

Figure 2. Comparison of Smad1/5 binding regions in HUVECs and PASCs. (A) A Venn diagram represents the overlaps of Smad1/5 binding regions of HUVECs treated with BMP-9 (Yellow) or BMP-6 (Blue) and PASCs treated with BMP-4 (Red). The number of binding regions of HUVECs treated with BMP-9 (Black) or BMP-6 (Blue) and that of PASCs treated with BMP-4 (Red) are also shown. The numbers of overlapped regions are not identical, since some of the peaks are not on a one-by-one correspondence. (B) ChIP-seq peaks of HUVECs treated with BMP-9 are ranked by peak height. Fraction of peaks overlapped with the peaks of HUVECs treated with BMP-6 is calculated for every 100 peak and plotted. Overlapped peaks are enriched in the high ranked peaks. (C) HUVECs were starved overnight and stimulated with the indicated concentration of BMP-6 or BMP-9 for 1.5 h and were subjected to ChIP assays with anti-Smad1/5 antibody. The ChIP samples were quantified by real-time PCR with locus-specific primers and normalized to input DNA. The dashed line indicates 0.01% of input. The data are the mean of triplicate values \pm SD.

enrichment of the motifs was compared (Figure 4B and Supplementary Figure S4B). Randomly selected genomic sequences ($n = 1000$) or non-overlapping matched regions ($n = 50$) were used as background controls. Four out of five motifs were significantly enriched in the Smad1/5 binding regions, and the MEME2 was the best.

TFAP2A (also known as AP-2 α) binding motif was a positive control and was found to be enriched in the Smad1/5 binding regions (Supplementary Table S3). No statistically significant differences were observed for motifs of transcription factors known to be expressed and functional in ECs, such as GATA2 (35). In contrast to the study of Chen and colleagues (16), the motifs for SOX2 and POU5F1 (also known as OCT4) were not enriched in the Smad1/5 binding regions, suggesting that different mechanisms or different enhancer complexes are adopted in differentiated ECs compared with mESCs. In addition, the incidence of the MEME motifs in the peaks was calculated. MEME2 occurred in about 45% of all Smad1/5 binding regions in HUVECs and PASCs (Figure 4C and Supplementary Figure S4C). Moreover, it was enriched in the higher ranked peaks in HUVECs treated with BMP-9 (Supplementary Figure S4D). Finally, the relative distribution of the motif around the peak summits, where Smad1/5 was expected to be located, was analyzed. MEME2 was enriched in the Smad1/5 binding regions, especially around the peak summits, while other MEME motifs were not (Figure 4D and Supplementary Figure S4E and F). We therefore designated MEME2 as GC-SBE because it is similar in sequence to the previously reported GC-rich sequences for BR-Smads (11–13).

Analysis of the frequency of GC-SBE sequence in Smad1/5 binding regions revealed that GGCGCC sequence was enriched in Smad1/5 binding regions shared with HUVECs and PASCs, while GGAGCC sequence was enriched in both HUVEC- and PASC-specific binding regions (Figure 5A). To validate the enhancer activity of the Smad1/5 binding regions and the effects of the newly identified GC-SBE on the cell type specificity, luciferase assays were performed in HUVECs. Both *BMPR2* and *JAG1* were HUVEC-specific target genes (Supplementary Table S4). Fragments from Smad1/5 binding regions in intron 3 of *BMPR2* and the *JAG1* promoter, which contain the GGAGCC sequence, were cloned into a luciferase reporter construct (Supplementary Figure S5A). Both BMP-9 and BMP-6 were able to activate these reporters in HUVECs, while BMP-4 induced only weak response in PASCs (Figure 5B and Supplementary Figure S5B and S5C). Consistent with ChIP data (Figure 1C), the Smad1/5 binding regions induced higher luciferase expression following treatment with BMP-9 compared with BMP-6. Even 1 ng/ml BMP-9 induced stronger luciferase activities in HUVECs than 50 or 200 ng/ml BMP-6 (Figure 5B). We also confirmed that these Smad1/5 binding fragments worked as transcriptional enhancers in the human microvascular endothelial cell line, HMEC-1 (Supplementary Figure S5D).

In order to compare the difference of enhancer activities between GGAGCC and GGCGCC sequence, a point mutation was introduced at the 'A' in the GGAGCC sequence. A mutation to GGCGCC induced higher luciferase expression compared with the GGAGCC wild-type. In contrast, a mutation to GGGGCC attenuated BMP responsiveness (Figure 5B and Supplementary Figure S5B). Interestingly, the fragments with GGAGCC

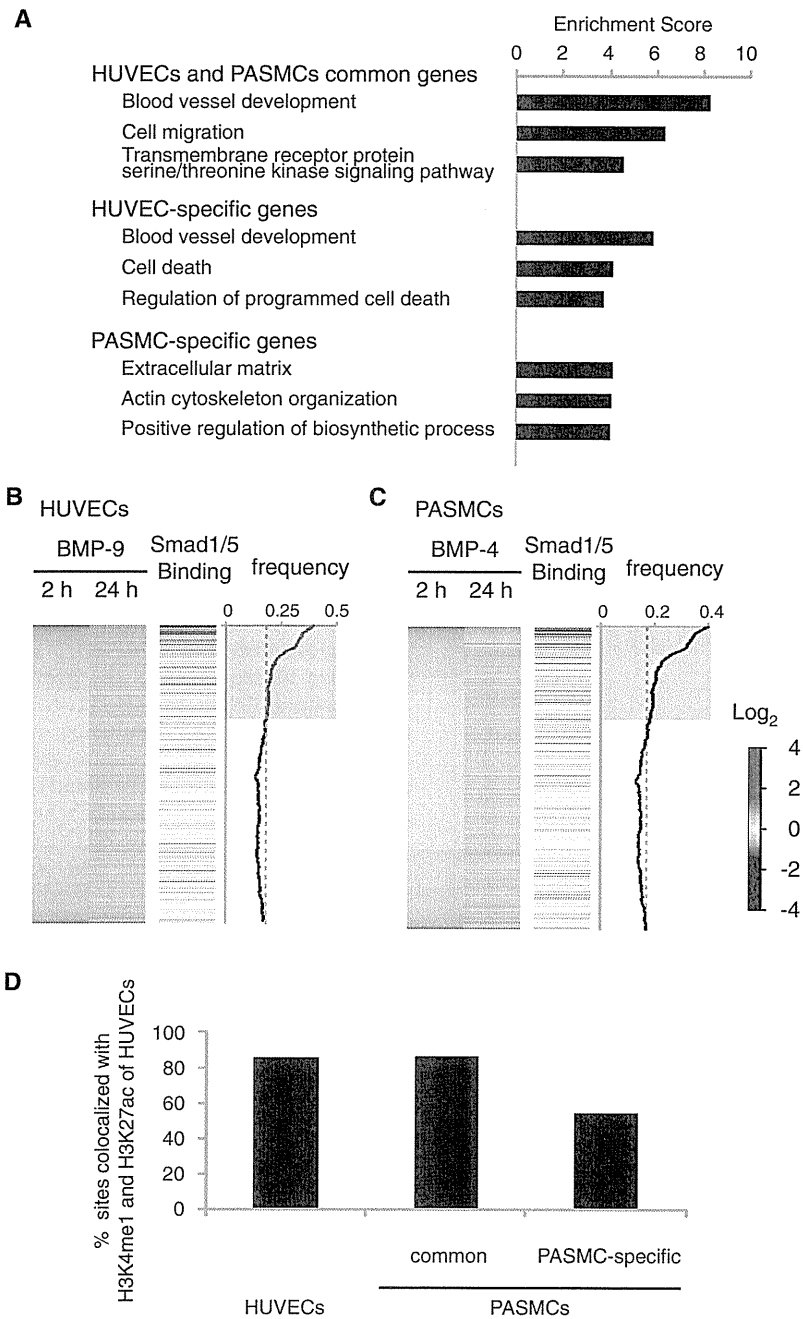


Figure 3. Genome-wide identification of cell type-specific BMP target genes and gene expression profiles. **(A)** GO enrichment analysis was performed to elucidate the biological processes and pathways associated with each gene cluster. The top three annotation clusters are shown in bar plots. The value reflects the Enrichment Score. Group names are based on interpretation of enriched GO annotations. **(B and C)** Gene expression profiles of HUVECs with BMP-9 stimulation or PASCs with BMP-4 are illustrated by heat map. Probes are sorted by fold change relative to time 0 at early phase (2 h after stimulation) (left panel). Increased or decreased mRNA expression is represented by red or blue, respectively. Black horizontal bars represent probes of genes associated with Smad1/5 binding regions (middle panel). Moving average of the frequency of probes with Smad1/5 binding is plotted in a 1000-probe sliding window (right panel). The red-colored areas indicate the probes, whose Smad binding frequency is higher than the expected average. The dashed line indicates the expected average. **(D)** Frequency of the Smad1/5 binding regions co-localized with enhancer regions of HUVECs. The Smad1/5 binding regions in PASCs are divided into two groups, those shared with HUVECs or PASC-specific sites.

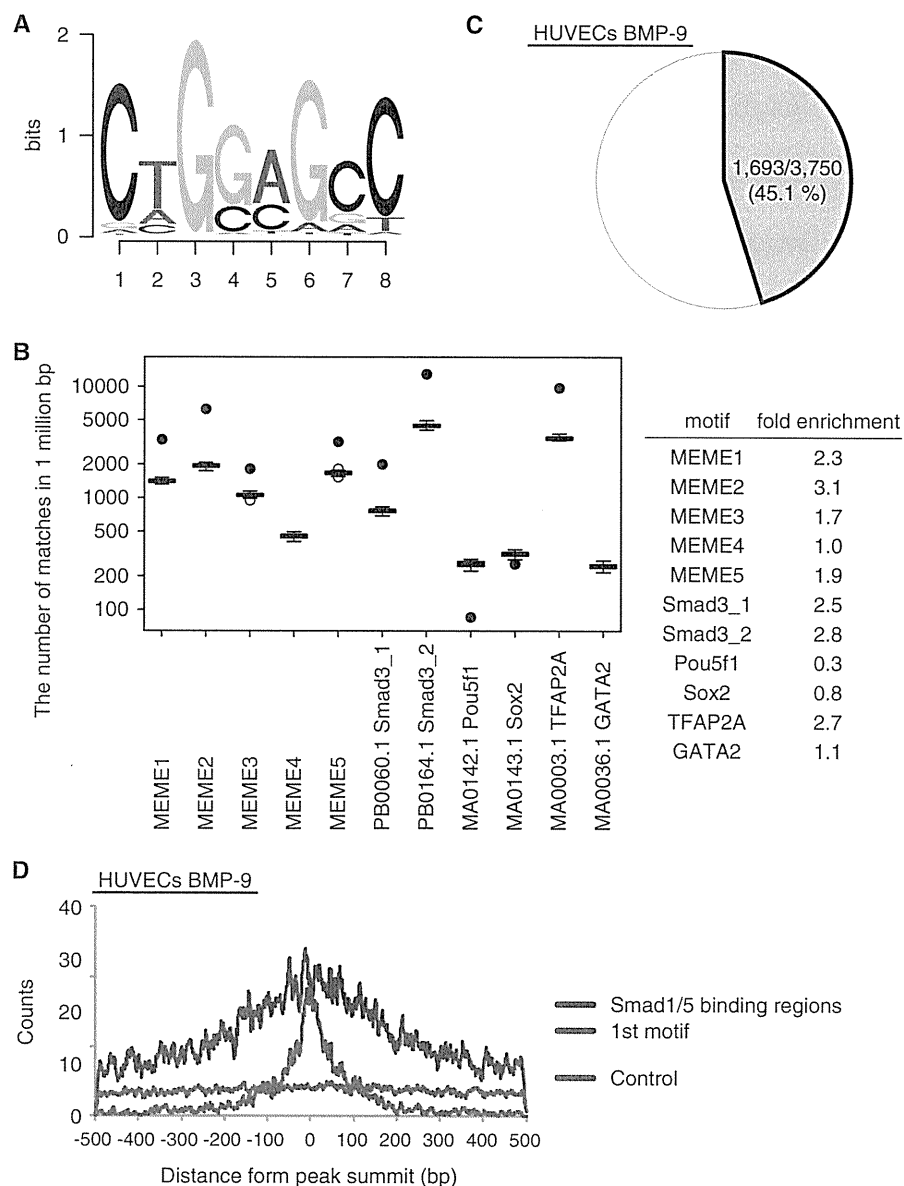


Figure 4. *De novo* prediction of Smad1/5 binding motif. Total 170 peak regions were analyzed for overrepresented motifs using MEME. (A) MEME2 is displayed as a sequence logo. (B) Enrichment of TFBS in the Smad1/5 binding regions. Fifty sets of non-overlapping matched genomic control sequences were used as background control. Data are given as boxplot. The circles represent outlier values. The black circles indicate the number of matched motifs observed in the Smad1/5 binding regions. (C) MEME2 motif occurs in about 45% of all Smad1/5 binding regions in HUVECs stimulated with BMP-9. (D) Distribution of MEME2 motif around the peak summits. The number of the MEME2 motif around the peak summits was counted and plotted in a 7 bp sliding window against the distance from the summits (within 500 bp from the summits) (blue). The motifs closest to the summits are located within 100 bp from the peak summit (First motif; green). Five separate matched control regions were randomly chosen by CisGenome and used as a control. The number of the MEME2 motif in those regions was counted and the average was plotted (red).

sequence did not respond to BMP stimulation in PSMCs, whereas mutation to GGCGCC showed a higher responsiveness (Supplementary Figure S5C). Luciferase assays were also performed in HepG2 cells to examine the cell type specificity of the fragments. Similarly, the GGCGCC mutant responded very well compared with wild-type and the T-mutant, while the

G-mutant had no enhancer activity in HepG2 (Supplementary Figure S5E).

We next showed the direct binding of recombinant human Smad1 MH1 (rhSmad1 MH1) to the GGAGCC sequence using EMSAs. The amino acid sequence of rhSmad1 MH1 is identical to the corresponding sequence of mouse Smad1 MH1, which was reported to bind to the

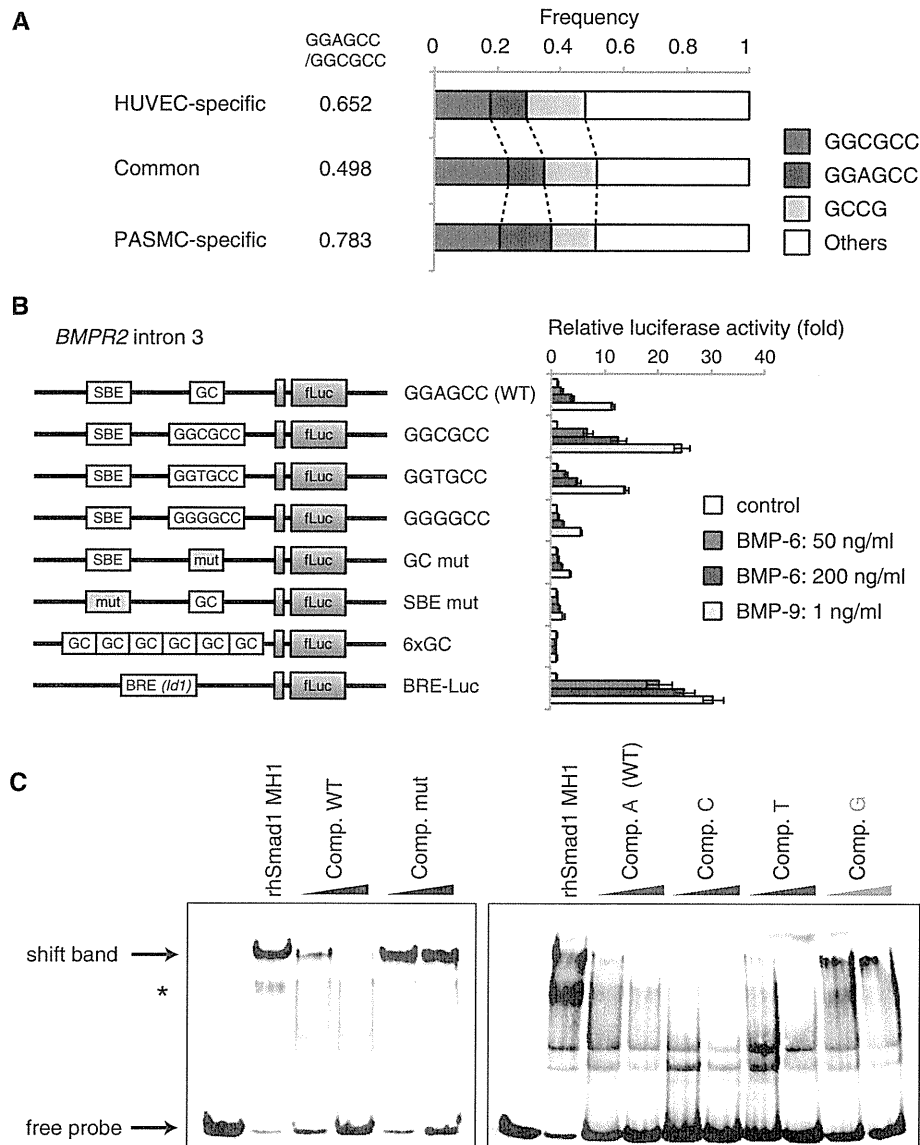


Figure 5. Validation of GGAGCC sequence as a novel BMP responsive element. (A) Frequency of GC-SBE sequences in the Smad1/5 binding regions. GGCGCC, GGAGCC and GCCG sequences were enriched in the Smad1/5 binding regions. The ratio of GGAGCC:GGCGCC is indicated. (B) pGL4-BMP2 reporter constructs were introduced into HUVECs using lentiviral vector system, in order to evaluate their enhancer activity. The cells were stimulated with indicated doses of BMP-9 or BMP-6 and then they were harvested and assayed for luciferase activity at 12h after stimulation. The data are the mean of triplicate values \pm SD. (C) Recombinant human Smad1 MH1 proteins interacted with GGAGCC sequence. (Left panel) rhSmad1 MH1 binding to the probe was competed with a 50-fold molar excess of the unlabeled wild-type competitor (Comp. WT), but not with the mutant competitor (Comp. mut). (Right panel) To evaluate the importance of 'A' in GGAGCC sequence, single-point mutant competitors were evaluated. An asterisk indicates background band. Full wild-type probe sequence was ACAGCTCT GGAGCC AGATGGCCTGG.

GGCGCC sequence (14). rhSmad1 MH1 was able to bind to the GGAGCC probe and this binding was blocked by wild-type oligonucleotide but not by the mutated one (Figure 5C). The effects of single-point mutation in the GGAGCC sequence were also examined. The GGCGCC sequence competed more efficiently than the GGAGCC sequence, suggesting that this sequence had higher affinity for binding to rhSmad1 MH1 (Figure 5C). Thus, these results showed that the GGAGCC sequence is also a

direct binding motif for Smad1/5 and that GC-SBE is a generalized form of the previously reported GC-rich sequences.

Both GC-SBE and SBE are required for full BMP responsiveness

In *Drosophila*, Dpp (Decapentaplegic; *Drosophila* BMP orthologs)-responsive elements are shown to contain a GC-rich Mad binding site and a flanking GTCT Medea

(*Drosophila* Smad4) binding site with a 5 bp spacer sequence (36). Indeed, Smad3 binding motifs were significantly enriched in the Smad1/5 binding regions found in our analysis (Figure 4B). The analysis of the spacer length between GC-SBE and SBE revealed that the 5 bp spacer was also prominent in HUVECs (Figure 6A), suggesting that the 5 bp spacer sequence has some beneficial effect for binding of the Smad complex, containing Smad1/5 and

Smad4, in mammalian cells too. On the other hand, expressions of genes associated with the GC-SBE/SBE composite motif with 5 bp spacer were not necessarily regulated by BMP-9 stimulation (Figure 6B).

Next, the roles of SBE sequences, which were located at different distances from GC-SBE, were examined. Evolutionarily conserved SBE/GC-SBE composite motifs with a 28 bp spacer sequence were found in the *BMPR2*

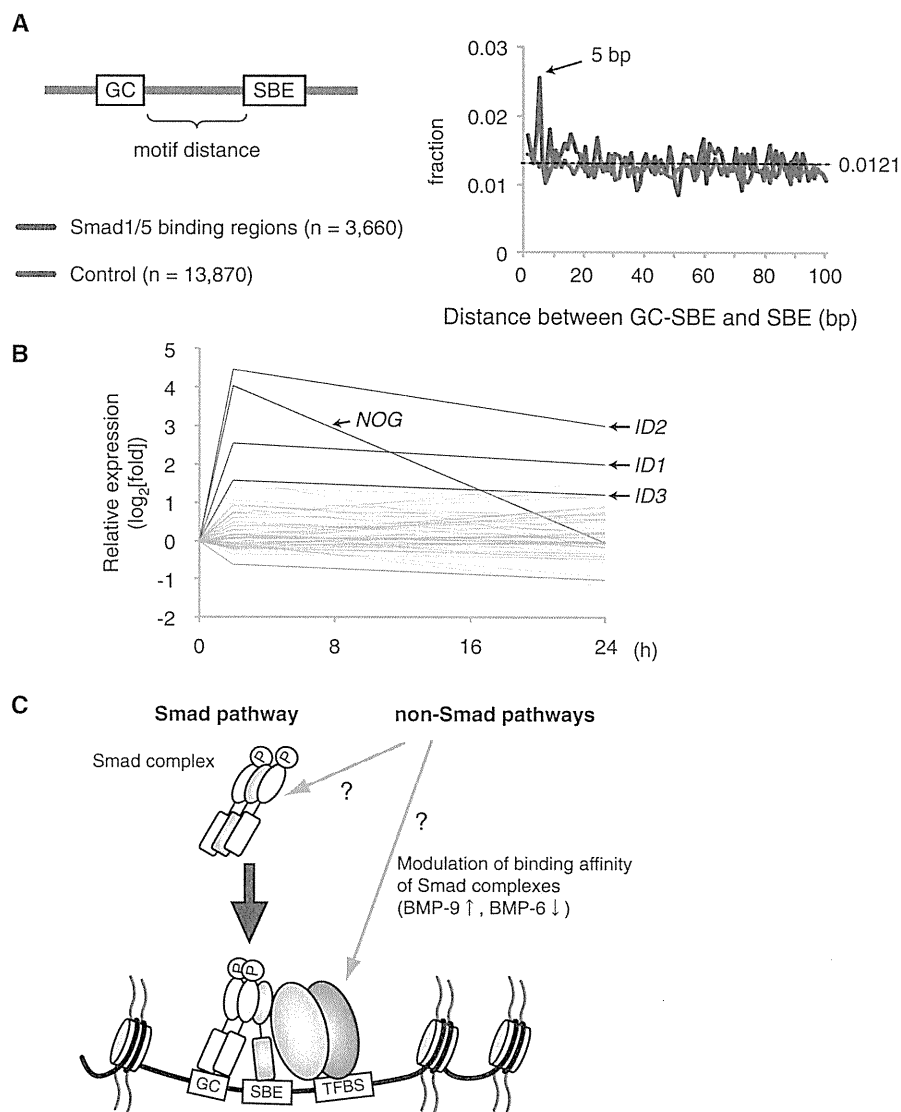


Figure 6. GC-SBE is required, but not sufficient, for full BMP responsiveness. (A) The distance between GC-SBE and GTCT-AGAC sequence in Smad1/5 binding regions is calculated and plotted (blue). Total 13 870 GC-SBEs in randomly-adapted control regions were used as a control (red). The dashed line indicates the expected average. (B) Graphical summary of expression microarray data of genes with GC-SBE and GTCT-AGAC composite motif with 5 bp spacer. The value of genes with GC-SBE/SBE composite motif with 5 bp spacer is represented as \log_2 -fold change relative to time 0. Several, but not all of these genes were induced more than 2-fold within 2 h, including the well-known Smad1/5 target genes *ID1*, *ID2*, *ID3* and *NOG* (Noggin) (red). (C) Schematic representation of Smad1/5 binding. Smad1/5 binding patterns appear to be predetermined by cell-specific differences in baseline chromatin accessibility patterns. Number and distribution of BR-Smad binding sites over the genome are primarily defined by the intensity of the Smad pathway. Each Smad1/5 binding site has different binding affinity for Smad complexes, which is determined by the affinities of GC-SBEs and SBEs. Non-Smad pathways were reported to affect the BR-Smad signaling through degrading Smad complexes or modulating binding affinity of Smad complexes. GC: GC-SBE, TFBS: transcription factor binding site.

intron 3 and the *JAG1* promoter (Supplementary Figure S5A). These sequences were able to drive luciferase expression in reporter assays in response to BMP stimulation (Figure 5B and Supplementary Figure S5B). Mutations in either GC-SBE or SBE sequence showed significant attenuation of BMP responsiveness (Figure 5B and Supplementary Figure S5B), indicating that the effective distance between GC-SBE and SBE was not restricted to 5 bp. The GC-SBE was not able to respond to BMP stimulation in luciferase reporter assays, even when present in six copies (Figure 5B). These findings clearly showed that both GC-SBE and SBE were required for full BMP responsiveness. Collectively, our results suggest that the binding affinity of Smad complexes to DNA is defined by the affinities of GC-SBEs and SBEs (Figure 6C).

JAG1 is a direct target gene of Smad1/5 in ECs and transactivates Notch signaling in the neighboring cells

EC-specific target genes contained well-known Notch-signal target genes and signaling components, including *HEY1*, *HEY2*, *HES1*, *FOXC1*, *LFNG*, *NRARP* and *JAG1* (Figure 7A and Supplementary Table S4). Synergic effects between Notch and BMP signaling on several Notch target genes, such as *HEY1* and *CDH2*, have been reported previously (13,37). However, little is known about direct expression regulation of Notch ligands by BMP signaling in ECs.

Two strong Smad1/5 binding regions were identified in the *JAG1* locus, in the promoter region at -500 bp from the TSS and in the second intron (Figure 7B), which were verified by ChIP-qPCR (Figure 1C). Both regions worked as transcriptional enhancers in HUVECs (Supplementary Figure S5B). Consistent with the results of the reporter assays, BMP-9 was able to induce expression of *JAG1* mRNA (Figure 7C). TNF- α has been shown to induce *JAG1* expression in ECs (38). The induction by BMP-9 was equivalent to that of TNF- α and also had some additive effects (Figure 7C). Western blot analysis and immunocytochemistry revealed that the *JAG1* protein was also upregulated by BMP-9 stimulation in ECs (Figure 7D and E). This *JAG1* mRNA induction was not affected by CHX, and siRNA against *SMAD4* (siSmad4) attenuated BMP-9-mediated upregulation of *JAG1* (Supplementary Figure S6A and B). These results showed that *JAG1* is a direct target gene of BMP-Smad1/5 pathway.

A HeLa reporter cell system was used to verify the function of *JAG1* as a Notch ligand. HeLa cells were transfected with the Notch-specific luciferase reporter construct (pGL4-12xCSL-Luc), and thus responsive to Notch activation (26). In the absence of HUVECs, BMP-9 did not induce reporter activity in the transfected HeLa cells (Figure 7F; lanes 1 and 3). In the presence of HUVECs, however, BMP-9 induced strong activation of reporter expression (Figure 7F; lanes 2 and 4), indicating that *JAG1* induced by BMP-9 in ECs was able to efficiently transactivate Notch signaling in neighboring cells.

DISCUSSION

In this study, genome-wide maps of Smad1/5 binding regions in human primary cells revealed how BR-Smads recognize and regulate their target genes. Both HUVECs and PSMCs express Smad1, Smad5 and Smad8 (Supplementary Figure S1E). However, redundant functions between Smad1 and Smad5 have been demonstrated *in vivo*, especially in the vasculature (39). *Smad1*^{+/-}; *Smad5*^{+/-} double heterozygous mutant mice are embryonic lethal and display defects, which closely resemble those seen in *Smad1*- or *Smad5*-null mice, whereas *Smad1* or *Smad5* single heterozygous mice show no overt phenotype. *Smad8*-null mice additionally lacking one copy of *Smad1* or *Smad5* did not exhibit overt phenotypes, and the tissue disturbances seen in *Smad1*- or *Smad5*-null embryos are not exacerbated in the absence of *Smad8*. These findings suggest that Smad1 and Smad5 possess equivalent biological functions especially in the vasculature, while Smad8 is dispensable.

The mapping data of Smad1/5 showed that $\sim 30\%$ of the binding sites were located in the introns of known genes. Smad1/5 binding peaks of 85.4% overlapped with enhancer regions in HUVECs, where histone modification markers in basal conditions were available. Motif analysis revealed that binding motifs for ETS, AP-1, AP-2 and SP-1 were enriched in Smad1/5 binding regions regardless of the cell types. These motifs were also enriched in the Smad4 binding regions in human keratinocyte HaCaT cells (31). Other motifs occurred only in a small proportion of sequences analyzed. Recently, John and colleagues (40) reported that cell type-specific glucocorticoid receptor binding patterns are comprehensively predetermined by cell-specific differences in baseline chromatin accessibility patterns, with secondary contributions from local sequence features. The similar motif occurrence patterns between HUVECs and PSMCs suggest that the binding regions of BR-Smad are also predetermined in the specific cell types.

Smad1/5 reproducibly bound to some target sites such as *ID1* and *ID3* loci with comparable enrichment after BMP-9 and BMP-6 stimulation, while the total number of Smad1/5 binding sites was dramatically lower in HUVECs treated with BMP-6 compared to those with BMP-9 (3750 versus 880). Increasing the dose of BMP-6 up to 200 ng/ml was not enough to elicit comparable level of enhancer activities as 1 ng/ml BMP-9 (Figures 2C and 5B). This suggests that each binding site has different binding affinity for Smad complex and that BR-Smad signaling through ALK-2 was not enough to occupy full sets of target sites in ECs. This is consistent with the facts that HHT2 is the result of haploinsufficiency of ALK-1 (6), and that ALK-2 signaling is not able to compensate for ALK-1 mutations in HHT patients even though BMP-9 can signal through ALK-2 (1).

In *Drosophila*, Ashe *et al.* (41) have reported that each enhancer element for Mad target genes has a different binding affinity for Smad/Mad. A gene with low-affinity Smad/Mad binding sites is transcribed only in response to high concentrations of Dpp, while a gene with higher affinity sites responds to a low dose of Dpp. Increasing

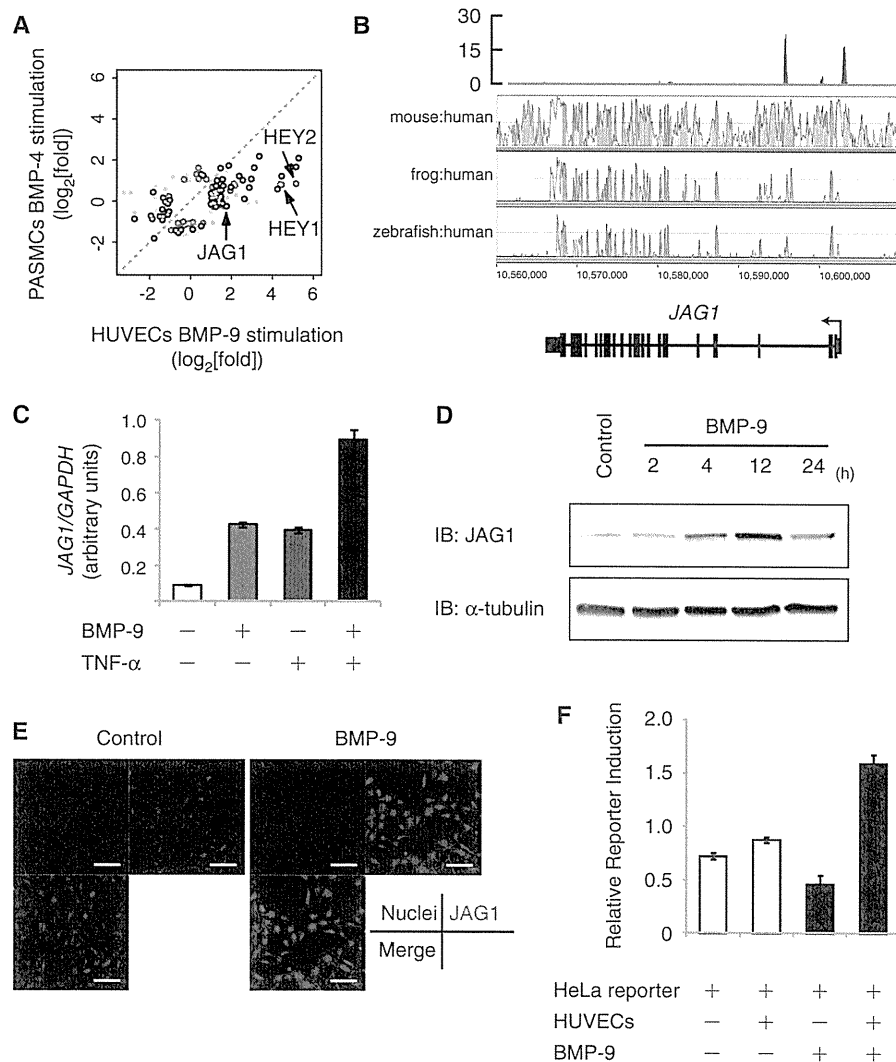


Figure 7. The Notch ligand JAG1 is a direct target gene of Smad1/5 and transactivates Notch signaling in the neighboring cells. (A) Scatter plot representation of differentially regulated genes between HUVECs and PASCs. Probes of genes with more than 2-fold change in expression relative to time 0 are plotted. If the genes are associated with Smad1/5 binding regions of HUVECs (green), PASCs (red) or both (black), the plots are colored. Signal intensities of HUVECs treated with BMP-9 for 2 h is plotted on the X-axis and those of PASCs treated with BMP-4 for 2 h is plotted on the Y-axis. (B) Visualization of *JAG1* locus with the result of BMP-9 ChIP-seq. Red peaks represent ChIP regions (top panel). The conservation plots for mouse/human, frog/human and zebrafish/human are derived from VISTA genome browser (middle panel), which represents the sequence conservation between species. (C) Induction of *JAG1* after BMP-9 stimulation in HUVECs. HUVECs were starved overnight, stimulated with 1 ng/ml BMP-9 and/or 10 ng/ml TNF- α for 2 h and subjected to qRT-PCR analysis for *JAG1*. Values were normalized to the amount of housekeeping *GAPDH* mRNA. The data are the mean of triplicate values \pm SD. (D) HUVECs were starved overnight and stimulated with 1 ng/ml BMP-9 for indicated time periods and subjected to immunoblot analysis to determine the JAG1 protein expression level. α -Tubulin was used as a loading control. (E) Immunocytochemistry of HUVECs treated with or without 1 ng/ml BMP-9 for 24 h. The cells were immunostained with anti-JAG1 antibody (green). Nuclei were labeled with TOTO-3 (blue). Scale bar, 100 μ m. (F) Endothelial JAG1 induced by BMP-9 stimulation transactivates Notch signaling in neighboring cells. HeLa cells were transiently transfected with pGL4-12xCSL-luciferase reporter construct and co-cultured with HUVECs. Cells were treated with or without 5 ng/ml BMP-9 for 24 h and subjected to luciferase assay. The data are the mean of triplicate values \pm SD.

the affinity of the Smad/Mad binding sites in the enhancer of the *Ance* (also known as *Race*) resulted in a wider expression pattern *in vivo* (42). We revealed that a mutation in our consensus GC-SBE sequences attenuates BMP responsiveness of target genes (Figure 5B and Supplementary Figure S5B). In addition, a mutation of

the *HAMP* promoter from GGCGCC to GGTGCC, which was identified in a hemochromatosis patient, impairs the BMP responsiveness *in vivo* and contributes to the severe phenotype (43). These results suggest that the binding affinity for Smad complex is the sum of the affinities of GC-SBEs, SBEs and other DNA binding proteins like

Sox2 and Oct4 in mESCs (16), and that unidentified mutations in the BR-Smad binding regions will be implicated in HHT or PAH.

Collectively, our findings support the notion that BR-Smad binding sites are predetermined in specific cell type and determined by the binding affinity of Smad complex to possible binding sites. It suggests that the strength of the BR-Smad pathway is converted to the number and distribution of BR-Smad binding sites over the genome. It does not necessarily exclude the possibilities that non-Smad pathways play important roles. Non-Smad pathways have been reported to affect the BR-Smad pathway through degrading BR-Smads or modulating binding affinity of Smad complexes [for review, see (44)]. It is possible that they modulate the intensity of BR-Smad pathway and affect the number and distribution of Smad1/5 binding sites in ECs (Figure 6C).

Dysregulation of Notch signaling has been reported to cause AVM [for review, see (45)] that is one of the major pathological features of HHT. JAG1 has been reported to cause differentiation of vascular smooth muscle cell (vSMC) precursor cells and induce vSMC-specific genes *in vitro* through the JAG1-Notch3 signaling pathway (46,47). EC-specific deletion of *Jag1* showed defects in vSMC coverage in mice (38,48). Interestingly, genetic and pharmacological inhibition of ALK-1 signaling showed a severe vascular phenotype including lack of differentiation and recruitment of vSMCs and defects in the maturation phase of angiogenesis (5,49,50). In the clinical settings, thalidomide has been shown to stimulate vessel maturation and have beneficial effects on HHT patients (51). Therefore, our results suggested the important roles of ALK-1-Smad-JAG1 pathway in the pathogenesis of the vascular lesions of the HHT. They also suggest that this pathway will be a novel therapeutic target for treatment of HHT.

ACCESSION NUMBERS

The microarray data from this study have been submitted to NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo>) under accession no. GSE27661, and the sequence data from this study have been submitted to the NCBI Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi>) under accession no. SRA030442.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Glioma-initiating Cells Retain Their Tumorigenicity through Integration of the Sox Axis and Oct4 Protein^{*S}

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Background: Glioma-initiating cells are underlying causes of development and progression of glioblastoma.

Results: Depletion of Oct4 expression suppresses tumorigenic activity of glioma-initiating cells through down-regulation of Sox2.

Conclusion: Oct4 maintains tumorigenicity of glioma-initiating cells in cooperation with the Sox axis.

Significance: This study uncovers the transcriptional network of stemness genes in cancer-initiating cells.

Although the concept of cancer stem cells or cancer-initiating cells had created a new paradigm for the treatment of malignant tumors, it remains unclear how cancer-initiating cells can be eradicated. We have previously reported that the transforming growth factor- β (TGF- β)-Sox4-Sox2 pathway is essential for glioma-initiating cells to retain their stemness, and inhibition of TGF- β signaling may lead to differentiation of glioma-initiating cells (Ikushima, H., Todo, T., Ino, Y., Takahashi, M., Miyazawa, K., and Miyazono, K. (2009) *Cell Stem Cell* 5, 504–514). Here we demonstrate that Oct4 plays essential roles in retention of the stemness properties of glioma-initiating cells through positive regulation of Sox2 expression. We also show that, in glioma-initiating cells, Oct4 is associated with Sox4 and that Oct4-Sox4 complexes cooperatively activate the enhancer activity of the *SOX2* gene. In contrast, in fetal neural progenitor cells, Sox2 expression is enhanced by transcriptional complex containing Sox2 protein itself, and this self-reinforcing loop of Sox2 appears to be disrupted in glioma-initiating cells, suggesting that Sox2 expression in glioma-initiating cells is differently regulated from that in neural progenitor cells. Our findings reveal differences between glioma-initiating cells and fetal neural progenitor cells and may open the way to depriving glioma-initiating cells of tumorigenic activity without affecting normal tissues.

Glioblastoma, also known as grade IV astrocytoma, is the most aggressive form of malignant glioma and is one of the

most malignant human cancers, with an estimated median survival of only ~1 year (1, 2). Despite past huge efforts, this statistic has not markedly improved over the past decades.

Cancer stem cells or cancer-initiating cells are tumor cells characterized by their ability to induce tumorigenesis and to self-renew (3). Similar to other types of tumor cells, glioma-initiating cells (or glioma stem cells) have been isolated from human glioblastoma tissues (4, 5). Following their identification, glioma-initiating cells have been intensively investigated and have been found to exhibit strong resistance to chemotherapy and radiotherapy (6, 7). It has been suggested that the failure to cure glioblastoma may be due to existing therapeutic strategies that affect only the tumor bulk and not glioma-initiating cells (8). These findings indicate the need for an innovative therapeutic strategy enabling functional eradication of glioma-initiating cells.

Although it has yet to be fully determined how the stemness of glioma-initiating cells is maintained, a few signaling pathways, including Hedgehog (9), bone morphogenetic protein 4 (10), and TGF- β (11–13), have been implicated to contribute to maintenance of the stemness properties of these cells. Although the transcriptional machinery required is under investigation, we have recently reported crucial roles for the Sox axis. Sox4 interacts with the *SOX2* enhancer region to induce Sox2 expression, and this “Sox4-Sox2” axis maintains stemness properties of glioma-initiating cells under the control of TGF- β signaling (11).

The POU class 5 transcription factor Oct4 (also known as Pou5f1) is essential for establishing and maintaining the pluripotent state of embryonic stem cells (14, 15). Deletion of Oct4 from embryonic stem cells results in trophoblast differentiation (16). Introduction of Oct4 together with Sox2, Klf4, and c-Myc into human or mouse adult fibroblasts results in the generation of induced pluripotent stem cells (17, 18). In addition, Oct4 has been detected in high grade glioma and specific types of testicular germ cell tumors (19–21). However, the role of Oct4 in cancer stem cells has yet to be fully determined.

Here, we report that Oct4 is a factor of crucial importance for the maintenance of tumorigenic activity of glioma-initiating

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cells. We have previously reported that, in contrast to Sox4 and Sox2, the expression of Oct4 is not regulated by TGF- β signaling in glioma-initiating cells (11). However, inhibition of Oct4 expression in glioma-initiating cells resulted in suppression of sphere formation *in vitro* and tumor formation *in vivo*. Oct4 knockdown also potentiated sensitivity to conventional chemotherapy. We also demonstrated that Oct4 interacted with Sox4 and cooperatively activated the SOX2 enhancer region to maintain stemness properties of glioma-initiating cells. Notably, Sox2 expression in glioma-initiating cells was induced by the Oct4-Sox4 complex acting on the SOX2 enhancer region to maintain stemness properties, whereas that in fetal neural progenitor cells was regulated by a transcriptional complex containing Sox2 protein itself through a self-reinforcing regulatory loop. These findings indicate that Oct4 plays a role in the tumorigenic activity of glioblastoma and suggest that the stemness properties of glioma-initiating cells are regulated by mechanisms different from those of neural progenitor cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Primary grade IV glioblastoma samples were obtained during surgery from consenting patients, as approved by the Institutional Review Board of the University of Tokyo Hospital. Spheres were cultured in DMEM/F-12 serum-free medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/ml EGF, and 20 ng/ml basic fibroblast growth factor (both from PeproTech). Characteristics of the glioma-initiating cells were evaluated in our previous study (11). Normal human fetal neural progenitor cells were obtained from Lonza and cultured in maintenance medium (NPM, Lonza). U373MG cells were maintained in DMEM containing 10% fetal bovine serum, 50 units/ml penicillin, 50 μ g/ml streptomycin, sodium pyruvate (1 mM), and non-essential amino acids (0.1 mM). The antibodies used were as follows: anti-Musashi (Chemicon), anti-Nestin (Chemicon), anti-gliial fibrillary acidic protein (Dako), anti-Tuj1 (Covance), anti-Oct4 (Santa Cruz Biotechnology), anti-Sox2 (R&D), anti-Sox4 (Santa Cruz Biotechnology), and anti- α -tubulin (Sigma-Aldrich).

Sphere-forming Assay—Glioma-initiating cells were cultured in non-tissue-culture-treated flasks (BD Biosciences) with vented caps (BD Biosciences) for 7 days. Floating spheres in five fields per sample were counted under a microscope (magnification, $\times 40$).

Limiting Dilution Assay—Sphere cells were dissociated and plated in 96-well plates in 200 μ l of serum-free medium. After a 7-day culture, the percentage of wells not containing spheres for each cell plating density was calculated and plotted against the number of cells per well.

RNA Interference—siRNAs (see supplemental Table S1 for sequences) were purchased from Invitrogen and introduced into cells using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions.

Immunostaining—Glioma-initiating cells were seeded on poly-L-ornithine (Sigma)- and fibronectin (Sigma)-coated slide glasses and cultured for 7 days with the indicated siRNA in serum-free medium. Cells were fixed with 3.7% paraformaldehyde, permeabilized with PBS containing 0.3% Triton X-100, and incubated with the indicated antibodies. Subsequently,

samples were incubated with secondary antibodies and stained with propidium iodide (Molecular Probes) for nuclear staining. Stained cells were observed with a confocal microscope (LSM510, Carl Zeiss).

Cell Lysis and Immunoblotting—Cells were lysed with a buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1% aprotinin, and 5 mM EDTA. Proteins in cleared cell lysates were subjected to SDS-PAGE and transferred to Fluoro Trans W membrane (Pall). Immunoblotting was performed using the indicated antibodies.

Quantitative Real-time PCR—Quantitative real-time reverse transcription-PCR was performed as described previously (22). All samples were run in triplicate in each experiment. The primers used are listed in supplemental Table S1. Values were normalized to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

ChIP and ChIP Re-IP—Chromatin immunoprecipitation (ChIP)³ was performed as described previously (11). PCR primers are listed in supplemental Table S1. For ChIP re-immunoprecipitation (Re-IP) assays, protein-DNA complexes were eluted from immunoprecipitation by incubation with 10 mM DTT at 37 °C for 30 min and diluted 1:50 in buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), followed by Re-IP with secondary antibodies.

Cell Viability Assay—Quantitation of cell viability was performed using a colorimetric assay for mitochondrial dehydrogenase activity (WST-8, Nacalai Tesque) after treatment with temozolomide (LKT Laboratories).

Luciferase Assay—The SOX2 enhancer region (+3553 through +4290) was cloned into a pGL4 vector (Promega, Madison, WI) with a minimal promoter, and a luciferase assay was performed as described previously (22). Values were normalized to *Renilla* luciferase activity under the control of thymidine kinase promoter.

Intracranial Proliferation Assay—Viable glioma-initiating cells (5×10^4) in 5 μ l of DMEM/F-12 medium were injected stereotactically into the right cerebral hemisphere of 5-week-old female BALB/c *nu/nu* mice at a depth of 3 mm. All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo.

RESULTS

Oct4 Is an Essential Factor for Retention of Stemness of Glioma-initiating Cells *in Vitro*—The transcriptional network essential for maintenance of glioma-initiating cells has not been fully determined. We used glioma-initiating cells obtained from two patients with glioblastoma, termed TGS-01 and TGS-04, and cultured in serum-free medium to study this network. The glioma-initiating capacities of these cells were characterized in our previous studies (11).

Oct4 is known to be one of the most crucial self-renewal gene products and to play pivotal roles in maintaining stemness of embryonic stem cells and neural stem cells. We have demon-

³ The abbreviations used are: ChIP, chromatin immunoprecipitation; Re-IP, re-immunoprecipitation; Tuj1, β III-tubulin; GATA1, -2, GATA-binding proteins 1 and 2.

Regulation of Glioma-initiating Cells by Oct4

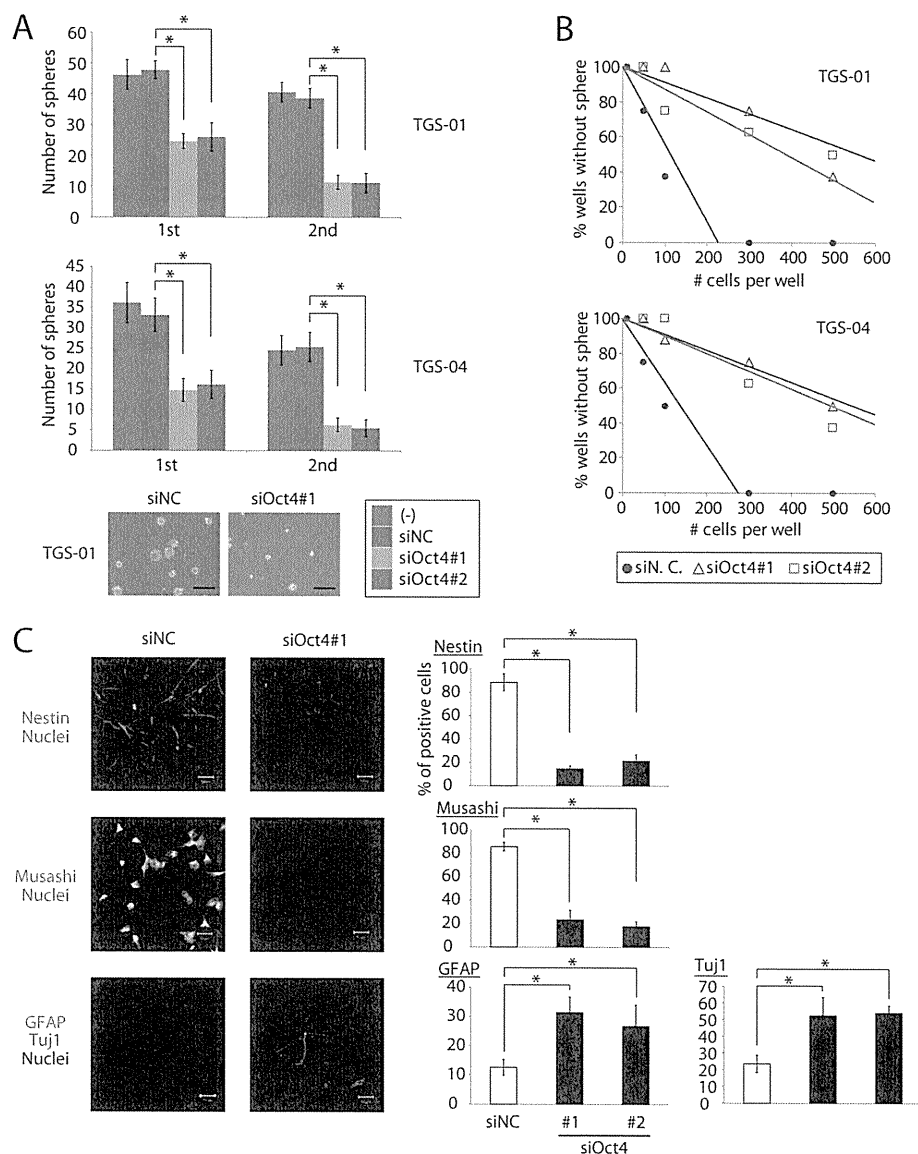


FIGURE 1. Oct4 is essential for retention of stemness of glioma-initiating cells. A, TGS-01 and TGS-04 cells were dissociated into single cell populations, transfected with control (NC) or Oct4 siRNA duplex, and cultured for 7 days (1st). After the 7-day culture, spheres were dissociated into single cell populations and equal numbers of cells were cultured for another 7 days (2nd). Values are the number of glioma spheres formed (means \pm S.E. of five fields). $^* p < 0.001$. Scale bars, 100 μ m. B, knockdown of Oct4 expression by siRNA in TGS-01 and TGS-04 cells resulted in a decrease of self-renewal capacity in limiting dilution assay. C, immunostaining of TGS-01 cells. Spheres were disaggregated, seeded on poly-L-ornithine- and fibronectin-coated slide glasses, and cultured in serum-free medium with control (NC) or Oct4 siRNA duplex for 7 days. Quantification of Nestin-, Musashi-, Tuj1-, or glial fibrillary acidic protein (GFAP)-positive cells is shown in the right graphs. $^* p < 0.01$. Scale bars, 50 μ m.

strated that expression of Oct4 is not affected by TGF- β signaling in glioma-initiating cells (11). To study the role of Oct4 in glioma-initiating cells, we first examined the effects of Oct4 knockdown on their biological properties. After Oct4 expression was knocked down (supplemental Fig. S1), glioma-initiating cells exhibited marked reduction of sphere-forming ability in serial sphere-forming assay (Fig. 1A), suggesting that Oct4 is required for self-renewal of glioma-initiating cells. In limiting dilution assay, TGS-01 or TGS-04 with Oct4 siRNA also showed less capacity for self-renewal than control cells (Fig. 1B). Similar results were obtained with the use of glioma-initiating cells, TGS-02, TGS-03, and TGS-05, derived from other patients with glioblastoma (supplemental Fig. S2). We also

examined the effects of Oct4 knockdown on proliferation and apoptosis of glioma-initiating cells. Treatment of siRNA against Oct4 did not significantly induce apoptosis but reduced proliferation of TGS-01 and TGS-04 cells (supplemental Fig. S3).

Glioma-initiating cells have been reported to express neural precursor cell markers, but to only minimally express neural or glial differentiation markers (11). To examine the expression of these marker proteins in each type of cell, spheres in serum-free medium were disaggregated and seeded on poly-L-ornithine- and fibronectin-coated slide glasses. Knockdown of Oct4 expression by siRNA decreased the number of cells positive for Nestin or Musashi (neural precursor cell markers) and

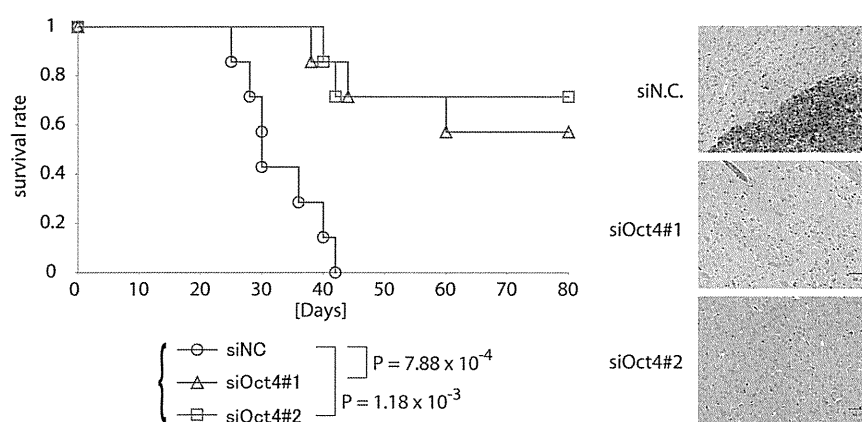


FIGURE 2. Development of brain tumors after intracerebral transplantation of 5×10^4 TGS-01 cells pretreated with control (NC) or Oct4 siRNA duplex for 7 days. Survival of mice ($n = 7$ mice for each condition) was evaluated by Kaplan-Meier analysis (left graph). p values were calculated by the log-rank test. The right panels show the results of histological examination of the samples dissected at 30 days after intracerebral transplantation. Tissue sections were stained with hematoxylin-eosin. Scale bars, 50 μ m. Experiments were repeated twice with essentially similar results.

increased that for glial fibrillary acidic protein (astrocyte differentiated marker) or Tuj1 (neuronal marker) (Fig. 1C). These results indicate that Oct4 is required for maintenance of the stemness properties of glioma-initiating cells *in vitro*.

Knockdown of Oct4 Expression Decreases Tumorigenicity of Glioma-initiating Cells *in Vivo*—To study the role of Oct4 in the tumorigenic activity of glioma-initiating cells *in vivo*, we next examined the effects of Oct4 knockdown on intracranial growth of glioma-initiating cells. We treated dissociated glioma-initiating cells with siRNA against Oct4. Cells from the newly formed glioma spheres were orthotopically inoculated into the brains of immunocompromised mice. The growth of glioma-initiating cells was inhibited by pretreatment with siRNA against Oct4, and the mice inoculated with the pretreated glioma-initiating cells survived significantly longer than those inoculated with control cells (Fig. 2). We also examined tumor formation in the brain 30 days after transplantation. Whereas mice with control glioma-initiating cells displayed large tumors in the brain, those with the pretreated glioma-initiating cells exhibited no evidence of tumor on histopathologic examination (Fig. 2). These results suggest that Oct4 is essential for the maintenance of tumorigenicity of glioma-initiating cells and that loss of tumorigenicity by Oct4 knockdown is an irreversible process.

Knockdown of Oct4 Expression in Glioma-initiating Cells Affects Sensitivity to Chemotherapy—As suggested by the cancer stem cell model, the resistance of glioma-initiating cells to conventional chemotherapy may be a major cause of the low cure rate for glioblastoma (3). Although oral administration of temozolomide, a new alkylating agent, has shown efficacy in patients with glioblastoma, it was found that glioma-initiating cells were resistant to temozolomide-induced cell death, causing tumors to recur (7). We examined the effects of Oct4 knockdown on the sensitivity of glioma-initiating cells to temozolomide-induced cell death. Control TGS-01 and TGS-04 cells exhibited low sensitivities to temozolomide treatment (Fig. 3 and supplemental Fig. S4). In contrast, treatment with increasing concentrations of temozolomide suppressed the viability of TGS-01 and TGS-04 cells pretreated with Oct4 siRNA in dose-

dependent fashion. These results suggest that Oct4 is involved in acquisition of drug-resistance properties of glioblastoma.

Oct4 Directly Induces Sox2 Expression to Maintain Stemness Properties of Glioma-initiating Cells—We next studied the molecular mechanisms that underlie the putative pathological roles of Oct4 in glioma-initiating cells. Oct4 regulates stemness properties of embryonic and neural stem cells via several mechanisms (23). Among them, Sox2, which has recently been reported to be a critical regulator of the stemness of glioma-initiating cells (11, 24), is known to be one of the major downstream targets of Oct4 in embryonic stem cells (25). We therefore examined whether Sox2 acts downstream of Oct4 in glioma-initiating cells. Oct4 knockdown in glioma-initiating cells resulted in down-regulation of Sox2 expression (Fig. 4A), indicating that Oct4 positively regulates Sox2 expression in glioma-initiating cells. To examine whether this regulation is directly mediated at the transcriptional level, we performed a ChIP assay using an antibody to Oct4. It has been demonstrated that the enhancer element located in the 3' flanking region of the *SOX2* gene is important for the regulation of Sox2 expression in embryonic stem cells (25, 26). Recruitment of Oct4 to the *SOX2* enhancer element was observed in glioma-initiating cells (Fig. 4B). In contrast, Oct4 was only minimally associated with the *SOX2* enhancer element in matched “differentiated” cells that were derived from the same patient but were cultured in media containing 10% fetal bovine serum to induce differentiation. These results appear to be due to the lower levels of expression of Oct4 in the “differentiated” cells compared with the “sphere” cells (Fig. 4C). These findings together indicate that Oct4 induces Sox2 expression in glioma-initiating cells through direct binding to the *SOX2* enhancer region.

Oct4 Induces Sox2 Expression Cooperatively with Sox4 and Activates the Sox4-Sox2 Cascade in Glioma-initiating Cells—In our previous study, another transcription factor, Sox4, was shown to associate with the *SOX2* enhancer region and maintain the stemness and tumorigenicity of glioma-initiating cells (11). In addition, consensus sequences of Sox proteins and Oct4 exist proximally in the *SOX2* enhancer region (Fig. 5A). These findings prompted us to study the interaction of the Sox axis

Regulation of Glioma-initiating Cells by Oct4

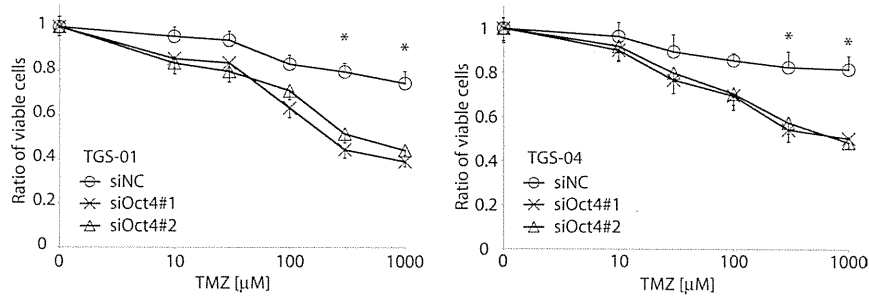


FIGURE 3. Knockdown of Oct4 expression enhances sensitivity to chemotherapy in glioma-initiating cells. TGS-01 and TGS-04 cells with control (NC) or Oct4 siRNA duplex were seeded in 96-well plates and treated with temozolomide (0, 10, 30, 100, 300, and 1000 μM) for 72 h. Cell viability was assessed by using a WST-8 assay. *, $p < 0.01$ (siNC versus siOct4#1 and siNC versus siOct4#2).

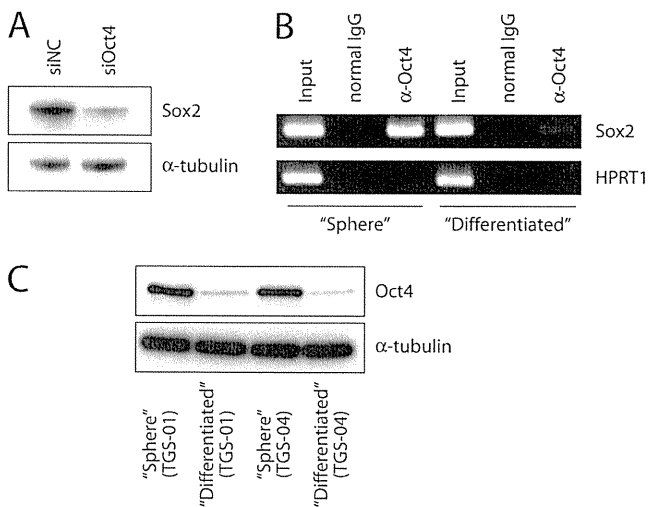


FIGURE 4. Oct4 is associated with the SOX2 enhancer region to up-regulate expression levels of Sox2 in glioma-initiating cells. *A*, effects of Oct4 knockdown on expression of Sox2. Amounts of Sox2 protein were determined after treatment with control (NC) or Oct4 siRNA #1 duplex for 24 h. α -Tubulin was used as a loading control. *B*, association of Oct4 with the SOX2 enhancer region. ChIP analysis was performed using TGS-01 cells ("Sphere") and matched "Differentiated" cells. Eluted DNAs were subjected to conventional RT-PCR. The first intron of hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was used as a negative control. *Input*: 1%. *C*, levels of expression of Oct4 protein in "Sphere" cells and "Differentiated" cells. α -Tubulin was used as a loading control.

and Oct4 in the maintenance of stemness properties of glioma-initiating cells. First, we examined the interaction between Sox4 and Oct4. As shown in Fig. 5*B*, endogenous Oct4 physically interacted with endogenous Sox4 in glioma-initiating cells. Moreover, ChIP Re-IP experiments demonstrated that Sox4 and Oct4 exist in the same transcription complex on the SOX2 enhancer region (Fig. 5*C*).

We next studied the effects of the Oct4-Sox4 complex on Sox2 expression in glioma-initiating cells. When Oct4 and Sox4 were both knocked down, Sox2 expression was more strongly down-regulated than it was by separate knockdown of Oct4 or Sox4 (Fig. 6*A*). Suppression of SOX2 enhancer activity by knockdown of Oct4 or Sox4 was also confirmed in luciferase assay using TGS-01 and TGS-04 cells (Fig. 6*B*). Moreover, the enhancer activity was synergistically activated by Oct4 and Sox4 overexpression in glioblastoma cell line U373MG (Fig. 6*B*), in which the levels of expression of Oct4 and Sox4 were significantly lower than in glioma-initiating cells (data not shown).

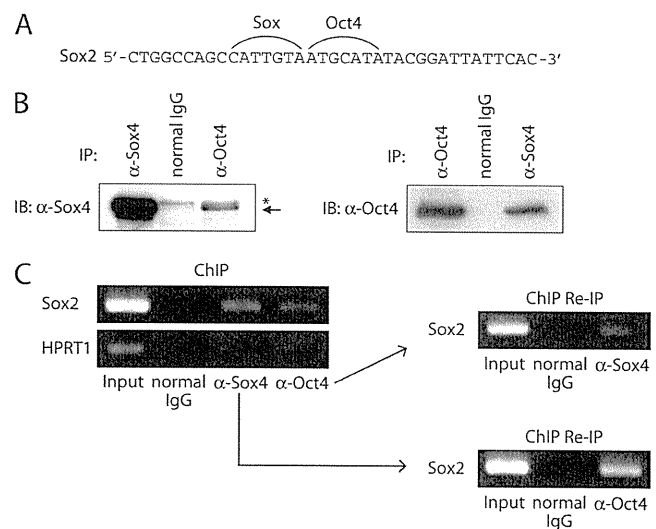


FIGURE 5. Oct4 physically interacts with Sox4 on SOX2 enhancer region. *A*, the sequences of Oct4-binding element and Sox-binding element in the SOX2 enhancer region. *B*, physical interaction of endogenous Oct4 with endogenous Sox4 in TGS-01 cells. Cell lysates were subjected to immunoprecipitation with anti-Oct4 antibody followed by immunoblotting with anti-Sox4 (left) or with anti-Sox4 antibody followed by immunoblotting with anti-Oct4 (right). Asterisk: nonspecific band. *C*, recruitment of the Oct4-Sox4 complex to the SOX2 enhancer region. Soluble chromatin was prepared from TGS-01 cells, and ChIP analysis was performed using anti-Sox4 and anti-Oct4 antibodies. Subsequently, ChIP Re-IP of protein-DNA complex eluted from the first immunoprecipitation was performed. Eluted DNAs were subjected to conventional RT-PCR.

To confirm a direct association of Oct4 and Sox4 with the SOX2 enhancer region, we generated luciferase constructs with mutated Oct4 and/or Sox4 binding elements in the SOX2 enhancer region (Fig. 6*C*). Mutation of one of the two elements led to a reduction of enhancer activity compared with the wild-type enhancer. When both binding elements were mutated, the enhancer activity was more strongly reduced. These results indicate that both Oct4 and Sox4 directly interact with the SOX2 enhancer region and synergistically induce Sox2 expression.

Transcription Factor Complexes on the SOX2 Enhancer Region in Glioma-initiating Cells Are Distinct from Those in Neural Progenitor Cells—As demonstrated here using glioma-initiating cells, Sox2 expression is also induced by Oct4 through interaction of Oct4 with the SOX2 enhancer region in embryonic stem cells. Furthermore, in embryonic stem cells, Sox2 is associated with Oct4 and the Oct4-Sox2 complex cooperatively

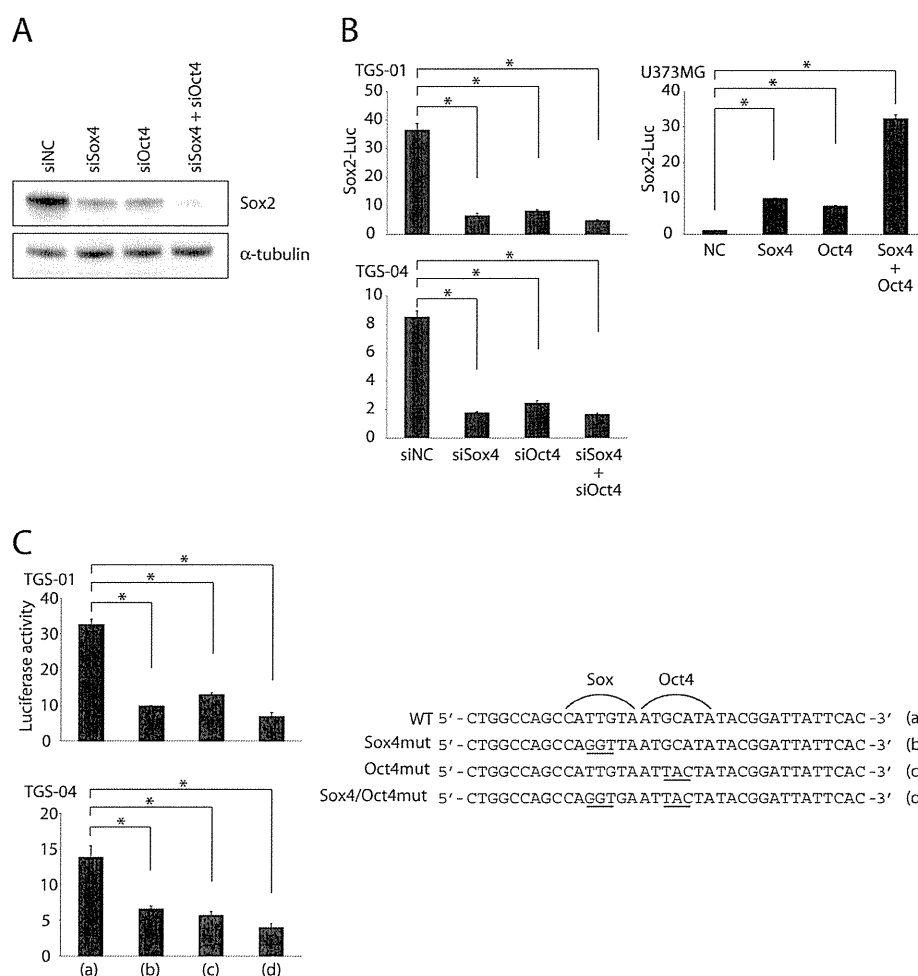


FIGURE 6. Oct4 acts in concert with Sox4 to potentiate SOX2 enhancer activity. *A*, effects of Oct4 and/or Sox4 knockdown on expression of Sox2. Amount of Sox2 protein was determined after treatment with indicated siRNA duplex for 24 h. *B*, roles of Oct4 and Sox4 in activation of the SOX2 enhancer region. Effects of Oct4 and/or Sox4 knockdown on SOX2 enhancer activity were examined in TGS-01 and TGS-04 cells (*left graphs*). Effects of Oct4 and/or Sox4 overexpression on SOX2 enhancer activity were examined in U373MG cells (*right graph*). Error bars represent \pm S.E. *, $p < 0.001$. *C*, TGS-01 or TGS-04 cells were transfected with luciferase constructs containing wild-type or mutated SOX2 enhancer region. The cells were collected 24 h after transfection, and luciferase activity was examined. *, $p < 0.001$. The *right panel* indicates the sequence of the SOX2 enhancer region and corresponding mutations (*underlined*) used in this study.

activates the SOX2 enhancer region to form a positive regulatory loop (25, 26). To determine whether this regulatory loop exists in neural progenitor cells and glioma-initiating cells, we examined recruitment of these transcription factors to the SOX2 enhancer region in a ChIP assay (Fig. 7). Anti-Oct4 antibody enriched the DNA fragments of SOX2 enhancer region equally well in fetal neural progenitor cells and glioma-initiating cells. In addition, anti-Sox2 antibody immunoprecipitated this region in neural progenitor cells. However, strong enrichment of the same region by anti-Sox2 antibody was not observed in glioma-initiating cells. These results indicate that transcription factor complex on the SOX2 enhancer region does not contain Sox2 in glioma-initiating cells and that Sox2 expression in glioma-initiating cells is regulated by mechanisms different from those in fetal neural progenitor cells.

We next examined recruitment of Sox4 to the SOX2 enhancer region in neural progenitor cells and glioma-initiating cells. In contrast to the experiment using anti-Sox2 antibody, anti-Sox4 antibody immunoprecipitated the DNA fragments of the SOX2 enhancer region in glioma-initiating cells,

whereas the enrichment observed in fetal neural progenitor cells was much weaker. These findings together indicate that Sox2 expression in glioma-initiating cells is potentiated by the Oct4-Sox4 complex acting on the SOX2 enhancer region to maintain tumorigenic activity, whereas that in neural progenitor cells may be promoted by transcriptional complex containing Sox2 protein itself through a positive regulatory loop.

DISCUSSION

Although the origin of glioma stem cells (or glioma-initiating cells) is controversial (27), several studies have suggested that glioma-initiating cells share characteristics with neural or glial stem/progenitor cells (28, 29). Glioma stem cells express neural stem cell markers, including Nestin, Musashi, and Prominin-1 (CD133). Like normal neural stem cells, glioma stem cells are located in specific niches surrounding the tumor vasculature. A recent study has shown that the perivascular niches control self-renewal of glioma stem cells through endothelial cell-derived factors (30). However, in terms of transcription factor complexes, the similarities and differences between glioma

Regulation of Glioma-initiating Cells by Oct4

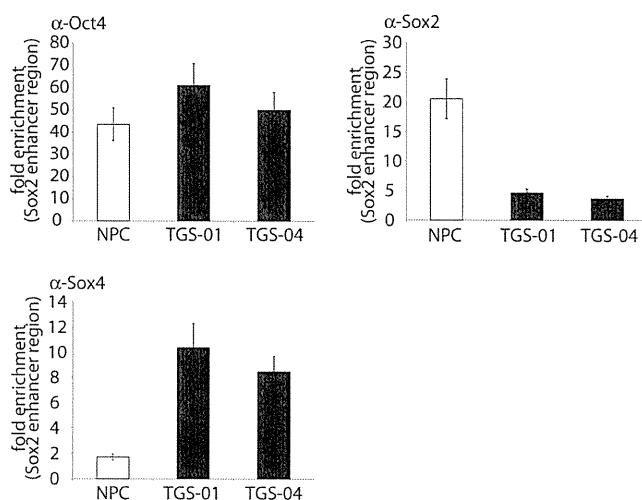


FIGURE 7. The partner of Oct4 on SOX2 enhancer region in glioma-initiating cells is distinct from that in neural progenitor cells. Soluble chromatin was prepared from glioma-initiating cells (TGS-01 and TGS-04) and neural progenitor cells (NPC). ChIP analysis was performed using anti-Oct4, anti-Sox2, and anti-Sox4 antibodies. Eluted DNAs were subjected to quantitative real-time PCR analysis. Values were normalized to the amount of the first intron of HPRT1. Error bars represent \pm S.E.

stem cells and neural stem cells have not been clearly determined.

Here we have shown that Oct4 expression is required for the maintenance of the self-renewal capacity of glioma-initiating cells. In addition, transient suppression of Oct4 by siRNA abolished the induction of Sox2 by TGF- β^4 and decreased the tumorigenic activity of glioma-initiating cells (Fig. 2), suggesting that impairment of stemness properties via Oct4 knockdown may be an irreversible process. We also demonstrated that Oct4 knockdown increases sensitivity to the chemotherapeutic alkylating agent, temozolomide.

Oct4 is essential for establishing and maintaining the pluripotent state of stem cells (14, 15). Moreover, Oct4 is one of the key factors in the generation of induced pluripotent stem cells (17, 18). However, the role of Oct4 in the development and progression of malignant tumors has not been fully determined. Our findings indicate that Oct4 is an essential factor for glioma-initiating cells and plays roles similar to those in embryonic stem cells.

One of the most intensively investigated topics in current cancer research is the identification of specific therapeutic compounds that can effectively eliminate cancer-initiating cells. Recent studies have identified factors essential for retention of cancer-initiating cells, including several growth factor signaling pathways such as Wnt, Hedgehog, Notch, PI3K-mTOR, TGF- β , and LIF (9, 31–36). Although new therapeutic targets have been intensively sought based on findings related to these pathways, one problem is that almost all of these signaling pathways are also indispensable for normal stem cells. Inhibitors of these signaling pathways may affect the characteristics of normal stem cells and impair maintenance of normal tissues. Thus, from a clinical standpoint, it is important to identify factors not only essential for the maintenance of cancer-

initiating cells but also different from those present in normal stem cells.

Here, we have demonstrated that Oct4-Sox4 complex activates the enhancer region of SOX2 genes to sustain stemness properties of glioma-initiating cells. Oct4 and Sox2 are also important for the maintenance of normal stem cells, and Oct4-Sox2 complex activates the SOX2 enhancer region to form a positive regulatory loop. However, in glioma-initiating cells, Sox2 is not predominantly present in the transcription factor complex on the SOX2 enhancer region. Instead, Sox4 forms a transcriptional complex with Oct4 in glioma-initiating cells to activate the enhancer region of SOX2, a gene essential for the maintenance of tumorigenicity of glioma-initiating cells. These findings suggest that Sox2 expression in glioma-initiating cells can be potentiated via up-regulation of Sox4, whereas Sox2 expression in neural progenitor cells is regulated by a self-reinforcing regulatory loop and is relatively self-contained (Fig. 7 and supplemental Fig. S5). In other words, the positive regulatory loop of Sox2 is not active in glioma-initiating cells, and alternatively, Oct4 acts with Sox4 to enhance Sox2 expression. We also confirmed that, in neural progenitor cells, Sox2 is only weakly induced by TGF- β stimulation (supplemental Fig. S6), whereas this cytokine activates the Sox4-Sox2 cascade in glioma-initiating cells (11). Loss of the regulatory loop of Sox2 expression may thus cause glioma-initiating cells to become susceptible to exogenous stimuli. However, we should bear in mind that our glioma-initiating cells were obtained from adult tumors, whereas neural progenitor cells were from a fetus. Further studies in neural progenitor cells from adults may be important to elucidate the differences between glioma-initiating cells and normal neural progenitor cells.

We examined combined effects of siRNAs against Sox4 and Oct4 in a limiting dilution assay but failed to observe any significant synergistic effects (supplemental Fig. S7). It may be because a defect of either factor in the Sox4-Oct4 complex results in significant inactivation of the SOX2 enhancer and/or because a single effect of siSox4 or siOct4 is strong enough to reduce sphere-forming ability of glioma-initiating cells.

It remains to be determined why the common Oct4-binding sequence and Sox-binding elements are differently regulated in neural progenitor cells and glioma-initiating cells. Upon differentiation of erythroid precursors into mature erythrocytes, GATA-binding protein 2 (GATA2) on some promoter regions is replaced by GATA1 (37). This process is termed the “GATA switch” and is an essential step in the maturation of erythrocytes and the expression of α -globin. One of the crucial mediators of this switching is Friend of GATA1 (FOG-1, also known as Zfp1), a multi-zinc-finger protein critical for the development of erythrocytes and megakaryocytes (38, 39), and GATA-FOG interaction is believed to be required for “GATA switch” (40). Like the “GATA switch,” the Sox-binding element on SOX2 enhancer region in glioma-initiating cells is differently regulated from that in neural progenitor cells, although the mechanism responsible for this remains to be determined.

Although Sox4 plays a crucial role in the retention of tumorigenicity of glioma-initiating cells through up-regulation of Sox2 expression (11), Sox4 $^{-/-}$ mice exhibit no neurological defects (41). This finding suggests that the mechanism of action

⁴ H. Ikushima and K. Miyazono, unpublished observation.

of Sox4 in glioma-initiating cells is distinct from that in neural stem/precursor cells. Because the self-renewal and proliferation of normal stem cells are likely strictly regulated, perhaps by genetic or epigenetic programs, the uncontrolled expansion of cancer-initiating cells may result from deregulation of such strict programs. In support of this conclusion, we found that the self-regulatory loop of Sox2 expression observed in neural progenitor cells was disrupted in glioma-initiating cells. This finding may enable the determination of a novel molecular target and eventually yield a therapeutic approach to eradication of glioblastoma without affecting the normal brain.

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TGF- β signal transduction spreading to a wider field: a broad variety of mechanisms for context-dependent effects of TGF- β

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Abstract Transforming growth factor (TGF)- β signaling is involved in almost all major cell behaviors under physiological and pathological conditions, and its regulatory system has therefore been vigorously investigated. The fundamental elements in TGF- β signaling are TGF- β ligands, their receptors, and intracellular Smad effectors. The TGF- β ligand induces the receptors directly to phosphorylate and activate Smad proteins, which then form transcriptional complexes to control target genes. One of the classical questions in the field of research on TGF- β signaling is how this cytokine induces multiple cell responses depending on cell type and cellular context. Possible answers to this question include cross-interaction with other signaling pathways, different repertoires of Smad-binding transcription factors, and genetic alterations, especially in cancer cells. In addition to these genetic paradigms, recent work has extended TGF- β research into new fields, including epigenetic regulation and non-coding RNAs. In this review, we first describe the basic machinery of TGF- β signaling and discuss several factors that comprise TGF- β signaling networks. We then address mechanisms by which TGF- β induces several responses in a cell-context-dependent fashion. In addition to classical

frames, the interaction of TGF- β signaling with epigenetics and microRNA is discussed.

Keywords TGF- β · Smad · ALK5 · Epigenetics · microRNA

Introduction

Cytokines are small secreted proteins that are produced by numerous types of cells and that play important roles in intercellular communication to maintain order in the organism. They elicit biological effects by binding to the extracellular domains of specific transmembrane receptors in the outer membrane of cells. Cytokines mediate intercellular communication via the regulation of cell growth and differentiation and are thus crucial for maintaining the homeostasis of multicellular organisms. Aberrant regulation of cytokine signaling can therefore result in various diseases.

The transforming growth factor (TGF)- β family is particularly prominent among these signals (Blobe et al. 2000; Feng and Derynck 2005; Massagué 2008). TGF- β signaling controls a diverse set of cellular processes, including cell growth, differentiation, apoptosis, survival, and specification of developmental fate, during embryogenesis and in mature tissues (Ikushima and Miyazono 2010a; Moustakas and Heldin 2009). To control TGF- β -induced cell responses, numerous factors tightly regulate this signaling pathway under physiological conditions (Ikushima and Miyazono 2010b; Bierie and Moses 2006). Loss of balance of TGF- β signaling thus leads to several pathological conditions, including malignant tumors, fibrotic diseases, and abnormal immune reactions (Levy and Hill 2006; Varga and Pasche 2009; Flavell et al.

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2010). Indeed, studies of clinical samples indicate that a distortion of TGF- β signaling is one of the major causes of several disorders. Here, we first discuss the way that (1) cells translate TGF- β signaling into cellular responses, and (2) TGF- β signaling and TGF- β -induced cell responses are tightly controlled. Possible and/or established mechanisms of the context-dependent diversity of TGF- β -induced cell responses are also addressed. In addition, recent research on TGF- β signaling has spread into novel fields, including epigenetics and non-coding RNAs. Thus, we also mention the involvement of epigenetic regulation and non-coding RNAs in the classical TGF- β signaling pathway.

Extracellular regulation of TGF- β signaling

Effects of TGF- β are mediated by three TGF- β ligands: TGF- β 1, TGF- β 2, and TGF- β 3 (Feng and Derynck 2005; Shi and Massagué 2003). Although each of these ligands is produced by distinct genes, they exhibit approximately 70%–80% sequence similarity. The TGF- β ligand is first synthesized as a dimeric pro-protein (pro-TGF- β), which is then cleaved to form the mature disulfide-bridged TGF- β dimer. The pro-peptide has high affinity for the cleaved mature TGF- β ligand, which is secreted from cells as a small latent complex (ten Dijke and Arthur 2007). Since TGF- β in this form does not have the ability to interact with its receptor, the pro-peptide is termed the latency-associated protein (LAP). The LAP dimer is also bound to the latent TGF- β binding proteins (LTBPs) by disulfide bonds, and the tri-molecular complex is termed the large latent complex (Rifkin 2005). The dissociation of TGF- β from the complex is a critical regulatory event and is achieved by integrin, shear force, thrombospondin-1 (TSP-1), some enzymes including plasmin, changes in pH, heat treatment, radiation, and other agents. Among the four different LTBPs, LTBP-1, 3, and 4 bind to small latent complexes and play key roles in targeting the large latent complex to the extracellular matrix, where active TGF- β is released by proteolytic cleavage. Although the synthesis of TGF- β is regulated by a variety of factors at the level of transcription and/or mRNA stability, the generation of active TGF- β from its latent form is also subject to regulation.

TGF- β receptors

Activated TGF- β ligands transduce their effects through TGF- β type I and II receptors (Ikushima and Miyazono 2010b; Wrana et al. 2008). The TGF- β type II receptor (T β RII) is the specific receptor for TGF- β ligands. Both

type II and type I receptors are comprised of an N-terminal extracellular ligand-binding domain, a transmembrane region, and a C-terminal intracellular serine/threonine kinase domain. TGF- β has high affinity for T β RII, and upon binding the ligand, the type I receptor forms a heteromeric complex consisting of two of each receptor type and is activated by the type II receptor (Fig. 1). The type I, but not type II, receptors contain a characteristic GS domain, located N-terminal to the kinase domain. Activation of the type I receptor involves the phosphorylation of its GS domain by the type II receptor. Although activin receptor-like kinase 5 (ALK5), also known as T β RI, mediates TGF- β signal transduction in most types of cells, ALK1 and other type I receptors also transduce TGF- β signaling in certain cells, including endothelial cells (Goumans et al. 2003; Daly et al. 2008).

TGF- β is also able to interact with proteins called TGF- β type III receptors, which do not have intrinsic kinase activity (Bernabeu et al. 2009). Betaglycan is a membrane-anchored proteoglycan that facilitates binding of TGF- β 2 to T β RII (Gatza et al. 2010). Endoglin, a glycoprotein expressed at high levels in endothelial cells, binds to T β RII and is thought to act as an accessory protein for the receptor complex (ten Dijke et al. 2008). Although the function of endoglin in TGF- β signaling is still controversial, mutations of it have been linked to hereditary hemorrhagic telangiectasia (McAllister et al. 1994; Abdalla and Letarte 2006). In addition, endoglin produced in a soluble form is associated with the pathogenesis of preeclampsia (Venkatesha et al. 2006). These findings indicate the central roles of endoglin in controlling vascular homeostasis.

Intracellular signal transduction through Smad proteins

Once the functional TGF- β receptor complex is formed, it regulates the activation of downstream signaling pathways. Although several substrates for the type I receptor kinases have been identified, the most important ones for the transduction of TGF- β stimulation are members of the Smad family proteins (Massagué et al. 2005; Schmierer and Hill 2007; Derynck and Zhang 2003). Phosphorylation and activation of the type I receptor enable the recruitment of receptor-regulated Smads (R-Smads). The type I receptor then phosphorylates R-Smads, allowing them to form hetero-oligomeric complexes with the common-partner Smad (Co-Smad) and to move into the nucleus. Of the five R-Smads in mammals, Smad2 and Smad3 are activated by the T β RII–ALK5 complex, whereas Smad1, Smad5, and Smad8 are activated by the T β RII–ALK1 complex. Interestingly, Liu et al. (2009) have recently reported that ALK5 can directly activate Smad1/5 in certain types of