Enhanced SMYD3 expression is essential for the growth of breast cancer cells

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We previously reported that upregulation of SMYD3, a histone H3 lysine-4-specific methyltransferase, plays a key role in the proliferation of colorectal carcinoma (CRC) and hepatocellular carcinoma (HCC). In the present study, we reveal that SMYD3 expression is also elevated in the great majority of breast cancer tissues. Similarly to CRC and HCC, silencing of SMYD3 by small interfering RNA to this gene resulted in the inhibited growth of breast cancer cells, suggesting that increased SMYD3 expression is also essential for the proliferation of breast cancer cells. Moreover, we show here that SMYD3 could promote breast carcinogenesis by directly regulating expression of the proto-oncogene *WNT10B*. These data imply that augmented SMYD3 expression plays a crucial role in breast carcinogenesis, and that inhibition of SMYD3 should be a novel therapeutic strategy for treatment of breast cancer. (*Cancer Sci* 2006; 97: 113–118)

Recent molecular studies have disclosed that breast carcinogenesis involves not only genetic alterations in oncogenes and tumor suppressor genes, but also epigenetic dysregulation of a number of genes associated with growth, survival, cell motility and differentiation.[1–3] For example, several oncogenes have been described as being amplified in breast cancer, including *HER2*, *EGFR*, *MYC*, *CCND1*, *MDM2* and *WNT10B*.[4–13] Although expression profile analysis using cDNA microarray or DNA chip technology has facilitated the identification of genes with altered expression in cancer, the mechanisms of deregulated expression need to be further investigated. A multistep model of mammary carcinogenesis has been proposed; transformation of normal cells leads to atypical ductal hyperplasia, ductal carcinoma *in situ* (DCIS), and eventually invasive ductal carcinoma (IDC). However, precise molecular mechanisms underlying breast cancer remain unresolved.

Chromatin is composed of genomic DNA and nuclear proteins including histones, and serves as the template for processing genetic information. The dynamic DNA–protein structure of chromatin is influenced by epigenetic modifications on both the DNA and nucleosomal histones. Under the euchromatin state where chromatin forms a relaxed state, the underlying DNA is accesible to transcription factors. In contrast, DNA in the heterochromatin state, where chromatin forms a condensed state, is transcriptionally restricted and thus untranscribed. Recent advances have shown that covalent histone modifications play critical roles in chromatin structure. One of the best-characterized modifications is acetylation, which is controlled by both histone acetyltransferases and deacetylases. Additionally, histone methylation has emerged as another modification that significantly impacts chromatin structure. We reported previously that SMYD3 shows histone H3-K4-specific methyltransferase activity, leading to transcriptional activation of downstream genes including *Nkx2.8*, and that elevated *SMYD3* expression is involved in the growth of human colorectal carcinoma (CRC) and hepatocellular carcinoma (HCC) cells.[14]

In the present paper, we show that expression of SMYD3 is frequently enhanced in human breast cancer tissues, and that its upregulation is also associated with proliferation of breast cancer cells. We further reveal that the *WNT10B* gene is a direct transcriptional target activated by SMYD3. These data should yield new insights into the molecular mechanisms of breast carcinogenesis, and contribute to the development of novel approaches for treatment of breast cancer.

Materials and Methods

Patients and tissue samples

Primary breast cancer tissues were obtained with informed consent from 81 patients (12 ductal carcinomas *in situ* and 69 T2-stage IDC, 2–5 cm in diameter), with a median age of 45 years (range 21–68 years), who were treated at the Department of Breast Surgery, Cancer Institute Hospital, Tokyo, Japan. A mixture of RNAs isolated from normal breast ductal cells of 15 pre-menopausal breast cancer patients served as a normal control.

Laser-Microbeam Micro-dissection, RNA extraction and T7-based amplication

We purified cancer and normal ductal cells that were clearly observed by a pathologist using the EZ Cut Laser-Microbeam Micro-dissection (LMM) system (MMI Molecular Machines & Industries, Glattbrugg, Switzerland) following the manufacturer's protocol with several modifications. We handled the cancer tissues carefully to minimize the effects of tissue collection and storage. Extraction of total RNA and T7-based

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amplification were carried out as described previously.[15,16] Antisense RNA amplified by this method accurately reflects the proportions of the original RNA source,[17,18] as we have confirmed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) experiments.[16]

Semiquantitative RT-PCR

To confirm the expression level of *SMYD3* in breast cancer, we carried out semiquantitative RT-PCR using the same RNA used for the microarray. RNA from each sample (1 μg) was reverse-transcribed into single-stranded cDNA using random primers and Superscript II (Invitrogen, Carlsbad, CA, USA). Expression of *GAPDH* served as a control. PCR reactions were optimized for the number of cycles to ensure product intensity at the linear phase of amplification. The primer sequences were as follows: *GAPDH* forward 5′-ACAACAGCCTCAAGATCATCAG-3′ and reverse 5′-GGTCCACCACTGACACGTTG-3′; *SMYD3* forward 5′-TTCCCGATATCAACATCTACCAG-3′ and reverse 5′-AGTGTGTGACCTCAATAAGGCAT-3′; and *WNT10B* forward 5′-GATACCCACAACCGCAATTCT-3′ and reverse 5′-GCATGCTCAGTCTTTTCCTCTTA-3′.

Immunohistochemical staining

Sections of human breast cancer were stained using the SAB-PO peroxidase immunostaining system (Nichirei, Tokyo, Japan). Briefly, the endogenous peroxidase activity of xylene-deparaffinized and dehydrated tissue sections was inhibited by treatment with 0.3% H_2O_2/methanol. Non-specific binding was blocked by incubating sections with 10% normal goat serum in a humidified chamber for 30 min at ambient temperature followed by overnight incubation at 4°C with a 1:1000 dilution of rabbit polyclonal antiSMYD3 antibody. The sections were washed twice with phosphate-buffered saline (PBS), incubated with 5 μg/μL goat antirabbit biotinylated IgG in PBS containing 1% bovine serum albumin for 30 min at ambient temperature, and then incubated with peroxidase-conjugating streptavidin for 30 min. Specific immunostaining was visualized using 3,3′-diaminobenzidine. Slides were dehydrated through a graded alcohol series with xylene washing and mounted on cover slips. Hematoxylin was used for nuclear counterstaining.

Gene silencing effect of SMYD3 small interfering RNA

Plasmids expressing SMYD3 small interfering RNA (siRNA) were prepared by cloning double-stranded oligonucleotides into the psiU6BX vector. The oligonucleotides used for SMYD3 siRNAs were 5′-CACCAACATCTACCAGCTGAA-GGTGTTCAAGAGACACCTTCAGCTGGTAGATGTT-3′ and 5′-AAAAAACATCTACCAGCTGAAGGTGTCTCTTGAAC-ACCTTCAGCTGGTAGATGTT-3′ for psiU6BX-SMYD3-12. The plasmids psiU6BX-SMYD3-12, psiU6BX-mock and psiU6BX-Luciferase plasmids were transfected into breast cancer cell lines using FuGENE6 reagent according to the supplier's recommendations (Roche, Basel, Switzerland).[14]

Chromatin immunoprecipitation assays

HEK293 cells were transfected with FLAG-SMYD3 and then fixed in 1% formaldehyde. The fixed chromatin samples were subjected to immunoprecipitation using a chromatin immunoprecipitation (ChIP) assay kit according to the manufacturer's instructions (Promega, Madison, WI, USA), with minor modifications.[18]

Results

Enhanced SMYD3 expression in breast cancer

We previously analyzed the global gene expression profiles of 92 breast carcinomas using cDNA microarray and identified a number of genes with altered expression in tumors compared to non-cancerous ductal cells.[19] The microarray data revealed that *SMYD3* expression was elevated in 36 of 69 IDC and in six of 11 DCIS when the cut-off value of > 2.0 was used for the tumor : normal tissue ratio (data not shown). To confirm its elevation in breast tumors, we carried out semiquantitative RT-PCR analysis and observed its elevated expression in seven of 12 IDC that were selected randomly, compared to normal mammary ductal cells (Fig. 1a). We further investigated expression of the SMYD3 protein by western blot analysis with antiSMYD3 antibody using protein extract from six breast cancer tissues and their corresponding non-cancerous mammary tissues. The analysis demonstrated a significant accumulation of SMYD3 in all six tumor tissues examined (Fig. 1b). Immunohistochemical staining of SMYD3 using the antibody also detected strong staining in breast carcinoma cells, but not in stromal cells or non-cancerous ductal cells in the four cancer tissues examined (Fig. 2). These data indicate that enhanced SMYD3 expression is involved in a majority of breast cancer tissues.

Growth suppression of breast cancer cells by SMYD3 siRNA

To test whether elevated expression of *SMYD3* is essential for the growth of breast cancer cells, we suppressed *SMYD3* using siRNA-SMYD3-12, which effectively suppressed *SMYD3* expression in colon and liver carcinoma cells,[14] and carried out cell viability assays using breast cancer cells. We used MDA-MB-231, MCF7 and T47D cells because western blot analysis revealed that SMYD3 was expressed abundantly in eight of 10 breast cancer cell lines, including BT-20, HBL-100, MDA-MB-231, MCF7 and T47D (Fig. 3a). Consistently, siRNA-SMYD3-12 markedly knocked down SMYD3 expression in these cells (Fig. 3b). We transfected MDA-MB-231, MCF7 and T47D cells with psiU6BX-SMYD3-12, psiU6BX-Luciferase or psiU6BX-mock, cultured them with an appropriate concentration of G418, and analyzed cell viability using a cell counting kit. As a result, psiU6BX-SMYD3-12 showed a significant growth inhibitory effect compared to psiU6BX-Luciferase or psiU6BX-mock in the three cell lines (Fig. 3c). Because psiU6BX-SMYD3-12 induced apoptosis in CRC and HCC,[14] it is likely that suppression of SMYD3 led to cell death in these breast cancer cells as well. We also tested whether this siRNA has some effect on the growth of HEK293 cells that do not express SMYD3. As a result, the siRNA did not show any growth inhibitory effect on HEK293 cells (data not shown), suggesting that the growth inhibitory effect in cancer cells resulted from suppression of SMYD3. Therefore, inhibition of SMYD3 may be a rational strategy to treat breast cancer.

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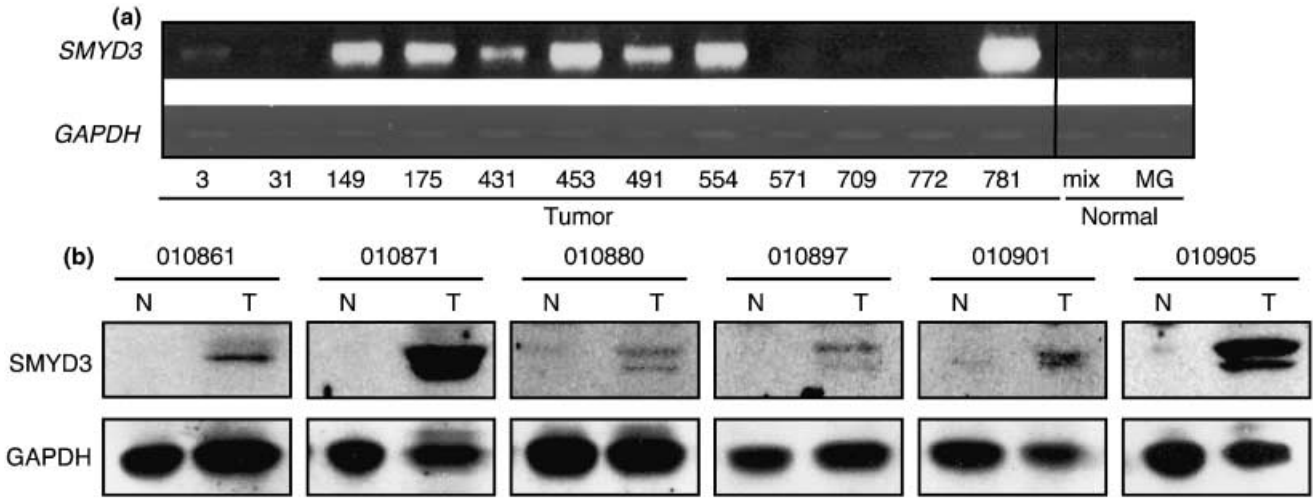

Fig. 1. Elevated SMYD3 expression in breast cancer tissues. (a) *SMYD3* expression in 12 breast tumors, a mixture of normal mammary gland cells from 15 premenopausal patients (mix), and normal mammary gland (MG) was analyzed by semiquantitative reverse transcription–polymerase chain reaction. Expression of *GAPDH* served as a quantitative control. (b) Western blot analysis of SMYD3 protein in six pairs of breast cancer tissue (T) and corresponding non-cancerous mammary tissue (N). Expression of GAPDH served as a control.

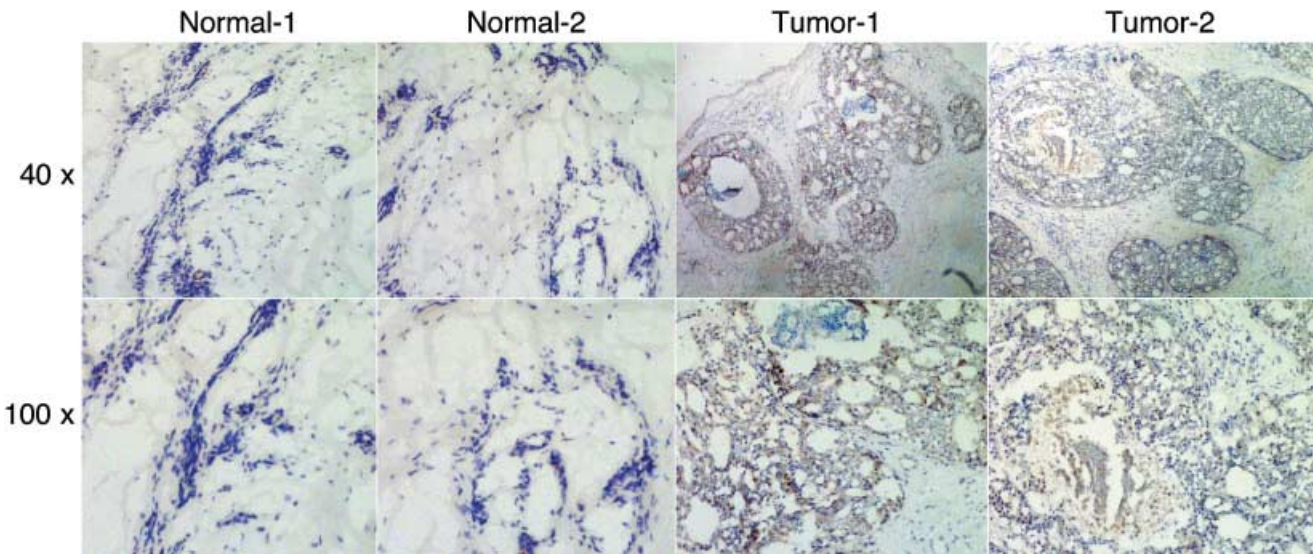

Fig. 2. Accumulated SMYD3 protein in breast cancer cells. Representative images of immunohistochemical staining of SMYD3 in breast cancer tissues and non-cancerous mammary gland.

WNT10B is a downstream gene of SMYD3

We previously identified 61 candidate downstream genes of SMYD3 by comparing the expression profiles of HEK293 cells expressing exogenous SMYD3 with profiles of control 293 cells. Among the 61 genes, our microarray data showed that *WNT10B* expression was elevated approximately eight-fold higher in HEK293 cells transfected with SMYD3 than in control cells (data not shown). As its overexpression was already reported in breast cancer,[13,20,21] we further focused on this molecule to test whether *WNT10B* is a direct target of SMYD3 or not. Semiquantitative RT-PCR corroborated its augmented expression in response to exogenous expression of *SMYD3* in HEK293 and HeLa cells (Fig. 4a). Conversely, MCF7 and BT20 breast cancer cells treated with siRNA-SMYD3-12 targeting SMYD3 resulted in a decrease of *SMYD3* and a reduction in *WTN10B* expression (Fig. 4b). We further carried out western blot analysis using the same six pairs of clinical tissues used for immunoblot analysis of SMYD3. Although WNT10B was significantly enhanced in three tumors (010861, 010897 and 010925) and slightly enhanced in one tumor (010871) among the cases examined, the remaining two tumors (010880 and 010901) showed undetectable or unchanged expression (Fig. 4c). Therefore, other factors may be involved in the regulation of WNT10B expression. Within 1 kb of the 5′ flanking region of the *WNT10B* transcription start site, we found one putative

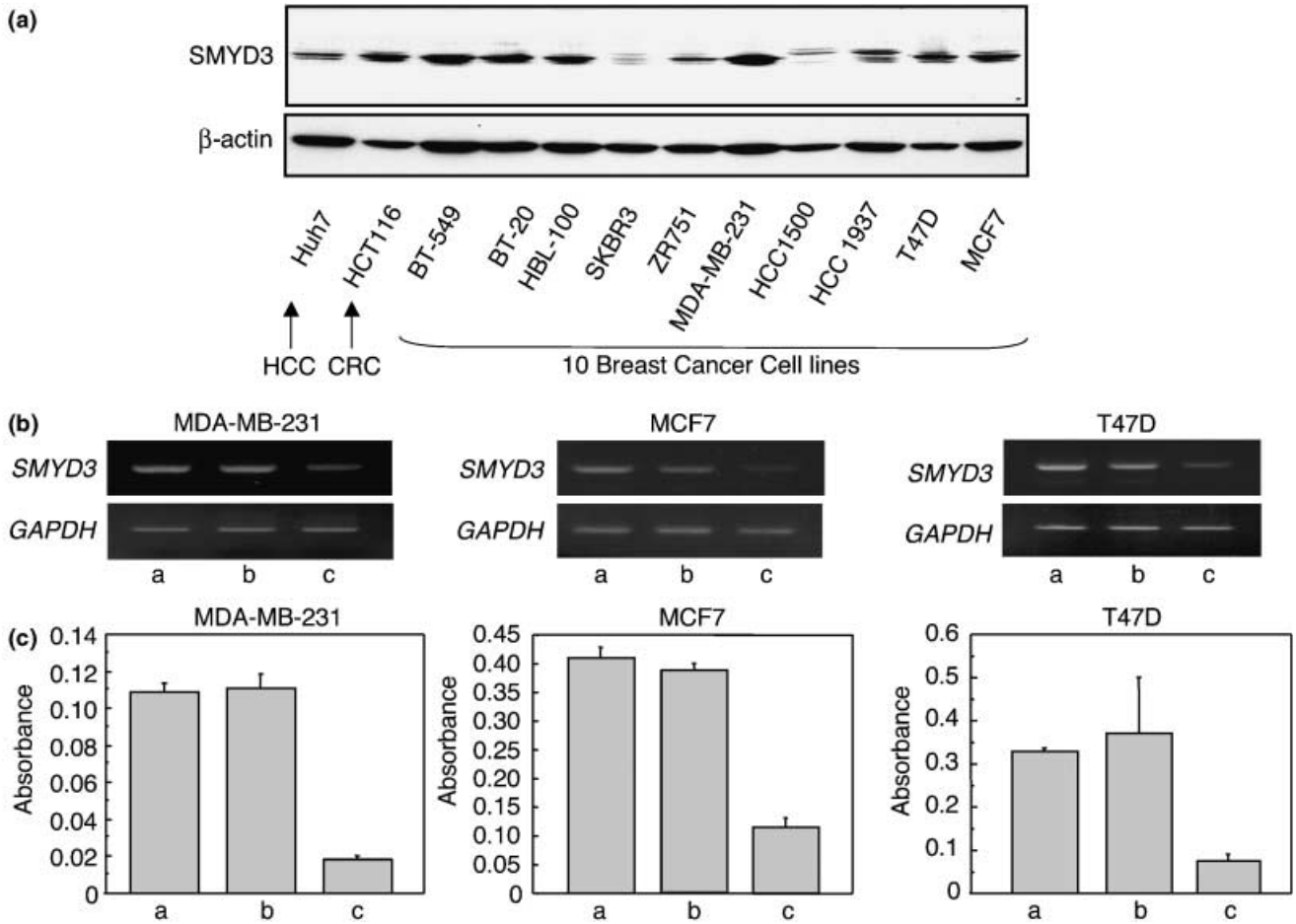

Fig. 3. Involvement of SMYD3 in the growth of breast cancer cells. (a) Western blot analysis of SMYD3 in 10 breast cancer cell lines, hepatocellular carcinoma (HCC) (Huh7), and colorectal carcinoma (CRC) (HCT116) cells. (b) Effect of SMYD3 siRNA and control siRNA on SMYD3 expression in MDA-MB-231, MCF7 and T47D breast cancer cells. Semiquantitative reverse transcription–polymerase chain reaction was carried out using RNA extracted from cells transfected with mock plasmids (a), plasmids expressing siRNA-Luciferase (b) and those expressing siRNA-SMYD3-12 (c). *GAPDH* served as a control. (c) Effect of the siRNAs on growth of the breast cancer cells was analyzed by MTT assay.

SMYD3 binding sequence (CCCTCCT) between −743 and −737 (Fig. 4d). ChIP assay using cells transfected with pFLAG-CMV and pFLAG-CMV-SMYD3 proved that a genomic segment (ChIP-1) containing the candidate SMYD3-binding sequence associates with SMYD3. The other genomic fragments (ChIP-2, ChIP-3 and ChiP-4) without possible SMYD3-binding sequences did not interact with SMYD3 (Fig. 4d, e). These results indicate that *WNT10B* is one of the direct downstream genes regulated by SMYD3.

Discussion

In our previous report, we revealed that SMYD3 has H3-K4-specific histone methyltransferase activity, and that its expression is frequently elevated in CRC and HCC.[14] In the present study, we have shown that SMYD3 expression is elevated in breast cancer tissues by semiquantitative RT-PCR, western blot analysis and immunohistochemical staining. Because its overexpression was observed in six of 11 DCIS (54.5%) and in 36 (52.2%) of 69 invasive tumors of the breast, SMYD3 may not be involved in the progression of tumors, but rather in the development of a subset of breast cancers at a relatively early stage. In line with our previous data, knockdown of SMYD3 by specific siRNA resulted in growth suppression of breast cancer cells. Therefore, enhanced SMYD3 may be implicated in the growth of breast cancer cells.

We here found that SMYD3 upregulates *WNT10B* as a direct downstream gene. Aberrant activation of the canonical Wnt/β-catenin pathway is one of the most frequent signaling abnormalities in human cancer.[13,22–24] Although colorectal cancer and hepatocellular carcinoma frequently involve mutations in molecules of the Wnt/β-catenin pathway, including *APC*, *β-catenin* and *AXIN1*, these mutations have not been reported in breast cancer so far. *Wnt1* (formerly *int-1*) is the first mouse Wnt gene identified as an oncogene. Insertion of mouse mammary tumor virus (MMTV) transactivates *Wnt1*, which results in tumor formation in the mammary glands of infected mice.[25] Its oncogenic activity was subsequently confirmed by the generation of transgenic mice expressing *Wnt1* under the control of an MMTV Long Terminal Repeat (LTR).[26] *Wnt10B* is also implicated in MMTV virus-induced

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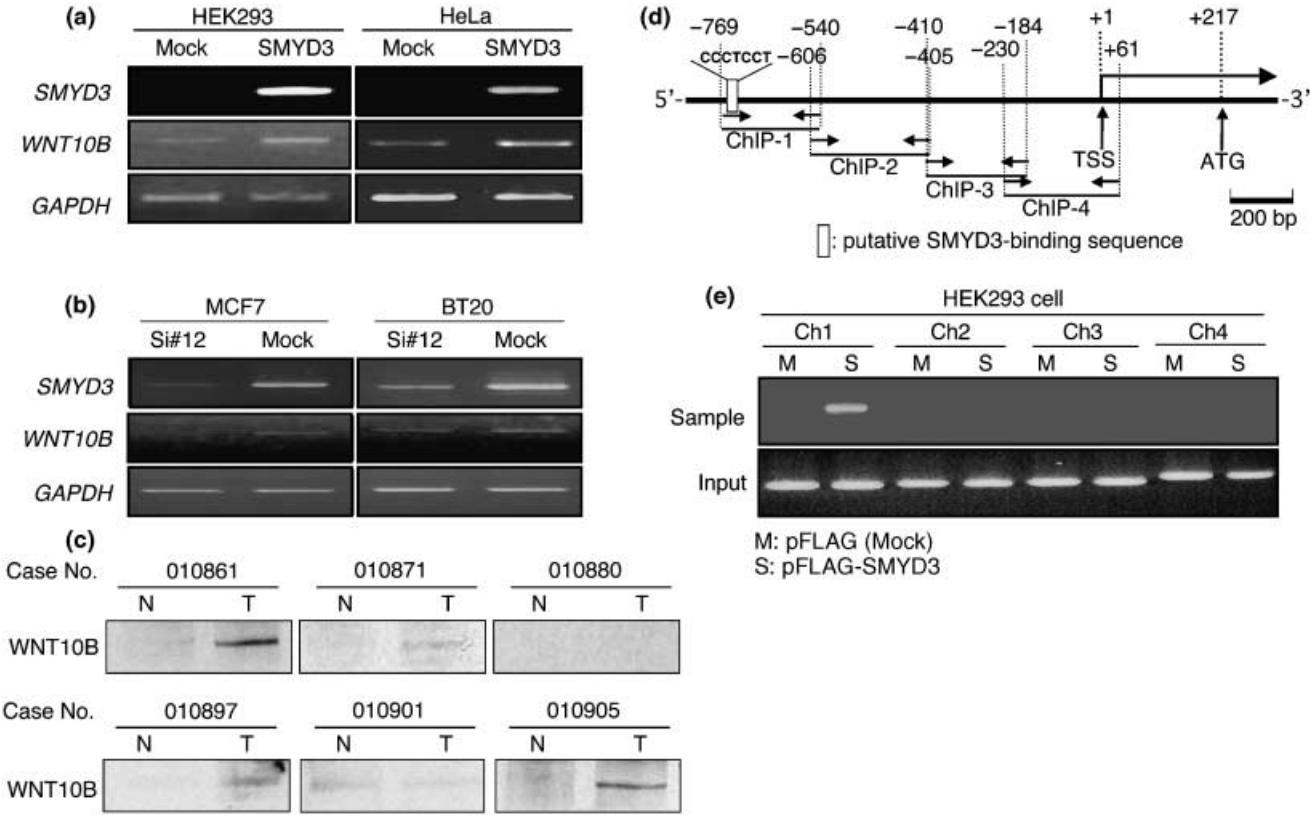

Fig. 4. *WNT10B* is a direct downstream gene transactivated by SMYD3. (a) Elevated expression of *WNT10B* in response to exogenous SMYD3 expression in HEK293 and HeLa cells. *WNT10B* expression was analyzed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). Expression of *GAPDH* served as a control. (b) Reduced expression of *WNT10B* by SMYD3-siRNAs (si-#12) in MCF7 and BT20 cells. WNT10B and SMYD3 expression was analyzed by semiquantitative RT-PCR. (c) Western blot analysis of WNT10B protein in six pairs of breast cancer tissue (T) and corresponding non-cancerous mammary tissue (N) with anti-WNT10B antibody. (d) Putative SMYD3-binding sequence in the 5′-flanking region of *WNT10B*. (e) ChIP assay using DNA from HEK293 cells transfected with mock (M) or pFLAG-tagged SMYD3 (S). DNA was immunoprecipitated with anti-FLAG M2 antibody.

tumorigenesis, and the phenotype of MMTV-*Wnt10B* transgenic mice is similar to that of the Wnt1 strain.[20,27] Therefore, activation of *Wnt10B* by enhanced expression of SMYD3 may lead to the development of human breast cancer in an autocrine manner. Generation of transgenic mice expressing SMYD3 may provide insights into its involvement in breast carcinogenesis.

These results recapitulate the importance of SMYD3 in human carcinogenesis. As reduction of SMYD3 expression by treatment with specific siRNA significantly decreased the growth of breast cancer cells, suppression of SMYD3 may be a promising therapeutic strategy to treat human breast cancer. Although future functional analysis of SMYD3 in breast tissues is required, the data provided here should contribute to a more profound understanding of breast carcinogenesis and to the development of novel therapeutic approaches.

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