Homeoprotein DLX-1 interacts with Smad4 and blocks a signaling pathway from activin A in hematopoietic cells

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In the transforming growth factor β (TGF-β) superfamily, activin A, TGF-β1, and bone morphogenic protein 4 (BMP-4) have various effects on hematopoiesis, including early mesodermo-hematogenesis. After these cytokines bind to their respective receptor, a regulatory Smad is phosphorylated and becomes associated with Smad4, the common Smad, and the resulting complex translocates to the nucleus to regulate transcription. DLX1 is the product of a member of the *distal-less* homeobox gene family, which is known to have important roles in embryogenesis, particularly in craniofacial development, and in GABAergic neurogenesis. DLX1 has been reported to be temporally and spatially coexpressed with BMP-4 during embryogenesis in selected contexts. We report here that, in addition to the previously reported regions/cells, DLX1 is expressed in hematopoietic cells in a lineage-dependent manner and that DLX1 interacts with Smad4 through its homeodomain. We show that it blocks multiple signals from TGF-β superfamily cytokines such as activin A, TGF-β1, and BMP-4, including differentiation of a hematopoietic cell line by activin A. Taken together, these data suggest that DLX1 may function as a regulator of multiple signals from TGF-β superfamily members in broad biological contexts during blood production.

The transforming growth factor β (TGF-β) superfamily comprises more than 30 members that have a broad array of biological activities (1). Once they bind to their respective type II receptor, the type I receptor phosphorylates one of the regulatory Smads, or R-Smads, each of which then associates with Smad4, which is called the common Smad or Co-Smad. The resulting R-Smad/Co-Smad complex translocates to the nucleus and regulates the transcription of target genes (2). Among R-Smads, Smad2 and Smad3 are used by the TGF-β/activin group, whereas Smad1, Smad5, and Smad8 are most often used by the bone morphogenic protein (BMP) group (3, 4). The transactivation is achieved through recruitment of coactivators such as p300 in the Smad complex and displacement of transcriptional repressors, whereas in other contexts, the complex can recruit molecules and act as a transcriptional repressor (5). Homeodomain-containing proteins are known to participate in such Smad complexes. Hox-C8, a classical homeoprotein and a known transcriptional repressor, is bound by Smad1 and is displaced from the promoter upon BMP-induced activation of Smad1, facilitating the transcriptional activation by BMPs (6). A ubiquitously expressed homeoprotein TGIF binds to Smad3 and acts as a corepressor by recruiting a histone deacetylase in TGF-β signaling (7).

The *DLX* genes constitute a subfamily of vertebrate homeobox genes that are structurally similar to the *Drosophila distal-less* gene. Six members in this family have been identified in mammals (*DXL1*, *DLX2*, *DLX3*, *DLX5*, *DLX6*, and *DLX7*) (8). *DLX1*, originally identified in the forebrain of the developing mouse embryo (9), is expressed in response to fibroblast growth factor-8 and is known to participate in craniofacial development (10), in teeth and jaw development (11–13), and in GABAergic neurogenesis (14–17). Although the phenotype of mice with disrupted *DLX1* or *DLX2* gene is fairly normal, mice lacking both genes exhibit abnormalities in facial structures and are neonatally fatal (10). It is known that *DLX1* and *DLX2*, as well as *MSX1* and *MSX2*, homeobox genes related to the *DLX* family genes, show a composite and complicated expression pattern in tooth cells during their ontogeny (11, 12) and are considered to be transcriptional targets of the BMP signaling (12, 13).

Recent evidence shows that TGF-β1, BMP-4, and activin A affect hematopoietic cells, particularly in early mesodermo-hematogenic cells and hematopoietic stem cells (18–22). Here, we report that DLX1 is expressed in hematopoietic cells with a lineage-dependent manner and that it interacts with Smad4 and blocks multiple signals from TGF-β1, BMP-4, and activin A. We propose that DLX1 may function as a regulator of multiple signals from TGF-β superfamily members in a broad range of biological contexts.

Materials and Methods

Library Screening. A cDNA library made from a human erythroleukemia cell line, TF-1 (23), was screened by a PCR-based strategy. Sequencing the multiple DLX1 cDNA clones isolated suggested the presence of several alternatively spliced mRNAs (unpublished data). The ORF used as the WT DLX1 cDNA (GenBank accession no. AY257976) in this study was defined based on published sequences of mouse and human *DLX* genes.

Northern Blot Analysis. Two micrograms of the poly(A)$^+$ RNA was electrophoresed in a formaldehyde gel, blotted to Hybond-N nylon membrane (Amersham Pharmacia), and hybridized with probes in 50% formamide/10% dextran sulfate/1 M NaCl/0.1% SDS/Tris-Cl, pH 7.5, at 45°C. The probes used were a 557-bp *Kpn*I-*Bam*HI fragment from human DLX1 cDNA (Fig. 1, depicted as 3′ probe), a 207-bp fragment from Smad7 (24), and a rat GAPDH cDNA. After hybridization, filters were washed at 65°C in 0.2× standard saline citrate (SSC, 1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) and 0.1% SDS.

Plasmid Construction. WT and deletion mutants (AA, BK, and SA) of DLX1 cDNAs with a Flag tag at the C terminus (Fig. 2*A*) were constructed in pSSRα (25) and pMX/IRES-EGFPpuro (26), whereas WT and deletion mutants (ΔC and ΔHDC) of DLX1 cDNAs with a 6 × Myc tag at the N terminus (see Fig. 5*B*) were constructed in pcdef3 (27) through a cassette vector,

Abbreviations: TGF-β, transforming growth factor β; BMP, bone morphogenic protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY257976).

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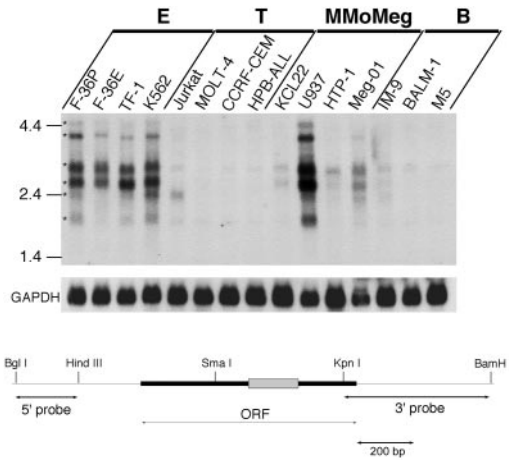

Fig. 1. Northern blot of human hematopoietic cell lines for DLX1. Asterisks indicate positions of mRNA signals. A schematic diagram of cDNA and the positions of probes and several restriction enzymes are depicted. The dotted box indicates the homeobox.

pcDNA3/6Myc [a gift from T. Imamura (Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo) and K. Miyazono (Cancer Institute of the Japanese Foundation for Cancer Research and University of Tokyo)]. Flag-Smad2, Flag-Smad3, and Flag-Smad4 (28) and mutants of Smad4, Smad4P102L, and Smad4Δ (483–552) (29) in pCMV5 have been described elsewhere. Human MSX2 cDNA was obtained by RT-PCR, sequenced, Myc-tagged, and inserted in pSSRα.

Cell Culture, Virus Transduction, and Selection. COS7, NIH 3T3, HepG2, and C2C12 were cultured in DMEM with 10% FCS. F5-5 was cultured in Ham's F-12 medium with 10% FCS. For retrovirus transduction of F5-5 cells, COS7 cells were transfected by a liposome-based method (SuperFect, purchased from Qiagen, Valencia, CA) with WT-inserted, BK-inserted, or mock pMX/IRES-EGFPpuro. The conditioned medium was used to transduce F5-5 cells. Individual clones were selected for puromycin resistance and expression of enhanced green fluorescence protein.

Immunostaining. NIH 3T3 and COS7 were transfected with WT and mutant DLX1-Flag-expressing pSSRα plasmids by using SuperFect and stained with anti-Flag (M2; Sigma) and fluorescein-labeled anti-mouse (The Jackson Laboratory) antibodies by a method described in ref. 30.

Transcriptional Activation Assay. NIH 3T3 was transfected with the WIP-Lux reporter plasmid (31) together with WT and mutant DLX1-Flag-expressing pSSRα plasmids and pSSRα/MSX2. F5-5 was transfected with A3-Lux (32) together with WT and mutant DLX1-Flag-expressing pSSRα plasmids and the FAST2-expressing plasmid (pCMV5B/Myc-FAST2) (33) in the presence or absence of various concentrations of activin A (a gift from Y. Eto, Ajinomoto, Tokyo). HepG2 was transfected with 3TP-Lux (34) together with Smad2-, Smad3-, and Smad4-expressing plasmids and pSSRα/DLX1-Flag in the presence or absence of 1 ng/ml TGF-β1 (R & D Systems). C2C12 was transfected with 3GC2-Lux (35) together with pSSRα/DLX1-Flag in the presence or absence of 100 ng/ml BMP-4 (R & D Systems). For transfection, SuperFect was used according to the manufacturer's instructions.

Immunoprecipitation and Western Blotting. COS7 was transfected with the Flag-Smad2-, Flag-Smad3-, or Flag-Smad4-expressing plasmid together with pcdef3/6Myc-DLX1 by using SuperFect. Flag-Smad4 was also introduced together with pcdef3/6Myc-ΔC or pcdef3/6Myc-ΔHDC. After 48 h, the cells were lysed in the TNE buffer (28). For immunoprecipitation, cell lysates were incubated with anti-Flag M2 affinity gel (Sigma) for 30 min at 4°C. Immunoprecipitates were washed five times with the TNE buffer and subjected to SDS/PAGE and Western blot analysis. Immunoblotting was performed with anti-Myc monoclonal antibody (9E10; Santa Cruz Biotechnology) and alkaline phosphatase-conjugated anti-mouse antibody (Promega).

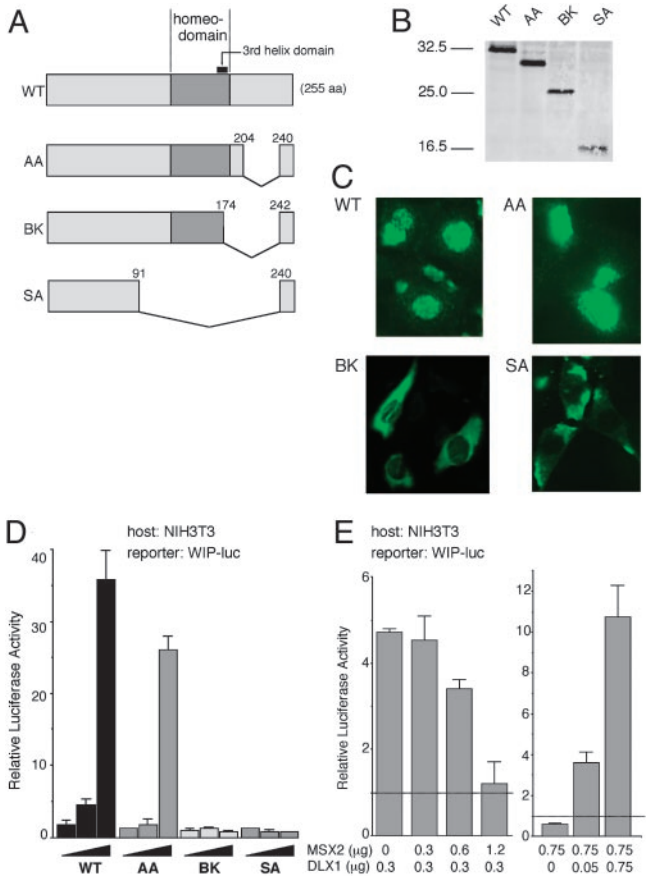

Fig. 2. Demonstration of WT and mutant DLX1 proteins (*A–C*), transactivation activity of DLX1 (*D*), and reciprocal transactivation and repression by DLX1 and MSX2 (*E*). (*A*) A schematic representation of the WT and mutant DLX1 expression constructs. Amino acid sequence is from NA 199 through 963 (GenBank accession no. AY257976). The hatched box and bold line indicate the homeodomain and third helix domain, respectively. (*B* and *C*) Western blot (*B*) and subcellular localization (*C*) of the WT and mutant DLX1 proteins. COS cells (*B*) and NIH 3T3 cells (*C*) were transfected with WT and deletion mutants (AA, BK, and SA) of DLX1 cDNAs with a Flag tag at the C terminus constructed in the pSSRα vector. Flag-tagged DLX1 proteins were detected by the M2 antibody and alkaline phosphatase-conjugated (*B*) and fluorescein-labeled (*C*) anti-mouse antibodies. (*D*) The WIP promoter is transactivated by DLX1 in a dose-dependent manner. NIH 3T3 was transfected with the WIP-Lux reporter plasmid together with increasing doses of pSSRα containing WT and deletion mutants (AA, BK, and SA) of DLX1 cDNAs. (*E*) Reciprocal transactivation and repression by DLX1 and MSX2, respectively, on the WIP promoter. NIH 3T3 was transfected with the WIP-Lux reporter plasmid together with a fixed dose of pSSRα/DLX1 and increasing dose of pSSRα/MSX2 (*Left*) and together with a fixed dose of pSSRα/MSX2 and increasing dose of pSSRα/DLX1 (*Right*). The luciferase activity with a control pSSRα was defined as 1 (horizontal line).

Results

DLX1 Is Expressed in Hematopoietic Cells in a Lineage-Dependent Manner. During our screening of homeobox gene expression in hematopoietic cells, we found that some human leukemia cell

lines expressed DLX1 using a degenerate RT-PCR method (data not shown). cDNA clones containing the entire coding region of human DLX1 was then isolated from a cDNA library made from an erythroleukemia cell line, TF-1 (36). A Northern blot with a probe comprising a 3′ end of coding and a flanking noncoding region showed the expression of multiple DLX1 transcripts (4.5, 4.0, 3.0, 2.5 kb) in human nonlymphoid hematopoietic cell lines (Fig. 1). The transcripts of 3.0 and 2.5 kb were also detected in some cell lines of T and B lineages, but at much lower levels. We obtained similar results with a probe from the 5′ noncoding region (Fig. 1 and data not shown). Different cDNA clones corresponding to various alternative splicing were isolated from the TF-1 library (data not shown). We further analyzed >100 human hematopoietic cell lines by RT-PCR for the expression of DLX1 mRNA and confirmed that 87% of the nonlymphoid cells expressed readily detectable levels of expression, whereas 45% of lymphoid cells expressed low levels and 55% of lymphoid cells showed no expression (data not shown).

DLX1 Is Localized to the Nucleus and Acts as a Transcriptional Activator. Expression vectors carrying cDNAs for WT DLX1 and several deletion mutants were prepared and used to examine their subcellular localization and transcriptional activity. The mutant AA lacks most of the region C-terminal to the homeodomain but preserves the intact homeodomain. In BK, the C-terminal region within the homeodomain has been further deleted, leading to the lack of the third helix, which generally serves as a DNA-contact domain in homeoproteins. In SA, the deletion extends to the N-terminal region to the homeodomain, leading to the complete lack of the homeodomain (Fig. 2*A*). We confirmed that these proteins with a Flag tag were expressed in COS7 cells transfected with the pSSRα vectors containing the respective cDNA (Fig. 2*B*). Subcellular localization of the WT and mutants of DLX1 was analyzed in COS7 cells (data not shown) and NIH 3T3 cells (Fig. 2*C*) transfected with those expression vectors. WT DLX1 and AA mutants were stained primarily in the nucleus, whereas the BK and SA mutants were localized primarily in the cytoplasm (Fig. 2*C*), suggesting that DLX1 is mainly localized in the nucleus and that the nuclear localization depends on the integrity of the homeodomain.

We next used WT and mutant DLX1 to study transcriptional activation of a luciferase gene driven by the regulatory element WIP, which was originally identified in the MSX1 homeodomain-binding fragment from the *Wnt-1* genome and shown as a responsive element for DLX2 and DLX5 (31). WT DLX1 significantly raised the luciferase activity in a dose-dependent manner. Similar transactivation was observed with the AA mutant, but not at all with BK and SA (Fig. 2 *A* and *D*), suggesting that the transactivation activity of DLX1 depends on the integrity of its homeodomain, particularly on its third helix (Fig. 2*A*), as expected. Because DLX2 and DLX5 compete at the WIP promoter with MSX1 and MSX2 (37), we examined for a similar competition between DLX1 and MSX2. The results showed that the DLX1-induced WIP activation was reversed by the increasing dose of MSX2 (Fig. 2*E Left*). In contrast, MSX2 repressed the luciferase activity, which was reversed by increasing the dose of DLX1 (Fig. 2*E Right*). These results indicate that DLX1 functionally interacts with the MSX proteins as DLX2 and DLX5.

DLX1 Blocks Activin A-Induced Erythroid Differentiation. Because it is known that DLX1 is temporally and spatially coexpressed with BMP-4 in development of teeth and jaw (12, 13), and because DLX1 is expressed in the hematopoietic compartment where activin A, TGF-β1, and BMP-4 are known to be important, we asked whether DLX1 modifies the biological responses triggered by these cytokines. Because DLX1 seemed to be preferentially expressed in erythroid cell lines, we chose the murine erythroleukemia cell line, F5-5, which is known to differentiate into hemoglobin-containing cells in response to activin A (38).

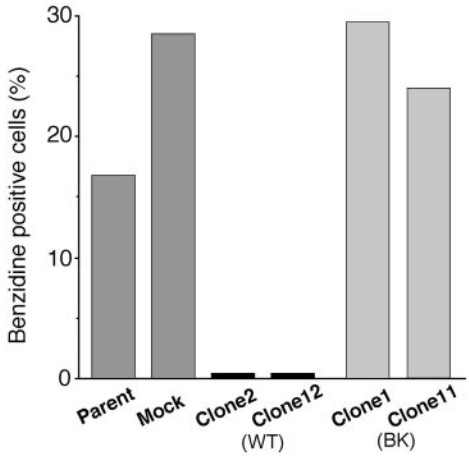

Fig. 3. Prevention of F5-5 cells from activin A-induced erythroid differentiation. After stimulating with activin A for 7 days, the ratio of benzidine-positive cells was evaluated. The data shown are representative of three independent experiments.

We obtained several clones transduced with WT or BK mutant DLX1 or the mock virus. In parental F5-5 and mock-transduced F5-5, activin A clearly induced erythroid differentiation as assessed by benzidine positivity in 20–40% of the cells. In contrast, the WT, but not BK mutant, DLX1-expressing clones were resistant to activin A-induced erythroid differentiation (Fig. 3). Because it has previously been reported that the absence of Smad7, an inhibitory Smad (I-Smad), is necessary for the activin A-induced erythroid differentiation in F5-5 (24), we examined for Smad7 mRNA expression in the parental and WT DLX1-transduced F5-5 cells. Unexpectedly, we observed prompt up-regulation of Smad7 mRNA in both parental and WT DLX1-transduced F5-5 cells (data not shown), in contrast to the previous report.

DLX1 Represses Activin A- and TGF-β1-Induced Transcriptional Activation. The above results raised the possibility that DLX1 affects the transcriptional responses induced by activin A. When F5-5 was transiently transfected with the luciferase gene driven by the activin-responsive element (A3-Lux), activin A induced the increase in the A3-Lux activity in a dose-dependent manner, reaching >20-fold at the concentration of 10 ng/ml (Fig. 4*A*). This increase was significantly reduced when WT DLX1 was cotransfected, in a manner dependent on the DLX1 dose (Fig. 4*A*). We then tested whether the similar repression was seen when FAST2, an activin-specific activation potentiator found in the Smad complex, was overexpressed. The level of transcriptional response to activin A was highly enhanced when F5-5 was cotransfected with FAST2 together with A3-Lux (Fig. 4*B*). However, DLX1 dramatically repressed the transactivation by activin A in the presence of FAST2 (Fig. 4*B*).

We then determined whether the transactivation by Smad2 and Smad3, which are effectors of the activin A stimulation, was indeed repressed by DLX1. Overexpression of Smad2 and Smad3 activated A3-Lux in F5-5 cells only at low levels (data not shown). Because it is well known that 3TP-Lux, a TGF-β-responsive reporter plasmid carrying the plasminogen-activator inhibitor-1 promoter, is highly responsive to overexpressed Smad2 and Smad3 in HepG2 cells (39), we used this system to evaluate the effect of DLX1 on transactivation by overexpressed Smad2 and Smad3 with or without overexpression of Smad4. The results clearly showed that cotransfection of DLX1 repressed the

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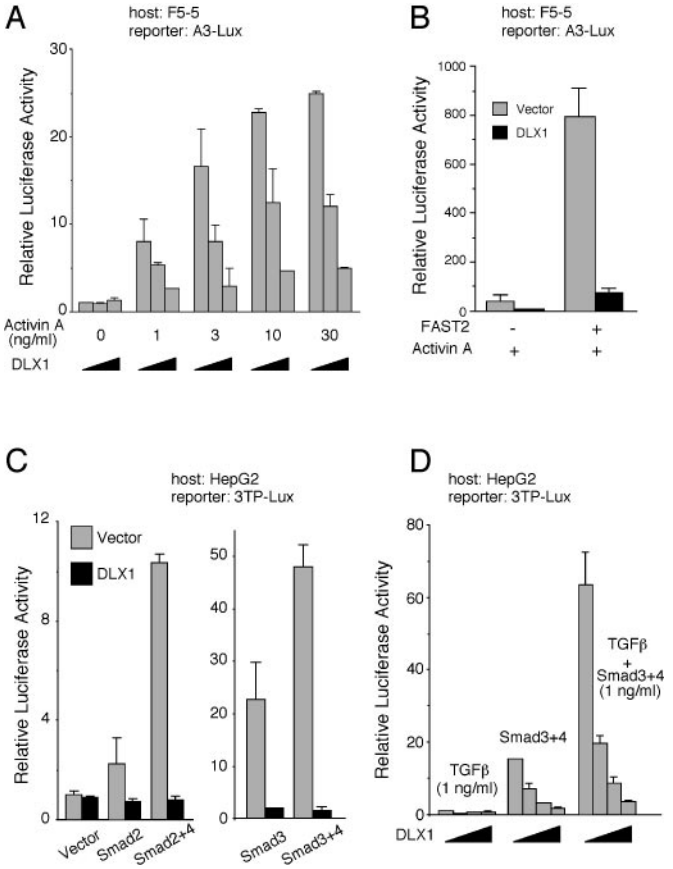

Fig. 4. DLX1 blocks activin A-induced, overexpressed Smads-induced, and TGF-β-induced transcriptional activations. (*A* and *B*) Block of activin A-induced transactivation in F5-5 cells without (*A*) or with (*B*) overexpressed FAST2. (*C*) Overexpressed Smad2- and Smad3-induced transactivation in HepG2 cells with or without Smad4 overexpression. (*D*) TGF-β-induced transcriptional activation.

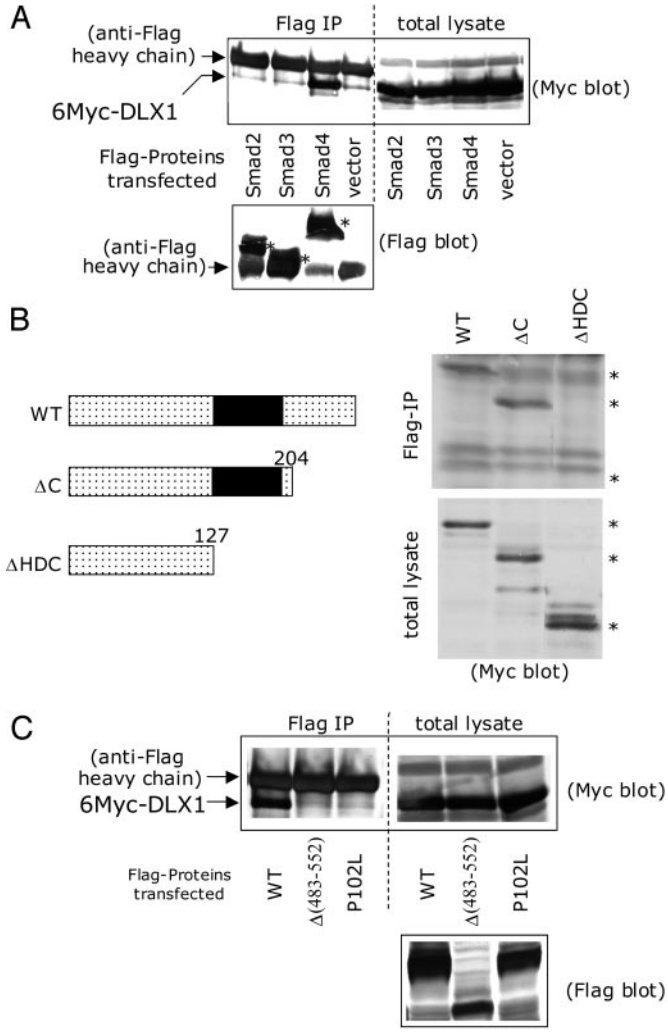

Fig. 5. Coprecipitation of DLX1 with Smad4 in COS7 cells. (*A*) Smad4 but not Smad2 or Smad3 coprecipitates DLX1. The asterisks indicate the positions of Smad2, Smad3, and Smad4. (*B*) Deletion of the homeodomain results in cancellation of coprecipitation. The asterisks indicate the positions of WT and mutant DLX1 proteins. (*C*) Mutant Smad4 proteins identified in leukemia cells do not coprecipitate DLX1.

3TP-Lux activity induced by Smad2 and Smad3 overexpression to almost the control level, irrespective of overexpression of Smad4 (Fig. 4*C*). Next, we examined whether DLX1 also repressed the transcriptional response induced by TGF-β1 stimulation. TGF-β1 raised the 3TP-Lux activity to only low levels in HepG2 cells. These cells, however, significantly responded to TGF-β1 stimulation when cotransfected with Smad3 and Smad4 (Fig. 4*D*). DLX1 again repressed the transcriptional activities induced by TGF-β1 stimulation and Smad3 and Smad4 overexpression (Fig. 4*D*). These results suggest that DLX1 represses the activity of activin A and TGF-β, either individually for Smad2 and Smad3 or through a pathway common to both Smad2 and Smad3.

DLX1 Physically Interacts with Smad4. To determine the target molecule through which DLX1 affects the activin A and TGF-β signaling, we explored whether DLX1 physically interacted with Smad proteins. In coprecipitation experiments using COS7 cells, Smad4, but not Smad2 or Smad3, coprecipitated with WT DLX1 (Fig. 5*A*). An experiment using DLX1 proteins truncated from the C terminus showed that the region C-terminal to the homeodomain was not required and that the domain N-terminal to the homeodomain alone was insufficient for DLX1 to interact with Smad4 (Fig. 5*B*), implying the DLX1 homeodomain in the interaction between DLX1 and Smad4.

To analyze the domain required for Smad4 to interact with DLX1, we used two mutant Smad4 proteins previously identified in the samples from a patient with acute myeloblastic leukemia (Smad4P102L) and from an acute monocytic leukemia cell line [Smad4Δ (483–552)] (29). Smad4P102L has an amino acid substitution in the N-terminal MH1 domain, and Smad4Δ (483–552) has a small C-terminal truncation. Both mutants are known to lack the transcriptional activity and block the normal Smad4 function at the DNA binding and the nuclear localization, respectively (29). Neither of these mutants coprecipitated WT DLX1 (Fig. 5*C*), indicating that both domains could contribute to the interaction with DLX1.

DLX1 Represses BMP-4-Induced Transcriptional Activation. If Smad4 is the molecule involved in the DLX1-Smad cross talk, it would be expected that DLX1 also represses the transcriptional activation induced by BMP-4. We assessed BMP-4-induced transcriptional activation using the 3GC2-Lux reporter system in C2C12 cells. We found that the DLX1 repressed 3GC2-Lux activation induced by BMP-4 in a dose-dependent manner (Fig. 6).

Discussion

We report that the *distal-less*-like homeodomain-containing protein DLX1 is expressed in the human hematopoietic cells and

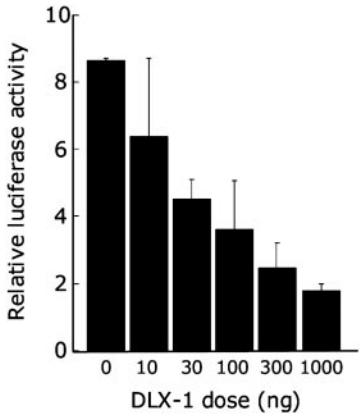

Fig. 6. DLX1 blocks BMP-4-induced transactivation in C2C12 cells. The ratio of luciferase activities with and without BMP-4 is plotted.

that it represses signaling pathways downstream to multiple TGF-β family cytokines by possibly interfering with the Co-Smad, Smad4.

Expression of DLX1. *DLX1* is the second member of DLX gene family to be found in hematopoietic cells, following the previous report of *DLX7* expression in leukemia cells (40, 41). *DLX1* is preferentially expressed in nonlymphoid cells to lymphoid cells, implying some role for DLX1 in nonlymphoid hematopoiesis.

Interference of TGF-β Pathways by DLX1. Hematopoietic expression of *DLX1* and a crucial role for TGF-β family cytokines in blood production is reminiscent of the temporal and spacial coexpression of *DLX/MSX* homeobox genes with BMP-4 in the developing embryo (12, 13). Although these homeobox genes have been considered to be a transcriptional target of BMP-4 signaling, it has also been reported that, among many molecules, some homeodomain-containing proteins such as TGIF (7) and Hox-C8 (6) physically interact with Smads and are involved in or modify Smad signaling pathways. Therefore, we asked whether the DLX1 protein is involved in the Smad pathways.

We demonstrate that the DLX1 protein does interfere with the Smad signaling from both biochemical and biological approaches. Some Smad inhibitors recruit a histone deacetylase complex, but trichostatin A, a histone deacetylase inhibitor, did not reverse the DLX1-mediated repression of the Smad activity (data not shown). DNA-binding of the Smad complex was not abolished, and formation of the Smad3/Smad4 complex was stably maintained if DLX1 was present (data not shown). Therefore, we speculate that DLX1 may sequester transcriptional activator complex from the Smad3/Smad4 complex by binding to Smad4, although positive data showing the recruitment of the histone deacetylase complex to the Smad complex was not available.

The biological significance of the interference between DLX1 and the Smad signaling is unknown. Activin A-induced erythroid differentiation in F5-5 could imply a role in primitive erythropoiesis in the yolk sac. It is also possible that there is an interaction between the activin A pathway and DLX1 in the bone marrow cells, as activin A is thought to play some role in the adult bone marrow microenvironment (22, 42, 43). Alternatively, high levels of DLX1 expression in nonlymphoid leukemia cells may reflect events occurring as part of the leukemic transformation of normal hematopoietic cells.

It was recently reported that the absence of one of the I-Smads, Smad7, was necessary for activin A-induced erythroid differentiation and that the forced expression of Smad7 blocked the activin A-induced erythroid differentiation in F5-5 cells (24). In our study, however, activin A rapidly induced the expression of Smad7 mRNA in F5-5 (data not shown). Given that the Smad7 expression is rapidly induced by various TGF-β-family cytokines in the target cells, induction of Smad7 in activin A-triggered differentiating F5-5 should not be unreasonable. Moreover, we observed that the level of Smad7 mRNA induction in DLX1-overexpressing F5-5 was the same as parental F5-5 (data not shown), indicating that Smad7 is not involved in the activin A resistance in DLX1-transduced F5-5.

Interaction of DLX1 and Smad4. Many transcription factors and cofactors are known to be involved in the Smad signaling. Many of these molecules directly associate with various Smad proteins (reviewed in ref. 44). Very few of them, however, associate with Smad4 rather than R-Smads and negatively regulate the Smad pathways (44). In this context, DLX1 represents an uncommon case among the many known Smad-associating proteins. For the interaction with Smad4, DLX1 exclusively uses the homeodomain, which is the primary site for the interaction with DNA. This is similar to the case of DLX2 and DLX5 (37), where the important region for interaction with other proteins is also present in the homeodomain.

We have demonstrated that integrity of both amino acids at the position 102 in the MH1 domain and C-terminal end of the MH2 domain in Smad4 are important for the interaction with DLX1, using two nonfunctioning Smad4 mutants isolated from human leukemia cells (29). Biological significance of these mutations in Smad4 and negative interaction with DLX1 remains to be elucidated.

Hisamaru Hirai died suddenly on August 23, 2003. His students, fellows, colleagues, and collaborators will greatly miss his energetic and nurturing leadership in the field of molecular hematology. We dedicate this paper in his memory. We thank Y. Eto for activin A; T. Kitamura for the TF-1 cDNA library and the pMX-puro retrovirus vector; T. Imamura and K. Miyazono for pcDef3 and pcDNA3/6Myc vectors and 3TP-Lux and 3GC2-Lux plasmids; M. Whitman for the A3-Lux plasmid; J. L. Wrana for a pCMV vector and Flag-Smad2 and Myc-FAST2 plasmids; R. Derynck for Flag-Smad3 and Flag-Smad4 plasmids; M. Hino for benzidine; and C. Kato for excellent technical help. This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Ministry of Health, Labor, and Welfare of Japan (to S.C. and H.H.), National Institutes of Health Grant R37GM09966 (to F.H.R.), the New York Community Trust (K.T.), the U.S.–Japan Cooperative Program for Cancer Research and the Donaghue Medical Research Foundation (S.C.), and the American Heart Association (K.T.).

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