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Original

Autoregulatory mechanism of Runx2 through the expression of transcription factors and bone matrix proteins in multipotential mesenchymal cell line, ROB-C26

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Abstract: Runx2 is essential for osteoblast differentiation and gene expression of bone matrix proteins, however, little is known about the mechanism regulating its activity. In this study, the role of Runx2 on gene expression of transcription factors, AJ18, Msx2, and Dlx5, was examined *in vitro*. It is known that AJ18 and Msx2 act as repressors to inhibit activity of Runx2, whereas Dlx5 promotes its activity. An expression vector inserted Runx2 cDNA was transiently overexpressed in a rat multipotential mesenchymal cell line, ROB-C26 (C26). Real time reverse transcription-PCR analysis showed that, in exogenous Runx2-overexpressing C26 cells (C26-Rx), AJ18 expression increased 1.8-fold, Msx2 expression increased 3.0-fold, and Dlx5 expression increased 2.7-fold compared to the cells transfected with vector alone (C26-Co). Luciferase assay also showed that, in C26-Rx, AJ18 promoter activity increased 2.1-fold compared to C26-Co. Furthermore, gene expression of alkaline phosphatase (ALP) and bone matrix proteins including type I collagen (Col1), osteocalcin (OC), osteopontin (OPN), and matrix Gla protein (MGP) was examined. In C26-Rx, MGP expression increased 1.8-fold, and OPN expression increased 1.4-fold compared to C26-Co. However, no significant difference in Col1, ALP, and OC expressions was detected between C26-Rx and C26-Co. These results suggest that the existence of autoregulatory feed back loops, which inhibit Runx2 activity through the interaction of AJ18, Dlx5, and Msx2 cooperating with that of MGP and OPN, interferes with the differentiation of C26 cells toward mature osteoblasts. (J. Oral Sci. 47, 199-207, 2005)

Keywords: Runx2; ROB-C26; transcription factor; bone matrix protein.

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Introduction

Skeletal tissue is composed of various types of mesenchymal cells such as osteoblasts, chondrocytes, myoblasts, and adipocytes. These cell lineages are thought to originate from common progenitors called multipotential mesenchymal stem cells. These progenitors acquire specific phenotypes depending on the maturation during differentiation (1). Osteoblasts express high alkaline phosphatase (ALP) activity (2) and synthesize bone matrix proteins including type I collagen (Col1) (3), osteocalcin (OC) (4), matrix Gla protein (MGP) (5), osteopontin (OPN) (6), and bone sialoprotein (BSP) (7), resulting in matrix maturation and mineralization. Although the molecular mechanisms that regulate their expressions have not been well elucidated, this process is involved in several transcription factors (8).

Runx2, also termed PEBP2αA/AML3/Cbfa1, is a transcription factor that belongs to the runt-domain gene family and has a DNA binding domain that is homologous

to pair-rule gene *runt* in *Drosophila* (9). *In vivo* studies using Runx2 knockout mice have demonstrated that homozygous mutants Runx2 (-/-) completely lack osteoblasts and bone formation (10), and heterozygous mutans Runx2 (-/+) show a phenotype similar to that of the heritable disease cleidocranial dysplasia in humans (11). Furthermore, overexpression of exogenous Runx2 up-regulates ALP activity and mRNA expression of Col1, OPN, BSP, and OC in non-osteogenic cell lines such as C3H10T1/2 mesenchymal cells and NIH3T3 fibroblasts (12-15). These regulations are thought to occur when the Runx2 binding domain interacts with its related DNA sequence, which is known as osteoblast-specific *cis*-acting element 2 (OSE2) (12). Overall, these findings demonstrate that Runx2 is an essential regulator of osteoblast differentiation.

It has been reported that transcription factors, Dlx5 and Msx2, which are mammalian homologues of the *Dorosophila* homeobox proteins, involve osteoblast differentiation (16,17). Msx2 acts as a transcriptional repressor, since its overexpression down-regulates ALP, Col1, OPN, and OC expressions and decreases mineralized matrix *in vitro* (18). Similarly, in transgenic mice overexpressed with exogenous Msx2, the number of osteoprogenitors increases through the inhibition of their differentiation in the growth center of the developing skull (19). In contrast, Dlx5 is a bone inducing transcriptional activator, thus its overexpression up-regulates ALP activity, OC mRNA expression, and mineralization *in vitro* (20). More importantly, Msx2 represses DNA binding activity of Runx2 to OSE2 by interacting with it, while Dlx5 activity interferes with the ability of Msx2 that bind to Runx2 (21). Furthermore, another transcription factor, AJ18, which belongs to the family of zinc finger protein, is thought to play a role for osteoblast differentiation at early and late stages of development (22). Notably, AJ18 antagonizes OC promoter activity interfering with the binding of Runx2 to a part of OSE2 and BMP-7-induced ALP activity (22). These findings suggest that gene expressions induced by Runx2 closely relate to the molecular interaction with AJ18, Msx2, and Dlx5 during osteoblast differentiation.

In order to increase understanding of the role of Runx2 in osteoblast differentiation, we examined mRNA expression of Msx2, Dlx5, and AJ18 together with that of ALP and bone matrix proteins, when exogenous Runx2 was overexpressed in rat multipotential mesenchymal cell line, ROB-C26 (C26). Here, we showed that overexpression of exogenous Runx2 up-regulated mRNA expression of AJ18, Dlx5, and Msx2 as well as OPN and MGP but not ALP, Col1, and OC in C26 cells. These results suggest that complex interaction induced by exogenous Runx2 restricts to the differentiation of C26 cells toward mature osteoblasts. This study provides new insights into the role of Runx2 in bone biology.

Materials and Methods

Cell culture

Undifferentiated clonal multipotential mesenchymal cell line, ROB-C26 (C26), which is established from newborn rat calvaria (23) and differentiates into osteoblasts when treated with bone morphogenetic protein (BMP)-2 (24), was kindly provided from Dr. Akira Yamaguchi (Tokyo Medical and Dental University, Japan). Cells were plated at a density of 2×10^5 on 100 mm^2 tissue culture dishes (Iwaki, Chiba, Japan) and cultured for 6 days at 37ºC in α-modified minimum essential medium (α-MEM; Invitrogen Carlsbad, CA, USA) supplement with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO_2. A medium change was performed at 3 days for culture period.

Transient transfection of exogenous Runx2 cDNA

An expression vector inserted murine Runx2 cDNA (pCMV-Osf2/Cbfa1) (12), which codes an approximate 1.8 kb transcript, was kindly provided by Dr. Gerard Karsenty (Baylor College of Medicine, Houston, TX, USA). C26 cells were plated at a density of 5×10^5 cells/well on a 6 well plate (Iwaki) and allowed to grow under normal culture conditions for 18 h. Cells were extensively washed with phosphate buffered saline (PBS), and incubated in 3ml of α-MEM containing 10 μl of lipofectamine™2000 (Invitrogen) with 4 μg of Runx2 expression vector or empty vector at 37ºC for 6 h. The medium was replaced with fresh antibiotic free α-MEM supplement with 10% FBS and further incubated for 48 and 96 h. Subsequently, total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instruction.

Northern blot analysis

Ten μg of total RNA was subjected to 1.2% agarose gel electrophoresis with formaldehyde and transferred to nylon membrane (Hybond N^+, Amersham Bioscience, Piscataway, NJ, USA). Hybridization with a ^{32}P-labeled full-length Osf2/Cbfa1 fragment was performed at 42ºC in a solution containing 50% formamide, 0.65 M NaCl, 5 mM EDTA, 0.1% SDS, 0.1 M 1, 4-piperazinediethanesulfonic acid (pH 6.8), 10% dextran sulfate, and 100 μg/ml salmon sperm DNA. After hybridization, the membrane was washed with 2 ×

SSC/0.1% SDS at 55ºC three times, and then exposed to X-ray film. As a control, a human full-length G3PDH cDNA fragment was used.

Real time reverse transcription (RT)-polymerase chain reaction (PCR)

First-stranded cDNA was synthesized from 1 μg of DNase I-treated total RNA in 20 μl of solution containing 1 × RT-buffer, 50 ng random primers, 10 mM dNTP mixture, 1mM dithiothreitol, and 0.5 units reverse transcriptase (SuperScript II™ RNase H^-, Invtrogen) at 42ºC for 1 h, and then diluted 5 times in sterile distilled water. Two μl of cDNA solution was mixed in 20 μl of PCR buffer containing 1 × SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA), 1.5 mM dNTP mixture, 15 mM Mg^{2+} solution, 0.25 units *Ex Taq* DNA polymerase (Takara Shuzo, Kyoto, Japan), and 20 μM primers (sense and antisense). Real time RT-PCR was performed using a Smart Cycler (Cepheid, Sunnyvale, CA, USA) under the following conditions (40 cycles); denaturation for 3 sec at 95ºC, annealing and extension for 20 sec at 68ºC. Sense and antisense primers used are shown in Table 1, which were designed on the basis of published nucleotide sequences deposited in DDBJ/EMBL/GenBank. All experiments were performed in triplicate, and mRNA expression levels were normalized by values obtained by that of β-actin mRNA.

Luciferase assay of AJ18 promoter region

A promoter construct, AJ-Cat (-1494 to +181bp), fused to firefly luciferase was kindly provided by Dr. Jaro Sodek (The University of Toronto, Canada). A plasmid, pGL3-Basic (Promega, Madison, WI, USA), was also used as a negative control. C26 cells were seeded on a 24 wells plate at a density of 1×10^5 cells/well and incubated for 18 h. Two μg of AJ-Cat or pBL3-Basic were co-transfected in the cells with 2 μg of Runx2 expression vector or empty vector using a lipofectamine™2000 as described above. Forty-eight hours after transfection, cells were rinsed with PBS, and scraped with lysis buffer. Luciferase assay was performed using Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Experiments were performed in triplicate, and values of luciferase activity of AJ-Cat and pGL3-Basic were normalized by values obtained by *renilla* luciferase activity as an internal control, respectively.

Statistical analysis

Statistical significance of the data was evaluated based on a Student's *t*-test, and *P* values less than 0.05 were considered to be significant.

Results

Detection of exogenous Runx2 mRNA overexpressed in C26 cells

Northern blot was performed to confirm the intensity of a 1.8 kb transcript derived from exogenous Runx2 cDNA (12). As shown in Fig. 1, its expression was strongly detected in C26 cells transfected with endogenous Runx2 (C26-Rx) but not in cells transfected with vector alone (C26-Co) at 48h. Exogenous Runx2 expression was diminished at 96 h, but much weaker signals originating from endogenous Runx2 were detected in both cells. Other transcriptions indicated by asterisks are thought to be artificial products driven by alternative splicing of Runx2 expression vector.

Table 1 Nucleotide sequences of sense and antisense primers used in real time RT-PCR

Gene	Sense	Antisense	Reference
OC	5'-TGCAAAGCCCAGCGACTCT-3'	5'-AGTCCATTGTTGAGGTAGCG-3'	M25490
OPN	5'-AGACCATGCAGAGAGCGAG-3'	5'-ACGTCTGCTTGTGTGCTGG-3'	M14656
MGP	5'-AATCTCACGAAAGCATGGAATC-3'	5'-GCAGGCTTGTTGAGTTCCCG-3'	J03026
BSP	5'-GATAGTTCGGAGGAGGAGGG-3'	5'-CTAACTCCAACTTTCCAGCGT-3'	X86100
ALP	5'-ATGTCAACCGAAACGCCTCAG-3'	5'-ATGGCGGAGTCGAACATGGCA-3'	J03572
Col1	5'-GTGGTAACGATGGTGCTGTC-3'	5'-CTTCACCCTTAGCACCAGC-3'	Z78279
Msx2	5'-TCACCACGTCCCAGCTTCTAG-3'	5'-AGCTTTTCCAGTTCCGCCTCC-3'	U12514
Dlx5	5'-GCGCTCAACCCATACCAGT-3'	5'-ACTCGGGACTCGGTTGTAGG-3'	AB073716
AJ18	5'-AAGCGTCGTCCCCTGAACAAG-3'	5'-CTGTCTCTAGCCTCTCATA-3'	AF321874
Osterix	5'-AGCTCTTCTGACTGCCTGCCTA-3'	5'-TGGGTGCGCTGATGTTTGCT-3'	AY177399
β-actin	5'-CTTTCTACAATGAGCTGCGTG-3'	5'-ATGGCTGGGGTGTTGAAGG-3'	V01217

Effect of exogenous Runx2 on mRNA expression of AJ18, Dlx5, and Msx2 in C26 cells

We first examined whether exogenous Runx2 regulates mRNA expressions of transcription factors, AJ18, Dlx5, and Msx2, using real time RT-PCR (Fig. 2). At 48 h after transfection, AJ18 expression increased 1.8-fold ($P < 0.05$), Msx2 expression increased 3.0-fold ($P < 0.01$), and Dlx5 expression increased 2.7-fold ($P < 0.02$) in C26-Rx compared with C26-Co. At 96 h after transfection, AJ18 expression increased 1.3-fold ($P < 0.05$) in C26-Rx compared with C26-Co, but no significant difference in Msx2 and Dlx5 mRNA was determined between them.

Effect of exogenous Runx2 on mRNA expression of ALP, Col1, OPN, MGP, and OC in C26 cells

The mRNA expression level of ALP, Col1, OPN, MGP, and OC was compared between C26-Rx and C26-Co (Fig. 3). At 48 h after transfection, OPN expression increased 1.4-fold ($P < 0.05$) and MGP expression increased1.8-fold ($P < 0.05$) in C26-Rx compared with C26-Co. At 96 h after transfection, MGP expression continued to increase 1.8-fold ($P < 0.05$) in C26-Rx compared with C26-Co. However, no significant difference of OC, Col1, and ALP was shown between C26-Rx and C26-Co.

Promoter activity of AJ18 in Runx2-overexpressing C26 cells

In order to examine whether Runx2 controls transcriptional activity of AJ18 in C26 cells, luciferase assay was performed using a reporter construct AJ-Cat. As shown in Fig. 4, luciferase activity increased 2.1-fold ($P < 0.05$) on AJ-Cat compared with a basal activity of pGL3-Basic (Basic) in C26-Rx, but no significant difference was observed between AJ18-Cat and Basic in C26-Co.

Discussion

The involvements of exogenous Runx2 on mRNA expression of ALP and bone matrix proteins have been shown clearly. For example, Ducy et al. (12) have demonstrated that transient overexpression of exogenous Runx2 up-regulates mRNA expression of OC and BSP in C3H10T1/2 mesenchymal cells and primary skin fibroblasts. Harada et al. (13) have also reported that its overexpression stably enhances mRNA expression of ALP, Col1, OC, and OPN in C3H10T1/2 cells. Furthermore, recent studies using adenoviral delivery systems have shown a high level of ALP activity and increased mRNA

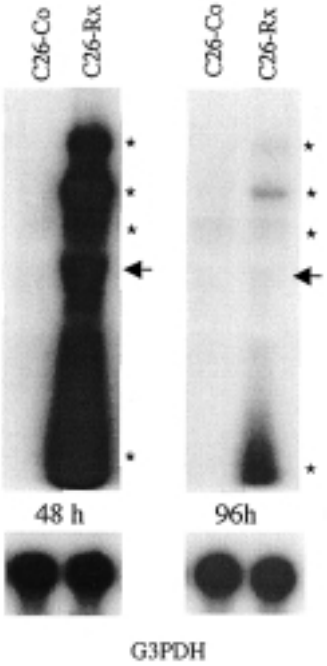

Fig. 1 Overexpression of exogenous Runx2 in C26 cells. Northern blot was performed using 10 μg of total RNA from C26-Rx and C26-Co at 48 h and 96 h. The probe used was a full-length mouse Osf2/Cbfa1 cDNA fragment. The arrows indicate a transcript, with approximate size of 1.8 kb, derived from Runx2 in expression vector. Asterisks indicate artificial splicing products driven by expression vector.

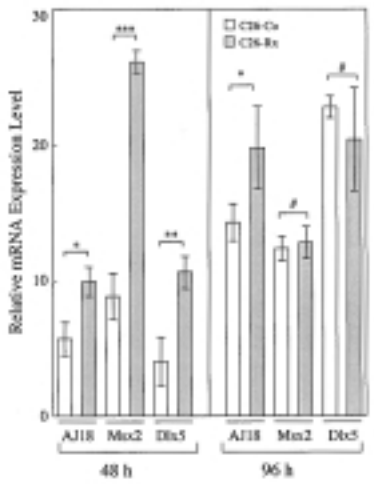

Fig. 2 Relative mRNA expression level of AJ18, Dlx5, and Msx2 in exogenous Runx2-overexpressing C26 cells. First stranded cDNAs from C26-Rx and C26-Co were subjected to real time RT-PCR. Relative mRNA expression levels were compared between C26-Rx and C26-Co at 48 h and 96 h, respectively. Each measurement is the mean of triplicate cultures. Standard deviation of the mean is shown by vertical bars. Statistical significance compared between C26-Rx and C26-Co, $*P < 0.05$, $**P < 0.02$, and $***P < 0.01$. Sharp (#) indicates no statistical significance $\#P > 0.05$.

expression of Col1, OC, and BSP with the appearance of mineralized matrix in exogenous Runx2-infected C3H10T1/2 (14) and bone marrow stromal cells (15). However, in contrast to this evidence, our data showed no inductive mRNA expression of ALP, Col1, and OC in C26 cells (Fig. 3). Although cell-type-specific difference is frequently focused on, we consider that this discrepancy is caused by inductive expression of AJ18, Msx2, and Dlx5 in exogenous Runx2-overexpressing C26 cells. Jheon et al. (22) have demonstrated the negative effects of AJ18 that its overexpression inhibits ALP activity in BMP-7-treated C3H10T1/2 and antagonizes the binding of Runx2 to OSE2. In addition, Shirakabe et al. (21) have reported competitive interactions that Msx2 represses the binding activity of Runx2 to OSE2 interacting with it, while Dlx5 interferes with the interaction between Msx2 and Runx2. Here, we showed an increased amount of AJ18, Msx2, and Dlx5 mRNA expressions by exogenous Runx2 (Fig. 2), suggesting that Runx2 stimulates Dlx5 expression, but the increased amount of AJ18 and Msx2 preferentially inhibits the DNA binding activity of Runx2. Therefore, the cooperative effect of AJ18 and Msx2 may contribute to no inductive mRNA expression of ALP, Col1, and OC in C26 cells. Furthermore, our data support the evidence observed in mice overexpressed with exogenous Runx2 that mRNA expression of OC, Col1, and ALP is slightly decreased. In these mice, immature osteoblasts are increased, but their maturation is completely blocked (25). These *in vivo* findings suggest the possibility that increased expressions of AJ18 and Msx2 by exogenous Runx2 restrict the differentiation of osteoprogenitors into osteoblasts interfering with its binding activity, whereas a decrease of AJ18 and Msx2 probably allows Dlx5 to activate their differentiation into mature osteoblasts.

It is thought that OSE2 is an important sequence for expressing target genes of Runx2 (12), implying that direct binding of Runx2 to OSE2 involves AJ18, Dlx5, and Msx2 expressions. Jheon et al. (26) have demonstrated two putative consensus sequences of OSE2 on proximal and distal promoter regions of rat AJ18 gene. Our data also showed an increase of promoter activity on AJ-Cat (Fig.4) that contains both OSE2s, suggesting that Runx2 directly regulates AJ18 expression. However, indirect effects of exogenous Runx2 on Msx2 and Dlx5 expressions cannot be excluded. For example, Lee et al. (27) have reported that, in C2C12 myoblastic cells, BMP-2 treatment markedly enhances Dlx5 mRNA expression. Similarly, increased expression of Msx2 mRNA has been shown in BMP-2-

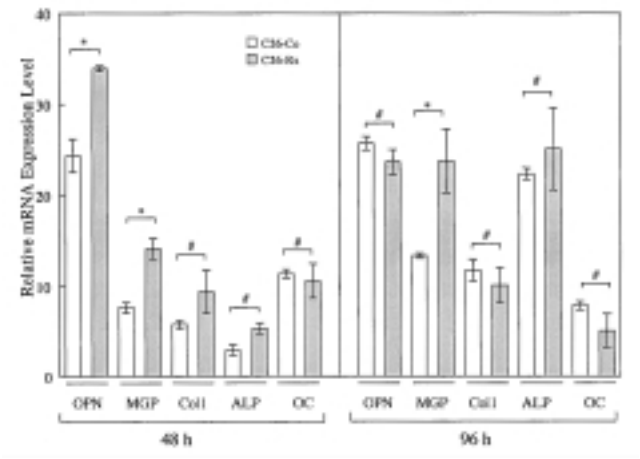

Fig. 3 Relative mRNA expression level of ALP, Col1, OC, OPN, and MGP in exogenous Runx2-overexpressing C26 cells. First stranded cDNAs from C26-Rx and C26-Co were subjected to real time RT-PCR. Relative mRNA expression levels were compared between C26-Rx and C26-Co at 48 h and 96 h, respectively. Each measurement is the mean of triplicate cultures. Standard deviation of the mean is shown by vertical bars. Statistical significance compared between C26-Rx and C26-Co, $*P < 0.05$. Sharp (#) indicates no statistical significance $\#P > 0.05$.

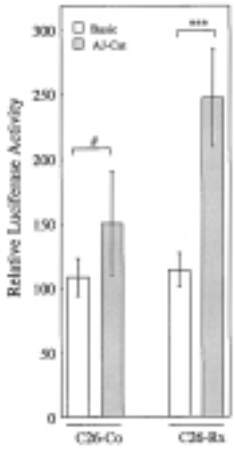

Fig. 4 Promoter activity of AJ18 in exogenous Runx2-overexpressing C26 cells. A promoter construct, AJ-Cat, was co-transfected into C26 cells with Runx2 expression vector (C26-Rx) or vector alone (C26-Co). After 48 h, luciferase activity was determined. A vector, pGL-Basic (Basic) was used as a negative control. Each measurement is the mean of triplicate cultures. Standard deviation of the mean is shown by vertical bars. Statistical significance compared between AJ-Cat and Basic in C26-Rx and C26-Co, respectively, $***P < 0.01$. Sharp (#) indicates no statistical significance $\#P > 0.05$.

treated C26 (28) and bone marrow stromal cells (29). These findings demonstrate that BMP-2 plays a role in Dlx5 and Msx2 expressions. Notably, Choi et al. (30) have reported that the disruption of Runx2 gene eliminates mRNA expression of BMP-2 with that of Dlx5 and Msx2 in the developing of the primordium of the cranial bone in mice, suggesting that Runx2 modulates BMP-2 expression. Since autocrine BMP-2-secretion occurs in C26 cells (31), it is considered that exogenous Runx2 stimulates secretion of BMP-2 to enhance Msx2 and Dlx5 expressions through the binding of its receptors on C26 cells. However, BMP-2-production is insufficient for the differentiation of C26 cells into mature osteoblasts, since osterix and BSP mRNA expressions were undetectable (data not shown). Osterix is a transcription factor that is induced by BMP-2 (32,33), and promotes BSP expression and terminal differentiation of osteoblasts (32).

Consistent with our results that overexpression of exogenous Runx2 up-regulates MGP mRNA expression in C26 cells (Fig. 3), a study using a differential display technique has shown inductive MGP mRNA expression in its overexpressing C3H10T1/2 cells (34), suggesting that Runx2 directly controls MGP expression. In contrast, Komori et al. (10) have reported that Runx2 does not link to MGP expression, since its expression is strikingly detected in chondrocytes in Runx2 (-/-) mice. Although we cannot fully explain the regulatory mechanism of MGP by AJ18, Msx2, and Dlx5, it seems that multiple signaling pathways modulate MGP expression. Interestingly, D'Alonzo et al. (35) have shown the binding of Runx2 to activator protein (AP)-1, which is composed of c-Fos and c-Jun, using a yeast two-hybrid system. They have also demonstrated that overexpression of exogenous Runx2 enhances c-Jun-mediated promoter activity of repeated AP-1 binding sequences *in vitro*. Furthermore, Farzaneh-Far et al. (36) have reported that c-Jun is able to interact with an AP-1 site on human MGP gene, resulting in a high promoter activity of MGP gene in cultured rat vascular smooth muscle cells. These findings suggest that OSE2 does not relate to MGP expression, while the interaction of c-Jun with AP-1 binding sequences with or without exogenous Runx2 may be crucial for MGP expression in C26 cells.

In contrast, OPN expression is thought to be regulated by Runx2 through the binding to OSE2 (12,37). Thus it is postulated that, in C26 cells, overexpression of exogenous Runx2 is restricted to OPN mRNA expression by increased amount of AJ18 and Msx2, similar to Col1 and OC expressions (Fig. 3). However, exogenous Runx2 up-regulates OPN mRNA expression (Fig. 3), suggesting the existence of additional factors that supplement activity of Runx2. Indeed, Runx2 can interact with a ubiquitous transcription factor, Ets-1, which binds Ets-1-related sequences on promoter region of human OPN gene, increasing its promoter activity (37). This evidence suggests that direct interaction of Runx2 with Ets-1 involves OPN expression without binding to OSE2. On the other hand, as MGP expression, other signaling pathways seem to play a role in OPN expression, since small amounts of its expression has been detected in embryos of Runx2 (-/-) mice (10).

Overexpression of exogenous OPN in MC3T3-E1 pre-osteoblastic cells inhibits the formation of mineralized matrix with a decrease of ALP activity and mRNA expression of OC and BSP, whereas overexpression of antisense OPN shows opposite effects (38). Similarly, in BMP-2-treated C3H10T1/2 cells, overexpression of exogenous MGP depresses OC mRNA expression and their differentiation into osteoblasts, while antisense MGP restores BMP-2 activity (39). Therefore, it is considered that OPN and MGP act as a negative regulator for osteoblast differentiation *in vitro*. OPN contains Arg-Gly-Asp (RGD) amino acid sequence that mediates cell-to-cell contact in a variety of cells such as fibroblasts and osteoblasts (40). The RGD sequence allows OPN to interact with different forms of integrin, such as $\alpha v\beta 3$ subunits (41). In contrast, MGP does not contain RGD, but is able to interact with integrin $\beta 1$subunit (42). Interestingly, Giuliani et al. (43) have shown the decrease of DNA binding activity of Runx2 along with that of ALP, ColI, and OC expressions in human osteoprogenitors co-cultured with myeloma cells. This phenomenon is more pronounced in the conditions of cell-to-cell contact through integrin $\alpha v\beta 1$ subunits known as very late antigen 4 (VLA4). Furthermore, Chen et al. (44) have reported that overexpression of integrin $\alpha v\beta 3$ in MC3T3-E1 cells stimulates cell proliferation but retards matrix mineralization with inhibition of ALP activity and mRNA expression of Col1 and OC. Taken together with this evidence, our data emphasize the existence of complicated signal transduction pathways which restrict the differentiation of C26 into mature osteoblasts; [1] transcription factors including AJ18 and Msx2 down-regulate the binding activity of exogenous Runx2 to OSE2, [2] excessive interaction of integrin subunits including VLA4 are mediated by OPN and MGP.

In conclusion, Runx2 up-regulates mRNA expression of transcription factors, AJ18, Msx2, and Dlx5, and bone matrix proteins, MGP and OPN, in C26 cells, suggesting the existence of autoregulatory feed back loops that inhibit the binding of Runx2 to OSE2 through increased expression of AJ18 and Msx2 cooperating with that of OPN and

MGP. These complex interactions may restrict the differentiation of C26 cells toward mature osteoblasts. However, clarification of the negative effect of Runx2 on osteoblast differentiation is necessary and will require the establishment of C26 cells stably overexpressed with exogenous Runx2.

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