Regulation of DU145 prostate cancer cell growth by Scm-like with four mbt domains 2

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Mammalian SFMBTs have been considered to be polycomb group repressors. However, molecular mechanisms underlying mammalian SFMBTs-mediated gene regulation and their biological function have not been characterized. In the present study, we identified YY1 and methylated histones as interacting proteins of human SFMBT2. We also found that human SFMBT2 binds preferentially to methylated histone H3 and H4 that are associated with transcriptional repression. Using DU145 prostate cancer cells as a model, we showed that SFMBT2 has a transcriptional repression activity on *HOXB13* gene expression. In addition, occupancy of SFMBT2 coincided with enrichment of di- and tri-methylated H3K9 and H4K20 as well as tri-methylated H3K27 at the *HOXB13* gene promoter. When *SFMBT2* was depleted by siRNA in DU145 prostate cancer cells, significant up-regulation of *HOXB13* gene expression and decreased cell growth were observed. Collectively, our findings indicate that human SFMBT2 may regulate cell growth via epigenetic regulation of *HOXB13* gene expression in DU145 prostate cancer cells.

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1. Introduction

The polycomb group (PcG) proteins are known to modify chromatin structure for the formation of transcription-resistant higher-order chromatin of target genes including the homeotic (HOX) gene in multicellular organisms (Morey and Helin 2010). In mammals, PcG proteins exist in distinct multiple complex, referred to as PRC1 and PRC2. PRC1 complex contains PcG proteins CBX, PHC, BMI, RING and several other proteins. RING ubiquitin E3 ligase mediates to transcriptional repression of PcG through mono-ubiquitination of lysine 119 of histone H2A (Wang *et al*. 2004). PRC2 complex is composed of three essential core subunits: EZH1/2, EED and SUZ12. Among these proteins, SET domain contains EZH1/2 di- and tri-methylate lysine 27 of histone H3, providing a mechanism for the spreading of PcG silencing (Kirmizis *et al*. 2004). Recently, a third PcG complex, PhoRC, has been identified in *Drosophila*. One of the PhoRC components, transcriptional repressor Pleiohomeotic (PHO), binds to polycomb response elements of *HOX* gene promoter together with dSFMBT (Scm-like with four mbt domains) (Klymenko *et al*. 2006). In addition, MBT (malignant brain tumour) domain of dSFMBT can recognize and bind to methylated lysine residue of histone tails that play important roles in gene regulation (Bonasio *et al*. 2010). Two mammalian SFMBTs (SFMBT1 and 2) have been postulated as a PcG protein because of the high degree of conservation of between dSFMBT and hSFMBTs (Wu *et al*. 2007).

Numerous reports indicate that PcG proteins are abnormally regulated, and play important roles in cancer development. PRC1 protein BMI1 promotes the B- and T-cell lymphomas by inhibiting c-MYC-induced apoptosis via INK4a/ARF, and regulates the growth and survival of cancer stem cells (Cui *et al*. 2007; Lukacs *et al*. 2010). PRC2 protein EZH2 is highly overexpressed in prostate carcinoma and breast cancer progression (Varambally *et al*. 2002; Kleer *et al*. 2003). Moreover, tri-methylation activity of EZH2 on H3K27 results in silencing tumour suppressor genes such as

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p16INK4alpha (Kotake *et al*. 2007). Mammalian ortholog of PHO, YY1, also is overexpressed in various cancers including breast and prostate (Begon *et al*. 2005; Seligson *et al*. 2005). The involvement of YY1 in tumorigenesis could be explained by the facts that YY1 negatively regulates tumour suppressor p53 by proteasome-dependent ubiquitination (Sui *et al*. 2004) or its interaction with the cell cycle regulators such as cyclin D, c-myc and retinoblastoma Rb (Gordon *et al*. 2006).

Although mammalian SFMBTs have similar structural features with PcG protein dSFMBT, biological function and target genes of SFMBT have been not investigated well. In this study, we investigated biological function of human SFMBT2 that interacted with YY1 using AR (androgen receptor)-negative DU145 prostate cancer cells. We found that human SFMBT2 represses *HOXB13* gene expression via its association with methylated histones H3 and H4 that are transcriptional repression marks. Furthermore, our findings indicate that SFMBT2 may be involved in regulation of cell growth of DU145 prostate cancer cells.

2. Methods

2.1 *Cell culture and antibodies*

DU145 cell and HEK293T cell lines were purchased from American Type Culture Collection (ATCC, Menassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Antibodies were purchased from commercial sources: anti-YY1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-dimethyl H3K9, anti-trimethyl H3K9, anti-dimethyl H4K20, anti-trimethyl H4K20, anti-acetyl H3K9, and anti-trimethyl H3K27 antibodies (Abcam, Cambridge, MA, USA), anti-dimethyl H3K4 antibody (Millipore, Temecula, CA, USA), anti-Flag antibodies (Sigma-Aldrich, St. Louis, MO, USA) and anti-HA antibody (Covance, Berkeley, CA, USA). Normal IgG (Santa Cruz Biotechnology) was used as a control.

2.2 *Production of polyclonal SFMBT2 antibodies*

The C-terminal region of human SFMBT2 (aa 796 to 811, NH_2-CPPTKPEGTEDTKQEE-COOH) which shows low homology to human SFMBT1 was chose as peptide antigen. To produce anti-SFMBT2 antibody, the peptide was synthesized and the antibodies were produced (Young In Frontier, Korea). The synthetic peptide antigen was dissolved in phosphate-buffered saline (PBS; pH 7.4) and coupled to keyhole limpet haemocyanin as described by Harlow and Lane (Harlow and Lane 1988). Aliquots equivalent to 1 mg of coupled peptide were emulsified in Freund's complete adjuvant and injected intradermally into two female New Zealand rabbits. After 28 days, the immunization was repeated with conjugate emulsified in Freund's incomplete adjuvant. Thereafter, three booster injections were performed at every 2 weeks and blood samples were taken from the heart at 7 days after the final injection. To investigate the specificity of each antiserum, Western blot analysis was performed with overexpressed HA-tagged SFMBT2 in 293T cells. In addition, the blot of the HA-tagged SFMBT2 was co-incubated with anti-SFMBT serum and the peptide antigen to test whether specific antibody–antigen interaction is inhibited by peptide antigen. The specificity of each antiserum was also confirmed by Western blot analysis with *SFMBT2*-depleted cells. Finally, the polyclonal SFMBT2 antibody was sequentially purified by affinity chromatography using peptide which was used for antigen and Protein A chromatography (Sigma-Aldrich) as described by Harlow and Lane (Harlow and Lane 1988).

2.3 *Transfection and immunoprecipitation*

DU145 and HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For immunoprecipitation, cells were rinsed in PBS, harvested, and sonicated in IP150 buffer (10% glycerol, 0.5 mM EDTA, 25 mM Tris-HCl, 0.1% NP40 150 mM NaCl and 1 mM DTT, pH 8.0) in the presence of protease inhibitors (Roche, Mannheim, Germany) and 1 mM phenylmethylsulphonylfluoride. To isolate nucleus, cells or tissues were lysed in buffer (10 mM HEPES-KOH, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9). After centrifugation, nuclear pellet was lysed in IP150 buffer using sonication. The extracts were incubated with the specific antibody for overnight at 4°C, followed by incubation with protein A/G agarose beads (Sigma-Aldrich), washed extensively and dissolved in SDS sample buffer. Western blotting was carried out by standard procedures.

2.4 *Immunocytochemistry*

HEK293T cells were fixed for 15 min with 2% paraformaldehyde in PBS and permeabilized with 0.05% Triton X-100 in PBS for 30 min. After blocking with PGBA solution (0.1% BSA, 0.1% gelatin, 0.1% FBS), cells were incubated with primary antibodies for overnight at 4°C. Antigen was detected with secondary antibodies conjugated to FITC or TRITC (Jackson ImmunoResearch Laboratory, West Grove, PA, USA). Cells were coverslipped using Vectasheild mounting media plus DAPI (Vector Laboratories, Burlingame, CA, USA).

2.5 *Luciferase activity assay*

HEK293T cells were transfected with various plasmid constructs such as 1.5 kb *HOXB13* gene promoter-driven firefly

luciferase (Ren *et al*. 2009), *thymidine kinase* promoter-driven renilla luciferase, *Flag-tagged YY1* and *HA-tagged SFMBT2.* After 48 h, cells were harvested for luciferase activity using the Dual-Luciferase Assay System (Promega, Madison, WI, USA) with a Lumat BL 9507 luminometer (Berthold technologies, Bad Wildbad, Germany). Renilla luciferase activity served as an internal control for normalizing firefly luciferase activity of *HOXB13* gene promoter.

2.6 *RNA isolation and quantitative PCR analysis*

Total RNA was extracted using Nucleospin RNA isolation kit (Macherey-Nagel, Düren, Germany). First strand cDNA synthesis was performed with PrimeScript II 1st strand cDNASynthesis Kit (Takara, Japan). The resulting cDNAs were subjected to real-time PCR with a Stratagene Mx3000P (Agilent Technologies, Waldbronn, Germany). Expression data were calculated from the cycle threshold (Ct) value using the ΔCt method for quantification. *GAPDH* mRNA levels were as used for normalization. Amplification was performed by using the following primers: 5′-TGACG TAGTCATCGCGGATTT-3′ and 5′-ACCAGTCAAGTC ACGTATGAGAA-3′ for human *SFMBT2*, 5′-CCGCAA GAAACGCATTCCG-3′ and 5′-GATGAACTTGTTAG CCGCATACT-3′ for human *HOXB13*, 5′-AGGTCGGTGT GAACGGATTTG-3′ and 5′-TGTAGACCATGTAGTTG AGGTCA-3′ for *GAPDH.*

2.7 *Chromatin immunoprecipitation (ChIP)*

ChIP assay was carried out using real time-PCR as described previously (Kotake *et al*. 2007; Ren *et al*. 2009). Amplification was performed by using the following primers: 5′-AAA TCCAGACCCTTCCACA-3′ and 5′-TAGCACCCCAGTT CATTCC-3′ for *HOXB13* promoter, 5′-TACTAGCGGTTT TACGGGCG-3′ and 5′-TCGAACAGGAGGAGC AGAGAGCGA-3′ for *GADPH* promoter. All PCR reactions were performed in triplicate and included negative controls (IgG) as well as positive controls (genomic DNA). The relative proportions of immunoprecipitated fragments were determined using the ΔCt comparative method based on the threshold cycle (Ct) value for each PCR reaction and normalized to input genomic DNA.

2.8 *RNA interference (siRNA)*

DU145 cells were transfected with siRNA against human *SFMBT2* (Dharmacon, Lafayette, CO, USA) or control siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen). The previous transfection protocol was followed (Raffo *et al*. 2004). At 60 h after transfection, cells were harvested for total RNA extraction or MTT assay. The efficiency of knock down of *SFMBT2* was confirmed with real time-PCR.

2.9 *MTT assay*

The MTT assay was performed to determine cell proliferation according manufacture's protocol (Daeillab Service, Korea). The previous MTT assay protocol was followed (Lee *et al*. 2011). At 60 h after siRNA transfection, optical density was measured at wavelength of 450 nm using a Spectra Max 250 microplate reader (Molecular Device, Sunnyvale, CA, USA).

2.10 *Statistical analyses*

All quantitative data are presented as mean±SEM (standard error of the mean) for three independent experiments. The differences between two groups were evaluated by a paired *t*-test. Analysis of variance (ANOVA) was used for multiple comparisons. Significance values were $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.005$.

3. Results

3.1 *Nuclear SFMBT2 interacts with YY1*

Although mammalian SFMBTs have been discovered as PcG proteins, biological functions of them have not been thoroughly characterized in tumorigenesis. Previously, SFMBT2 was identified as an interacting protein of YY1, which is known to mammalian ortholog of *Drosophila* PHO (Kuzmin *et al*. 2008). In addition, YY1 participated in transcriptional repression of the *HOXB13* gene that is involved in growth arrest of AR- negative DU145 prostate cancer cells (Ren *et al*. 2009). Therefore, we decided to study molecular mechanism and biological function of SFMBT2 using DU145 prostate cancer cells as a model. We first further confirmed the interaction between human SFMBT2 and YY1 using HEK293T cells overexpressing Flag-tagged YY1 and HA-tagged SFMBT2. While immunoprecipitation with control IgG showed no specific SFMBT2 interaction, overexpressed YY1 clearly interacted with SFMBT2 *in vivo* (figure 1A). When cell extracts were reciprocally immunoprecipitated and immunoblotted, we also detected a significant interaction of SFMBT2 with YY1 (figure 1A). In addition, we examined the cellular sublocalization of endogenous SFMBT2 in HEK293T cells. The cells immunostained with anti-SFMBT2 antibody indicated SFMBT2 mainly localizes in the nucleus of HEK293T cells (figure 1B). Moreover, SFMBT2 colocalized with nuclear YY1. These results indicate that human SFMBT2 interacts with YY1 in the nucleus of HEK293T cells *in vivo*.

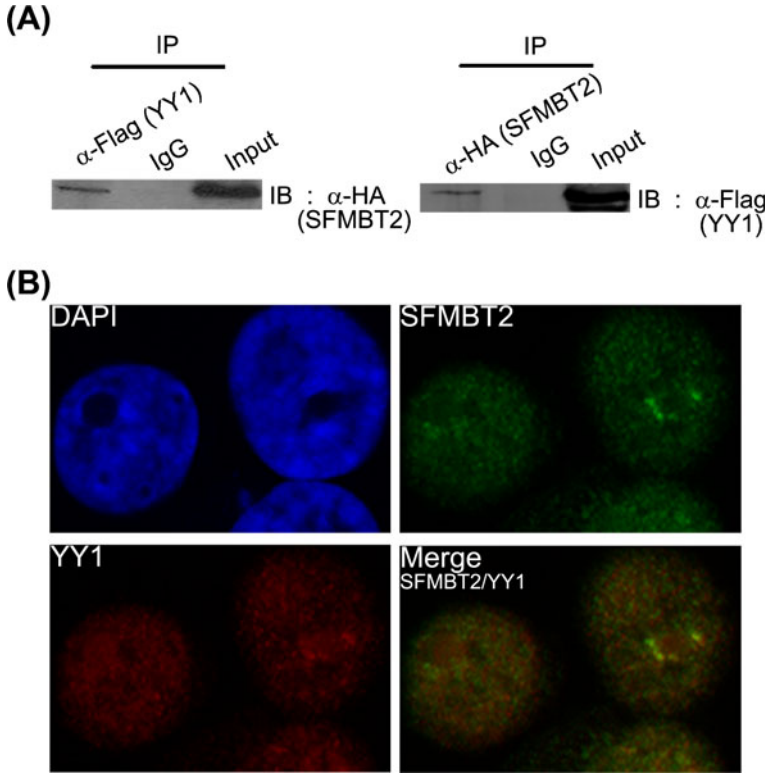

Figure 1. SFMBT2 interacts with YY1 in the nucleus. (**A**) SFMBT2 interacts significantly with YY1 *in vivo*. The lysates from HA-tagged SFMBT2 and Flag-tagged YY1 overexpressing HEK293T cells were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-HA antibody (left) or immunoprecipitated with anti-HA antibody and immunoblotted with anti-Flag antibody (right). Immunoprecipitiation with normal IgG was used for control. (**B**) SFMBT2 (green) colocalizes with YY1 (red) in the nucleus. HEK293T cells were immunostained with anti-SFMBT2 antibody and anti-YY1 antibody. Nuclei were identified using DAPI staining.

3.2 *SFMBT2 interacts with methylated histones*

Next, we investigated whether SFMBT2 interacts with histones *in vivo* since previous structural studies indicated that the MBT domain is evolutionary related to chromatin binding domains such as tudor, chromo and PWWP domain that can bind histones (Maurer-Stroh *et al*. 2003). HEK293T cells were co-transfected with HA-tagged SFMBT2 and Flag-tagged histones (H2A, H2B, H3, H4), and then immunoprecipitated with the anti-Flag antibody, which was followed by Western blot analysis with the anti-HA antibodies. As shown in figure 2A, overexpressed SFMBT2 bound specifically to histone H3 and H4. However, we failed to detect significant interaction of SFMBT2 with histone H2A and H2B (figure 2A). Previously, the MBT domain of dSFMBT has been reported to be a binding module that binds to methylated lysine residue of histone tails (Bonasio *et al*. 2010). Therefore, we further tested whether human SFMBT2 can bind to methylated histones *in vivo*. As a result, SFMBT2 interacted specifically with histone H3 when di-methylated on lysine 9 (H3K9me2) and histone H4 when di-methylated on lysine 20 (H4K20me2) (figure 2B). In contrast, overexpressed SFMBT2 was not associated with active histone marks such as di-methylated H3K4 (H3K4me2) and acetylated H3K9 (H3K9Ac) (figure 2B). Consistent with previous *in vitro* findings, these results suggest that human SFMBT may bind to less methylated histones to ensure that they become tri-methylated and are in an inactive state (Klymenko *et al*. 2006; Grimm *et al*. 2009).

3.3 *SFMBT2 participates in transcriptional repression of HOXB13 gene*

To gain insight into the molecular mechanism underlying SFMBT2-mediated transcriptional regulation of the *HOXB13* gene, we tested transcription inhibitory activity of SFMBR2 using the *HOXB13* gene promoter luciferase-reporter containing YY1 binding sites (Ren *et al*. 2009). As shown in figure 3A, *HOXB13* gene promoter activity decreased in YY1-overexpressed HEK293T cells. In addition, overexpression of SFMBT2 augmented the transcriptional repression activity of YY1 (figure 3A).

We next examined recruitment of SFMBT2 to the *HOXB13* gene promoter in DU145 cells by ChIP assay.

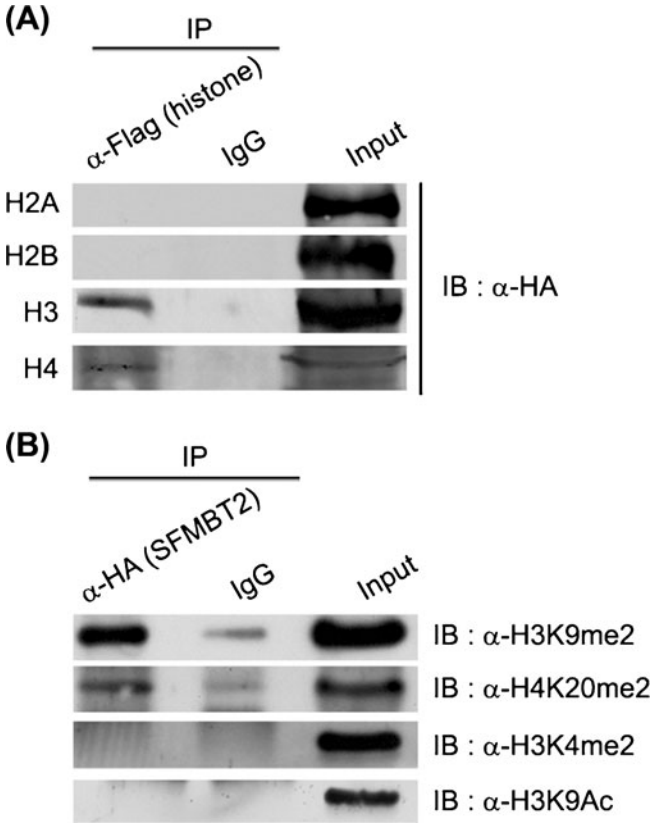

Figure 2. SFMBT2 binds to methylated histones. (**A**) SFMBT2 interacts preferentially with histones H3 and H4. The lysates from HEK293T cells overexpressing HA-tagged SFMBT2 in combination with Flag-tagged histone (H2A, H2B, H3, H4) were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-HA antibody. Immunoprecipitiation with normal IgG was used for control. (**B**) SFMBT2 has specific affinity to di-methylated histone H3K9 (H3K9me2) and H4K20 (H4K20me2). The lysates from HEK293T cells overexpressing HA-tagged SFMBT2 were immunoprecipitated with anti-HA antibody and immunoblotted with anti-dimethyl H3K9 antibody, anti-dimethyl H4K20 antibody, anti-dimethyl H3K4 antibody, and anti-acetyl H3K9 antibody, respectively. Immunoprecipitiation with normal IgG was used for control.

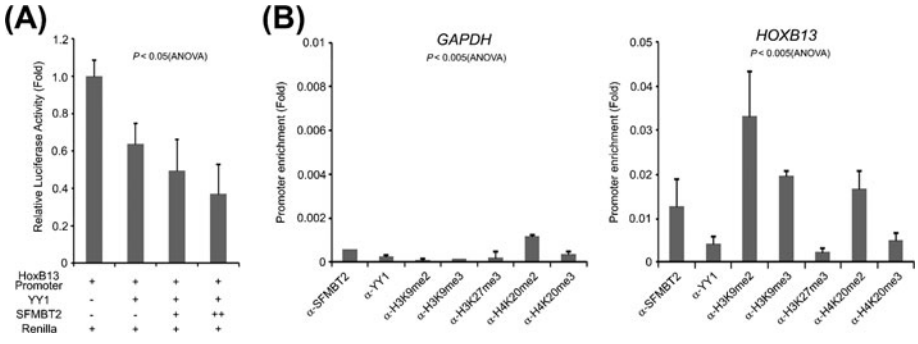

Figure 3. SFMBT2 is involved in transcriptional repression of the *HOXB13* gene. (**A**) Suppression of the *HOXB13* gene activation by overexpression of SFMBT2 and YY1. HEK293T cells were transiently transfected with the human *HOXB13* gene promoter driven-firefly luciferase reporter vector (100 ng) in conjunction with a control renilla luciferase expression vector. Expression vectors for YY1 (200 ng) and SFMBT2 (250 ng, 500 ng) were transfected alone or in combination. Reporter activity is represented as the fold activation relative to the renilla luciferase activity. (**B**) ChIP assay was performed in DU145 cells using anti-SFMBT2 antibody, anti-YY1 antibody, anti-dimethyl H3K9 antibody, anti-trimethyl H3K9 antibody, anti-dimethyl H4K20 antibody, anti-trimethyl H4K20 antibody, and anti-trimethyl H3K27, respectively. The occupancy of each protein was quantified with quantitative PCR in promoter of *GAPDH* and *HOXB13* genes in DU145 cells. ChIP using normal IgG was performed as the negative control.

While we failed to detect significant recruitments of SFMBT2 and YY1 to promoter of the *GAPDH* gene that is expressed constitutively, SFMBT2 and YY1 bound significantly to the *HOXB13* gene promoter (figure 3B). Since SFMBT2 bound preferentially to repressive histone marks, and acts as a transcriptional repressor of the *HOXB13* gene (figures 2B and 3A), we further investigated the association of SFMBT2 with enrichment of methylated histones at the *HOXB13* gene promoter. We found that di- and trimethylated H3K9 (H3K9me2 and me3) and di- and trimethylated H4K20 (H4K20me2 and me3) as well as trimethylated H3K27 (H3K27me3) are significantly enriched at the *HOXB13* gene promoter, while no enrichment of such methylated histones was observed at the *GAPDH* gene promoter (figure 3B). These results indicate that human SFMBT2 may be involved in the repression of the *HOXB13* gene via an association with repressive histone marks in DU145 prostate cancer cells.

3.4 *SFMBT2 affects cell growth of DU145 prostate cancer cells*

To further confirm that transcriptional repression activity of SFMBT2, we examined effects of *SFMBT2* depletion by siRNA transfection on *HOXB13* gene expression in DU145 cells. As shown in figure 4, *SFMBT2* siRNA efficiently depleted *SFMBT2* transcript. Expression of the *HOXB13* gene was significantly up-regulated in *SFMBT2*-depleted DU145 cells compared to control groups (no transfection and control siRNA transfection) (figure 4A). In the following experiments, we investigated whether depletion of SFMBT2 affects DU145 prostate cancer cell growth by MTT assay. We observed significant decrease of cell growth in *SFMBT2* siRNA-transfected cells, while control siRNA-transfected cell showed neglectable change with cell growth (figure 4B).

Collectively, our findings suggest that human SFMBT2 interacts with YY1 and methylated histones H3 and H4 that are associated with gene inactivation. Furthermore, SFMBT2 may regulate growth of DU145 prostate cancer cells by inhibition of *HOXB13* gene expression and maintenance of the inactive state of the *HOXB13* gene.

4. Discussion

Fly SFMBT (dSFMBT) was first identified as a PHO-interacting protein and mediates the *HOX* gene silencing in *Drosophila* early development (Klymenko *et al*. 2006). Mammalian SFMBT (SFMBT1 and 2) has also been considered as a PcG protein. However, the biological function and molecular mechanism of mammalian SFMBT-mediated gene regulation have not been characterized. In the present study, we found that human SFMBT2 interacts with YY1 in the nucleus. Consistently, overexpressed mouse SFMBT2 interacts with YY1, and these genes are co-expressed in early mouse embryos and extraembryonic tissues (Donohoe *et al*. 1999; Frankenberg *et al*. 2007; Kuzmin *et al*. 2008). MBT domains of dSFMBT bind preferentially to mono- and di-methylated histone H3K9 and H4K20 peptides *in vitro*, suggesting that MBT domains maybe required for ensuring hypermethylation of histones which are associated with transcriptional repression (Klymenko *et al*. 2006; Wu *et al*. 2007; Grimm *et al*. 2009). Similarly, we found that human SFMBT2 binds selectively to methylated lysine residue of histone H3 and H4 such as H3K9me2 and H4K20me2 *in vivo* that are frequently found in inactive genes (Rosenfeld *et al*. 2009; Izzo and Schneider 2011).

We also showed that SFMBT2 negatively regulates *HOXB13* gene expression via an association with repression histone marks in AR-negative DU145 prostate cells. These results may support previous hypothesis that MBT domain of SFMBT can recognize and bind to methylated lysine residue of histones that play an important role in the maintenance of inactive state of gene (Klymenko *et al*. 2006; Grimm *et al*. 2009). When we depleted *SFMBT2*, we

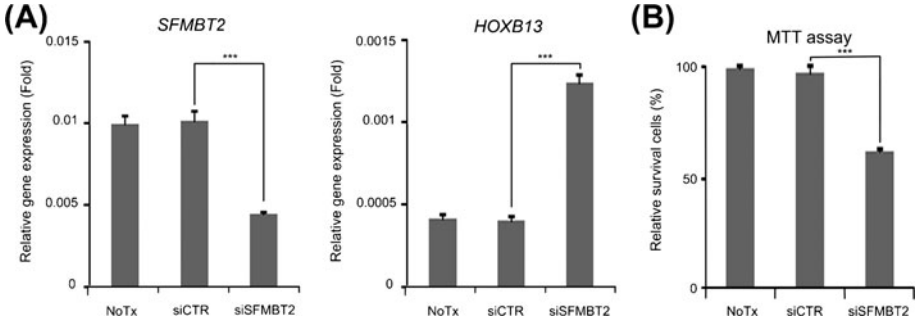

Figure 4. SFMBT2 regulates DU145 cell growth. (**A**) Up-regulation of the *HOXB13* gene expression in *SFMBT2*-depleted DU145 cells. Total RNA was isolated from DU145 cells transfected with control siRNA or *SFMBT2* siRNA. Transcripts of *SFMBT2* and *HOXB13* were determined by quantitative PCR. *GAPDH* transcript was used for normalization. (**B**) DU145 cell growth is inhibited in *SFMBT2*-depleted cells. MTT assay was performed in DU145 cell transfected with control siRNA and *SFMBT2* siRNA.

observed significant increased *HOXB13* gene expression as well as decreased DU145 cell growth. In fact, several reports indicate that inverse relationship between *HOXB13* gene expression and prostate cancer cell growth (Jung *et al*. 2004a, b; Ren *et al*. 2009). Similarly, depletion of YY1 that interacts with human SFMBT2 results in DU145 cell growth arrest (Ren *et al*. 2009).

Although further studies are needed to identify genome-wide target genes of SFMBT2 and determine alternations of the exact chromatin structure of target gene, our results suggest that human SFMBT2 may induce a formation of inactive chromatin structure since we found significant enrichment of tri-methylated H3K9, H4K20 and H3K27 at the gene promoter. In addition, SFMBT2 may be involved in cell growth regulation by direct repression of the *HOXB13* gene expression in DU145 prostate cancer cells.

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