BMP-2-induced Runx2 Expression Is Mediated by Dlx5, and TGF-β1 Opposes the BMP-2-induced Osteoblast Differentiation by Suppression of Dlx5 Expression*

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Intramuscular injection of BMP-2 induces ectopic bone formation in vivo. Similarly, BMP-2 treatment blocks myogenic differentiation and induces osteoblastic transdifferentiation of premyoblastic C2C12 cells. Previous reports suggested that BMP-2-stimulated Runx2 expression could play a pivotal role in transdifferentiation. However, increased Runx2 expression by TGF-β1 did not support osteoblast differentiation in vitro. These results indicate that the induction of Runx2 is not sufficient to explain the BMP-induced transdifferentiation. We found that Dlx5 is specifically expressed in osteogenic cells, and is specifically induced by BMP-2 or -4 signaling but not by other osteotrophic signals or other TGF-β superfamily members. Cycloheximide treatment indicated that Dlx5 was immediately induced by BMP signaling, while Runx2 required de novo protein synthesis. In addition, blocking or overexpressing each transcription factor indicated that Dlx5 is an indispensable mediator of BMP-2-induced Runx2 expression but is not involved in TGF-β1-induced Runx2 expression. Moreover, TGF-β1 opposed BMP-2-induced osteogenic transdifferentiation through Dlx5 suppression by de novo induction of AP-1. Taken together, these results indicate that Dlx5 is an indispensable regulator of BMP-2-induced osteoblast differentiation as well as the counteraction of the opposing TGF-β1 action.

Bone morphogenetic proteins (BMPs)§ were originally identified from demineralized bone matrix as factors that induce ectopic bone formation when implanted into muscular tissue (1). In vitro models of bone formation by BMP have also been established using myoblastic lineage cells (2, 3). Using this system, the molecular mechanism of the BMP-2-induced ectopic bone formation has been investigated. BMP-2 is not only a potent inducer of osteogenesis. It can also block the differentiation of C2C12 myoblasts into mature muscle cells by suppressing the master control genes for myoblast differentiation (3). Subsequently, expression of osteoblast phenotypic marker genes, such as alkaline phosphatase and osteocalcin, is induced by continuous BMP-2 treatment of C2C12 cells (3, 4). BMPs exert their diverse biological effects through two types of transmembrane receptors; BMP receptor type I (BMPR-I) and type II (BMPR-II), which possess intrinsic serine/threonine kinase activity (5). BMPR-I is further subclassified into BMPR-IA (also called ALK3) and BMPR-IB (also called ALK6). It has been shown that both the inhibition of myoblast differentiation and the induction of osteoblast differentiation by BMP-2 involve the activations of BMPR-I receptors (6, 7), their intracellular transducers Smad1 and Smad5 (8, 9), and the osteogenic master transcription factor Runx2 (4, 10).

Runt-related transcription factor 2 (Runx2), previously known as Chfa1/Pebp2α/AML3, plays an essential role in osteoblast differentiation (11). Runx2 knockout mice display complete absence of bone due to arrested osteoblast maturation (12). Our previous results demonstrated that Runx2 plays a central role in BMP-2- and TGF-β1-induced transdifferentiation of C2C12 cell at an early restriction point to divert the cells from the myogenic pathway. However, commitment and progression of osteogenesis appeared to require interactions with the BMP-2 signaling machinery, but not with TGF-β1 (4). A subsequent study suggested that BMP-specific Smads might play an indirect role in inducing Runx2 and that an additional step of de novo protein synthesis was required (10). Moreover, it has been suggested that BMP-specific Smads interact with the Runx2 protein and that the interaction between Runx2 and BMP-specific Smads could determine ligand-specific activities (10, 13). Although both BMP-2 and TGF-β1 stimulate Runx2 expression, only BMP-2 induces osteogenic marker genes. Therefore, Runx2 alone is insufficient to mediate BMP-induced osteoblast differentiation but requires collaboration with other signaling molecules that are stimulated by BMP signaling. This study addresses two unsolved questions concerning the mechanism of BMP-2-induced osteoblast differentiation. First, what is the protein (possibly a transcription factor) that mediates the stimulation of the Runx2 expression in response to BMP-specific Smads activation (10)? Second, both BMP-β1 and BMP-2 commonly stimulate Runx2 expression, however, TGF-β1 opposes the stimulated osteogenic differentiation by BMP-2. To resolve these questions, we have explored the pos-
sible involvement of Dlx5 in BMP-2-induced osteoblast differentiation because Dlx5 is induced by BMP-2 treatment (14, 15) and is known to play crucial roles in osteoblast differentiation (14, 16–18).

Dlx5 is a bone inducing transcription factor that is expressed in later stages of osteoblast differentiation (17). Forced expression of Dlx5 leads to osteocalcin expression and a fully mineralized matrix in cell culture (14, 18). Normally, Dlx5 expression is detected in discrete neuronal tissues and developing skeletal elements such as cartilage, bone, and tooth (19, 20). Moreover, Dlx5-deficient mice demonstrate severe craniofacial abnormalities with a delayed ossification of the cranium and abnormal osteogenesis (21), and Dlx5/Dlx6 double knockout mouse showed much more comprehensive bone defects (22). These results strongly suggest that Dlx5 plays important and evolutionarily conserved roles in the development of mineralized tissues even if there is a functional compensation by other members of the Dlx family. In this study we demonstrate that Dlx5 is the proximal target of BMP-signaling and in BMP-2-induced osteoblast differentiation, Dlx5 plays a pivotal role in stimulating downstream osteogenic master transcription factor Runx2 which in turn work sequentially and/or work together to induce the expression of bone marker genes that represent osteoblast differentiation, especially in the BMP-2-induced osteogenic differentiation because Dlx5 is induced by BMP-2 treatment (14, 15).

**Experimental Procedures**

**Materials**—Bioactive recombinant human BMP-2, -4, growth, and differentiation factor (GDF)-5, -6, -7 (23) were from Genetics Institute Inc. (Cambridge, MA). Recombinant human TGF-β1 and fibroblast growth factor (FGF)-2 were purchased from R&D Systems Inc. (Minneapolis, MN). Vitamin D3, ascorbic acid, dexamethasone, alkaline phosphatase staining kit, and cycloheximide were purchased from Sigma Chemical Company. Recombinant sonic hedgehog protein was generously provided by Dr. Masahiro Iwamoto (24). Superscript™ first-strand synthesis system for reverse transcription and LipofectAMINE plus were from Invitrogen (Carlsbad, CA). Taq polymerase, dNTP mix, and G418 were from Promega (Madison, WI). Megaprime DNA labeling system kit was from Amersham Biosciences. Express hybridization solution was from Clontech (Palo Alto, CA), and Zetaprobe membrane was from BioRad (Melville, NY).

**Cell Culture**—Mouse myogenic C2C12 cells, osteoblast-like MC3T3-E1 cells, and rat osteosarcoma cell line ROS17/2.8 were maintained as previously described (4, 17). ST2 cells (murine bone marrow-derived stromal cells) and C3H10T1/2 cells (stem cell-like fibroblasts) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Mouse chondrogenic ATDC5 cells (Riken, Japan) and adipogenic 3T3L1 cells were maintained as previously described (25, 26). Cells were inoculated at a density of 1 × 10^5 cells/100 mm culture dishes. To examine the effects of BMP-2 or TGF-β1 on cell differentiation, the cells were cultured for indicated period with or without treatment of indicated amount of the factors, in the respective media.

**RNA Extraction and Reverse Transcription**—Total cellular RNA was extracted from the cells and the concentration was measured by spectrophotometer (4). RNA integrity was assessed by the ratio of 28S/18S ribosomal RNA after electrophoresis in 1% agarose/5.5% formaldehyde gels. Reverse transcription was performed with a Superscript™ first-strand synthesis system for RT-PCR. 1 μg of total cellular RNA as a template, 0.5 μg of oligo(dT)_12-18, 200 units of Superscript II reverse transcriptase, 0.1 volume of 10× reverse transcription buffer, 0.5 mM of dNTP mixture (each of dATP, dCTP, dGTP, and dTTP), 10 μM of dithiothreitol used for first strand cDNA synthesis for 60 min at 42 °C. To eliminate contamination by RNA, the reverse-transcribed cDNA mixture was incubated with 2 units of RNase H for 20 min at 37 °C.

**Polymerase Chain Reaction (PCR)** and Northern/Southern Blot Analysis—Oligonucleotides for the PCR of mouse Dlx5 and mouse GAPDH were synthesized (TakaraKorea, Seoul, Korea). The nucleotide sequences of the sense strand are listed in Table I. Dlx5 and GAPDH were PCR amplified with reverse-transcribed cDNA as template, 0.2 μM each forward and reverse primer set, 0.4 mM dNTP mixture (each of dATP, dCTP, dGTP, and dTTP), 1 unit of 10× PCR buffer, 1 unit of polymerase, dNTP mix, dATP, dCTP, dGTP, and dTTP, and dithiothreitol were used for first strand cDNA synthesis. PCR products were separated on agarose gel electrophoresis and checked by ethidium bromide staining. The PCR products were subcloned into pBluescript KS vector and used as a probe for Northern or Southern blot hybridization after sequencing. Northern blot analysis for Dlx5, Runx2, and osteoblast marker genes was performed as previously described (4, 10).

**Plasmid Constructs**—The Dlx5 RT-PCR product was subcloned into pcDNA3.1 (Invitrogen) and was used for the Dlx5 expression construct, pcDNA3.1-Dlx5. The entire coding region of mouse Dlx5 cDNA was amplified by PCR with the Dlx5 primers in Table I. Transfection initiation and stop codons in the Dlx5 primers are underlined. To produce Dlx5 antisense expression construct, pCMV5-Dlx5-AS, Dlx5 PCR product was subcloned into pCMV5 expression vector generating antisense Dlx5.

**Transient Transfection and Establishment of Stable Cell Line**—To establish Dlx5 stable cell line (C2C12-pcDNA3.1-Dlx5) and Dlx5 antisense stable cell line (C2C12-pcDNA5-Dlx5-AS), C2C12 cells were transfected with pcDNA3.1-Dlx5 and pcDNA5-Dlx5-AS. Constitutive active BMPR-IA and -IB stable cells were established by transfecting ALK-3 QD and ALK-6 QD expression vectors (27) into C2D12 cells, respectively. BMPR-related expression vectors were generously provided by Drs. Heldin and ten Dijke. Dlx5 stable cell line (C2C12-pCMV5-Dlx5-AS) was transduced with Dlx5, Runx2, and osteoblast marker genes was performed as previously described (4, 10). As Dlx5 expression was very low and obscure by 18 S ribosomal RNA in non-osteoblastic cells, it is difficult to detect by Northern blot analysis. Thus, Dlx5 expression in non-osteoblastic cells was determined by RT-PCR. When the RT-PCR results required a quantitative value, the RT-PCR products (less than 25 cycles) were separated on agarose gel and Southern blot analysis was performed.

**Alkaline Phosphatase Staining**—Alkaline phosphatase (ALP) activity has been widely accepted as a simple and easiest way to determine the osteogenic differentiation, especially in the BMP-2-induced osteogenic differentiation in C2C12 cells (3, 6, 7). Cells were washed with phosphate-buffered saline twice, fixed with 2% paraformaldehyde, and stained for alkaline phosphatase according to the manufacturer’s instructions (Sigma).

**Western Blot Analysis**—Proteins from cell lysates or nuclear extracts were resolved by 13% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Schleicher & Schuell, Dassel, Germany). All procedures of Western blot analysis
agarose gel, and Southern blot analysis was performed. Products with lower cycles (25 cycles or less) were separated in 1% agarose, and Southern blot analysis was performed.

A

B

were done as previously described (10). Dlx5 antibody was raised against a 19-amino acid peptide located at the C terminus 268–286 of the deduced polypeptide sequence (GenBankTM accession number AAC52843). Anti-Dlx5 antiserum against the synthetic peptide was generated from rabbit and purified by affinity chromatography using IgG (Fc)-specific resin (TakaraKorea, Seoul, Korea). Anti-c-Jun rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). After primary antibody reaction and washing, the blot was incubated with the horseradish peroxidase-conjugated goat anti-rabbit antibody (Advanced Biochemicals Inc., Seoul, Korea) for 1 h at room temperature. After washing, the signal was detected by ECL plus (Amersham Biosciences).

RESULTS

Dlx5 Is Specifically Expressed in Osteoblastic Cells and Is a Direct and Specific Target of BMP Signaling—Dlx5 expression was examined in osteoblast lineage cells, MC3T3-E1, ROS 17/ 2.8, and ST2 cells, and in a chondrogenic cell line, ATDC5 cells. In the absence of BMP-2 treatment, Dlx5 was basally expressed in these osteogenic and chondrogenic cells (Fig. 1A). Although basal Dlx5 expression was very strong in ROS 17/2.8 cells as previously reported (17) Dlx5 expression was stimulated by BMP-2 treatment but not by TGF-β1. In ST2 cells, Dlx5 expression was even suppressed by TGF-β1 (Fig. 1A). In contrast to these results from osteo-chondrogenic cells, basal Dlx5 expression was undetectable in non-osteoblastic cells, such as myogenic C2C12 cells, fibroblastic C3H10T1/2 cells (10T1/2), and adipogenic 3T3-L1 cells. BMP-2 treatment induced Dlx5 expression in these cells whereas TGF-β1 did not (Fig. 1B).

To determine whether Dlx5 is a necessary and commonly employed component for the osteogenic shift of non-osteoblastic stem cells, we treated C2C12 myogenic cells with several osteotrophic hormones or growth factors (Fig. 2A). Vitamin D3 or dexamethasone did not induce Dlx5 expression in these cells nor did osteogenic growth factors such as FGF-2 and sonic hedgehog, or ascorbic acid. Among the TGF-β superfamily members, neither TGF-β1 nor GDF-5, -6, -7 induced Dlx5 expression in C2C12 cells. Only BMP-2 or BMP-4 treatment induced Dlx5 expression. Coincident with Dlx5 expression, alkaline phosphatase activity was strongly activated by BMP-2 or BMP-4 treatment (Fig. 2A). These results indicated that Dlx5 is a specific target of BMP-signaling.

BMP-2-induced Dlx5 expression was not detected at 3 ng/ml but was dose-dependently induced by 30–300 ng/ml of BMP-2 treatment (Fig. 2B) similar to the levels required to induce alkaline phosphatase (10). Dlx5 expression was initially detected at 1 h after BMP-2 treatment, gradually increased, and then continuously detected through 7 days of BMP-2 treatment (Fig. 2C). This time-dependent expression pattern indicates that Dlx5 is an early response gene and could be a direct target of BMP-signaling. In order to confirm the idea, cycloheximide (CHX), a protein synthesis inhibitor, was pretreated for 30 min and then BMP-2 was treated for additional 1 h, 3 h and 1 day. The CHX pretreatment could not block BMP-2-induced Dlx5 expression (Fig. 2D), indicating that Dlx5 is the direct target of BMP-signaling.

Activation of the BMP-Signaling Components Induced Dlx5 Expression without Treatment of BMP—Activation of BMP signaling is initiated by the binding of the ligand to the BMP receptor II and BMP receptor IA or IB. The hetero-tetramization of two type I- and two type II-receptors activates the serine/threonine kinase in the cytoplasmic domain of BMPR-I. Next, Smad1 and Smad5 are activated by the receptor kinase through the phosphorylation of their C-terminal serines of SSXS. To determine whether activation of the BMP-signaling components was sufficient to induce Dlx5 expression, we established stable cell lines expressing constitutively active BMPR-IA (ALK-3 QD) and BMPR-IB (ALK-6 QD), dominant negative BMPR-IA (ALK-3 KR), and BMPR-IB (ALK-6 KR), and wild-type Smad1 and Smad5. Then we selected two representative clones from each molecule of stable transfection by ALP staining. Forced expression of the constitutively active form of BMPR-IA or -IB strongly induced Dlx5 expression even in the absence of BMP-2 treatment (Fig. 3A). Positive Dlx5 expression was strongly correlated with positive ALP staining in these cells (Fig. 3A). In contrast, forced expression of the dominant negative BMPR-IA or -IB partially or completely blocked Dlx5 expression even in the presence of 50 ng/ml BMP-2, which is a sufficient dose to induce Δn5 in mock-transfected cells (Fig. 3B). In some clones, Dlx5 expression in these cells was not completely blocked by the forced expression of dominant negative BMPR-I expression vectors, suggesting that the dominant negative form of BMPR-I might not completely block endogenous BMPR-I action in these clones. Over-expression of Smad1 or Smad5, mediators of BMP-signaling, also induced Dlx5 expression even in the absence of BMP-2 treatment concomitant with positive ALP staining (Fig. 3C).

Dlx5 Is an Upstream Regulator of Runx2 Expression in BMP-Signaling—Because the expression of both osteogenic transcription factors, Runx2 and Dlx5, was commonly stimulated by BMP-2, we investigated their hierarchical regulatory relationship. Since CHX did not influence on the initial induction of Dlx5 (1 h) (Fig. 2D) and our previous report showed that the initial induction of Runx2 was blocked by pretreatment with CHX (10), we hypothesized that Dlx5 is involved in Runx2 expression. To test this hypothesis, we established two cell lines; one is C2C12-stably expressing Dlx5 (C2C12-Dlx5), and the other is C2C12 stably expressing Dlx5 antisense (C2C12-Dlx5-AS). These cells were selected by ALP staining and further confirmed Dlx5 protein expression by Western blot analysis. The Dlx5 C-terminal peptide antibody recognized well the

FIG. 1. The expression pattern of Dlx5 in osteogenic and non-osteogenic cells. A, rat osteoarcoma cells (ROS17/2.8, ROS), mouse calvarial osteoblast-like cells (MC3T3-E1, MC), mouse chondrogenic cells (ATDC5), mouse bone marrow stromal cells (ST2) were cultured without (C) or with treatment of BMP-2 (B, 300 ng/ml) or TGF-β1 (T, 5 ng/ml) for 36 h after reaching visual confluence. Total cellular RNA was purified and Dlx5 expression was determined by Northern blot analysis with 10 ng of total cellular RNA. Arrows and arrowheads indicate Dlx5 and 18S ribosomal RNA, respectively. B, myogenic stem cells from fetal mouse muscle tissue (C2C12), mouse fibroblastic stem cells (C3H10T1/2, 10T1/2), mouse adipocyte cells (3T3L1) were cultured without (C) or with treatment of BMP-2 (B, 300 ng/ml) or TGF-β1 (T, 5 ng/ml) for 36 h after reaching visual confluence. Total cellular RNA was purified and Dlx5 expression was determined by RT-PCR using GAPDH as an internal control. PCR products with lower cycles (25 cycles or less) were separated in 1% agarose gel, and Southern blot analysis was performed.
roughly 32-kDa protein present in the nuclear extracts in C2C12-Dlx5 cells (Fig. 4A). The size of this protein was in agreement with the predicted size from the deduced amino acid sequence of Dlx5 (289 amino acids in mouse, accession no. AAC52843). Conversely, C2C12-Dlx5 AS cells did not express Dlx5 protein even in the presence of BMP-2 (Fig. 4A, lane 5). Dlx5 antisense (Dlx5-AS) overexpression completely blocked the BMP-2-stimulated Runx2 expression but interestingly it could not block the TGF-β1-induced Runx2 expression (Fig. 4B, lanes 7–9). Consistent with the lack of Runx2 expression, ALP staining was negative even in the presence of BMP-2 in the cells (Fig. 4B, lane 8). Meanwhile, Dlx5 overexpression strongly activated Runx2 expression in the absence of BMP-2 treatment (Fig. 4B, lanes 1 and 4). These Dlx5 gain- or loss-of-function results strongly indicate that Runx2 is a downstream target of Dlx5.

As Runx2 is widely accepted as a key transcription factor for the initiation of osteogenesis, there is a possibility that Runx2 can regulate Dlx5 expression. In order to confirm this possibility, we established two cell lines; one is C2C12-stably expressing Runx2, and the other is Runx2(−/−) cell clone that is developed from the calvaria of Runx2 knockout mouse (10). In contrast to Dlx5 overexpression, the forced expression of Runx2 alone was not sufficient to induce Dlx5 expression (Fig. 5, lane 4) but the Dlx5 expression was much more strongly enhanced by BMP-2 treatment compared with the expression transfected with empty expression vector (Fig. 5, lanes 2 and 5). Because the Runx2(−/−) cells were derived from calvaria, basal Dlx5 expression was detected in the absence of BMP-2 treatment (Fig. 5, lane 7). Expression of Dlx5 was stimulated by BMP-2 treatment even in the absence of endogenous Runx2 function (Fig. 5A, lane 8). Consistent with previous results, Dlx5 was not induced by TGF-β1 treatment in the cells (Fig. 5, lane 9).

**Dlx5 Overexpression Stimulated Osteocalcin and Other Bone Marker Gene Expression**—BMP-2 induces ectopic bone formation in vivo, and stimulates transdifferentiation of myogenic cells into osteoblasts in vitro (2, 3, 4). Moreover, our results above indicate that Dlx5 is a specific target of BMP-2 signaling. Therefore, we investigated the role of Dlx5 on osteoblast marker gene expression using the same cells of Dlx5 gain- or loss-of-function. As Dlx5 expression increased, the downstream Runx2 expression was subsequently increased (Fig. 4B, lane 4). At this time point (3 days culture after visual confluence), ColI and FN expression was somewhat increased by Dlx5 overexpression. However, the expression of osteogenic marker genes, such as ALP and OC, was not increased by Dlx5 overexpression (Fig. 4B, lane 4). Although the expression of the latter two bone marker genes was not induced by Dlx5 alone, the induction of these two genes by BMP-2 treatment was much stronger than that detected in control cells (Fig. 4B, lanes 2 and 5). Moreover, prolonged culture of Dlx5-overexpressing cells resulted in detectable ALP and OC expression at day 6 and day 14, respectively, whereas the expression of myogenic master control transcription factor MyoD was vanished in the respective time (Fig. 4, C and D). Antisense Dlx5 completely suppressed BMP-2-stimulated expression of bone marker genes but could not suppress TGF-β1-stimulated collagen and fibronectin expressions (Fig. 4B, lanes 8 and 9).
TGF-β1 Opposes the Osteogenic Activity of BMP-2 through the Inhibition of Dlx5—BMP-2 induces or enhances the expression of the osteoblast differentiation markers, ALP and OC in C2C12 cells (3, 4). In contrast, TGF-β1 was not only unable to induce these markers, but it dramatically inhibited BMP-2-mediated OC gene expression and ALP activity (29). As Dlx5 expression increased with BMP-2-induced osteoblast differentiation, we investigated whether the inhibitory role of osteoblast differentiation by TGF-β1 was also mediated by the BMP-specific target, Dlx5. The induction of Dlx5 gene expression by constitutively active BMPR-IA or IB was completely or partially blocked by TGF-β1 treatment (Fig. 6A). To determine whether the inhibition of Dlx5 mRNA expression is dependent on new protein synthesis or not, CHX experiment was performed in both constitutively active BMPR-IA (no. 4) and IB (no. 11) stable cells because, in these clones, BMPR-induced Dlx5 gene expression was completely abrogated by TGF-β1 treatment. Interestingly, pretreatment of CHX completely blocked TGF-β1-induced suppression of the Dlx5 expression in both cells (Fig. 6B). Since Dlx5 induction by BMP-2 is observed as early as 1 h after stimulation (Fig. 2C) and a new protein synthesized by TGF-β1 suppresses Dlx5 expression before being induced by BMP-2, we assumed that an immediate early gene product, such as AP-1, might be involved in the Dlx5 suppression by TGF-β1.

In order to examine a possible involvement of AP-1 in the suppression of BMP-induced Dlx5 expression by TGF-β1, we introduced dominant negative c-Fos, termed as A-fos, for the experiment (28, 30). The A-fos protein has a much higher binding activity to Jun protein than any other form of Fos proteins so that seques ters all Jun proteins, then consequently demonstrates dominant negative AP-1 action. Overexpression of A-fos by itself did not influence on the BMP-induced Dlx5 mRNA expression (Fig. 6C, lanes 2 and 4). However, A-fos significantly interrupted TGF-β1-induced suppression of Dlx5 (Fig. 6C, lanes 3 and 5). Among several AP-1 components, c-Jun protein showed a differential expression pattern between TGF-β1 and BMP-2; c-Jun protein level was significantly increased by TGF-β1 but not by BMP-2 (Fig. 6D). Consistent with these results, the overexpression of c-Jun alone mimicked the antagonistic action of TGF-β1 in BMP-stimulated Dlx5 expression (Fig. 6C, lanes 2, 3, 6, and 7).

**DISCUSSION**

The identification of the involvement of BMP receptors (6), intracellular mediator Smads (8, 9) and osteogenic master transcription factor, Runx2 (12), was important for our understanding in BMP-2-induced osteogenic transdifferentiation of myogenic cells in vitro (3) and ectopic bone formation in muscle tissue (1). Despite the information available regarding the role of individual components in this differentiation model, we still do not have a complete picture integrating BMP-signaling molecules and the key transcription factors. Here, we suggest that Dlx5 is the critical linker between BMP-signaling molecules and the downstream Runx2 for the transdifferentiation, and a possible antagonistic target of TGF-β1 action in the BMP-2-induced osteogenic transdifferentiation.

**Dlx5 Is an Osteoblast-specific and BMP-Signaling-specific Transcription Factor**—In the present study, Dlx5 expression was detected in osteogenic lineage cells including chondro progenitor cells but not in other mesenchymal cells such as adipocytes, myoblasts, and fibroblasts. It is highly correlated with previous reports that showed the role of Dlx5 is developmentally highly conserved in skeletal tissue development (19, 20) and osteoblast differentiation (14, 17, 18). In addition, Dlx5 expression was specifically induced by stimulation of the BMP signaling pathway, such as direct application of BMP-2 or BMP-4 ligands, overexpression of constitutively active BMPR-I receptors, and overexpression of BMP-regulated Smads. In contrast, Dlx5 expression was not induced by other osteogenic stimulators, such as vitamin D3, FGF-2, dexamethasone, ascorbic acid, and other TGF-β superfamily members. Together, these results indicate that Dlx5 is a skeletal tissuespecific and BMP-signaling-specific transcription factor.

**Dlx5 Mediates BMP-2-stimulated Runx2 Expression**—Our previous report demonstrated that the stimulation of Runx2 expression by BMP-2 required new protein synthesis, and the initial stimulation has occurred between 2–6 h in C2C12 cells but decreased to basal levels by 24 h after treatment (10). Based on these findings, we suggested that Runx2 expression by BMP-2 is mediated by newly synthesized transcription factors. In this work, we hypothesized that Dlx5 could be a candidate that mediates Runx2 expression in response to BMP-signaling for the following reasons; first, Dlx5 is osteoblast-specific and BMP-signaling-specific transcription factor.
Dlx5 Mediates BMP-2-induced Runx2 Expression

**Fig. 4. The effect of Dlx5 overexpression or antisense blocking on the expression of Runx2 and osteoblast markers in C2C12 cells.**

A. C2C12 cells were stably transfected with Dlx5 expression vector or Dlx5 antisense expression vector. Dlx5 and Dlx5-AS stable cells were treated with 300 ng/ml of BMP-2 for 3 days and the Dlx5 expression was determined from the nuclear extracts by Western blot analysis using anti Dlx5 rabbit polyclonal antibody. B. Dlx5 or Dlx5 antisense stable cell clones were treated without (lanes 1, 4, and 7) or with 300 ng/ml of BMP-2 (lanes 2, 5, and 8) or 5 ng/ml of TGF-β1 (lanes 3, 6, and 9) for additional 36 h. The mRNA levels of Runx2, osteocalcin (OC), alkaline phosphatase (ALP), type I collagen (Col I), and fibronectin (FN) were analyzed by Northern blot hybridization and ALP activity was assayed after culture for 6 days and determined by histochemical staining. C. The delayed expression of ALP by Dlx5 overexpression was determined. Dlx5 stable cells were cultured for 6 days, and ALP and MyoD mRNA levels were determined by Northern blot analysis. Consistent RNA loading was represented by GAPDH. D, the delayed expression of OC by Dlx5 overexpression was determined. Dlx5 stable cells were cultured for 14 days, and OC and MyoD mRNA levels were determined by Northern blot analysis.

Second, both transcription factors employ the same signaling machineries (ligands, receptors, and R-Smads) for the induction. Third, upon the stimulation by BMP-2, the expression timing of Dlx5 is earlier than that of Runx2. Finally, Dlx5 expression does not require de novo protein synthesis.

The hypothesis was further confirmed by the results that blocking Dlx5 expression completely suppressed Runx2 expression even in the presence of strong BMP-2 stimulation and Dlx5 overexpression stimulated Runx2 even in the absence of BMP-2 signaling (Fig. 4). On the contrary, the forced expression of Runx2 alone did not induce Dlx5 expression and the deficiency of Runx2 did not abrogate BMP-induced Dlx5 gene expression (Fig. 5). Moreover, the induction of Dlx5 is BMP-specific while Runx2 expression can be regulated by other growth factors, such as FGF-2, -4, or -8 (31, 32) or TGF-β1 (4, 10) that did not induce Dlx5 gene. In other words, the Runx2 induction by factors other than BMP is not mediated by Dlx5 (Fig. 5). Thus, we can summarize that Dlx5 is a specific and indispensable upstream component in BMP-induced Runx2 expression, even if Dlx5 is not a universal upstream regulatory molecule of the Runx2 gene expression.

**Fig. 5. Effect of Runx2 for Dlx5 expression in BMP-2 signaling in C2C12 cells.** Runx2 expression vector (Runx2) or empty vector (Mock) was stably introduced in C2C12 cells. Fetal mouse calvarial cell clone was established from Runx2(-/-) mouse. The cells were cultured without (lanes 1, 4, and 7) or with the treatment of 300 ng/ml of BMP-2 (lanes 2, 5, and 8) or 5 ng/ml of TGF-β1 (lanes 3, 6, and 9) for additional 36 h. Dlx5 expression was determined by RT-PCR and subsequent Southern blot analysis, or by Northern blot analysis. Arrowhead and arrow indicate 18 S rRNA and Dlx5, respectively. ALP activity was assayed after a 6-day culture by histochemical staining.

BMP-2 and TGF-β1 Regulate Runx2 Expression via Different Pathways; One by Dlx5-dependent and the Other by Dlx5-independent Pathway—Several lines of evidences indicate that TGF-β1 opposes osteoblast differentiation that is stimulated by BMP-2-signaling (10, 29, 33). However, each suggested different mechanism, especially, in terms of Runx2 involvement in the process. Our previous report showed that Runx2 expression is commonly stimulated by BMP-2 or TGF-β1, and the initial stimulation occurred between 2–6 h in C2C12 cells but decreased to the basal levels by 24 h after treatment (10). Based on the result, we suggested interaction of Runx2 with respective R-Smad would be important for the ligand specific action. On the other hand, Alliston et al. (33) suggested that TGF-β1 inhibits osteoblast differentiation of primary cultured mouse calvarial cells or ROS 17/2.8 cells by repression of Runx2 expression through Smad3 activation. In addition, Spinella-Jeagle et al. (29) showed that TGF-β1 did not sensibly modify the increase of Runx2 gene expression mediated by BMP-2, thus demonstrating that the inhibitory effect of TGF-β1 on osteoblast differentiation and maturation stimulated by BMP-2 was independent of Runx2 gene expression. The latter two reports concerning Runx2 regulation by TGF-β1 are quite different from our previous reports (4, 10) in which we consistently demonstrated TGF-β1 stimulated Runx2 expression.

The discordance in the regulation of Runx2 expression by TGF-β1 could be explained as follows; promoter analysis of the Runx2 gene indicated that Runx2 gene expression is autoregulated in part by negative feedback inhibition of Runx2 protein,
Runx2 expression 2–6 h after TGF-β1 treatment of C2C12 cells is reproducible. In addition, overexpression or antisense blocking of Dlx5 did not influence the stimulation of Runx2 expression by TGF-β1 (Fig. 4B, lanes 3, 6, 9) while greatly influencing that by BMP-2 (Fig. 4B, lanes 2, 5, 8). The result not only further confirms that TGF-β1 stimulates Runx2 expression but also indicates TGF-β1 regulates Runx2 expression by Dlx5 independent pathway. In view of our previous report (10) and present results, it is likely that Runx2 expression induced by TGF-β1 can occur independently of Dlx5.

**TGF-β1 Opposes BMP-2 Action through Dlx5—**Even if Runx2 expression is regulated differently by TGF-β1 in different contexts, TGF-β1 clearly opposes BMP-2-induced osteoblast differentiation in vitro, which suggests that Runx2 might not be the key target that mediates the opposing action of TGF-β1 in BMP-2-induced bone formation. In this study, we demonstrated that TGF-β1 treatment strongly or completely suppressed the Dlx5 expression that was induced by constitutively active BMPR-IA or -IB overexpression (Fig. 6A). Moreover, the cycloheximide experiment indicated that the opposing action of TGF-β1 occurs through newly synthesized proteins rather than the direct activation of preexisting factors (Fig. 6B). The blocking of AP-1 induced by TGF-β1 using dominant negative A-fos overexpression suppressed the antagonizing action of TGF-β1 on Dlx5 expression (Fig. 6C), which indicates AP-1 is involved in the TGF-β1-mediated suppression of BMP-induced Dlx5. We further confirmed that c-Jun among several AP-1 components was induced by TGF-β1 but not by BMP-2 in our cell type and the exogenous overexpression of c-Jun could mimic the opposing action of TGF-β1. The up-regulation of c-Jun transcript by TGF-β1 occurs in a wide range of cell lines and the evidence of c-Jun promoter analysis indicated that this gene is a primary target of TGF-β1 (35–38). Although we could not exclude the involvement of the other AP-1 components, we can suggest c-Jun is a good candidate for the mediator of antagonistic action of TGF-β1 by following reasons; first, we showed c-Jun protein level was stimulated by TGF-β1 but not by BMP-2. Second, not only the c-Jun but some other AP-1 components also could suppress Dlx5 expression even in the absence of TGF-β1 (data not shown), but most of them were conversely stimulated by BMP-2 and TGF-β1 treatment (28, 39). Further studies would be required for a comprehensive understanding of the involvement of other AP-1 components and upstream signaling pathways.

In this study, we identified Dlx5 as a specific target of BMP-2-induced osteoblast differentiation in C2C12 cells. Dlx5 is a crucial component that links BMP-signaling molecules to the downstream master transcription factor of osteogenesis, Runx2. Both transcription factors work sequentially and/or work together to induce components of extracellular matrix. Dlx5 alone or together with Runx2 is still insufficient for the immediate induction of OC expression, which requires time for further propagation of the regulatory cascades triggered by Dlx5. In addition, we identified TGF-β1 stimulates Runx2 expression via Dlx-5-independent pathway, and Dlx5 seems to be a key switch on which osteogenic BMP-2 signals and opposing TGF-β1 signals conflict.

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**Fig. 6. BMP-2-stimulated Dlx5 expression was downregulated by TGF-β1.** A, C2C12 cells were stably transected with constitutive active forms of BMPR-IA (ALK-3) and BMPR-IB (ALK-6) and 5 selected clones were treated with TGF-β1 (5 ng/ml) for 36 h after visual confluency. Total cellular RNA was purified and Dlx5 expression was determined by RT-PCR and subsequent Southern blot analysis. GAPDH represents an internal control. B, stably transfected constitutive active forms of BMPR-IA (ALK-3) clone 4 and BMPR-IB (ALK-6) clone 11 were pretreated with 10 μg/ml CHX for 30 min, and then 5 ng/ml TGF-β1 was added for additional 24 h. Dlx5 expression was determined as described above. C, C2C12 cells were transfected with vectors expressing constitutively active BMPR IB (ALK6 QD), and/or dominant negative c-Fos (A-fos) and/or c-Jun. On the second day, 5 ng/ml TGF-β1 was treated for 48 h and Dlx5 expression was determined as described above. D, C2C12 were cultured without (C) or with treatment of BMP-2 (B, 300 ng/ml) or TGF-β1 (T, 5 ng/ml) for 1 h after reaching visual confluency. The harvested nuclear extracts were examined by Western blot analysis using the anti-c-Jun rabbit polyclonal antibody. Coomassie Blue staining showed that each nuclear extract includes consistent amount of total protein.
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