Epigenetic Inactivation of the Canonical Wnt Antagonist SRY-Box Containing Gene 17 in Colorectal Cancer


Abstract

**SRY-box containing gene 17 (Sox17)** is a member of the high mobility group (HMG) transcription factor superfamily, which plays critical roles in the regulation of development and stem/precursor cell function, at least partly through repression of Wnt pathway activity. Modulators controlling aberrant Wnt signaling activation are frequently disrupted in human cancers through complementary effects of epigenetic and genetic changes. Our recent global analysis of CpG island hypermethylation and gene expression in colorectal cancer (CRC) cell lines revealed that **SOX17** gene silencing is associated with DNA hypermethylation of a CpG island in the promoter region. Here, we report that CpG island methylation-dependent silencing of **SOX17** occurs in 100% of CRC cell lines, 86% of colorectal adenomas, 100% of stage I and II CRC, 89% of stage III CRC, 89% of primary esophageal cancer, and 50% of non–small cell lung cancer. Overexpression of **SOX17** in *HCT116* CRC cells inhibits colony growth and β-catenin/T-cell factor–dependent transcription. Structure-based deletion analysis further shows the presence of a Wnt signaling repression domain in the **SOX17** HMG box. Together, our studies suggest that **SOX17** is a negative modulator of canonical Wnt signaling, and that **SOX17** silencing due to promoter hypermethylation is an early event during tumorigenesis and may contribute to aberrant activation of Wnt signaling in CRC. [Cancer Res 2008;68(8):2764–72]

Introduction

The **Sox** gene family was first identified by virtue of its strong homology (>50%) to the high mobility group (HMG) box of the sex-determining gene **SRY** (1). There are at least 30 members of the **Sox** family expressed in many different cell types and tissues, and at multiple stages during development (2). **Sox** genes have been classified into seven groups based on their amino acid sequence and genomic organization, and **Sox17**, together with **Sox7** and **Sox18**, belongs to **Sox** group F (2). **Sox17** encodes a HMG box transcription factor and has been implicated in oligodendrocyte development (3), vascular development (4), formation of definitive endoderm (5), and embryonic hematopoiesis (6). **Sox17** binds to a common **Sox** target DNA sequence 5’-(A/T)(A/T)CA(A/T)G-3’ in the minor groove (7) and is known to regulate the transcription of a number of target genes, including **Foxa1** and **Foxa2** via the physical interaction of its COOH-terminal transcriptional activation domain with β-catenin (8). The importance of **Sox17** for embryonic development has been shown by two knockout experiments in mice. **Sox17-null** embryos exhibit a deficiency of gut definitive endoderm, which leads to embryonic lethality before day E10.5 (9). A recent conditional knockout study reveals that **Sox17** is critical for the generation or maintenance of fetal blood stem cells (6).

A growing body of evidence suggests that an important function of **Sox17** is to inhibit canonical Wnt pathway signaling. Injection of **Sox17** mRNA can effectively suppress the induction of a second axis in *Xenopus* embryos induced by Wnt activators, but failed to do so when coinfected with mRNAs encoding Wnt targets (10). **Sox17** is also indispensable for the specification of cardiac mesoderm in embryonic stem cells by inactivating the canonical Wnt pathway (11). A recent study suggests that mouse **Sox17** suppresses canonical Wnt signaling by GSK3β-independent protein degradation of β-catenin and T-cell factor/lymphoid enhancer factor (TCF/LEF) in human SW480 colorectal cancer (CRC) cells (12).

Mutations in the intracellular components of the Wnt/β-catenin pathway, such as **APC**, **Axin2**, and β-catenin, are thought to cause constitutive activation of downstream signaling independent of extracellular Wnt ligands in CRC (13). Our previous studies revealed that epigenetic gene silencing of secreted frizzled-related proteins (SFRPs), which encode secreted Wnt antagonists, occurs aberrantly in CRC and enhances constitutive Wnt signaling (14). Furthermore, another two extracellular Wnt inhibitors, Wnt inhibitory factor-1 (**WIF-1**) and DICKKOPF-1 (**DKK-1**), are also silenced in cancer cell lines and primary tumors (15, 16).

Here, we show that **SOX17** is frequently silenced by promoter hypermethylation in colonic neoplasia and CRC. Reexpression of **SOX17** in CRC cells leads to a significant reduction in colony formation, suggesting a potential role as a tumor suppressor. Additionally, we show that overexpression of **SOX17** suppresses β-catenin/TCF–regulated transcription in a dose-dependent manner. Deletion analysis in this present study, when combined with previous work of others (10, 12), further suggests that the HMG box of **SOX17**, but in our hands, not the COOH-terminal transcription activation domain, is essential for this transcriptional repression in colon cancer cells. In view of these and other findings, we conclude that **SOX17** gene silencing is an
early frequent event associated with aberrant Wnt signaling in CRC, and SOX17 inhibits Wnt signaling through the NH2-terminal HMG box.

Materials and Methods

Cell culture. HCT116, DKO, and SW480 CRC cells were cultured in McCoy’s 5A modified medium; RKO and Caco-2 cells were maintained in MEM; HEK293T cells were maintained in DMEM. All media (Cellgro) were supplemented with 10% fetal bovine serum (HyClone) and antibiotics and grown at 37°C in 5% CO2 atmosphere. For drug treatments, log phase CRC cells were cultured in the above-described medium supplemented with 1 μmol/L 5-aza-2-deoxycytidine (DAC; Sigma) for 96 h, with replacement of medium and DAC every 24 h.

Vector constructs. SOX17 (Genbank accession number NM_022454) was cloned by reverse transcription-PCR (RT-PCR) from cDNA derived from normal colon mucosa. To generate SOX17 expression constructs, the entire encoding region of its cDNA was subcloned in frame into the pcDNA3.1/V5-His B vector (Invitrogen) via KpnI and EcoRI sites. Truncated SOX17 mutants were generated by PCR. All constructs were verified in each case by DNA sequencing.

Gene expression analysis. RNA was isolated with TRIzol reagent (Invitrogen). One microgram RNA was treated with DNase I–treated RNA without reverse transcription.

Figure 1. Identification of SOX17 as a hypermethylated gene in CRC cells. A, the distribution of gene expression changes for HCT116 cells treated with trichostatin A (X axis) or DAC (Y axis) is analyzed and displayed (18). Black dots, individual genes; red dot, SOX17, which is a candidate DNA hypermethylated gene. SOX17 is identified by its position in a zone where gene expression did not respond to trichostatin A (<1.4-fold) but increased >2-fold with DAC treatment. B, RT-PCR analysis for expression of SOX genes in HCT116, DKO, and normal colonic mucosa. Gene symbols are indicated on the left; cell lines are indicated above the data; and NC indicates normal colon. β-Actin is used as a internal control. C, RT-PCR analysis for expression of SOX genes before and after treatment of cells with 1 μmol/L DAC (+) for 96 h. Restoration of SOX17 expression is observed in seven CRC cells. As a control for SOX17 expression, PCR was performed on DNase I-treated RNA without reverse transcription. D, Western blot analysis of SOX17 in different CRC cells. The positive control for SOX17 protein detection uses whole-cell lysate from HCT116 cells transfected with the pcDNA3.1-SOX17 expression vector. Left, of the five cell lines tested, endogenous SOX17 protein was only detected in DKO cells. β-Actin is shown as a gel loading control for the different cell lysates. Right, SOX17 protein is seen after treatment with 1 μmol/L DAC (+) for 96 h in HCT116 and Caco-2 cells.
PCR fragments were verified by culture plate dishes 24 h before transfection. Ten micrograms of empty previously described (14). Briefly, 5/C2 FuGENE6 reagent (Roche Applied Science). Twenty-four hours later, the GAGG-3 oligonucleotides were used as PCR primers: 5\'-ATATGAAGGTGAGGAGG-GAG-GGAGG-3\' (SOX17 BS 5\'), 5\'-CTCACCCCTGTCCTCCTC-3\' (SOX7 forward), 5\'-TTCACTGTTACTACGGCGGAG-3\' (SOX17 forward), 5\'-ATGTGCGAATTAATACGGCGGAG-3\'; SOX17 reverse, 5\'-GATTTTGTTGTGTTAGTTGT-3\'; SOX7 reverse, 5\'-GAATCCCGTATCCGACG-3\'; SOX17 forward, 5\'-AGAGGAGTCCGTGGAAGACG-3'; SOX9 reverse, 5\'-AGTTCGATA-GGGGGCTGTCT-3'; SOX7 forward, 5\'-GAGGAAGTCCGTAAGAAGACG-3'; SOX9 forward, 5\'-ATGTGCGAATTAATACGGCGGAG-3'; SOX17 reverse, 5\'-ATGTGCGAATTAATACGGCGGAG-3'; SOX18 forward, 5\'-TGAAGCGCTTCATTGGTGTGGACAA-3'; SOX18 reverse, 5\'-GGGAGTGGGGTGGTCGC-3'.

Western blots and antibodies. Antibodies used for Western blots were anti-SOX17 (R&D Systems) and anti-\(\beta\)-actin (Sigma).

Methylation-specific PCR and bisulfite sequencing. Genomic DNA from primary colonic, esophageal, and lung tissue samples and from the CRC cell lines was prepared using the proteinase-K method (17). After chloroform/phenol extraction, DNA was precipitated in ethanol and later dissolved in low TE buffer and stored at -20°C. Genomic DNA was bisulfite treated using the EZ DNA methylation Kit (Zymo Research). Methylation-specific PCR (MSP) primers specific for the unmethylated and methylated promoter sequences were included using MSPPrimer.\(^5\) MSP primers are as follows: SOX17-M forward, 5\'-CAAAAAC-CAAAAAC-CAAAAAC-CAAAAAC-CAAAAAC-3'; SOX17-M reverse, 5\'-CCATATCCAACA-3'. Each MSP was done by using 100 ng of bisulfite-treated DNA, 25 pmol of each primer, 100 pmol deoxynucleotide triphosphates, 50 pmol of each primer, 100 pmol deoxynucleotide triphosphates, 2.5\(\mu\)l 10\(\times\) PCR buffer, and 1 unit of JumpStart Red Taq Polymerase (Sigma) in a final reaction volume of 25 \(\mu\)l. Amplifications were done as follows: 95°C 5 min; 35 cycles (95°C 30 s, 60°C 30 s, 72°C 30 s); 72°C 5 min. MSP products were analyzed using 6% PAGE. Bisulfite sequencing, we first did PCR on bisulfite-treated DNA using the primers that amplify both methylated and unmethylated alleles. The following oligonucleotides were used as PCR primers: 5\'-ATATGAAGGTGAGGAGG-GAG-GGAGG-3' (SOX17 BS 5') and 5\'-CTCACCCCTGTCCTCCTC-3' (SOX7 BS 3'). PCR products were gel purified and cloned into the vector pCR2.1-TOPO according to the manufacturer's protocol (Invitrogen). Integrated PCR fragments were verified by EcoRI digestion, and sequenced with the Mi13 reverse primer by the Johns Hopkins Medical Institutions DNA sequencing facility.

 Colony formation assay. HCT116 cells were plated in 10-cm plate culture dishes 24 h before transfection. Ten micrograms of empty control vector or SOX17 expression vector were transfected using 50 \(\mu\)l FuGENE6 reagent (Roche Applied Science). Twenty-four hours later, the transfected cells were diluted, replated, and selected in 10-cm plates containing 0.4 mg/ml G418 for 10 d. Staining, visualization, and counting of triplicate wells were done as previously described (14).

Luciferase reporter assays. Reporter gene assays were done as previously described (14). Briefly, 5 \(\times\) 10\(^4\) cells were seeded in 24-well tissue culture plates 24 h before transfection. The TOPFLASH or FOPFLASH reporter vectors (gifts from Dr. Bert Vogelstein, Cancer Biology Division, The Sydney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, Baltimore, MD) were transfected at 70 ng/well, and the pRL-TK control vector (Promega) was cotransfected at 7 ng/well as an internal control reporter. For the cotransfection in 293T cells, pCI-neo-\(\beta\)-catenin expressing wild-type \(\beta\)-catenin (70 ng/well) was used to activate the reporter gene. Increasing amounts of pcDNA3.1-SOX17 wild-type, mutants, or the empty vector were transfected into cells using FuGENE 6 (Roche Applied Science). Forty-eight hours posttransfection, cells were washed and lysed in Passive Lysis Buffer (Promega). We measured luciferase activity in a luminometer (BD Biosciences) and transfection efficiency was normalized using the paired Renilla luciferase activity by using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions.

Results

SOX17 is a candidate for aberrant gene silencing in CRC cells. Our earlier studies described a novel approach to identify genes silenced by DNA hypermethylation in CRC based on a genome-wide microarray expression assay (18). After treatment of HCT116 CRC cells with either DAC, a drug that inhibits DNMT-mediated hypermethylation of promoter CpG islands, or the histone deacetylase inhibitor trichostatin A, we identified SOX17 as a candidate promoter CpG island hypermethylated gene by the criteria previously outlined (18). In this approach, candidate DNA hypermethylated genes are identified by selecting those which have no basal expression on the microarray, have a promoter CpG island, and reside in a zone where gene expression did not respond to trichostatin A (<1.4-fold), but increased >2-fold with DAC treatment (Fig. 1A).

To validate the expression array results, we first did RT-PCR to examine the expression of SOX17 and the other SOX group F genes, including SOX7 and SOX18 in the CRC cell line HCT116. Two additional SOX genes, SOX3 and SOX9, are also included in this study because they have been shown to inhibit the TCF-mediated Wnt signaling activity (10, 12). In addition, we studied normal human colon cells and a cell line isogenic to HCT116 cells in which two DNA methyltransferases (DNMT1 and DNMT3b) have been genetically disrupted (DKO HCT116 cells). The latter cells have ~95% reduction in the genomic 5-methylcytosine (19). Of the five genes we examined, SOX3 and SOX7 are not expressed in normal colon whereas SOX9, SOX17, and SOX18 are expressed in normal colon and CRC cells. Strikingly, SOX17 is the only gene exhibiting loss of expression in HCT116 cells. Furthermore, this gene is reexpressed in the HCT 116 isogenic DKO cells (Fig. 1B).

To address the role of epigenetic gene silencing, we analyzed SOX17 expression in the absence or presence of the demethylation agent DAC. Using RT-PCR, we found that SOX17 mRNA was undetectable or expressed at extremely low levels in seven CRC cell lines (HCT116, RKO, SW480, Caco-2, SW480, LOVO, and DLD1). After treating these cells with 1 \(\mu\)mol/L DAC for 96 h, SOX17 was reexpressed in all of the seven cell lines (Fig. 1C). As a control for SOX17 expression, RT-PCR without reverse transcription showed absence of SOX17 bands (Fig. 1C). Consistent with the RT-PCR results, Western blots established that endogenous SOX17 protein is detectable in DKO cells, but not in the HCT116, SW480, Caco-2, and RKO cells (Fig. 1D). These data are consistent with a previous expression analysis in which SW480 cells lacked SOX17 expression (20). In addition, expression of SOX17 protein was restored in HCT116 and Caco-2 cells with DAC treatment (Fig. 1D). Together, these data strongly suggest that SOX17 expression is down-regulated in CRC cells in association with abnormal promoter region DNA methylation.

Silencing of SOX17 is associated with its promoter CpG island hypermethylation. Genomic DNA sequence analysis of SOX17 5' regulatory regions shows that there is a CpG island encompassing its transcription start site (CpG frequency 68%; Fig. 2A). To analyze the methylation status of the SOX17 promoter–associated CpG island, we first designed a set of primers, in a region downstream to the transcription start site, to screen human CRC cell lines by MSP (18). As predicted by our RT-PCR and Western blot results, hypermethylation of SOX17 promoter was detected in seven of the studied colon cancer cell lines (Fig. 2B). In contrast, there is no methylation detected in isogenic DKO cells (Fig. 2B). We obtained the same results with a second set of MSP

\(^5\) http://www.mspprimer.org
primers encompassing the transcription start site (supplementary Fig. 1). We then analyzed methylation status in a series of human colon specimens from unselected patients to determine whether aberrant methylation of the \textit{SOX17} gene in human CRC cell lines reflects an epigenetic process of colon cancer initiation and progression in humans. Normal colon (\(n = 20\)), tubular adenomas (\(n = 15\)), villous adenomas (\(n = 22\)), and stage I (\(n = 35\)), stage II (\(n = 36\)), and stage III (\(n = 28\)) CRCs were included in this study. Strikingly, \textit{SOX17} hypermethylation was found in 12 of 15 (80\%) colorectal tubular adenomas and 20 of 22 (91\%) villous adenomas (Fig. 2). Overall, the frequency of \textit{SOX17} methylation in adenomas was 86\% (32 of 37). Similarly, extensive methylation at the same loci was seen in primary CRCs: 35 of 35 (100\%) in stage I CRC, 36 of 36 (100\%) in stage II CRC, and 25 of 28 (89\%) in stage III CRC (Fig. 2C). Notably, promoter methylation has not been detected in 20 samples of normal colon. These data strongly suggest that hypermethylation of \textit{SOX17} is established at a very early stage in the initiation of colorectal carcinogenesis.

Hypermethylation of the \textit{SOX17} promoter region was not limited to colorectal neoplasms. We observed \textit{SOX17} methylation in 17 of 19 (89\%) esophageal squamous cancers and 6 of 12 (50\%) non–small cell lung cancer samples (Fig. 2D). To address the functional significance of \textit{SOX17} gene silencing, we tested its ability to suppress tumor growth in HCT116 cells, in which endogenous expression is silenced by DNA hypermethylation. Colony formation assays were carried out following transient transfection of a vector with the cDNA for wild-type \textit{SOX17} (pcDNA3.1-SOX17) or the corresponding empty vector into HCT116 cells. The cells were selected in the presence of 400 \(\mu\)g/mL G418, and the number of colonies was counted 10 days later. As shown in Fig. 4A and B, reexpression of \textit{SOX17} markedly decreased colony formation, which is consistent with a similar assay done by overexpressing mouse Sox17 in SW480 cells (12). Furthermore, seven surviving clones transfected with \textit{SOX17} expression plasmids were randomly selected for subculture. Western blots indicate that there is no detectable \textit{SOX17} protein among these colonies (data not shown). These results reveal that \textit{SOX17} can inhibit colony growth in CRC cells.

\textbf{Restoration of \textit{SOX17} expression suppresses tumor cell growth.} To address the functional significance of \textit{SOX17} gene silencing, we tested its ability to suppress tumor growth in HCT116 cells, in which endogenous expression is silenced by DNA hypermethylation. Colony formation assays were carried out following transient transfection of a vector with the cDNA for wild-type \textit{SOX17} (pcDNA3.1-SOX17) or the corresponding empty vector into HCT116 cells. The cells were selected in the presence of 400 \(\mu\)g/mL G418, and the number of colonies was counted 10 days later. As shown in Fig. 4A and B, reexpression of \textit{SOX17} markedly decreased colony formation, which is consistent with a similar assay done by overexpressing mouse Sox17 in SW480 cells (12). Furthermore, seven surviving clones transfected with \textit{SOX17} expression plasmids were randomly selected for subculture. Western blots indicate that there is no detectable \textit{SOX17} protein among these colonies (data not shown). These results reveal that \textit{SOX17} can inhibit colony growth in CRC cells.

\textbf{SOX17 inhibits \(\beta\)-catenin/TCF–driven transcription.} Previous studies have shown that several members of the \textit{Sox} gene family can antagonize the canonical Wnt signaling pathway (10, 12, 21, 22). For Sox17, the mechanisms identified, to date, includes the following: (a) \textit{Xenopus} Sox17\(\beta\) protein competes with TCF/LEF for \(\beta\)-catenin binding (10), and (b) mouse Sox17 induces degradation of \(\beta\)-catenin.
of both β-catenin and TCF/LEF in SW480 cells (12). We tested the ability of SOX17 to repress Wnt signaling in the context of our findings. To examine whether SOX17 can inhibit wild-type β-catenin-mediated Wnt activation, HEK293T cells were transfected with the TOPFLASH or FOPFLASH reporter constructs, concomitantly with a wild-type β-catenin expression vector and increasing amounts of pcDNA3.1-SOX17. The TOPFLASH transfection plasmid contains three consensus TCF binding sites fused to a minimal FOS promoter and firefly luciferase gene. The FOPFLASH plasmid is identical except that the TCF binding site sequences are mutated to serve as a negative control (23). Wild-type β-catenin expression induced a 60-fold activation of TOPFLASH activity, which can be repressed by overexpression of SOX17 in a dose-dependent manner (Fig. 5A). In contrast, SOX17 did not affect transcription from the plasmid FOPFLASH (Fig. 5A). We also examined whether SOX17 could inhibit β-catenin/TCF activity in CRC cells that have a high basal transcriptional activity driven by this protein complex as measured by the TOPFLASH assay (23, 24). In keeping with our observation in HEK293T cells, inhibition of TOPFLASH reporter activity by SOX17 was seen in HCT116 cells (Fig. 5C), which contain the high basal activity driven by an endogenously mutated β-catenin gene (24).

To better understand the mechanisms by which human SOX17 represses β-catenin/TCF activity, segments of SOX17 that are necessary for inhibition were mapped by structure-based deletion analysis (Fig. 5B). An NH₂-terminal deletion (yielding SOX17 fragment 50–414) did not affect the inhibitory function of the protein at all (Fig. 5C). The deletion of the HMG domain (yielding SOX17 fragment 135–414) led to a complete loss of repression of TCF/β-catenin activity (Fig. 5C), which is consistent with the result of reporter gene assays acquired in studies of the Xenopus Sox17β protein (10). In contrast to a recent study that suggests that the COOH terminus of mouse Sox17 is also required for the repressive ability on β-catenin/TCF activity, our COOH-terminal deletion

Figure 3. Bisulfite genomic DNA sequencing results of SOX17 in normal colon, DKO, HCT116, and SW480 cells. ○, unmethylated CpG sites; ●, methylated CpG sites. Locations of CpG sites are given relative to the transcription start site.
construct (human SOX17 fragment 1–353) exhibited a similar repression effect as wild-type SOX17 expression vector. Interestingly, alignment of mouse and human SOX17 protein sequences reveals that they share the identical HMG box located in the NH2 terminus (residue 67–135), and that the COOH-terminal fragments are strongly homologous. Previous studies suggest that *Xenopus* Sox17 and mouse Sox17 have a β-catenin interaction domain at their COOH termini (8, 10). Because the deletion construct (SOX17 1–353) does not contain this domain, our results suggest that the repressive function of SOX17 does not depend on β-catenin binding. The inhibitory capability of the SOX17 HMG domain on TOPFLASH reporter transcription was also seen in SW480 cells (Fig. 5D), which harbor a mutant APC gene (24). These data collectively show that SOX17 can inhibit canonical Wnt signaling triggered by mutations in either APC or β-catenin, and that the HMG domain is required for this effect.

**Discussion**

It is now well established that loss of proper gene function by gene silencing contributes heavily to CRC initiation and progression. In this regard, several important Wnt signaling inhibitors such as SFRPs, WIF-1, and DKK-1 have been previously reported to be frequently hypermethylated in primary colorectal tumors (14–16). Our results suggest that epigenetic silencing of SOX17 is another important step in activation or amplification of aberrant Wnt signaling in CRC. This conclusion is supported by our observations that (a) methylation of SOX17 is strongly associated with the loss of gene expression in seven CRC cell lines; (b) SOX17 is unmethylated in normal colon but methylated with high frequency in a variety of human primary tumors including CRC; (c) methylation of SOX17 occurs frequently in premalignant colonic neoplasms; (d) restoration of SOX17 function reduces colony formation in colon cancer cells; and (e) SOX17 can efficiently suppress both wild-type and mutant β-catenin–mediated transcriptional activity. These findings suggest that SOX17 hypermethylation may play an important role in the early steps of cancer formation.

Our current findings for SOX17 make yet another addition of epigenetic inactivation events to the growing list of DNA hypermethylated Wnt antagonist genes in colon and other cancers. The question arises as to why so many potentially redundant steps for Wnt pathway activation would simultaneously be present in a single tumor. The answer may be 2-fold, as we and others have postulated (14, 25, 26). First, that each of these individual gene epigenetic inactivation steps alone may be less potent than single mutations for driving the Wnt pathway and that they are required to summate to yield the full epigenetic drive for tumorigenesis. Second, this summation may be additionally necessary to give Wnt pathway mutations their full effect to help drive abnormal activation of the Wnt pathway.

Figure 4. Suppression of cancer cell growth by SOX17. A, expression vectors encoding wild-type SOX17 or empty control vectors were transfected into HCT116 cells, which were then selected for G418 resistance. After 10 d, the cells were fixed with 10% formaldehyde and stained with Giemsa. B, quantitative analysis of surviving colonies after G418 selection. Each experiment was repeated three times and the average number of colonies is indicated with error bars on the histogram.
In this regard, silenced Wnt antagonist genes can be divided into three broad classes, each contributing to individual steps in amplifying the effects of increasing nuclear β-catenin function as the final readout for the active Wnt pathway. The first class, including the secreted SFRPs, WIF-1, and DKK, acts at the level of the cell membrane to prevent ligand-receptor interactions. The inactivation of this class, as we and others have shown for SFRPs (14, 27, 28), can up-regulate the Wnt pathway at the cell membrane and this leads to increased cellular levels of β-catenin. When these increases meet a crippled cytoplasmic degradation complex for this protein, such as in colon cancer cells with APC mutations, or when this leads to increased levels of mutant β-catenin, which can evade the complex, then more β-catenin reaches the nucleus to transcriptionally drive Wnt pathway target genes (23, 24). The second class comprises certain members of the cytoplasmic degradation complex for β-catenin and the example here is the APC gene. Thus, APC promoter hypermethylation is an alternative mechanism to mutations for inactivation of this key gene in colon cancer development (29) and can, especially in the setting of inactivation of the above membrane Wnt antagonists, result in Wnt nuclear activation. Finally, we now show epigenetic inactivation of another class of Wnt antagonist. Certain nuclear proteins, including SOX17, inhibit Wnt signaling at the level of the nuclear complex between β-catenin and TCF. Inactivation of SOX17 is then added to abnormal activation of the pathway by stabilizing and/or facilitating this key Wnt-driven transcription complex. In summary, we hypothesize that simultaneous epigenetic down-regulation of SFRPs, WIF-1, DKK, and SOX17, especially in the setting of key pathway mutations, act in a complementary manner for the constitutive activation of Wnt signaling, which can drive tumor initiation and progression.

Our detection of SOX17 hypermethylation now adds this gene to the repertoire of DNA hypermethylated genes that may

Figure 5. SOX17 inhibits Wnt stimulated transcription. A, SOX17 inhibits wild-type β-catenin–activated transcription. HEK293T cells were transfected with 70 ng of TOPFLASH or FOPFLASH plasmids, 7 ng of pRL-TK, and increasing amounts of pcDNA3.1-SOX17 or empty vector control (0, 30, 50, and 100 ng; black triangle, increasing dose), and stimulated for 48 h with cotransfection of 70 ng wild-type β-catenin expression vectors. The results are normalized to those for empty control vectors and are expressed as a relative ratio of firefly luciferase to Renilla luciferase. Bars, +1 SD. B, deletion analysis of SOX17. Individual SOX17 deletion mutants are depicted. The HMG box of SOX17 is shown in schematic form. C, SOX17 inhibits endogenous TCF/β-catenin–mediated transcription through the NH2-terminal HMG box. HCT116 cells were transfected with 70 ng of TOPFLASH, 7 ng of pRL-TK, and increasing amounts of pcDNA3.1-SOX17 or the different deletion mutants (SOX17 constructs 50–414, 135–414, and 1–353) or empty vectors (2, 10, 30, 50, and 100 ng; black triangle, increasing dose). Transfection of TOPFLASH reporter vectors reveals that HCT116 cells have high levels of endogenous β-catenin/TCF transcription activity (lane marked “None,” and set at 100% for normalization of data in all other lanes). Columns, mean of three independent experiments; bars, SD. D, a similar suppression effect mediated by the SOX17 HMG box is observed in SW480 cells. The SW480 cells were transfected with 10, 30, 50, or 100 ng SOX17 expression constructs, respectively. Data are expressed relative to the high basal activity in nontransfected cells as in C.
Hypermethylation of Wnt Antagonist SOX17 in CRC

Correction

Correction: Hypermethylation of Wnt Antagonist SOX17 in Colorectal Cancer

In the article on hypermethylation of SOX17 in colorectal cancer in the April 15, 2008 issue of Cancer Research (1), the SOX17-M-reverse primer for methylated promoter sequence was listed incorrectly. The correct sequence appears below.

SOX17-M-reverse, 5’-TTGCGTTAGTCGTTTGCGTTC-3’

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