# Cell Type-specific Target Selection by Combinatorial Binding of Smad2/3 Proteins and Hepatocyte Nuclear Factor 4α in HepG2 Cells*[S]◆

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**Specific regulation of target genes by transforming growth factor-β (TGF-β) in a given cellular context is determined in part by transcription factors and cofactors that interact with the Smad complex. In this study, we determined Smad2 and Smad3 (Smad2/3) binding regions in the promoters of known genes in HepG2 hepatoblastoma cells, and we compared them with those in HaCaT epidermal keratinocytes to elucidate the mechanisms of cell type- and context-dependent regulation of transcription induced by TGF-β. Our results show that 81% of the Smad2/3 binding regions in HepG2 cells were not shared with those found in HaCaT cells. Hepatocyte nuclear factor 4α (HNF4α) is expressed in HepG2 cells but not in HaCaT cells, and the HNF4α-binding motif was identified as an enriched motif in the HepG2-specific Smad2/3 binding regions. Chromatin immunoprecipitation sequencing analysis of HNF4α binding regions under TGF-β stimulation revealed that 32.5% of the Smad2/3 binding regions overlapped HNF4α bindings. *MIXL1* was identified as a new combinatorial target of HNF4α and Smad2/3, and both the HNF4α protein and its binding motif were required for the induction of *MIXL1* by TGF-β in HepG2 cells. These findings generalize the importance of binding of HNF4α on Smad2/3 binding genomic regions for HepG2-specific regulation of transcription by TGF-β and suggest that certain transcription factors expressed in a cell type-specific manner play important roles in the transcription regulated by the TGF-β-Smad signaling pathway.**

Transforming growth factor-β (TGF-β) has multiple roles in growth arrest, apoptosis, differentiation, epithelial-to-mesenchymal transition, and extracellular matrix deposition in various types of cells and is related to embryonic development and various human diseases (1). In cancer cells, TGF-β is known to possess conflicting tumor-suppressive and pro-metastatic functions; TGF-β inhibits cancer progression by cell cycle arrest and apoptosis, although it also helps cancer cells to evade anti-tumor immune response and metastasize to distant organs by epithelial-to-mesenchymal transition. Understanding the precise regulatory mechanisms downstream of this signaling pathway is required for the control of diseases.

Smad family proteins are the principal and specific molecules that transduce signals from the ligand-bound active receptor complexes on the cell surface membrane to the nucleus (2–4). Smad2 and Smad3 form hetero-oligomers with Smad4 after phosphorylation of the C terminus of Smad2 or Smad3 by the receptor complex, and the Smad complex serves as a transcription factor on the genome. The Smad complex was reported to bind to the sequences containing "GTCT" (Smad-binding element) by *in vitro* screening of the binding sequences and structural analysis of the Smad complex bound to the DNA (5, 6). However, this very simple motif is present everywhere in the genome. It has also been suggested that the binding affinity of the Smad complex to Smad-binding elements is not high. Interaction with other transcription factors and cofactors has been shown to be important to provide functional specificity of TGF-β signaling, and these transcription factors and cofactors facilitate binding of the Smad complex to the favorable positions in the genome. Expressions of these transcription factors and cofactors are often regulated in a cell- or tissue-specific manner, and a subset of these molecules indeed has been shown to be important for the context-dependent Smad binding to the genome and transcriptional regulation of target genes. Target genes of TGF-β that are regulated by the same cofactors are designated as a synexpression group (7), as reported in the regulation of several genes such as *CDKN1A/p21* and *GADD45A* by FOXO family proteins (8).

High throughput analyses of transcription factor binding regions using either an oligonucleotide tiling microarray or massively parallel sequencing are now widely used to understand the roles of transcription factors (9, 10). We have identi-

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fied Smad2/3 binding regions and Smad4 binding regions using a promoter tiling array in the HaCaT normal human epidermal keratinocyte cell line (11, 12). We found Smad2/3 binding regions at the previously analyzed regions as well as many unrecognized binding regions. Activator protein-1 (AP-1), v-Ets erythroblastosis virus E26 oncogene homolog, and transcription factor AP-2-binding motifs were identified as enriched motifs in the Smad2/3 binding regions in HaCaT cells (11). However, it remains to be determined whether the identified Smad2/3 binding regions are shared with those in other cells and tissues.

Hepatocyte nuclear factor 4α (HNF4α)[4] is a member of the hepatocyte nuclear factor family, which includes well conserved nuclear receptors among mammals. HNF4α is expressed in the liver, kidney, small intestine, and pancreas and is essential for the organogenesis of the liver (13, 14). HNF4α is also required for the differentiation of hepatocytes and is engaged in hepatocyte-specific gene regulation related to the synthesis of apolipoproteins, acute phase reactive proteins, and other secreted proteins. HNF4α is located in the nucleus, forms a homodimer, and functions as a transcription factor by binding to DR1 elements in the genome (15).

Several groups have identified a functional relationship between HNF4α and TGF-β signaling. TGF-β down-regulates the expression of variant 1 of HNF4α, one of the transcriptional variants of HNF4α, which has an AF1 transcriptional activation domain in their N terminus (16). On the contrary, the expression of the transcriptional activation domain lacking variant 8 is repressed by TGF-β in normal murine mammary gland (NMuMG) epithelial cells (17). TGF-β has also been reported to regulate the HNF4α expression by proteasome-dependent degradation (16). The effect of HNF4α on TGF-β-induced transcription has also been analyzed for the *APOC3* promoter, where HNF4α interacts with Smad3 and Smad4 to induce the *APOC3* expression (18, 19). The HNF4α-binding motif in the *APOC3* promoter has been shown to be important for TGF-β-induced transcriptional activity, and a mutant of Smad3 that lacks the DNA binding property to Smad-binding elements still interacts with HNF4α to synergistically transactivate the *APOC3* promoter (19). Because HNF4α binds to the MH1 DNA binding domain of Smad3 through both its N and C termini (19), Smads may indirectly bind to the *APOC3* promoter through HNF4α. However, it is still unclear whether the reported interaction with Smads and mechanisms of transcriptional regulation are generally important for the function of both HNF4α and Smads in hepatocytes.

Here, we identified Smad2/3 binding regions in the HepG2 hepatoblastoma cell line and compared them with the binding regions in HaCaT cells and hepatocellular carcinoma Hep3B cells to elucidate the mechanisms of context-dependent regulation of TGF-β-induced transcription. We found HNF4α as an important factor for HepG2-specific Smad2/3 binding regions and analyzed its regulatory mechanism using a new target gene of HNF4α, *MIXL1*, under TGF-β stimulation.

[4] The abbreviations used are: HNF4α, hepatocyte nuclear factor 4α; qPCR, quantitative PCR; ChIP-seq, ChIP sequencing; FDR, false discovery rate; CEAS, cis-regulatory element annotation system.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—Human hepatoblastoma HepG2 cells and hepatocellular carcinoma Hep3B cells were obtained from the American Type Culture Collection and were cultured in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Rockford, IL), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin G, and 100 μg/ml streptomycin. HaCaT cells were maintained in Dulbecco's modified Eagle's medium (DMEM; catalog no. 11965; Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were grown in a humidified atmosphere with 5% $CO_2$ at 37 °C.

*Antibodies and Chemicals*—We used the following commercially available antibodies: mouse anti-Smad2/3 (BD Biosciences), anti-α-tubulin (DM1A) (Sigma), rabbit anti-phospho-Smad2 (Cell Signaling Technology, Beverly, MA), anti-phospho-Smad3 (Cell Signaling Technology), and anti-HNF4α (Santa Cruz Biotechnology, Santa Cruz, CA). TGF-β3 was from R & D Systems (Minneapolis, MN).

*RNA Interference and Oligonucleotides*—Stealth small interfering RNA (siRNA) targeting HNF4α (5′-AAAGCGGCCACGCGAGUCAUACUGG-3′) was purchased from Invitrogen. As a negative control, we used a predesigned siRNA (12935–200, sequence not available). siRNAs were introduced into HepG2 cells using the Lipofectamine RNAi MAX reagent (Invitrogen) according to the manufacturer's instructions (reverse transfection method), using 3 nM siRNA per $1 \times 10^5$ cells/ml per well of 12-well plates.

*Immunoblotting*—SDS-gel electrophoresis and immunoblotting were performed as described, using a LAS-4000 lumino-image analyzer (Fujifilm, Tokyo, Japan) (20). RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA) was used for cell lysis.

*Chromatin Immunoprecipitation (ChIP), ChIP-chip, and ChIP Sequencing (Seq)*—Sample preparation for ChIP-chip analysis was performed as described previously, using anti-Smad2/3 (BD Biosciences) (11, 21, 22). Briefly, ChIP and control input DNA samples were amplified by two cycles of *in vitro* transcription and hybridized on separate Affymetrix human promoter 1.0R oligonucleotide tiling arrays (Affymetrix, Santa Clara, CA). Enrichment values (ChIP/control input DNA) were calculated using the MAT algorithm, and Smad2/3 binding regions were determined using detection $p$ values of $10^{-4}$ (23). For conventional quantitative anti-Smad2/3 ChIP-quantitative PCR (ChIP-qPCR) analyses, cells were cross-linked with 10 mM dimethyl adipimidate (Thermo Scientific) for 30 min at room temperature before formaldehyde fixation. Bioruptor UCW-301 (output: H, 2 cycles of 30 s of sonication with 30-s intervals; Cosmobio, Tokyo, Japan) was used for sonication of ChIP-qPCR samples. Sample preparation for ChIP-seq was performed according to the manufacturer's instructions (Illumina, San Diego). The obtained read tags were mapped on the NCBI/hg18 human genome assembly using ELAND (Illumina). Cis-Genome was used for the calculation of significant HNF4α

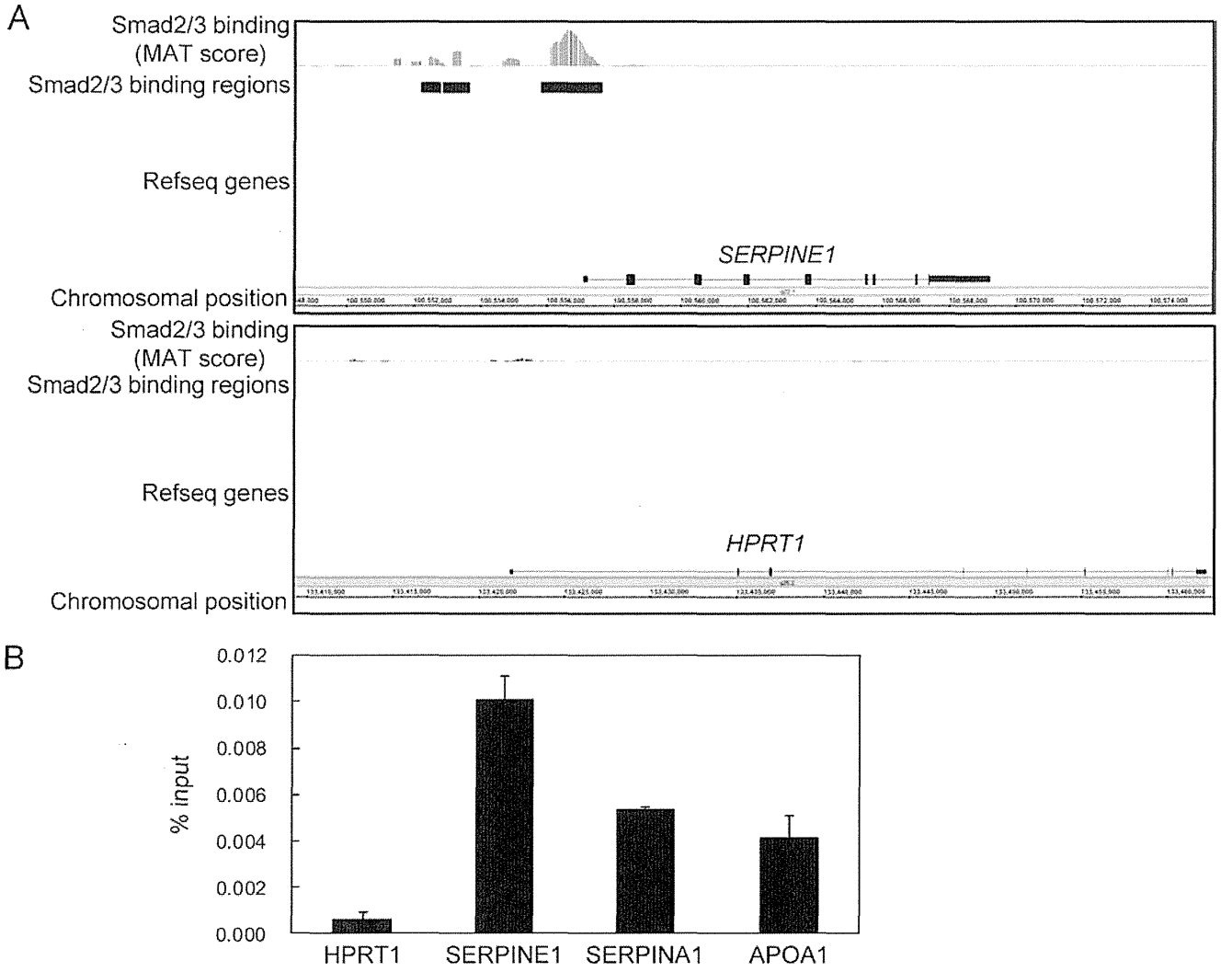



FIGURE 1. **Identification of Smad2/3 binding regions in HepG2 cells.** *A*, Smad2/3 binding to the *SERPINE1/PAI-1* locus in HepG2 cells. MAT scores were plotted at the *SERPINE1* and *HPRT1* loci to obtain a graphical representation of Smad2/3 binding in these regions. Significant Smad2/3 binding regions as determined by detection of $p$ values of $10^{-4}$ are shown by *black bars*. *B*, percent input values of Smad2/3 binding compared with input genome as determined by ChIP-qPCR. Cells were treated with 120 pM TGF-β for 1.5 h. Cells were cross-linked sequentially with dimethyl adipimidate and formaldehyde. *Error bars* represent S.D.

binding regions (one-sample analysis, with default parameters and a false discovery rate (FDR) of less than 0.1%) (24).

*Motif Prediction and Mapping*—CisGenome was used for both *de novo* motif prediction and motif mapping of Smad2/3 ChIP-chip and HNF4α ChIP-seq binding regions. Cis-regulatory element annotation system (CEAS) was also used for identification of known motifs in Smad2/3 and HNF4α binding regions (25).

*Quantitative PCR Analysis*—Quantitative real time PCR analysis was performed as described previously (26). Amplification data were quantified using the standard curve method. Detected signals were confirmed to be specific by a dissociation protocol. All samples were run in duplicate or triplicate, and the results were averaged. Sequences of the primers are available in supplemental Tables S1 and S2.

*Reverse Transcription-PCR and Expression Microarray Analysis*—Total RNAs were extracted as described previously (26). First strand cDNAs were synthesized using the PrimeScript2 reverse transcriptase (TakaraBio, Shiga, Japan). The experimental procedures for GeneChip (Affymetrix) were performed as described previously (11) using the GeneChip human U133 plus 2.0 oligonucleotide array (Affymetrix). Microarray Suite software 5.0 (Affymetrix) was used with a target intensity of 100. Data from one array were obtained for each sample.

*Promoter Reporter Constructs and cDNA Constructs*—The human *MIXL1* promoter reporter (MIXL1-WT-luc, −583 to −8) and its mutants were constructed by a PCR-based approach and cloned into the pGL4.10 (Promega, Fitchburg, WI) vector with a minimal luciferase promoter sequence (11). Primer sequences used for the construction of MIXL1-mut1-luc were 5′-GCAGGGGTGGTAAATAAATTTAGGGTTATCGGGACAGACGGGAC-3′ and 5′-GTCCCGTCTGTCCGATAACCCTAAATTTATTTACCACCCCTGC-3′. The primer sequences for the construction of MIXL1-mut2-luc were 5′-TCCCCGAGCCCTTAGGGTATTACACCGCCCCGCCTTC-3′ and 5′-GAAGGCGGGGCGGTGTAATACCCTAAGGGCTCGGGGA-3′. MIXL1-luc reporters with mutations in Smad-binding elements were also constructed by

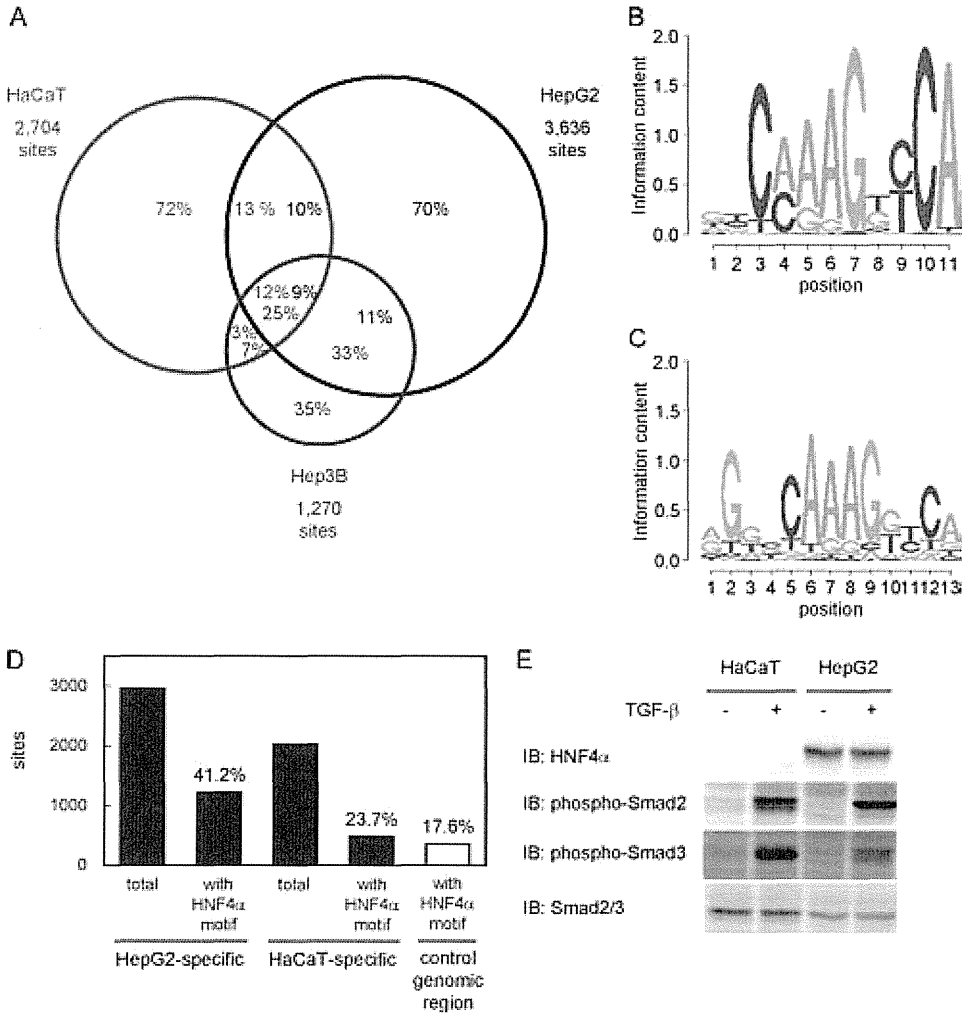



FIGURE 2. **Comparison of the Smad2/3 binding regions among different cell lines.** *A*, Venn diagrams showing the overlaps of Smad2/3 binding regions in HaCaT, HepG2, and Hep3B cells. *Numbers* in the *circles* indicate percentages of the Smad2/3 binding region of each cell line (*red*, HaCaT; *black*, HepG2; *blue*, Hep3B). *B*, identification of a motif conserved in HepG2-specific Smad binding regions. Partial genomic sequences within 250 bp from the peak positions of HepG2-specific Smad2/3 binding regions ($n$ = 2,955) were analyzed using the CisGenome Gibbs motif sampler. Default parameters were used for the calculation, except for the numbers of motifs to be identified ($n$ = 10). Matrix datum of the motif calculated by CisGenome was graphically shown using the SegLogo function of the R software. *C*, HNF4α-binding motif that matched the predicted motif in HepG2-specific Smad2/3 binding regions. The JASPAR CORE data base was used to identify known transcription factor binding motifs similar to the calculated matrix data in *B*. An HNF4α motif (ID: MA0114.1) was identified as the most similar motif with a comparison score of 21.3, which reached 96.9% of the potential maximal score. *D*, frequencies of the HNF4α-binding motif in Smad2/3 binding regions. Presence of the HNF4α-binding motif in each Smad2/3 binding region (within 250 bp from the peak signal position) was determined using CisGenome. Frequencies of the motif in either HepG2- or HaCaT-specific Smad2/3 binding regions were then calculated. As a control, matched genomic regions to HaCaT-specific Smad2/3 binding regions were obtained using CisGenome, and the frequency of the HNF4α motif was determined. *E*, expression of the HNF4α protein and phosphorylation of Smad2/3 in HaCaT and HepG2 cells. Cells were treated with TGF-β for 1.5 h, and the expression of each protein was determined by immunoblotting (*IB*).

PCR (see Fig. 6*D* for sequences of the mutants). HNF4α and its C115R mutant were constructed by a PCR-based approach using the first strand cDNA of HepG2 cells as a template. The sequences of all cDNAs constructed were verified by sequencing.

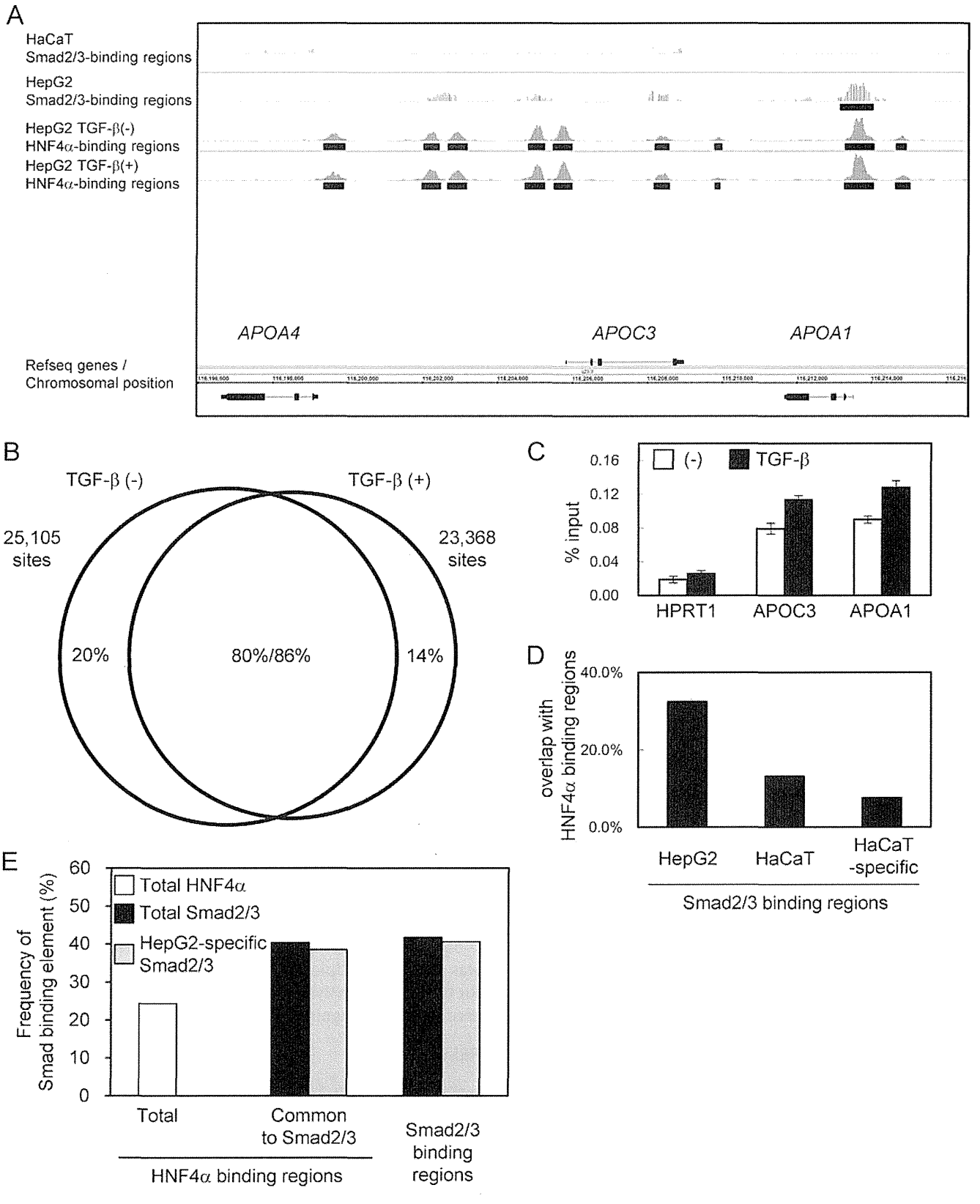
*Luciferase Assay*—Cells in 24-well plates were transfected with different combinations of promoter reporter constructs and expression plasmids by using Lipofectamine LTX (Invitrogen). The total amount of transfected DNA was adjusted to the same amount using an empty vector. Twenty four hours later, cells were treated with TGF-β for an additional 24–48 h and lysed. Luciferase activities in the lysates were measured using the Dual-Luciferase® reporter system (Promega) as described previously. For normalization, cells were co-transfected with pGL4.75-SV40-hRluc. Where indicated, siRNAs were transfected 24 h before reporter transfection. All samples were prepared in triplicate, and results were averaged.

*Statistical Analysis*—The Tukey-Kramer test of R program was used for multiple comparisons of data. The $t$ test was used for two-sample analysis. $p$ values of less than 0.05 were considered to indicate significance for each experiment.

## RESULTS

*Identification of HNF4α-binding Motif in HepG2-specific Smad2/3 Binding Regions*—To determine Smad2/3 binding regions in HepG2 hepatoblastoma cells, we stimulated the cells with TGF-β for 1.5 h and fixed them with formaldehyde to cross-link genome-bound molecules to DNA. ChIP on microarray analysis (ChIP-chip) was performed according to the established protocol using an Affymetrix human promoter

array (22). Obtained image data were analyzed using the MAT algorithm, which provided enrichment values of the ChIP samples for every promoter region, compared with the input genomic sample. There was significant Smad2/3 binding to the promoter region of the *SERPINE1/PAI-1* gene as observed previously in the ChIP-chip analysis of Smad2/3 bindings in HaCaT cells (Fig. 1*A*, *upper panel*) (11). In contrast, there was no significant Smad2/3 binding to the *HPRT1* locus that served



as a negative control region (Fig. 1*A*, *lower panel*). We confirmed significant enrichment of Smad2/3 binding to the *SERPINE1*, *SERPINA1*/α-antitrypsin, and *APOA1* loci using ChIP-qPCR (Fig. 1*B*). We identified 3,636 significant Smad2/3 binding regions that had detection $p$ values of less than $10^{-4}$ within the promoter regions of known genes.

Next, we compared the identified Smad2/3 binding regions in HepG2 cells to those in HaCaT cells (11). We also obtained 1,270 Smad2/3 binding regions in Hep3B hepatocellular carcinoma cells to determine their overlaps (Fig. 2*A*). We found that only 25.2% of the Smad2/3 binding regions in HaCaT cells ($n$ = 682) were shared with those in HepG2 cells. In contrast, 58.3% of the binding regions in Hep3B cells overlapped with those identified in HepG2 cells, although the number of overlapping binding regions ($n$ = 741) was similar to that between HaCaT and HepG2 cells. Many of the Smad2/3 binding regions were thus unique to each cell type. We determined the candidate target genes of Smad2/3 using the dataset of Smad2/3 binding regions that were either common to HepG2 and HaCaT, HepG2-specific, or HaCaT-specific (supplemental Tables S3-S5), and performed gene ontology analysis of each category by DAVID (27). We did not observe remarkable differences in the top five enriched annotation clusters between the common Smad2/3 binding regions and HepG2-specific Smad2/3 binding region. Conversely, enrichment of cell death and cytoskeleton-related annotations was found in HaCaT-specific binding regions (supplemental Table S6).

To identify specific motifs in the Smad2/3 binding regions in HepG2 cells, *de novo* motif prediction was performed using the CisGenome Gibbs motif sampler (supplemental Fig. S1*A*). We searched for known motifs that had similarity to the calculated motifs using the JASPAR data base (28). As shown in Fig. 2*C*, we found that one predicted motif (Fig. 2*B*) was strongly similar to the HNF4α-binding motif (Fig. 2*C*, 96.9% score). The frequency of the HNF4α motif in HepG2-specific Smad2/3 binding regions was 41.2%, although that in HaCaT-specific Smad2/3 binding regions and its matched random genomic regions was 23.7 and 17.6%, respectively (Fig. 2*D*). We also analyzed the sequences in Smad2/3 binding regions using the CEAS analysis tool as we did in our previous report (11, 12, 25), and the HNF4α-binding motif was identified as one of the top three enriched motifs in the binding sequences (supplemental Fig. S1*B*) (25). It should be noted that canonical Smad-binding element (M00974.SMAD, "CAGAC") was also identified as enriched motif through CEAS analysis and present in 40.6% of the HepG2-specific Smad2/3 binding regions (data not shown). These findings suggested that the HNF4α motif was enriched in HepG2-specific Smad2/3 binding regions and had roles for cell type specificity of Smad2/3 binding and TGF-β-induced transcription in HepG2 cells. HNF4α is one of the "master genes" of hepatocytes and is essential for hepatocyte-specific gene expressions and functions. Because HNF4α was not expressed in HaCaT cells (Fig. 2*E*), we decided to determine HNF4α binding regions *in vivo* in the presence of TGF-β.

*HNF4α Binding to Its Binding Regions Is Not Extensively Altered by TGF-β*—ChIP-chip and ChIP-seq studies of HNF4α binding regions have been reported using HepG2 cells (29–31). We retrieved data (30) from the data base and found that 20.7% of the Smad2/3 binding regions were common to HNF4α binding regions *in vivo*. However, no reports have yet determined the changes in the binding of HNF4α by extracellular stimulations, including that by TGF-β. We therefore acquired the HNF4α binding data using the newly available ChIP-seq technology to compare the Smad2/3 binding with the HNF4α binding under TGF-β stimulation. We identified 25,105 significant HNF4α binding regions in the absence of TGF-β and 23,368 regions in the presence of TGF-β, at an FDR of less than 0.1%. The *APOA4/APOC3/APOA1* gene cluster that is a known target of HNF4α is shown in Fig. 3*A*. We observed significant HNF4α binding to several of these regions in the absence of TGF-β, and the binding was not extensively changed following stimulation. We also found that there was significant Smad2/3 binding to the *APOA1* promoter, which was absent in HaCaT cells. Our data showed that Smad2/3 binding to the *APOC3* promoter was not significant.

We then examined the changes in HNF4α binding by TGF-β stimulation. More than 80% of the HNF4α binding regions overlapped between TGF-β-treated and untreated cells. However, there were also specific binding regions for both TGF-β-treated and untreated cells (Fig. 3*B*). In addition, we calculated the changes in the normalized read numbers within the HNF4α binding regions by TGF-β stimulation and found that some regions indeed had either decreased or increased sequence reads following TGF-β stimulation (data not shown). Percent input values of HNF4α binding to the *APOC3* and *APOA1* loci were also up-regulated to some extent (Fig. 3*C*), suggesting that there were some, if limited, roles of TGF-β for HNF4α binding. Using the HNF4α binding data with TGF-β stimulation, we determined the frequency of HNF4α binding in Smad2/3 binding regions *in vivo*. We found that 32.5% of the Smad2/3 binding regions in HepG2 cells were indeed common to HNF4α binding regions. In contrast, only 13.2% of Smad2/3 binding regions in HaCaT cells were common, and the frequency decreased to 7.7% when HaCaT-specific Smad2/3 binding regions were examined (Fig. 3*D*). These results suggested that HNF4α and Smad2/3 binding regions are located in close prox-

FIGURE 3. **Identification of HNF4α binding regions in the presence and absence of TGF-β stimulation.** *A*, graphical representation of HNF4α binding to the *APOA4/APOC3/APOA1* gene loci. Sequence read numbers of 100-bp sliding window were plotted for HNF4α ChIP-seq samples. Smad2/3 bindings as determined by ChIP-chip analysis were shown in the *upper two panels* as in Fig. 1*A*. *Black bars* represent significant binding regions (FDR, <0.1%). *B*, Venn diagrams showing overlap between TGF-β-treated and untreated HNF4α binding regions. HNF4α binding regions were determined for each sample (FDR, <0.1%). HNF4α binding regions that have overlapping regions within 500 bp from their positions of maximum read numbers were considered as shared binding regions. *C*, changes in the HNF4α binding to *APOC3* and *APOA1* loci were quantitatively determined by ChIP-qPCR analysis. *Error bars*, S.D. *D*, frequencies of *in vivo* HNF4α binding to the Smad2/3 binding regions. Percentages of HNF4α binding within 250 bp from the peak signal position of Smad2/3 binding regions were calculated for the indicated Smad2/3 binding groups. *E*, frequencies of canonical Smad-binding elements in HNF4α binding regions compared with Smad binding regions in HepG2 cells. A Smad-binding element, M00974.SMAD that was identified as an enriched motif in HepG2-specific Smad2/3 binding regions using CEAS (see text), was selected for calculation. CisGenome was used for mapping of the motif. Presence of the motif for each HNF4α binding region and Smad2/3 binding region was determined using PerlScript.

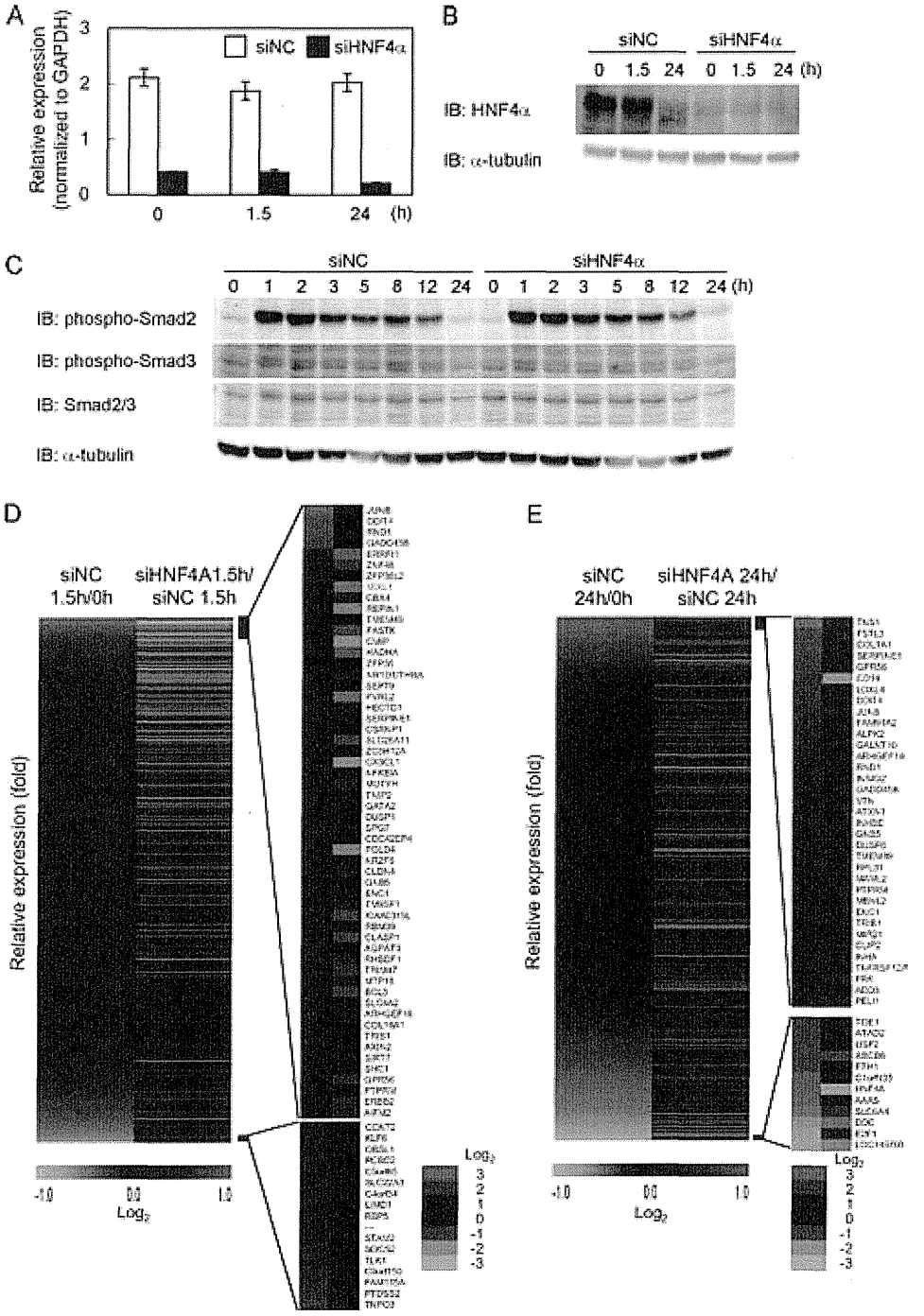



FIGURE 4. **Effect of knockdown of HNF4α on TGF-β-induced gene expression in HepG2 cells.** *A,* confirmation of HNF4α-knocked down samples for microarray analysis. HepG2 cells were transfected with HNF4α siRNA and treated with 120 pM TGF-β for the indicated times and harvested. Expression of HNF4α was determined by RT-qPCR and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *siNC*, negative control siRNA. *B,* down-regulation of HNF4α protein expression by siRNA. *C,* phosphorylation levels of Smad2/3 by using HNF4α siRNA. The *top two panels* show phosphorylation of Smad2 and Smad3. The *3rd panel* indicates the expression of total Smad2/3, and the *bottom panel* is a loading control. *IB*, immunoblot. *D,* heat map of the TGF-β-induced expression of target genes of Smad2/3 and HNF4α and effect of HNF4α siRNA. Target genes that have overlapping binding regions of Smad2/3 and HNF4α were sorted by their induction of probe signal values by TGF-β stimulation for 1.5 h and are represented by *color bars* in the *1st column*, using the TM4 microarray software (60). Relative expression of these genes in HNF4α siRNA samples to the control siRNA is shown in the *2nd column*. In addition, a list of genes whose expressions changed more than 1.5-fold is shown in the *right panel* with their expression changes. *E,* heat map of target genes of TGF-β with Smad2/3 binding regions common to HNF4α at 24 h after TGF-β stimulation are shown as in *D*. Genes whose expressions were changed more than 2-fold are shown in the *right panel*.

imity to each other in HepG2 cells, although we could not determine whether HNF4α- and Smad2/3-binding "elements" overlapped within the binding regions because of the limited resolution of ChIP-chip- and ChIP-seq-based assays. We then calculated the frequency of Smad-binding element CAGAC in HNF4α binding regions. 40.4% of the binding regions common to HNF4α and Smad2/3 had Smad-binding elements, compared with 24.3% in the total HNF4α binding regions (Fig. 3*E*).

**TABLE 1**

**TGF-β-induced changes in gene expression in relation to Smad2/3 binding**

Expression array data transfected with control siRNA and stimulated with TGF-β were compared with Smad2/3 ChIP-chip data. A total of 8,653 genes that had values of more than 100 at least at one time point for one of their probes ($n$ = 13,720) was used for the analysis. Up-regulated or down-regulated genes were determined compared with 0-h values. The positions of peak signals of Smad binding regions (SBRs) relative to the nearby RefSeq genes were first determined, and regions within 5 kb upstream from the transcription start site and the first intron were selected. *a indicates number of genes analyzed by microarray. *b indicates number of genes which have Smad2/3 binding regions.

| | | All genes | | Genes with SBRs | | |
|---|---|---|---|---|---|---|
| | | *a | % | *b | % | *b/*a (%) |
| Total | | 8653 | 100.0 | 1941 | 100.0 | 22.4 |
| Increase | | | | | | |
| >2-Fold | 1.5 h | 25 | 0.3 | 14 | 0.7 | 56.0 |
| | 24 h | 223 | 2.6 | 59 | 3.0 | 26.5 |
| >1.5-Fold | 1.5 h | 273 | 3.2 | 89 | 4.6 | 32.6 |
| | 24 h | 837 | 9.7 | 250 | 12.9 | 29.9 |
| Decrease | | | | | | |
| >2-Fold | 1.5 h | 16 | 0.2 | 2 | 0.1 | 12.5 |
| | 24 h | 174 | 2.0 | 25 | 1.3 | 14.4 |
| >1.5-Fold | 1.5 h | 217 | 2.5 | 47 | 2.4 | 21.7 |
| | 24 h | 877 | 10.1 | 160 | 8.2 | 18.2 |

*Effect of HNF4α on the Expression of Smad2/3 Target Genes*—To elucidate the effect of HNF4α on TGF-β-induced transcriptional regulation, we knocked down HNF4α by using siRNA (Fig. 4, *A* and *B*). The phosphorylation of Smad2 and Smad3 was not affected by the siRNA under the applied condition (Fig. 4*C*). We obtained expression microarray data and calculated the changes in the expression of genes with binding regions shared by Smad2/3 and HNF4α in the presence of TGF-β and siRNA. We first analyzed the data of cells transfected with control siRNA. Twenty four hours after TGF-β stimulation, 4.3 and 21.1% of the genes with Smad2/3 binding regions were regulated (either up- or down-regulated) more than 2- and 1.5-fold, respectively (Table 1). We observed that Smad2/3 binding regions were weakly enriched in the genes up-regulated by TGF-β at 1.5 h (supplemental Fig. S2). Many of the genes with Smad2/3 binding regions were not transcriptionally regulated by TGF-β, and these findings were essentially similar to those in our previous analysis in HaCaT cells (11). We then found that HNF4α siRNA inhibited the expression changes of common target genes of HNF4α and Smad2/3 by TGF-β 1.5 h after stimulation (Fig. 4*D*). This result underscored the general roles of HNF4α in hepatocyte-specific transcriptome regulation by TGF-β. In contrast, the effect of HNF4α silencing was not so obvious in the TGF-β-induced expression changes 24 h after stimulation, compared with the setting after 1.5 h of TGF-β stimulation, although TGF-β-induced expression changes of a subset of genes appeared to be rather enhanced by HNF4α knockdown (Fig. 4*E*). We focused on the changes at 1.5 h, when we obtained Smad2/3 and HNF4α binding data, and we listed target genes of TGF-β and the effect of HNF4α knockdown (Table 2). We identified *MIXL1* as both TGF-β- and HNF4α-regulated gene with no Smad2/3 binding regions in HaCaT cells.

*HNF4α Provides a New Mechanism of TGF-β-induced MIXL1 Expression*—As shown in Fig. 5*A*, significant binding of Smad2/3 and HNF4α to the *MIXL1* promoter was observed (Fig. 5*A*). We confirmed the binding of these transcription factors by ChIP-qPCR (Fig. 5, *B* and *C*) and changes in the expression of *MIXL1* by HNF4α siRNA by RT-qPCR (Fig. 5*D*). We then determined the sequence of the *MIXL1* promoter bound by Smad2/3 and HNF4α. We first found two possible HNF4α-binding motifs (Fig. 6*A*). Using a promoter reporter assay, we found

**TABLE 2**

**TGF-β-induced genes with Smad2/3 and HNF4α binding at 1.5 h**

Target genes of TGF-β in HepG2 cells that were induced more than 2-fold at 1.5 h and that have common binding regions for Smad2/3 and HNF4α were sorted by their expression changes in the presence or absence of HNF4α siRNA. Presence of Smad2/3 binding regions in HaCaT cells is also shown in the 2nd column.

| Gene symbol | Smad2/3 binding in HaCaT cells | Relative expression (siNC/siHNF4α) | Induction by TGF-β |
|---|---|---|---|
| | | *-fold* | *-fold* |
| *CMIP* | + | 0.3 | 2.1 |
| *REPIN1* | + | 0.3 | 2.2 |
| *MIXL1* | − | 0.3 | 2.3 |
| *ERRFI1* | + | 0.4 | 2.7 |
| *FASTK* | + | 0.5 | 2.1 |
| *ZNF48* | − | 0.5 | 2.6 |
| *CBX4* | − | 0.6 | 2.2 |
| *TMEM49* | − | 0.8 | 2.1 |
| *ZFP36L2* | − | 0.9 | 2.6 |
| *JUNB* | + | 1.0 | 9.8 |
| *DDIT4* | + | 1.1 | 5.6 |
| *GADD45B* | + | 1.3 | 4.9 |
| *RND1* | − | 1.4 | 5.5 |

that the transcriptional activity of the reporter containing the Smad2/3-HNF4α binding regions was up-regulated by TGF-β, which was significantly repressed by mutations in the HNF4α-binding sequences (Fig. 6*B*). We next searched for canonical Smad-binding elements conserved between mouse and human. We identified three Smad-binding elements between the two HNF4α motifs, and one just upstream of the distal HNF4α motif, termed SBE1 to -4 (Fig. 6*C*). Of them, only a mutation in SBE2 lost TGF-β-induced transcription (Fig. 6*D*). These results suggested that both HNF4α-binding motifs and SBE2 are required for *MIXL1* reporter activity induced by TGF-β.

The transcriptional activity of the reporter was inhibited by HNF4α siRNA, which was observed even without TGF-β, suggesting that preceding binding of HNF4α to its binding motifs as observed in Fig. 5*A* was important both for the basal and TGF-β-induced transcriptional activation of *MIXL1* promoter (Fig. 7*A*). We also investigated the effect of forced HNF4α expression in HaCaT cells to determine whether HNF4α was able to activate the *MIXL1* transcriptional activity in these cells. As shown in Fig. 7*B*, HNF4α induced the transcriptional activity of the *MIXL1* promoter reporter in HaCaT cells. We then examined the effect of a mutant of HNF4α that cannot bind to DNA (HNF4α CR mutant) (32) and found that DNA binding

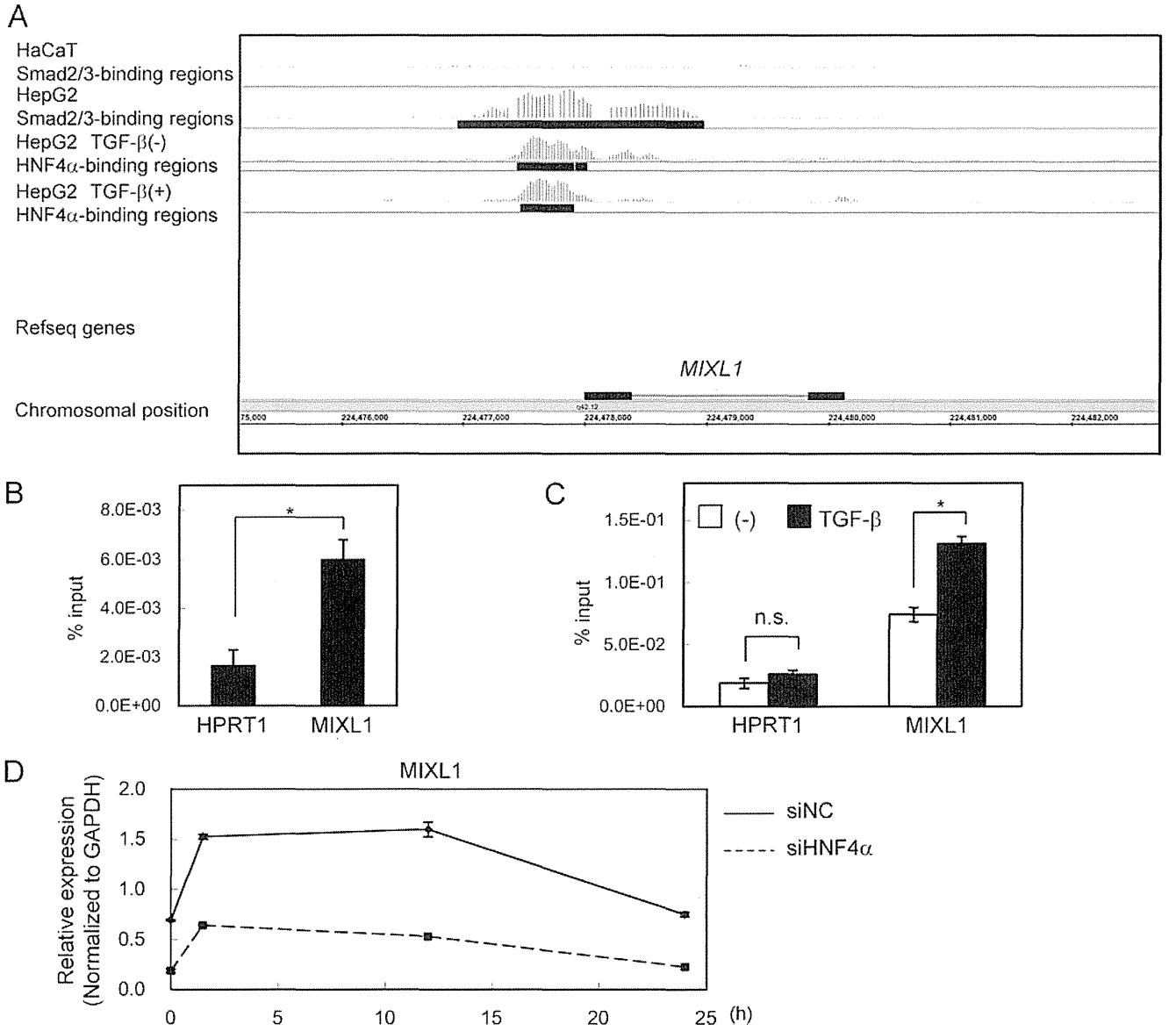



FIGURE 5. **Smad2/3 and HNF4α bindings in the *MIXL1* locus.** *A*, Smad2/3- and HNF4α-enriched regions in the *MIXL1* locus are shown as in Fig. 3*A*. *B*, HepG2 cells were treated with 120 pM TGF-β for 1.5 h, fixed in formaldehyde, and harvested. Smad2/3 binding to the *MIXL1* locus was verified by ChIP-qPCR. *HPRT1* served as a negative control. *C*, HepG2 cells were treated with or without 120 pM TGF-β for 1.5 h, and ChIP-qPCR analysis of the *MIXL1* locus using anti-HNF4α was performed as in *B*. *n.s.*, not significant. *D*, effects of knockdown of HNF4α on TGF-β-induced expression changes of *MIXL1*. HepG2 cells were transfected with control siRNA (*siNC*) or siHNF4α, treated with 3 ng/ml TGF-β for the indicated times, and harvested. HNF4α expression was quantified by RT-qPCR. *, $p < 0.05$; *error bars*, S.D.

activity was required for its TGF-β-induced transcriptional activation (Fig. 7*C*). Finally, the effect of HNF4α siRNA on Smad2/3 binding to the *MIXL1* promoter was determined. HNF4α siRNA inhibited the TGF-β-induced Smad2/3 binding to the *MIXL1* promoter, indicating that the recruitment of Smad2/3 was one of the mechanisms of transcriptional regulation by HNF4α under TGF-β stimulation (Fig. 7*D*).

Taken together, these findings propose that the preceding binding of HNF4α on *MIXL1* promoter enables the recruitment of Smad2/3 to this promoter after TGF-β stimulation and confers TGF-β-mediated HepG2-specific *MIXL1* induction.

## DISCUSSION

Recent technological advances, including ChIP-chip and ChIP-seq, provide a functional platform for comprehensive understanding of transcriptional regulation. This study revealed that Smad2/3 binding regions specifically observed in HepG2 cells were enriched in HNF4α binding regions. HNF4α was also expressed in Hep3B cells, and HNF4α-binding motif was identified in Smad2/3 binding regions in Hep3B cells by CEAS analysis (data not shown), which suggests that the functional relation between Smad2/3 and HNF4α is commonly observed in hepatocyte-derived cells. Based on the findings on the HNF4α-Smad interaction (18), physical interaction between HNF4α and Smads is important, at least in part, for TGF-β-induced Smad2/3 binding and transcriptional activation in HepG2 cells. It is also possible that HNF4α has additional indirect interactive functions for TGF-β signaling. Many regulatory mechanisms control the expression of a proper set of genes in various cells and tissues. At the genome level, CpG methylation plays a cen-

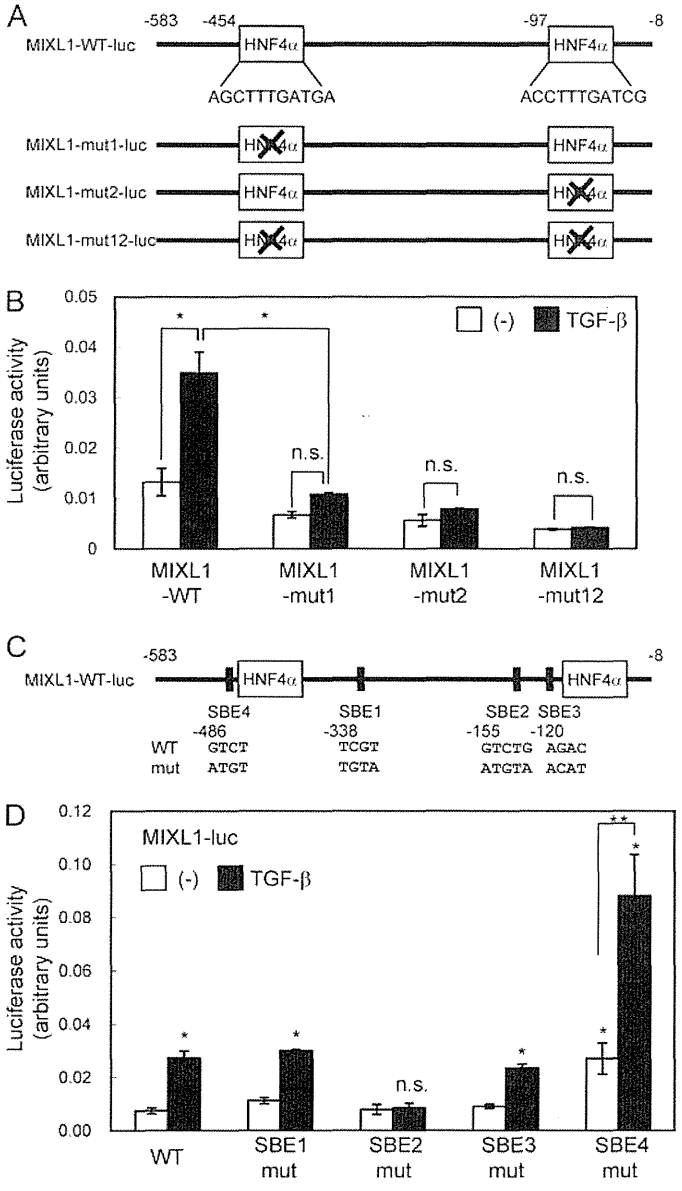



FIGURE 6. **Identification of regulatory elements important for TGF-β-induced transactivation of the *MIXL1* promoter.** *A*, schematic representation of HNF4α-binding motifs in the Smad2/3 and HNF4α binding region of the *MIXL1* promoter. Promoter reporters with mutations in their HNF4α-binding motifs are shown in the *lower panel*. *B*, activation of the *MIXL1* gene promoter by TGF-β and effects of mutations in putative HNF4α-binding motifs. HepG2 cells were transfected with the *MIXL1* promoter and its mutants and treated with TGF-β for 24 h. *C*, conserved Smad-binding elements (*SBEs*) of the *MIXL1* promoter. Four Smad-binding elements that were conserved between mouse and human (SBE 1–4) are shown with their relative positions from the transcription start site. Nucleotide sequences of Smad-binding elements and their mutations used in *D* are also shown. *WT*, wild-type; *mut*, mutant. *D*, effect of mutations in Smad-binding elements on TGF-β-induced transcriptional activity of *MIXL1* promoter. Cells were treated as in *B*, and luciferase activities were determined. *, $p < 0.05$ compared with WT without TGF-β; **, $p < 0.05$ compared with SBE4 mutant, without TGF-β; *n.s.*, not significant compared with WT and SBE2 mutant, without TGF-β; *error bars*, S.D.

tral role to avoid unintended expression of genes that are not suitable for the given tissue (33). Modification of the histone tail is also well known to lead to the formation of either euchromatin or heterochromatin. These modifications of the genome or histones allow transcription factors and cofactors to access the cell- and tissue-specific genomic loci to exert their actions. Modifications of the genome and histones are sometimes induced by *trans* factors during differentiation of the cells and tissues (34, 35). HNF4α physically interacts with the histone acetyltransferase complex and chromatin remodeling complex (29), and it is thus possible that HNF4α induces such epigenomic changes in the liver and indirectly provides Smad2/3 to access to hepatocyte-specific binding regions.

Identification of Smad binding regions downstream of the TGF-β/activin signaling by ChIP-chip analysis has been performed using several cell lines. Recently, Fei *et al.* (36) reported promoter analysis of Smad2 binding regions in mouse embryonic stem cells by ChIP-chip. We and Qin *et al.* (12, 37) analyzed Smad4 binding regions under TGF-β stimulation using HaCaT and ovarian surface epithelial cells, respectively. It has been reported that transcription factor binding regions in the same target gene loci differ among the five vertebrate species (38); it is thus difficult to compare the ChIP-chip or ChIP-seq data obtained from mouse and human. Differences in the ChIP efficiencies of the antibodies also make the comparison of the data difficult (12). Importantly, we used the same antibody and sample preparation procedures for HaCaT cells and HepG2 cells. Our present analysis thus revealed for the first time that Smad binding regions greatly differ among cell lines. Analysis of HaCaT-specific *trans* factors will facilitate our understanding of cell type-specific TGF-β-induced transcription in the future. However, comparison of the number of binding regions in different cell types is still difficult. We found a greater number of Smad2/3 binding regions in HepG2 cells than in HaCaT cells. Because the phosphorylation of Smad3 was weaker and the percent input value of the Smad2/3 ChIP sample was smaller in HepG2 than HaCaT cells, we cannot conclude that HepG2 cells have more Smad2/3 binding regions than HaCaT. It should also be noted that we cannot fully exclude that the antibody recognizes unknown genome-bound molecules in addition to Smad2/3.

Comparison of ChIP-chip and ChIP-seq data of the same transcription factor has been reported (39). In general, ChIP-seq is reported to be more sensitive and specific than ChIP-chip. Oligonucleotide-based array analysis has a potential risk of cross-hybridization and false discovery. Conversely, ChIP-seq also has difficulty in identifying GC-rich sequences (10, 39). We primarily focused on the comparison of our previously reported Smad2/3 binding regions to those of different cell types by the same platform. However, based on the known problems as described above, comparison of the Smad2/3-HNF4α binding regions will be more accurately performed by the ChIP-seq in the future.

Interaction of several transcription factors at the same enhancer positions has been recognized, and the complex is called "enhanceosome." Structure of such complex and their binding DNA motifs have been analyzed in the interferon-β promoter as reviewed by Panne (40). In enhanceosome, each transcription factor physically interacts with others to provide its adequate surface that can bind to the series of their corresponding DNA motifs. Several reports have identified HNF4α binding regions by ChIP-chip and ChIP-seq analyses (29–31, 38, 41–43). Many transcription factors, *e.g.* FOXA2, GABP, HNF1α, HNF4γ, HNF6, cohesin, and CDX2, were identified to

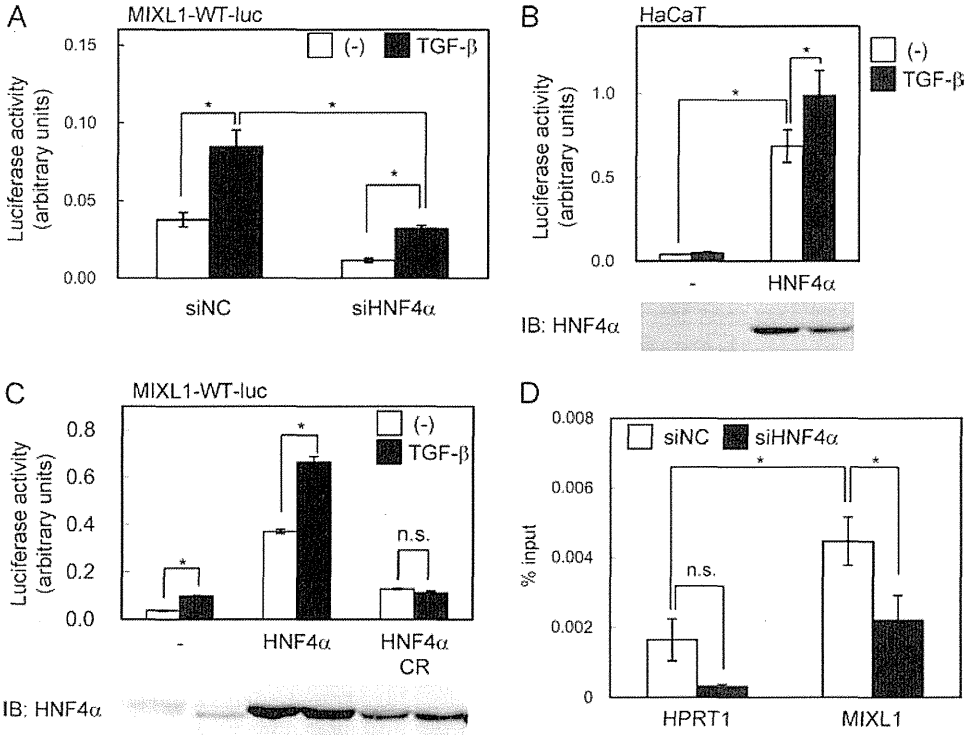



FIGURE 7. **Roles of HNF4α on Smad2/3 binding and transcriptional activity of *MIXL1* promoter.** *A*, effects of HNF4α knockdown on transactivation of the *MIXL1* promoter. HepG2 cells were transfected with siRNAs 1 day before transfection with the reporter constructs. *siNC*, negative control siRNA. *B*, effects of exogenous HNF4α on transactivation of the *MIXL1* promoter. HNF4α was exogenously expressed in HaCaT cells, and transcriptional activity of MIXL1 reporter was determined. The *lower panel* shows the protein expression of HNF4α. *IB*, immunoblot. *C*, HNF4α (variant 2, RefSeq ID: NM_000457) or its C115R (*CR*) mutant, which does not bind to DNA, was overexpressed in HepG2 cells. The *lower panel* shows the protein expression of HNF4α and its mutant. *D*, effect of HNF4α siRNA on Smad2/3 binding to the *MIXL1* locus. HepG2 cells were transfected with siRNAs 24 h before TGF-β stimulation. Cells were fixed 1.5 h after treatment, and ChIP-qPCR was performed as in Fig. 1*B*. *Error bars*, S.D.; *, $p < 0.05$; *n.s.*, not significant.

co-localize with HNF4α through these analyses. Other reports also revealed interaction of FOXO1 or retinoic acid receptor/retinoid X receptor with HNF4α on specific promoters (44, 45). These findings clearly revealed steady-state binding regions of HNF4α on the genome and suggested that transcription factors that co-localize or interact with HNF4α may form enhanceosome with HNF4α. Changes in the HNF4α binding regions were found during differentiation of an intestinal epithelial cell line CaCo2 (43); however, to our knowledge, the effect of single extracellular stimulation on genome-wide HNF4α binding regions has not yet been elucidated. Our present analysis provides the data of HNF4α binding regions following TGF-β stimulation, which were compared with the Smad2/3 binding regions in HaCaT cells that lack the expression of HNF4α. We have found that large proportions of HNF4α binding regions in HepG2 cells were unchanged by TGF-β stimulation. However, some changes in HNF4α binding regions were observed with regard to their positions and their strength, suggesting that TGF-β might regulate a subset of HNF4α binding regions. de Boussac *et al.* (46) reported that hepatocyte growth factor inhibited HNF4α binding to the *ABCC6* promoter, which together suggest the importance of changes in the HNF4α binding positions by external stimuli. We also found that the effect of HNF4α on the TGF-β-induced gene expression after 24 h of TGF-β stimulation was different from that after 1.5 h of TGF-β stimulation. Studies on the changes in the genome-wide HNF4α and Smad2/3 binding after TGF-β stimulation at several time points and ChIP-seq analysis of HNF4α with other interactive factors in relation to their binding DNA sequences will reveal new mechanisms of the regulation of HNF4α-induced transcription in the context of the enhanceosome.

MIXL1 is an ortholog of *Xenopus* Mix.1, a transcription factor rapidly induced by activin during the early stage of *Xenopus* development (47). There are six known homologs that have been identified in *Xenopus* to engage in the formation of mesoderm and endoderm (48, 49). However, only one ortholog of Mix.1 is known in human and mouse (50). MIXL1 is required for the development of the chordamesoderm, heart, and gut in mouse (51). Forced expression of MIXL1 in embryonic stem cells resulted in the differentiation of the cells to endoderm (52). TGF-β is reported to induce *Mix.2* promoter activity by formation of a Smad2/Smad4/FAST-1 (FoxH1) complex (53). In mouse, Smads and FAST-1 interact to up-regulate the transcriptional activity of the *MIXL1* promoter (54, 55). However, FAST-1 is not expressed in HepG2 cells (56). Our finding of TGF-β-induced *MIXL1* expression in HepG2 cells suggests a previously unrecognized regulatory mechanism of its expression by HNF4α in the absence of FAST-1. During development, HNF4α is expressed in the visceral endoderm during the gastrulation stage and plays a role in the differentiation of the embryonic mesoderm (57). MIXL1 is also expressed in the visceral endoderm and induces migration of the embryonic endoderm. HNF4α-null mice embryo showed impaired development of mature visceral endoderm, indicating that HNF4α acts upstream of MIXL1, at least in the visceral endoderm. Notably, both HNF4α and MIXL1 positively reg-

ulate the E-cadherin expression (52, 58), and the HNF4α expression was repressed in a model of progression of hepatocellular carcinoma (59). Functional analysis of MIXL1 in liver fibrosis and hepatocellular carcinoma in relation to TGF-β signaling might reveal the roles of MIXL1 in the adult liver in the future.

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# Homozygously deleted gene DACH1 regulates tumor-initiating activity of glioma cells

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**Loss or reduction in function of tumor suppressor genes contributes to tumorigenesis. Here, by allelic DNA copy number analysis using single-nucleotide polymorphism genotyping array and mass spectrometry, we report homozygous deletion in glioblastoma multiformes at chromosome 13q21, where *DACH1* gene is located. We found decreased cell proliferation of a series of glioma cell lines by forced expression of DACH1. We then generated U87TR-Da glioma cells, where DACH1 expression could be activated by exposure of the cells to doxycycline. Both ex vivo cellular proliferation and in vivo growth of s.c. transplanted tumors in mice are reduced in U87TR-Da cells with DACH1 expression (U87-DACH1-high), compared with DACH1-nonexpressing U87TR-Da cells (U87-DACH1-low). U87-DACH1-low cells form spheroids with CD133 and Nestin expression in serum-free medium but U87-DACH1-high cells do not. Compared with spheroid-forming U87-DACH1-low cells, adherent U87-DACH1-high cells display lower tumorigenicity, indicating DACH1 decreases the number of tumor-initiating cells. Gene expression analysis and chromatin immunoprecipitation assay reveal that fibroblast growth factor 2 (FGF2/bFGF) is transcriptionally repressed by DACH1, especially in cells cultured in serum-free medium. Exogenous bFGF rescues spheroid-forming activity and tumorigenicity of the U87-DACH1-high cells, suggesting that loss of DACH1 increases the number of tumor-initiating cells through transcriptional activation of bFGF. These results illustrate that DACH1 is a distinctive tumor suppressor, which does not only suppress growth of tumor cells but also regulates bFGF-mediated tumor-initiating activity of glioma cells.**

neural differentiation | gliomagenesis

Glioblastoma multiformes (GBMs), the most frequent primary malignant brain tumor in adults, are aggressive and highly invasive tumors (1). Genetic alterations of GBMs, including aberration of DNA copy number such as gene amplifications, loss of heterozygosity (LOH), and homozygous deletions, leads to activation of oncogenes and inactivation of tumor suppressor genes (1–3). DNA copy number analysis by single-nucleotide polymorphism (SNP) genotyping enables the high-resolution analysis of allelic DNA copy number and has been used to obtain a genome-wide view of DNA copy number alterations in human cancers (4–10). Particularly, pairwise analysis of normal and tumor DNAs is crucial in detecting homozygous deletion in clinical specimens, because infiltrating nontumorous cells are significant in GBMs.

In this study we examined the allelic copy number of paired glioma and blood DNAs by SNP genotyping array analysis by using Genome Imbalanced Map (GIM) algorithm (5, 11), which could calculate the signal ratio of SNP genotyping array in an allelic manner. We identified a unique homozygous deletion at *DACH1* gene region on chromosome 13q21, and we demonstrated forced expression of DACH1 reduced proliferation of cultured glioma cells and in vivo tumor growth in ortholotopic xenograft model. We also found that DACH1 inhibited formation of tumor-initiating spheroids, presumably by directly repressing expression of fibroblast growth factor-2 (*FGF2*), suggesting *DACH1* is a unique tumor suppressor of glioblastoma, which not only suppresses tumor growth but also inhibits generation of tumor-initiating cells.

## Results

**DACH1 Gene on Chromosome 13q21 Is Homozygously Deleted in Glioblastoma.** To identify genomic alterations involved in gliomagenesis, we performed DNA copy number analysis of eight GBMs by using SNP genotyping array (Fig. 1*A* and Fig. S1*A*), as well as analysis of a corresponding normal blood DNA for highlighting tumor-specific alterations. We observed high-level amplification at chromosome 7q21 (inferred total copy number >8) and copy number reduction within chromosome arm 4q, 10p, 13q, 16q, 17q, and 18q, and we detected homozygously deleted loci at chromosome 9q21 and 10q23, which spanned known tumor suppressor genes *CDKN2A* and *PTEN*, respectively. In addition, we found a unique homozygous deletion at chromosome 13q21 in GBM case 4 and LOH at the region in GBM case 1 and 3 (Fig. 1*A* and Fig. S1*A*). Although loss of chromosome 13q14.2 spanning *RB1* gene is frequently observed in human malignancies including GBMs (12, 13), the homozygous deletion of chromosome 13q21 has not been reported.

To examine allelic DNA copy numbers at the chromosome 13q21 region in additional GBM cases, we performed targeted genotyping analysis of 28 paired GBMs and blood cells by high-density mass spectrometric analysis using MassARRAY (14, 15). The chromosomal losses were found at least in 11 samples (GBM case 1, 3, 4, 5, 18, 19, 24, 25, 26, 27, and 28; 39.3%), and three of them (GBM case 4, 5, and 27; 10.7% of GBMs) displayed homozygous deletion (Fig. 1*B* and Fig. S1*B*). By combination of SNP genotyping array with MassARRAY analysis, homozygously deleted region at chromosome 13q21 of GBM case 4 was restricted to rs1999603 (probe S10)–rs1326684 (probe M8), which might be extended from rs9542598 (probe M2) to rs1421280 (probe S15). In two additional GBM cases, homozygous deletions found by MassARRAY at this locus were from rs10492537 (probe M6) to rs3818437 (probe M7), which might be extended

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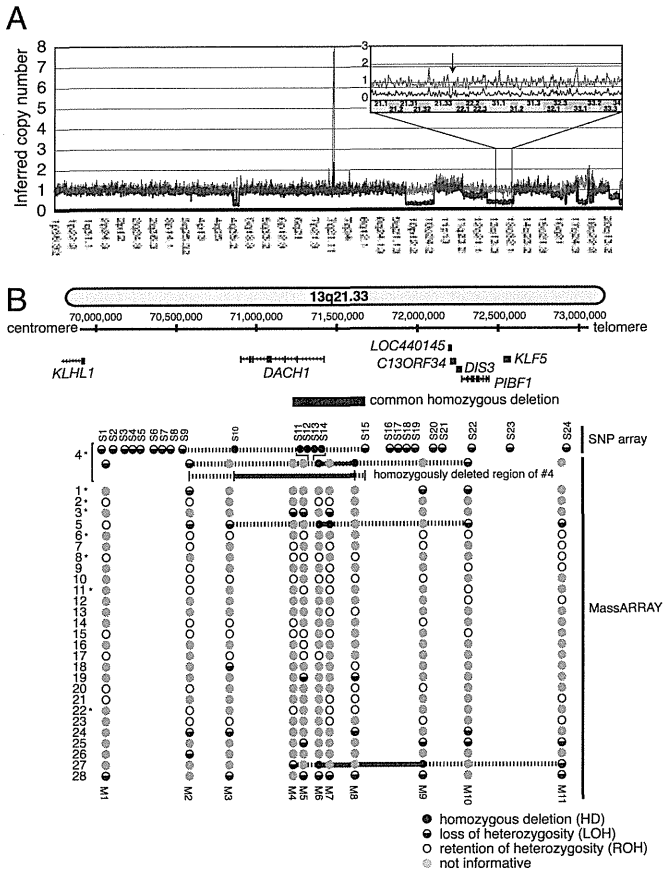


**Fig. 1.** DACH1 is homozygously deleted in GBMs. (*A*) An integrated view of DNA copy numbers and allelic alternations of GBM case 4. Scatter plot of inferred allelic copy numbers (red and blue) was estimated by GIM algorithm. An arrow indicated homozygously deleted loci. (*Right*) Magnified view of DNA copy number of GBM case 4 from chromosome 13q21 to 13q34. One allele showed DNA copy number reduction in whole region (blue), whereas the other allele (red) showed copy number reduction only at chromosome 13q21. (*B*) Summary of SNP genotyping in the 13q21 region. S1-24 and M1-11 are SNP ID available in SNP genotyping array and MassARRAY, respectively. Black line, the homozygously deleted region; dotted line, the possible extended region of homozygous deletions; *, also examined by SNP arrays shown in Fig. S1*A*.

to rs1931443 (probe M3)–rs7332388 (probe M10) in GBM case 5, and from rs10492537 (probe M6) to rs1981186 (probe M9), which might be extended to rs10507796 (probe M4)–rs1886452 (probe M11) in GBM case 27. The boundary of the common homozygously deleted region on the centromeric side was estimated at rs10507796 (probe M4), which was genotyped as LOH in case 27, whereas the telomeric boundary was estimated at rs1421280 (probe S15), which was genotyped as LOH in case 4 (Fig. 1*B*). Because the homozygously deleted region from rs10507796 to rs1421280 overlapped with *DACH1* gene and did not span the adjacent genes to *DACH1* such as *LOC440145* and *KLHL1*, we thought *DACH1* was a target of these deletions and could be a potential candidate as a tumor suppressor gene of GBMs. Quantitative PCR analysis demonstrated that DNA copy numbers of GBM samples with a homozygous deletion at *DACH1*-region were much lower than that of whole brain and normal fibroblast cells KMS-6, which has a normal karyotype as (46, XX) (Fig. S2*A*). In addition, we confirmed protein expression of DACH1 was reduced in glioma cells with a homozygous deletion at *DACH1* region, whereas it was detectable in vascular endothelial cells (Fig. S2*B*).

**DACH1 Expression Reduces Growth of Glioma Cells.** We screened an expression level of *DACH1* gene in a series of glioma cell lines. Forced expression of lentivirus-carrying *DACH1* reduced cell proliferation of SF188, U87MG, T98G, and LNG-308 glioma cell lines, where endogenous expression of *DACH1* was not detectable (Fig. S3 *A* and *B*). We then generated two U87MG-derived cell lines: U87TR-Da clone-16 and -18, where DACH1 expression could be induced by the addition of doxycycline (Fig. 2*A* and Fig. S4*A*). DACH1 decreased viability of the cells at 4 and 6 d after addition of doxycycline (Fig. 2*B*) and also abrogated anchorage-independent growth of the cells in soft agar (Fig. S3 *C* and *D*). We next examined the impact of DACH1 expression on in vivo growth of s.c.-injected tumors of U87TR-Da clone-16 and -18. Increased expression of DACH1 was observed at 8 d after replacement of doxycycline-free drinking water by doxycycline-supplemented water (Fig. 2 *C* and *D*). Growth of the U87TR-Da clone-16 and clone-18 tumors was significantly decreased by administration of doxycycline compared with U87TR-Da tumors supplemented with normal drinking water (Fig. 2 *E* and *F* and Fig. S4*B*). Tumor formation was reproduced by stereotactic intracerebral inoculation of DACH1-nonexpressing U87TR-Da cells, whereas tumor was not detectable by the injection of DACH1-expressing U87TR-Da cells (Fig. 2 *G* and *H* and Fig. S3*E*). These results demonstrated that *DACH1* decreased growth and proliferation of glioma cells both ex vivo and in vivo, supporting that *DACH1* is a tumor suppressor gene of GBMs.

**DACH1 Inhibits Formation of Tumor-Initiating Spheroids of Glioma Cells.** DACH1 is structurally related to c-Ski and SnoN, which act as transcriptional repressors of the transforming growth factor-β (TGF-β) signaling pathway through the interaction with Smad proteins (16). Previous studies showed that human DACH1 inhibited TGF-β signaling through repressing cyclin D1 (*CCND1*) expression and decreased proliferation of breast cancer cells (17, 18). We examined whether DACH1 expression affected TGF-β signaling in glioma cells. Induced DACH1 expression reduced the proliferation of the U-373MG cells, which are widely used as a cell model for analyzing TGF-β signaling (19), and repressed TGF-β3–stimulated $(CAGA)_9$- and p800-luciferase activity (Fig. S5 *A*–*C*). However, the expression of cyclin D1, which was proposed as a transcriptional target of DACH1 in breast cancer cell lines (18), was not affected by induced expression of DACH1 (Fig. S5*D*). Because DACH1 might repress expressions of the other target genes, but except for cyclin D1, we sought DACH1-regulated genes, which could affect growth of glioma cells. By global expression analysis of DACH1-high and -low cells, we found *FGF2* expression was repressed by DACH1 (Table S1, Fig. 3*A*, and Fig. S4*A*). Because FGF2, also named basic FGF (bFGF), is an essential factor for maintenance of self-renewal of glioma-initiating cells (20, 21), we thought DACH1 affected manintenance of self-renewal of glioma-initiating cells. We discovered DACH1, grown in serum-free neurobasal (NBE) medium (22), blocked spheroid formation of U87TR-Da cells, whereas cell morphology in serum-containing medium was not changed by DACH1 expression (Fig. 3*B*). Because glioma-initiating cells have been proposed to form spheroids (21), we thought that DACH1-nonexpressing U87TR-Da cells, which formed spheroids in NBE medium, showed high tumorigenicity compared with DACH1-expressing U87TR-Da cells in NBE medium. We confirmed that spheroid of DACH-nonexpressing cells cultured in NBE medium showed high expression of *CD133*, which has been reported as a marker of cells that are capable of tumor initiation (21), and a neural stem cell marker Nestin (Fig. 3 *C* and *D*). We then performed s.c. injection of U87TR-Da cells to examine the relationship between spheroid formation and tumorigenicity of the cells. We found tumor formation of DACH1-nonexpressing U87TR-Da cells (3 of 4 mice with $5 \times 10^3$ cells and all mice with $2 \times 10^4$ and $1 \times 10^5$ cells), whereas DACH1-

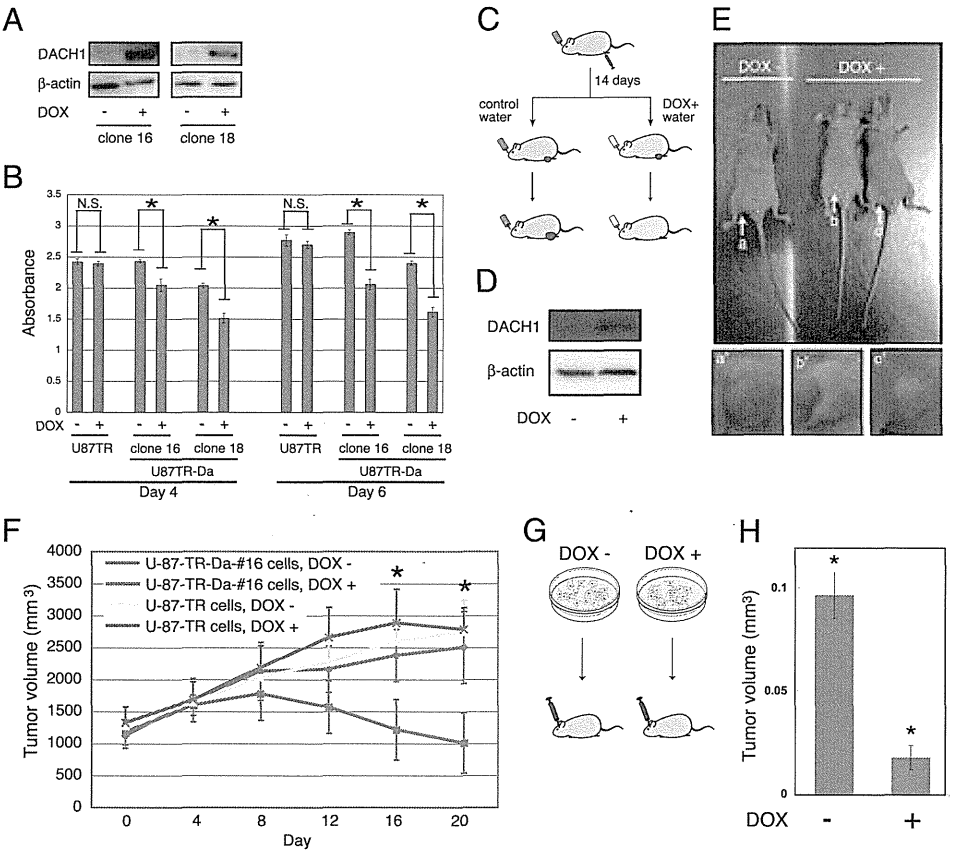


**Fig. 2.** DACH1 expression repressed growth of glioma cells. (*A*) Immunoblotting of U87TR-Da clone-16 and clone-18 cells with an anti-DACH1 antibody. (B) Cell proliferation of U87TR-Da cells, counted by WST-8 assay at 4 or 6 d after induction of *DACH1* by doxycycline. (*C*) Experimental model of tumor progression affected by DACH1 expression. Serially diluted U87TR-Da cells were injected s.c. into the backs of BALB/c nude mice, and the tumor formation was observed at 28 d after s.c. injection of the cells. (*D*) Expression of DACH1 in U87TR-Da tumor. DACH1 was detected by immunoblotting of xenografted U87TR-Da clone-16 tumors with an anti-DACH1 antibody (*Upper*). Tumor tissues were resected from mice drinking doxycycline-supplemented (left lane) or control water (right lane). β-actin was detected as a loading control (*Lower*). (*E*) Tumor formation of xenografted DACH1-nonexpressing or -expressing U87TR-Da clone-16 cells at 20 d after inoculation. (*F*) Growth of xenografted U87TR-Da clone-16 or U87TR tumors. DOX, doxycycline (1 μg/mL); points, mean ($n$ = 6); bars, SEM of 6 amimals on per group; N.S., not statistically significant; *$P < 0.05$ (two-way ANOVA), statistically significant compared with doxycycline minus control. (*G*) Orthotopic xenograft model for assessing the effect of DACH1 expression on tumor progression. U87TR-Da clone-16 cells were precultured in doxycycline-free (*Left*) or doxycycline-containing (*Right*) medium. (*H*) Size of tumors of the brain stereotactically implanted with DACH1-nonexpressing (DOX-) or -expressing (DOX+) cells at 5 wk after implantation. Bars, SEM of 4 amimals per group; N.S., not statistically significant; *$P < 0.05$ (two-way ANOVA), statistically significant compared with doxycycline minus control.

expressing U87TR-Da cells did not form any tumors (Fig. 3*E*). Because DACH1-expressing U87TR-Da cells in serum-containing DMEM did not form tumors with so few cells ($2 \times 10^4$ to $1 \times 10^5$ cells), tumor-initiating cells might be enriched in spheroids of DACH1-nonexpressing U87TR-Da cells grown in serum-free NBE medium.

**FGF2 Rescues DACH1-Repressed Tumorigenicity.** *FGF2* expression was highly induced under serum-free culture condition, however, DACH1 repressed *FGF2* expression at low levels (Fig. 4*A*). We verified that DACH1 reduced a reporter activity of luciferase cis-regulated by *FGF2* promoter (Fig. S6*A*) and directly bound to FGF2 promoter region in DACH1-expressing U87TR-Da cells cultured in both serum-containing DMEM and serum-free NBE medium by ChIP analysis with an anti-DACH1 antibody (Fig. S6*B*). Because overexpression of *FGF2* was frequently observed in high-grade gliomas and involved in malignant progression of gliomas (23, 24) and a previous study showed that bFGF enhanced tumor-initiating spheroid formation of glioma cells (20), we examined whether spheroid formation of DACH1-expressing U87TR-Da cells was enhanced by exposure of cells to bFGF. DACH1-expressing U87TR-Da cells did not form spheroid, but exogenous bFGF-induced spheroid formation of DACH1-expressing U87TR-Da cells, indicating that bFGF, which was repressed by DACH1, increased the number of spheroid-forming tumor-initiating cells (Fig. 4*B* and Fig. S4*C*). Morphology of primary tumor spheroid, which did not express *DACH1*, was not markedly different under culture conditions with or without bFGF. However, by lentiviral expression of DACH1, the spheroid formation was partially disrupted, and most spheroid-forming cells started to differentiate even under bFGF-supplemented condition (Fig. S4*D*). We confirmed the reduced expression of a glioma stem cell marker CD133 in primary tumor-derived cells with DACH1 expression under both bFGF2-supplemented and -unsupplemented culture conditions (Fig. S4*E*). To examine whether bFGF overexpression can confer an increased tumorigenicity, the intracerebral implantation of the cells ectopically overexpressing DACH1 and FGF2 was performed (Fig. 4*C*). Tumor formation in DACH1-expressing cells with ectopic expression of FGF2 was much higher than that in control DACH1-expressing cells, showing that rescue of FGF2 repression in DACH1-expressing cells increases intracerebral tumor formation (Fig. 4 *D* and *E* and Fig. S6*C*). These results suggested that DACH1 suppress tumor formation through transcriptional repression of *FGF2*.

## Discussion

Loss of chromosome 13q has been reported to occur frequently in GBMs (12, 25, 26). Homozygous deletions, LOH, and mutations in *RB1* gene, which is located at chromosome 13q14.2, 23 Mb centromeric to *DACH1*, are found in human cancers including GBMs (13, 26). In this study, we found another locus with a homozygous deletion at 13q21 by SNP genotyping array and targeted genotyping analysis with a mass spectrometer. A previous study reported LOH of chromosome 13q was more common in secondary than in primary glioblastomas (3), and the mutations of *IDH1* and *IDH2* genes have suggested to associate with development of secondary glioblastomas (27–30). However, because significant relationship between loss of *DACH1* and mutations of *IDH1* and *IDH2* were not found, we think that the mechanisms of *DACH1* loss and gliomagenesis categorized into primary or secondary glioblastoma are different (Table S2).

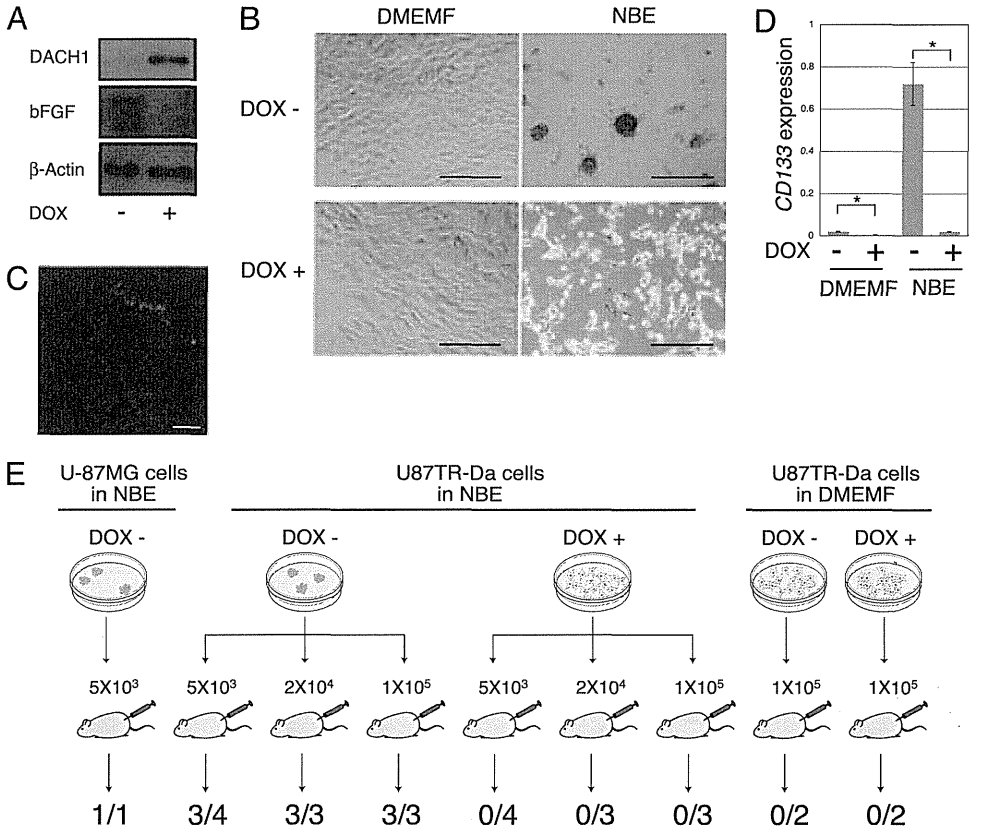


**Fig. 3.** Spheroid formation was inhibited by DACH1 expression. (*A*) Protein expression levels of DACH1 and bFGF in U87TR-Da clone-16 cells. (*B*) Images of cultured cells. U87TR-Da clone-16 cells were first cultured in FBS-containing DMEM in the absence (*Upper Left*) or presence (*Lower Left*) of doxycycline for 72 h, and then culture medium was replaced into serum-free NBE or serum containing DMEMF medium. Branched projection of adherent cells was observed in DACH1-nonexpressing cells (red arrows). (Scale bars: 500 μm.) (*C*) Nestin staining of spheroid (green). Nucleus was stained with DAPI (blue). (*D*) CD133 expression of DACH1-nonexpressing (DOX-) or -expressing (DOX+) cells under serum-containing (DMEMF) or serum-free (NBE) culture condition. Columns, mean ($n = 3$); bars, S.D.; *, **$P < 0.05$. (*E*) Tumor formation of DACH1-expressing or DACH1-nonexpressing U87TR-Da cells cultured in NBE medium. Tumor formation in 3 of 4 mice with $5 \times 10^3$ DACH1-nonexpressing U87TR-Da clone-16 cells and in all mice with $2 \times 10^4$ and $1 \times 10^5$ DACH1-nonexpressing U87TR-Da clone-16 cells was observed, whereas s.c. injection of DACH1-expressing U87TR-Da clone-16 cells did not form any tumors. DOX-, mice with U87TR-Da clone-16 cells cultured in doxycycline-free NBE medium; DOX+, mice with U87TR-Da cells cultured in doxycycline-supplemented medium.

*DACH1*-spanning region is indicated as a target of copy number variations (CNV), where healthy individuals also exhibit copy number changes. Although it is hard to detect tumor-specific small deletions by a general copy number analysis using an algorithm based on moving window, our GIM algorithm can normalize aberrant copy number change even in CNV region by using signal data of both tumor DNA and corresponding normal DNA (4, 11). We overcame CNV effect and could detect small homozygous deletions at DACH1 region by an improved algorithm for SNP genotyping array with the targeted genotyping analysis using a mass spectrometer.

To explore additional mechanisms for *DACH1* inactivation other than genomic loss, we first examined sequencing of *DACH1* exonic regions by using genomic DNA from 25 GBM tumors and 8 glioma cell lines and did not observe any somatic mutations, whereas GBM case 25 showed LOH and the remained allele with nonsynonymous polymorphism, which could be translated into amino acid-substituted DACH1, V464M (Fig. S2*C*). It should require further examination such as loss-of-function of the protein and frequency of mutational events with amino acid substitution of DACH1 protein in GBMs. We then examined methylation status of *DACH1* promoter region, because a CpG island existed in the vicinity of transcription start site (TSS) of *DACH1*. Because DNA hypermethylation at *DACH1* promoter region was frequently observed (Fig. S2*D*), hypermethylation of the *DACH1* promoter could be an additional mechanism for DACH1 inactivation in gliomagenesis.

Homozygous deletions observed in all three cases contained a deduced promoter region and the first exon with ATG start codon of *DACH1* gene, indicating the homozygous deletions cause functional disruption of DACH1. Forced expression of DACH1 decreases growth of glioma cells and inhibits formation of spheroids, which are proposed as a tumor-initiating cell population and chemoresistant population of glioma cells. Even though downstream signaling of bFGF is well described, only a few reports have provided the mechanism of transcriptional regulation of *FGF2* (31, 32). Here, we demonstrated that DACH1, a member of corepressor complexes, repressed *FGF2* expression. Campanelli et al. found the expression level of DACH1 in glial precursors was much higher than that in neural stem cells (NSCs) (33). Withdrawal of bFGF, which is known as a critical component of culture medium for human embryonic and neural stem cells (34), drives astrocytic differentiation (2, 34, 35). So we think that loss or mutation of DACH1 may interrupt astrocytic differentiation of glial-restricted progenitor cells through sustained expression of bFGF and, subsequently, trigger gliomagenesis by convertion of progenitor cells to tumor-initiating cells.

Although loss of *DACH1* was detected in some GBM specimens and glioma cell lines at genomic and/or transcriptional level, there were other glioma cell lines that exhibited detectable DACH1 expression, such as U251MG (Fig. S3*A*). When we silenced *DACH1* expression of U251MG by RNA interference (RNAi) (Fig. S7*A*), slightly increased cell proliferation was observed (Fig. S7*B*). *FGF2* and *CD133* expression was slightly upregulated when *DACH1* expression was silenced by RNAi (Fig. S7 *C* and *D*). We also observed increased spheroid formation in *DACH1*-silenced cells under serum-free culture condition (Fig. S7*E*). However, these effects of *DACH1* silencing in U251MG cells were relatively slight. Because U251MG exhibited cell-autonomous growth even with high DACH1 expression, there must be additional alterations in oncogenes/tumor suppressor genes other than *DACH1*, which might obscure the effect of *DACH1* silencing on tumor suppressing activity.

In the analysis with expression microarrays, we observed that DACH1 decreased expression of secreted factors including TGF-β2, leukemia inhibitory factor (LIF), and interleukin-6 (IL-6) (Table S1). Recent reports demonstrated that TGF-β enhanced the self-renewal property of glioma-initiating cells by activating

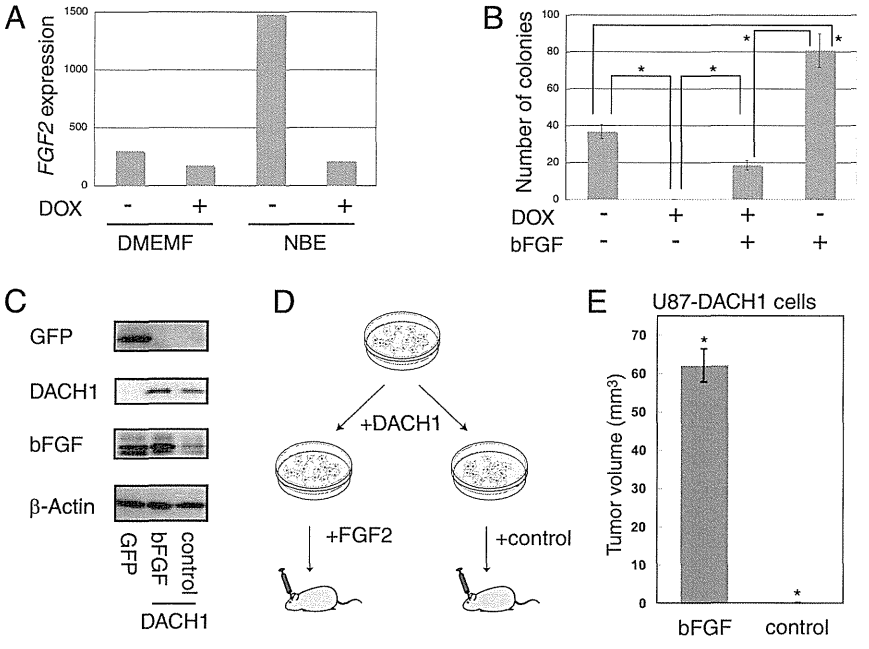


**Fig. 4.** Ectopic DACH1 expression decreases FGF2 expression, spheroid formation, and tumor growth. (*A*) Repressed *FGF2* expression by DACH1. DMEMF, serum-containing DMEM; NBE, serum-free Neurobasal medium. (*B*) Spheroid formation in U87TR-Da in the absence and presence of bFGF. Columns, mean ($n$ = 3); bars, SEM of three experiments; $*P < 0.05$ (unpaired $t$-test), statistically significant compared with doxycycline minus control. (*C*) Protein expression of green fluorescent protein (GFP), DACH1, and bFGF. (*D*) Orthotopic xenograft model for assessing the effect of *FGF2* on tumor progression of *DACH1*-expressing cells. U87MG cells were lentivirally transduced with DACH1 and cultured in zeocin-containing medium. Then, DACH1-expressing cells were infected with lentivirus carrying FGF2 (*Left*) or control (*Right*) vector. (*E*) Size of tumors of the brain implanted with *FGF2*-expressing or control U87MG cells. Bars, SEM of 5 amimals on per group; N.S., not statistically significant; $*P < 0.05$ (two-way ANOVA), statistically significant compared with doxycycline minus control.

LIF-JAK/STAT pathway (36), and autocrine TGF-β signaling is involved in maintenance of tumorigenicity of glioma-initiating cells (19). Both LIF and IL-6 also are activators of JAK/STAT pathway. So we presumed that DACH1 may suppress tumorigenesis through repression of not only *FGF2* but also the above tumor-initiating factors. Potential tumor-initiating factors, such as bFGF, TGF-β, LIF, and IL-6, will also be good candidates drug targets for GBMs.

The previous integrated genomic analysis of glioblastoma identified genomic alterations in genes belonging not only to a variety of cellular process pathways, which were likewise altered in many types of cancers, but also to nervous system-specific cellular pathways (27). From our results, we think that *DACH1* may function as one of tumor suppressor genes regulating through nervous system-specific cellular pathways. Little is yet known about the origin of glioblastoma cells, but some reports suggested that NSCs in the subventricular zone (SVZ) of the adult brain might be a candidate origin of cancer stem cells of glioblastoma (37, 38). Although we could not detect DACH1 expression at adult SVZ, a high expression of DACH1 was observed at the ventricular wall of fetus (Fig. S2 *E* and *F*), implicating involvement of DACH1 in maturation of neural cells. Li and colleagues demonstrated murine Dach1 regulated retinogenesis and pituitary development through tissue-specific gene regulation by recruiting the corepressors (39, 40). Functional relevance of *DACH1* expression in development and neural differentiation of both invertebrates and vertebrates has been reported (39–46). DACH1-expressed embryonic and postnatal brain-derived cells displayed neural stem cell-like property (44), suggesting that DACH1 functions in neural differentiation. From these facts together, we think that DACH1 act as a guardian of differentiation in the glial lineage, and loss of DACH1 would result in dysregulation of normal differentiation and drive gliomagenesis.

Taking advantages of our allelic DNA copy number analysis for tumors and corresponding normal cells by using SNP genotyping array as well as MassARRAY, we provide evidence that DACH1 is homozygously deleted in GBMs. As far as we know, this is the first study showing loss of DACH1 gene function in tumor cells at the genomic level. Our observation supports recent studies indicating that DACH1 scarcely expressed in tumor-initiating cells and such low expression correlated with poor prognosis of breast cancers (17, 18, 47). We showed that DACH1 expression decreases proliferation of glioma cells and suppresses tumorigenicity through inhibition of bFGF-dependent spheroid formation. We here propose DACH1 is a unique tumor suppressor gene, which does not only suppresses tumor growth but also inhibits generation of tumor-initiating cells during neural differentiation. Understanding molecular basis of DACH1-mediated epigenetic regulation may provide mechanism of both neural differentiation and gliomagenesis.

## Methods

**Materials.** All clinical samples were obtained with the informed consent of the patients after permission by the ethics committees of Tokyo University Hospital. Tumors were diagnosed according to World Health Organization classification (48).

**SNP Genotyping Array.** SNPs of peripheral blood cells or GBM samples were genotyped by 50K Xba SNP mapping arrays (Affymetrix) according to GeneChip Mapping 50K Assay Manual (4). Allelic and total DNA copy numbers were calculated by GIM algorithm (11).

**MassARRAY Analysis.** SNP genotyping of 28 GBM samples, including the 8 samples used in the initial screening by SNP mapping array analysis, was performed with MassARRAY Genotyping system (Sequenom). Paired DNA samples from blood or tumor were genotyped in duplicate. Quantitation of the peak area was performed by Sequenom's MassARRAY RT software. The threshold for LOH was defined as 40% reduction of one allele in tumor sample, as described (14).

**Gene Expression Analysis.** Total RNA was extracted with TRIzol (Life Technologies). Biotin-labeled cRNA was synthesized and hybridized to GeneChip U133 plus 2 (Affymetrix) according to the manufacturer's instruction. Gene expression data were normalized by using the MAS5 algorithm. Data were deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (GEO accession no. GSE19678; www.ncbi.nlm.nih.gov/geo/).

**Immunoblotting.** An anti-DACH1 antibody was generated by immunization of GST-fused DACH1 protein and purified by affinity-purification with Immunogen-bound beads. Immunoblot was performed by using anti-DACH1 or anti–β-actin (AC-40; Sigma) antibody according to a previous report (49). Cell lysates at 3 d after the addition of doxycycline or RNAi treatment were used.

**Spheroid Formation Assay.** Expression of DACH1 was induced by exposure of U87TR-Da-clone16 or -clone18 cells to doxycycline for 72 h and then cells were grown in NBE medium (22, 50) composed of Neurobasal Medium (Life

Technologies), *N*-2, B-27 supplement (0.5× each; Life Technologies), human recombinant bFGF (50 ng/mL; PeproTech), and EGF (50 ng/mL; Life Technologies). At 10 d after medium change, the number of spheroid-forming colonies was counted. Paraformaldehyde-fixed spheroids were stained with an anti-Nestin monoclonal antibody (BD Biosciences) and Alexa488-conjugated anti-mouse IgG antibody (Life Technologies).

**In Vivo Studies.** Male 6- to 8-wk-old male athymic (nude) mice (nu/nu) of BALB/c were purchased from Charles River Laboratory Japan. For assessment of in vivo tumor growth, U87TR-Da cells were injected s.c. into the flank of mice. At the start point (day 0), drinking water was replaced with doxycycline-containing water for induction of DACH1 expression. For assessment of tumorigenicity, before injection, DACH1 expression was induced by the exposure of the U87TR-Da clone-16 cells with doxycycline for 10 d. The tumor formation was observed after s.c. injection of the serially diluted cells. For intracerebral stereotactic inoculation, cells in 6 μL of PBS were implanted into the right corpus striatum of the anesthetized mouse brain. Animal studies were performed according to institutional guidelines. See details of in vivo tumor growth and tumorigenicity assay in *SI Methods*.

Full methods and any associated references are available in *SI Methods*. Primers used were listed in Table S3.

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# ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif

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## ABSTRACT

**Dysregulated bone morphogenetic protein (BMP) signaling in endothelial cells (ECs) and pulmonary arterial smooth muscle cells (PASMCs) are implicated in human genetic disorders. Here, we generated genome-wide maps of Smad1/5 binding sites in ECs and PASMCs. Smad1/5 preferentially bound to the region outside the promoter of known genes, and the binding was associated with target gene upregulation. Cell-selective Smad1/5 binding patterns appear to be determined mostly by cell-specific differences in baseline chromatin accessibility patterns. We identified, for the first time, a Smad1/5 binding motif in mammals, and termed GC-rich Smad binding element (GC-SBE). Several sequences in the identified GC-SBE motif had relatively weak affinity for Smad binding, and were enriched in cell type-specific Smad1/5 binding regions. We also found that both GC-SBE and the canonical SBE affect binding affinity for the Smad complex. Furthermore, we characterized EC-specific Smad1/5 target genes and found that several Notch signaling pathway-related genes were induced by BMP in ECs. Among them, a Notch ligand, JAG1 was regulated directly by Smad1/5, transactivating Notch signaling in the neighboring cells. These results provide insights into the molecular mechanism of BMP signaling and the pathogenesis of vascular lesions of certain genetic disorders, including hereditary hemorrhagic telangiectasia.**

## INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) family, which regulate a variety of cellular processes including differentiation, proliferation, migration and cell death in a cell type-specific and context-dependent manner (1). Perturbations of BMP signaling pathways have been implicated in a diverse set of developmental disorders, tumorigenesis and diseases including ectopic ossification and cardiovascular diseases. Mutations in *ENG*, *ACVRL1* or *SMAD4* genes have been shown to cause hereditary hemorrhagic telangiectasia (HHT) (2–4), which is a multisystemic vascular disorder characterized by epistaxis, telangiectases and arteriovenous malformation (AVM). The *ACVRL1* gene encodes an endothelial-specific type I receptor for TGF-β members, ALK-1, whose signals are transmitted through BMP-specific receptor-regulated Smads (BR-Smads; Smad1/5/8) (5). Recent work has indicated that haploinsufficiency of ALK-1 causes HHT (6). The *ENG* gene encodes Endoglin, which is an endothelial expressed co-receptor and modulates ALK-1 signaling (7). The *SMAD4* gene encodes a common mediator Smad (co-Smad), which makes a heterotrimeric complex with BR-Smads and regulate transcription of specific target genes (8). Therefore, dysregulated BMP signaling through ALK-1 in endothelial cells (ECs) is implicated in the pathogenesis of HHT. Interestingly, BMP signaling activated by BMP type I receptors, other than ALK-1 in ECs, are not able to compensate for the loss of function of ALK-1. On the other hand, aberrant BMP signaling through BMP type II receptor (encoded by *BMPR2*), especially in pulmonary arterial smooth muscle cells (PASMCs), are implicated in the pathogenesis of pulmonary arterial hypertension (PAH) (9,10). Therefore, readout of BMP signaling depends on the strength of BMP signaling, types I and II receptors and co-receptors, and cell types.

A binding sequence for BR-Smad was originally identified in *Drosophila*. Kim and colleagues (11) indicated that GCCGnCGC is a consensus binding sequence for Mad (*Drosophila* Smad1). In mammals, similar GC-rich

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