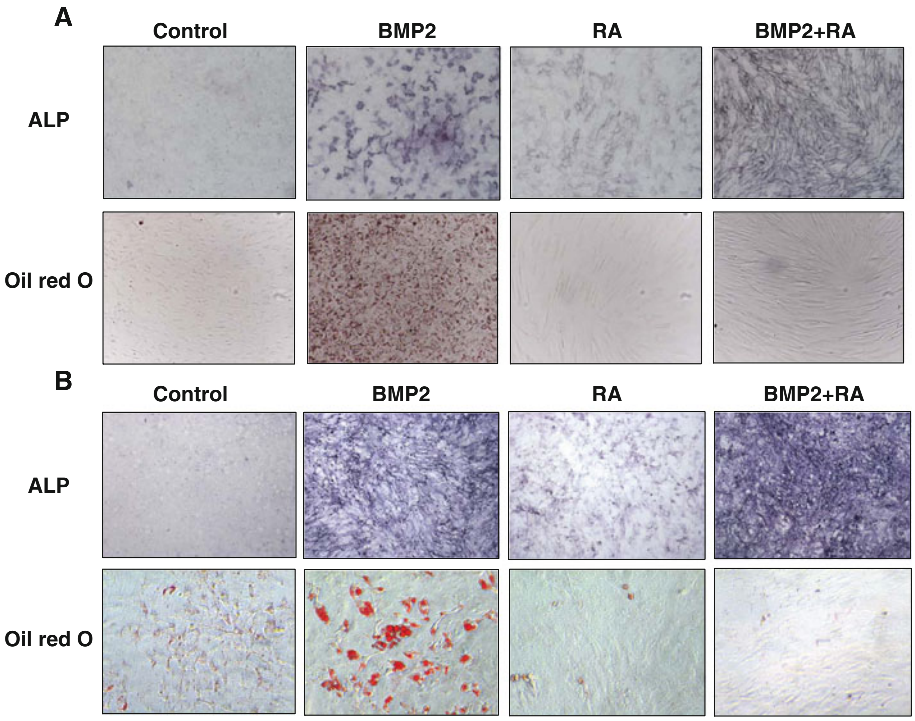

Fig. 1 Effect of retinoic acid (*RA*) on osteogenic and adipogenic differentiation of precursor cells. C3H10T1/2 (**a**) or 3T3-F442A cells (**b**) were cultured in the presence or absence of bone morphogenetic protein (*BMP*)2 (300 ng/ml) and/or RA (10^{-6} M) for 7 days before alkaline phosphatase (*ALP*) or *oil red O* staining

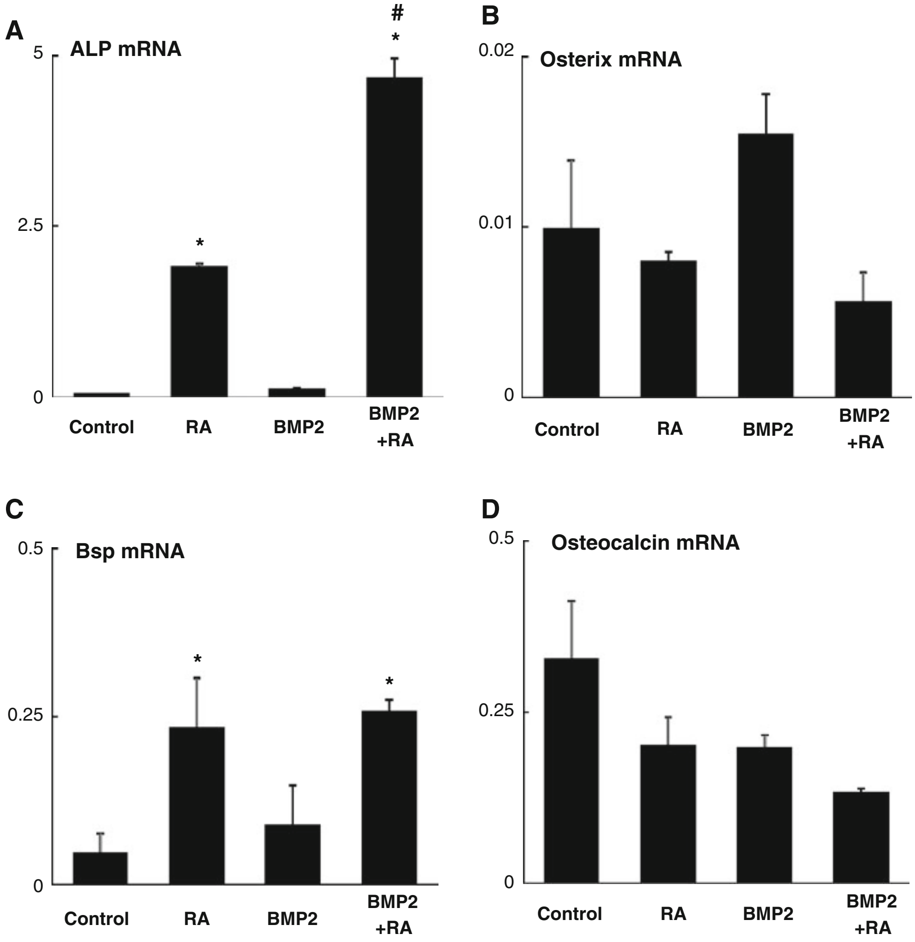

Fig. 2 Effects of RA on osteoblastogenic markers. C3H10T1/2 cells were cultured in the presence of BMP2 (300 ng/ml), RA (10^{-6} M), or a combination of both for 3 days. Real-time polymerase chain reaction (PCR) was used to analyze gene expression of ALP (**a**), Osterix (**b**), bone sialoprotein (*Bsp*) (**c**), and osteocalcin (**d**), normalized against β-actin expression. *$P < 0.01$ vs. *Control* by Dunnett's; $^{\#}P < 0.01$ vs. RA and BMP2 groups by Tukey's

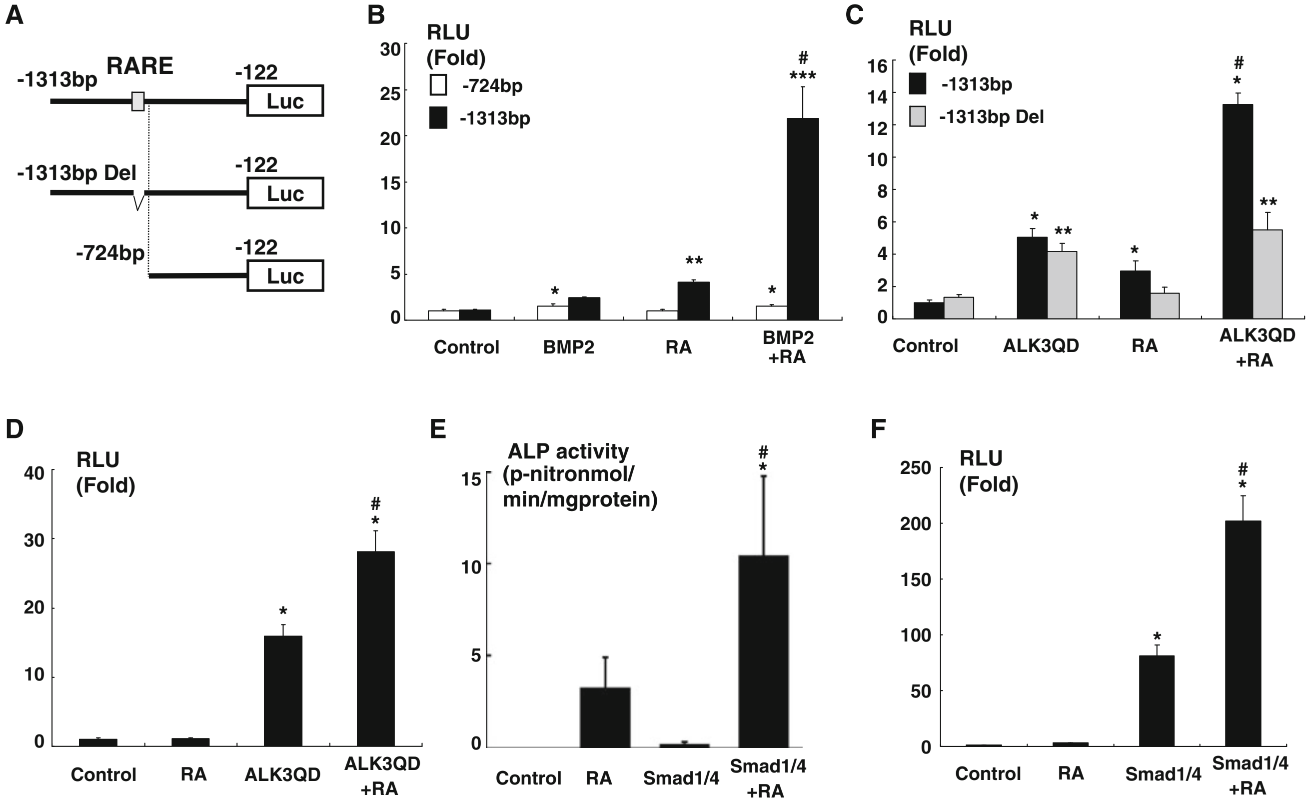

Fig. 3 Relationship between RA and BMP signaling. **a** Schematic of the ALP gene promoter constructs. *RARE* RA receptor-binding element, *Del* deleted RARE, *RLU* relative luminescence units. **b** C3H10T1/2 cells were transfected with the ALP gene promoter luciferase (*luc*) constructs, −1,313 or −724 bp, and cultured with BMP2 (300 ng/ml) and RA (10^{-6} M) as indicated for 2 days. Luciferase activity of the lysates was determined. $*P < 0.01$ vs. Control (−724 bp) by Dunnett's; $**P < 0.05$ vs. Control (−1,313 bp) by Dunnett's; $***P < 0.01$ vs. Control (−1,313 bp) by Dunnett's; $^{\#}P < 0.01$ vs. BMP2 (−1,313 bp) and RA groups (−1,313 bp) by Tukey's. **c** C3H10T1/2 cells were transfected with the ALP gene promoter luciferase constructs, −1,313 or −1,313 bp Del, together with either pcDNA3 (Control) or ALK3QD expression vector, before culture in RA (10^{-6} M) as indicated for 2 days. Luciferase activity of the lysates was determined. $*P < 0.01$ vs. Control (−1,313 bp) by Dunnett's; $**P < 0.05$ vs. Control (−1,313 bp Del) by Dunnett's; $^{\#}P < 0.01$ vs. ALK3QD (−1,313 bp) and RA groups (−1,313 bp) by Tukey's. **d** C3H10T1/2 cells were transfected with a luciferase reporter construct containing Smad-binding elements together with either pcDNA3 (control) or ALK3QD expression vector before culture in the presence or absence of RA (10^{-6} M) for 2 days. Luciferase activity of the lysates was determined. $*P < 0.01$ vs. Control by Dunnett's; $**P < 0.01$ vs. RA and ALK3QD groups by Tukey's. **e** C3H10T1/2 cells were infected with control adenovirus or both Smad1 and Smad4 adenoviruses before culture in the presence or absence of RA (10^{-6} M) for 7 days. ALP activity in the cells was measured. $*P < 0.01$ vs. Control by Dunnett's; $^{\#}P < 0.01$ vs. RA and Smad1/4 groups by Tukey's. **f** C3H10T1/2 cells were transfected with the ALP gene promoter fused to the luciferase reporter construct containing a RARE region (−1,313 bp) together with pcDNA3 (control) or both Smad1 and Smad4 expression vectors. Cells were then cultured in the presence or absence of RA (10^{-6} M) for 2 days. Luciferase activity of the lysates was determined. $*P < 0.01$ vs. Control by Dunnett's; $^{\#}P < 0.01$ vs. RA and Smad1/4 groups by Tukey's

inhibited upregulation of ALP expression by Smad1/4 (Fig. 4e). These results indicate that Msx2 plays a role in cooperation of RA and BMP2 signaling during osteoblast differentiation.

To understand the molecular mechanism of the RA inhibitory effect on adipocyte differentiation (Fig. 1), we next examined the effect of RA on PPARγ and C/EBP family members, both of which play an important role in adipogenesis. When 3T3-F442A cells were treated with insulin, oil red O-positive adipocyte differentiation was induced with upregulation of PPARγ expression (Fig. 5a, b). Interestingly, RA strongly inhibited insulin-induced adipocyte differentiation and PPARγ expression (Fig. 5a, b), suggesting that RA inhibits adipocyte differentiation through inhibition of PPARγ. To understand this further, we investigated the effect of RA on C/EBPβ and C/EBPδ, both of which directly control PPARγ expression [3, 38]. Overexpression of C/EBPβ and C/EBPδ promoted adipocyte differentiation and induced PPARγ expression (Fig. 5c, d). RA treatment strongly inhibited adipocyte differentiation and PPARγ expression induced by both C/EBPβ and C/EBPδ (Fig. 5c, d). These data suggest that RA inhibits adipocyte differentiation by blocking C/EBPβ and C/EBPδ-mediated PPARγ expression. We next

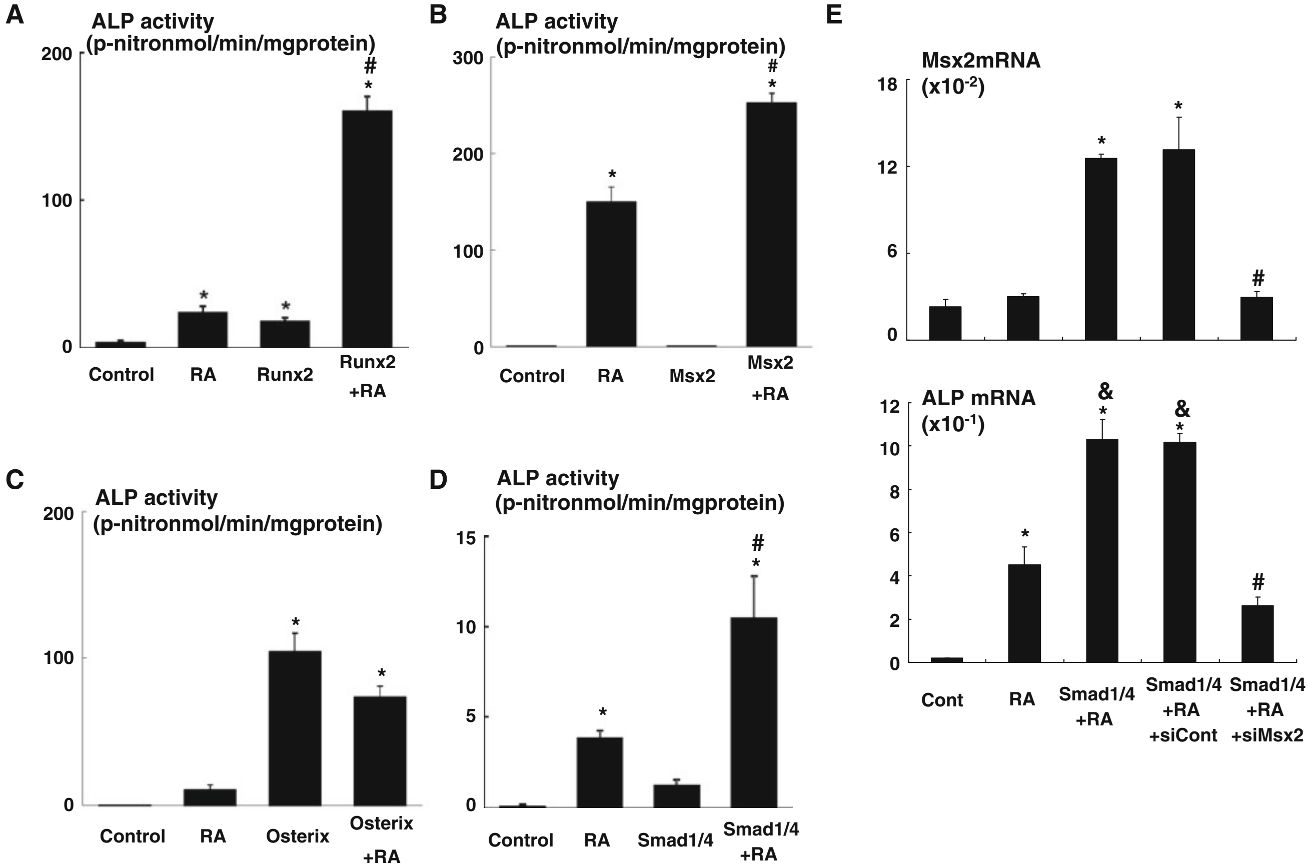

Fig. 4 Relationship between RA and Runx2, Osterix, and Msx2. **a** C3H10T1/2 cells were infected with control or Runx2 adenovirus and cultured in the presence or absence of RA (10^{-6} M) for 7 days before determining ALP activity. $*P < 0.01$ vs. Control by Dunnett's; $^{\#}P < 0.01$ vs. RA and Runx2 groups by Tukey's. **b** C3H10T1/2 cells were infected with control or Msx2 adenovirus and cultured in the presence or absence of RA (10^{-6} M) for 7 days before determining ALP activity. $*P < 0.01$ vs. Control by Dunnett's; $^{\#}P < 0.01$ vs. RA and Msx2 groups by Tukey's. **c** C3H10T1/2 cells infected with control or Osterix adenovirus were cultured in the presence or absence of RA (10^{-6} M) for 7 days before determining ALP activity. $*P < 0.01$ vs. Control by Dunnett's. **d** RD-C2 cells infected with control adenovirus or both Smad1 and Smad4 adenoviruses were cultured in the presence or absence of RA (10^{-6} M) for 7 days before determining ALP activity. $*P < 0.01$ vs. Control by Dunnett's; $^{\#}P < 0.01$ vs. RA and Smad1/4 groups by Tukey's. **e** RD-C2 cells were transfected with control siRNA (*siCont*) or *siMsx2* for 8 h before infection with control adenovirus or both Smad1 and Smad4 adenoviruses: 12 h after infection, cells were cultured in the presence or absence of RA (10^{-6} M) for 4 days. Real-time PCR was used to analyze gene expression of Msx2 (*upper panel*) and ALP (*lower panel*), normalized against β-actin expression. $*P < 0.01$ vs. Control by Dunnett's; $^{\&}P < 0.01$ vs. RA group by Tukey's; $^{\#}P < 0.01$ vs. Smad1/4 + RA and Smad1/4 + RA + siCont groups by Tukey's

examined whether RA affects expression of C/EBP family members. As shown in Fig. 5e, RA markedly inhibited expression of C/EBPα and C/EBPδ in 3T3-F442A cells. However, RA marginally increased C/EBPβ expression (Fig. 5e). These results suggest that RA inhibits not only adipogenic function of C/EBP family members, but also expression of C/EBPδ and C/EBPα, which function in the late stages of adipocyte differentiation [38].

It remains possible that RA inhibits not only expression but also function of PPARγ. To address this, we exogenously introduced PPARγ adenovirus into 3T3-F442A cells and examined the effect of RA on adipogenic function of PPARγ. As expected, PPARγ overexpression induced adipocyte differentiation (Fig. 6a, b). As shown in Fig. 6b, RA significantly inhibited PPARγ-induced adipocyte differentiation. Consistent with this, RA inhibited transcriptional activity of PPARγ (Fig. 6c). Based on these findings, RA suppresses adipocyte differentiation by inhibiting both expression and function of PPARγ (Fig. 6d).

Discussion

A number of studies suggest that several cytokines and transcription factors are involved in regulating the balance between osteoblast and adipocyte differentiation of MSCs [3]. RA has been shown to stimulate osteoblast differentiation of the 3T3-F442A pre-adipocyte cell line when used in combination with BMP2 [33], suggesting that RA has

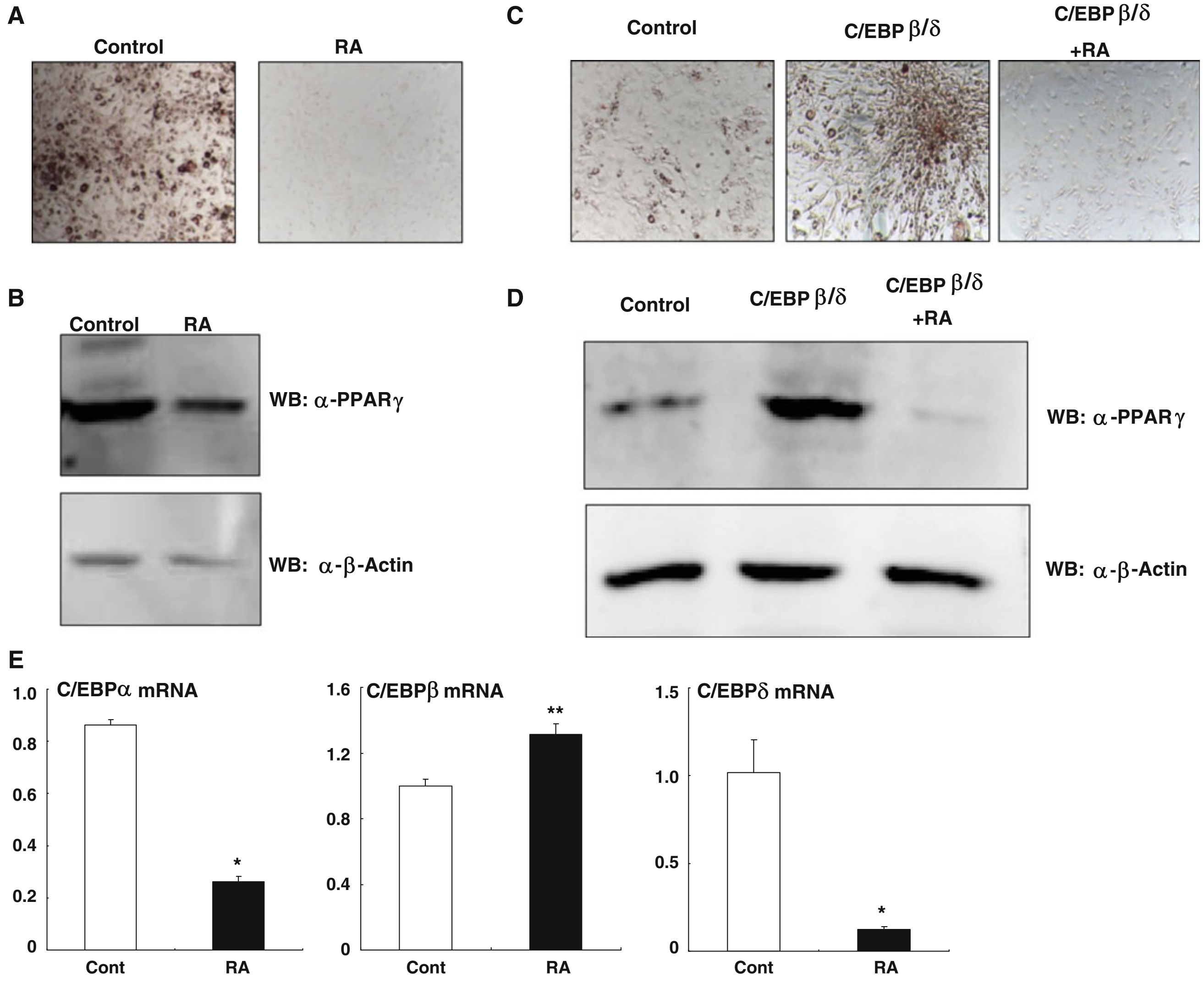

Fig. 5 Effects of RA on adipocyte differentiation and peroxisome proliferator-activated receptor-γ (PPARγ) expression. **a** 3T3-F442A cells were cultured with insulin for 7 days in the presence or absence of RA (10^{-6} M) before oil red O staining. **b** 3T3-F442A cells were cultured with insulin in the presence or absence of RA (10^{-6} M) for 4 days. Cell lysates were analyzed by immunoblotting with anti-PPARγ (*upper panel*) and anti-β-actin (*lower panel*) antibody. **c** 3T3-F442A cells infected with control adenovirus or both C/enhancer-binding protein (EBP)β and C/EBPδ adenoviruses were cultured in the presence or absence of RA (10^{-6} M) for 7 days before oil red O staining. **d** 3T3-F442A cells infected with control adenovirus or both C/EBPβ and C/EBPδ adenoviruses were cultured in the presence or absence of RA for 4 days. Cell lysates were analyzed by immunoblotting with anti-PPARγ (*upper panel*) and anti-β-actin (*lower panel*) antibody. **e** 3T3-F442A cells were cultured in the presence or absence of RA (10^{-6} M) for 4 days. Real-time PCR was used to analyze gene expression of C/EBPα (*left panel*), C/EBPβ (*middle panel*), and C/EBPδ (*right panel*), normalized against β-actin expression. *$P < 0.01$ vs. control by Student's *t* test; **$P < 0.05$ vs. control by Student's *t* test

osteoblastogenic activity. However, it is still unknown how RA is involved in regulating the balance between osteoblast differentiation and adipocyte differentiation. In this study, we investigated whether RA plays a role in regulating the direction of differentiation of MSCs into osteoblasts and adipocytes. We found that RA is an important regulator that preferentially stimulates osteoblast differentiation but inhibits adipocyte differentiation. We found that RA stimulates expression and activity of ALP, an effect markedly enhanced in the presence of BMP2. We also found that RA upregulates ALP gene promoter activity through the RARE present within the ALP gene promoter region. In contrast, we found that RA inhibited BMP2-induced adipocyte differentiation. RA inhibited PPARγ expression induced by treatment of insulin or by overexpression of both C/EBPβ and C/EBPδ. In addition, RA suppressed expression of C/EBPα and C/EBPδ. Although RA marginally increased C/EBPβ expression, the degree of increase in C/EBPβ expression was much weaker than the degree of inhibition of C/EBPδ expression, which would be

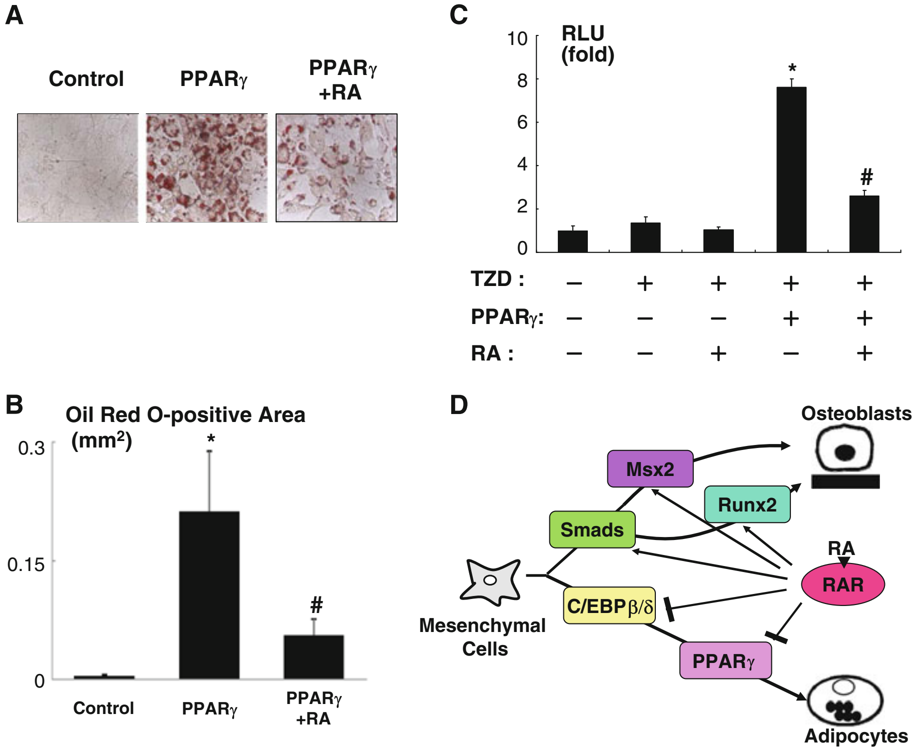

Fig. 6 Effects of RA on adipogenic function and transcriptional activity of PPARγ. **a**, **b** 3T3-F442A cells infected with control or PPARγ adenovirus were cultured in the presence or absence of RA (10^{-6} M) for 7 days before oil red O staining (**a**). Areas stained with oil red O were quantitatively measured as described in the "Materials and methods" (**b**). **c** 3T3-F442A cells were transfected with the aP2 gene promoter luciferase construct together with pcDNA3 or PPARγ expression vector before culture with RA (10^{-6} M) and troglitazone (*TZD*) (10^{-6} M) as indicated. Luciferase activity of the lysates was determined. $^{*}P < 0.01$ vs. *lane 1* [TZD (−), PPARγ (−), RA (−)] by Dunnett's; $^{\#}P < 0.01$ vs. *lane 4* [TZD (+), PPARγ (+)] by Tukey's. **d** Schematic summary of study. RA stimulates osteoblast differentiation through the BMP2–Smad–Runx2 and BMP2–Smad–Msx2 signaling pathways. Conversely, RA inhibits adipocyte differentiation by inhibiting C/EBPβ- and C/EBPδ-induced PPARγ expression and also the function of PPARγ

involved in the suppressive effect of RA on adipocyte differentiation. Moreover, RA suppressed PPARγ-induced adipocyte differentiation. Collectively, these results indicate that RA is an important factor in regulating commitment of MSCs into osteoblasts and adipocytes (Fig 6d).

RA synergistically promoted osteoblast differentiation and markedly increased ALP gene promoter activity with BMP2. We found that RA and Smad1/4 signaling cooperatively stimulate ALP activity and ALP gene promoter activity. In contrast, BMP2 or ALK3QD failed to stimulate ALP gene promoter activity when we used the −724-bp ALP gene promoter construct or −1,313-bp Del ALP gene promoter construct, both of which lack a RARE. These results suggest that upregulation of the ALP gene promoter by Smad1/4 might require RAR. Because co-immunoprecipitation experiments failed to detect physical interaction between RAR and Smad1/4, we speculate that the interaction of RAR and Smad1/4 is transient or very weak.

It is well known that cell linage-specific transcription factors play a critical role in differentiation programs of numerous types of cells. We found that Runx2, an essential transcription factor for osteoblastogenesis and bone formation [43–45], cooperatively stimulates osteoblast differentiation with RA treatment. Interestingly, in Runx2-deficient MSCs, RA stimulates ALP activity and overexpression of Smad1/4 further enhances the effect of RA, suggesting other BMP-related transcription factors associate with RA to stimulate BMP2-dependent osteoblastogenesis. Consistent with this, we found that Msx2 cooperatively stimulates ALP activity with RA, in contrast to Osterix, which does not appear to interact with RA. Furthermore, in Runx2-deficient cells, knockdown of Msx2 suppressed the stimulating effects of Smad signaling on ALP expression. Together, our results suggest that RA stimulates osteoblast differentiation in association with Runx2 and Msx2, both of which function downstream of BMP2/Smad signaling (Fig. 6d).

With respect to RA regulation of expression of other osteoblastogenic genes, RA did not increase expression of Osterix, Bsp, or osteocalcin, even in the presence of BMP2. Interestingly, the putative RAREs are not present in the promoter region of these genes. It is therefore likely that

RA is a specific regulator for ALP expression during osteoblastogenesis. Another possibility is that RA would be involved with other early-stage markers for osteoblast differentiation. Microarray analysis would be useful in addressing this question, although this is beyond the scope of this study. It is also possible that RA is involved in early stages of osteoblast differentiation but not in late stages of the process when Bsp and osteocalcin are involved. This concept is consistent with our hypothesis that RA is a switching regulator that defines the lineage of MSCs. Because it has been reported that vitamin A deficiency retards skeletal growth [47] and that long-term excess intake of vitamin A reduces bone mass with increased fracture risk [48], an appropriate amount of RA is necessary for normal bone development. It is possible that RA has opposing roles in early- and late-stage osteoblastogenesis. Indeed, we found that RA inhibited calcification of mouse primary osteoblasts (data not shown). However, in one study it was reported that there is no association between intake of vitamin A or retinol and fractures associated with osteoporosis [49]. Because RA has been shown to inhibit NFATc1 expression and osteoblastogenesis [50], RA seems to play very complex roles in bone metabolism. Further investigation into stage-dependent roles of RA in osteoblast differentiation would contribute to understanding the relationship between RA and osteoblastogenesis.

Acknowledgments We thank Dr. Minoru Morikawa, Dr. Yoshiaki Ito, Dr. Shizuo Akira, Dr. Bruce M. Spiegelman. and Dr. Takeshi Imamura for providing 3T3-F442A cell line cells, Runx2 cDNA, C/EBPβ and C/EBPδ cDNA, aP2 gene promoter construct, and SBE luciferase construct, respectively. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Uehara Memorial Foundation and the Astellas Foundation for Research on Metabolic Disorders.

Conflict of interest All authors have no conflicts of interest.

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