WNT5A Exhibits Tumor-Suppressive Activity through Antagonizing the Wnt/β-Catenin Signaling, and Is Frequently Methylated in Colorectal Cancer

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Abstract

Purpose: Aberrant activation of the Wnt/β-catenin signaling pathway is associated with multiple tumors including colorectal cancer (CRC). *WNT5A* is a member of the nontransforming Wnt protein family, whose role in tumorigenesis is still ambiguous. We investigated its epigenetic alteration in CRCs.

Experimental Design: We examined its expression and methylation in normal colon, CRC cell lines, and tumors. We also evaluated its tumor-suppressive function and its modulation to Wnt signaling in CRC cells.

Results: *WNT5A* is silenced in most CRC cell lines due to promoter methylation, but is expressed in most normal tissues including the colon, and is unmethylated in normal colon epithelial cells. *WNT5A* expression could be reactivated by pharmacologic or genetic demethylation, indicating that methylation directly mediates its silencing. *WNT5A* methylation was frequently detected in CRC tumors (14 of 29, 48%), but only occasionally in paired normal colon tissues (2 of 15, 13%; $P = 0.025$). Ectopic expression of *WNT5A*, but not its nonfunctional short-isoform with the WNT domain deleted, in silenced CRC cells resulted in substantial inhibition of tumor cell clonogenicity, which is associated with down-regulated intracellular β-catenin protein level and concomitant decrease in β-catenin activity.

Conclusions: *WNT5A* is frequently inactivated in CRC by tumor-specific methylation, and thus, is a potential biomarker. *WNT5A* could act as a tumor suppressor for CRC by antagonizing the Wnt/β-catenin signaling.

Wingless-type mouse mammary tumor virus integration site family (Wnt) proteins are a large family of cysteine-rich, secreted signaling glycoproteins that control essential developmental and normal physiologic processes (reviewed in refs. 1, 2). Vertebrate Wnts are divided into canonical signaling and noncanonical members (3, 4). Activation of the canonical Wnt signaling pathway (Wnt/β-catenin/TCF) leads to the tumorigenesis of multiple carcinomas including colorectal cancer (CRC; refs. 5, 6).

WNT5A is located at 3p14, a commonly deleted tumor suppressor locus in multiple tumors. *WNT5A* has been classified as a noncanonical and nontransforming Wnt protein (3), with its role in tumorigenesis still ambiguous. There is evidence indicating that increased *WNT5A* expression is important for cancer progression, and that *WNT5A* was initially proposed as a proto-oncogene (7). *WNT5A* has been shown as a potent enhancer of cell motility and invasiveness of melanoma (8), up-regulated in cancers of the lung, breast, stomach, and prostate (9–12). On the other hand, in other tumor models, including hematopoietic tissues, brain, breast, thyroid, and uroepithelial cancers, *WNT5A* has been shown to inhibit tumor cell proliferation (13–17), with its expression as a good prognostic marker for patients with breast and colon cancer (18, 19). These results suggest that dysregulation of *WNT5A* expression is involved in tumor pathogenesis, although its exact role is still controversial.

Epigenetic silencing of tumor suppressor genes by promoter methylation represents an important mechanism of tumor suppressor gene inactivation during tumorigenesis. Multiple tumor suppressor genes participating in various biological processes and pathways have been shown to be silenced by aberrant CpG methylation in virtually all tumor types (20, 21). In addition to genetic mutations of certain genes such as *APC*, epigenetic silencing of Wnt signaling molecules such as SFRPs activate this pathway, thus its involvement in CRC pathogenesis (22). Here, we report the frequent epigenetic

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Note: Genbank accession numbers. The sequence of the WNT5A short isoform (accession no. EF028086) has been deposited to Genbank.

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inactivation of *WNT5A* in CRC. We also found that *WNT5A* expression resulted in significant suppression of colony formation of CRC cells, at least partially mediated by the down-regulation of intracellular β-catenin protein levels and a decrease of β-catenin/TCF transcriptional activity.

Patients and Methods

Cell lines and primary tumors. Six CRC cell lines (HCT116, HT29, SW480, LoVo, SW620, and Caco-2) were used. Cell lines were routinely maintained in cRPMI 1640. HCT116 cells with genetic knockout of DNA methyltransferases (DNMT): HCT116 DNMT1-/- (1KO), HCT116 DNMT3B-/- (3BKO), and HCT116 DNMT1-/- DNMT3B-/- (DKO; a gift from Dr. Bert Vogelstein, Johns Hopkins) were also used (23). DNA and total RNA were extracted using TRI REAGENT (Molecular Research Center). Genomic DNAs of another five CRC cell lines (HCT15, DLD-1, RKO, SW48, and Colo205) and one transformed normal colon epithelial cell line, CCD-841, were also used. DNA extraction from paired CRC tumor samples have been described previously (24).

5-Aza-2′-deoxycytidine treatment. HCT116 with silenced *WNT5A* was treated with 5 μmol/L of 5-Aza-2′-deoxycytidine (Sigma) for 3 days as described previously (25). After the treatment, cells were pelleted and extracted for DNA and RNA.

Semiquantitative reverse transcription-PCR. Reverse transcription-PCR was done using the GeneAmp RNA PCR kit (Applied Biosystems; refs. 26, 27), with GAPDH as a control (20 PCR cycles only). The primers used included: WNT5AF, 5′-caggcttaacccggtcgc, and WNT5AR, 5′-ctggcattctttgatgcctg; CCND1F, 5′-tgctgcgaagtggaaaccat, and CCND1R, 5′-gcggtccaggtagttcatg. The PCR program included initial denaturation at 95°C for 10 min, followed by 35 cycles (for *WNT5A*) or 32 cycles (for *CCND1*) of reaction (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s), using the Go-Taq polymerase (Promega), with a final extension at 72°C for 10 min.

Bisulfite treatment and methylation analysis. Bisulfite modification of DNA was done using 2.4 mol/L of sodium metabisulfite (26, 27). Methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) were conducted as previously described (26–28). MSP primers targeting two different regions of the *WNT5A* promoter for methylated sets included: WNT5Am1, 5′-gttgtttgcgcgttttcgtc, and WNT5Am2, 5′-caaaaataaaaaaaacgcgaacg; or WNT5Am3, 5′-ttgtttttgttgtcggttcgc, and WNT5Am4, 5′-aactacgaaatcctccgacg. For the unmethylated sets: WNT5Au1, 5′-aatagttgtttgtgtgtttttgtt, and WNT5Au2, 5′-cccaaaaataaaaaaaacacaaaca; or WNT5Au3, 5′-ttttgtttttgttgttggtttgt, and WNT5Au4, 5′-aaaactacaaaatcctccaaca. MSP was conducted at 95°C for 10 min, followed by 40 cycles of reaction [94°C/30 s; 60°C (for methylated) or 58°C (for unmethylated), 30 s; and 72°C/30 s], and ended at 72°C for 5 min. Methylated and unmethylated MSP primer sets targeted the same CpG sites, and were tested for not amplifying any non–bisulfite-treated genomic DNA and were thus specific. The MSP products of selected samples were confirmed by direct sequencing. The bottom strand–specific BGS primers for the *WNT5A* promoter were: WNT5ABGSb1, 5′-tggggttggaaagttttaattat, and WNT5ABGSb2, 5′-actaaacacctaccttcataac. Amplified products were cloned into pCR4-Topo (Invitrogen), with six to eight colonies randomly chosen and sequenced.

Construction of expression plasmids. The full-length cDNA encoding *WNT5A* was PCR-cloned using the high-fidelity AccuPrime Taq DNA polymerase (Invitrogen) and human testis RNA (BD Clontech), sequence-verified, and then subcloned into pcDNA3.1(+) (Invitrogen) to generate pcDNA3.1-*WNT5A*. During the cloning process, we also discovered a novel short isoform of *WNT5A* (submitted to the National Center for Biotechnology Information, with accession no. EF028086) with deletion of most of the WNT domain (nonfunctional). The expression level of this variant is very low (barely seen or absent) in all normal tissues and cell lines. We also cloned its cDNA, pcDNA3.1-*WNT5A*-SI (for short isoform), as a control for the functional assays of *WNT5A*.

Colony formation assay. Cells (1.5×10^5/well) were plated in a 12-well plate and transfected with either expressing plasmids or the empty vector (0.8 μg each), using FuGENE 6 (Roche). Forty-eight hours posttransfection, cells were collected and plated in a six-well plate, and selected for ~2 weeks with G418 (0.4 mg/mL). Surviving colonies (≥50 cells/colony) were counted after staining with gentian violet. Total RNA from transfected cells was extracted, treated with DNase I, and analyzed by RT-PCR to confirm *WNT5A* expression. All the experiments were done thrice in triplicate wells.

Protein preparation and Western blot. Transfected cells were lysed in ice-cold Tris buffer (20 mmol/L Tris; pH 7.5) containing 137 mmol/L of NaCl, 2 mmol/L of EDTA, 1% Triton X, 10% glycerol, 50 mmol/L of NaF, 1 mmol/L of DTT, and a protease inhibitor cocktail (Roche). The protein lysates were then separated by SDS-PAGE and electroblotted onto Hybond-P membranes (Amersham). After blocking with 5% nonfat milk and 0.1%Tween 20 in TBS, the membranes were incubated with mouse anti–β-catenin (DAKO) or mouse anti–α-tubulin (Cell Signaling) antibodies. The blots were visualized using enhanced chemiluminescence (Amersham Biosciences) and quantitated.

Luciferase activity assay. TCF luciferase construct (pTOPFLASH; 0.1 μg/well) containing TCF-binding sites (kindly provided by Prof. Christof Niehrs, German Cancer Research Center DKFZ, Heidelberg, Germany), with an internal control (0.01 μg/well pRL-TK Renilla luciferase vector), were cotransfected with either pcDNA3.1-*WNT5A*, pcDNA3.1-*WNT5A*-SI, or empty vector (0.1 μg/well) into HCT116 cells in a 96-well plate, using FuGENE 6. Transfection was carried out in triplicate. Forty-eight hours posttransfection, the cells were washed with PBS, and lysed in 1× passive lysis buffer (Dual Luciferase kit; Promega). The cell lysates were transferred into an OptiPlate 96-well plate (Perkin-Elmer) and assayed in a 1420-Multilabel counter luminometer, VICTOR^3 (Perkin-Elmer) using the Dual-Luciferase kit (Promega). Relative TOPFLASH luciferase units were measured and normalized against Renilla luciferase activity. Data were expressed as mean of triplicate values (±SD) of the normalized TOPFLASH activity of pcDNA3.1-*WNT5A* or *WNT5A*-SI–transfected cells, relative to the vector-transfected cells (set as 100%).

Statistical analysis. Statistical analysis was carried out using the χ^2 test (the web χ^2 calculator) and Student's t test. $P < 0.05$ was considered statistically significant.

Results

Frequent loss of WNT5A in CRC cell lines. *WNT5A* is one of the genes identified during our genome-wide screening of epigenetically silenced cancer genes,[5] located at an important tumor suppressor locus, 3p14. We further examined its expression in CRC cell lines and normal tissues. Semiquantitative RT-PCR showed that *WNT5A* was ubiquitously expressed in all normal tissues including the colon (Fig. 1B), in agreement with previous reports of normal colon epithelium by *in situ* hybridization and immunohistochemistry (18, 29). In contrast, *WNT5A* was silenced in four of six cell lines, and down-regulated in another one (LoVo; Fig. 1D). Moreover, screening the CGAP gene expression database (National Cancer Institute Cancer Genome Anatomy Project)[6] in normal and tumor tissues also showed that *WNT5A* is down-regulated in multiple tumors of colon, breast, lung, liver, prostate, and thyroid, whereas it is up-regulated in tumors of brain, kidney, skin, and stomach (Fig. 1C), indicating important cancer-related functions for *WNT5A*.

WNT5A is silenced in cell lines due to promoter methylation. The *WNT5A* promoter contains a typical CpG island

[5] Ying & Tao, manuscript in preparation.
[6] http://cgap.nci.nih.gov/

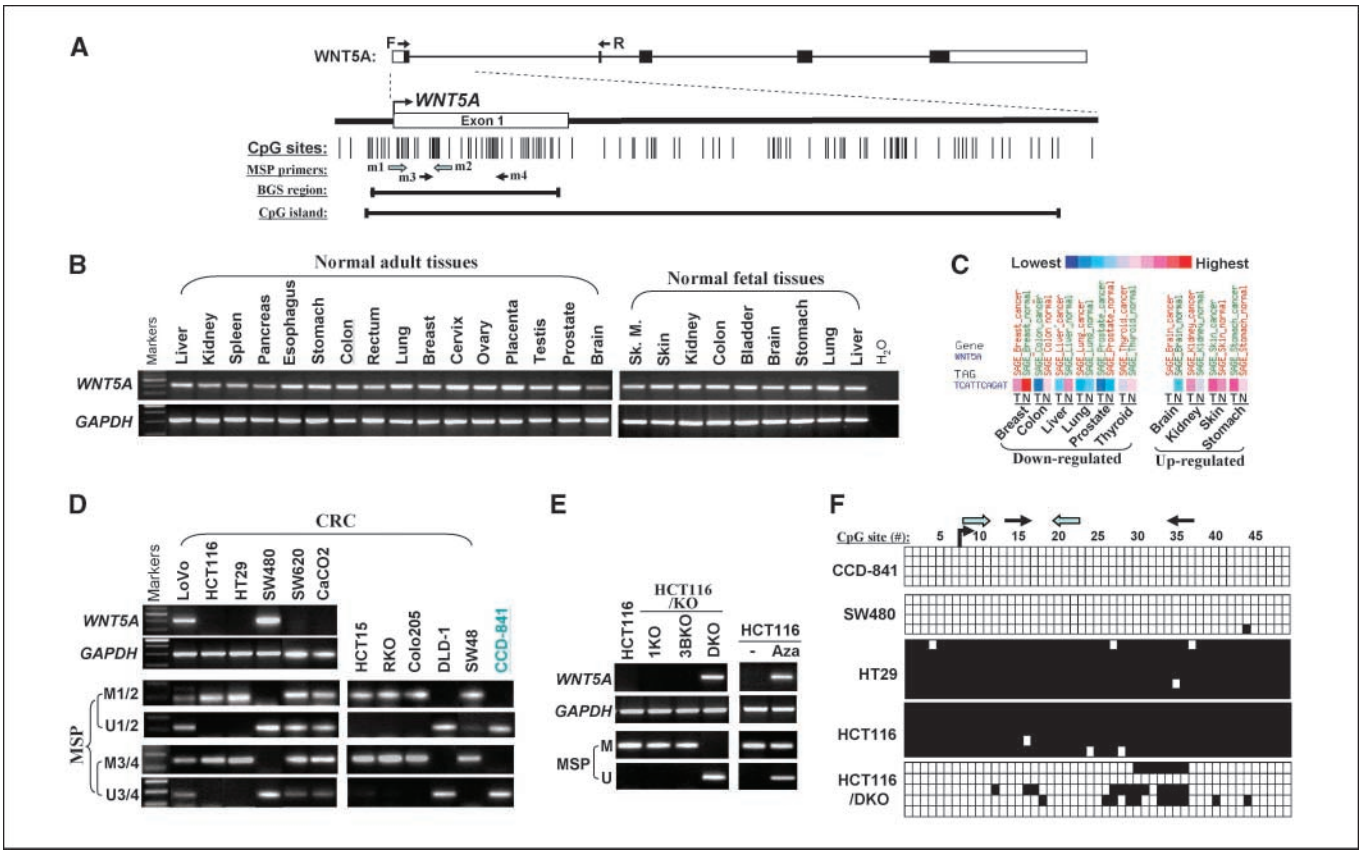

Fig. 1. Methylation-associated silencing of *WNT5A* in CRC cell lines. *A,* schematic structure of *WNT5A* transcript and its promoter CpG island (CGI). Locations of exon 1 (*long rectangle*), CpG sites in the CGI (*short vertical lines*), coding exons (*filled rectangles*), and the transcription start site (*curved arrow*). The MSP and BGS regions analyzed and the positions of primers used are also indicated. *B,* broad expression of *WNT5A* in human normal adult and fetal tissues as detected by semiquantitative RT-PCR, with *GAPDH* as a control. Sk.M., skeletal muscle. *C,* down-regulation or up-regulation of *WNT5A* in multiple malignancies compared with their normal counterparts, by analyzing the gene expression databases CGAP (http://cgap.nci.nih.gov/). *D,* expression (*top*) and methylation status (*bottom*) of *WNT5A* in a panel of CRC cell lines. MSP results of two promoter regions. CCD-841 is a transformed normal colon epithelial cell line. M, methylated; U, unmethylated. *E,* pharmacologic and genetic demethylation induces *WNT5A* expression in methylated and silenced cell line HCT116. 5-Aza-2′-deoxycytidine demethylation activated *WNT5A* expression (*right*), whereas genetic double knockout (*KO*) of both *DNMT1* and *DNMT3B* in HCT116 also resulted in demethylation and induction of *WNT5A* (*left*). MSP was done using primers m1/m2 and u1/u2. *F,* high-resolution mapping of the methylation status of individual CpG sites in the *WNT5A* promoter by BGS in CRC cell lines. Methylation status of each individual promoter allele was shown as a row of CpG sites sequenced from each bacterium colony. The locations of four MSP primers (*arrows*). The transcription start site (*curved arrow*).

(CGI), spanning the core promoter, exon 1 and part of intron 1 (Fig. 1A). We suspect that *WNT5A* silencing might be mediated by epigenetic regulation, and thus, analyzed its promoter methylation status. MSP showed that *WNT5A* was methylated in silenced cell lines (HCT116, HT29, SW620, and Caco-2) with weak methylation in the down-regulated cell line LoVo, whereas it is unmethylated in SW480 with strong expression (Fig. 1D). Results using two independent sets of MSP primers targeting two different promoter regions were identical. Thus, *WNT5A* methylation status is inversely well-correlated with its expression levels. Furthermore, *WNT5A* was methylated in another four of five CRC cell lines (in total 9 of 11, 82%), but is unmethylated in normal colon epithelial cell line CCD-841 (Fig. 1D).

Further detailed methylation analyses of individual CpG sites of the *WNT5A* promoter using BGS was done for four CRC cell lines and CCD-841 (Fig. 1F). Densely methylated CpG sites were detected in HCT116 and HT29, which showed complete methylation by MSP and no expression by RT-PCR. In expressing cell lines with unmethylated MSP results, virtually no methylated CpG sites (CCD-841 and SW480) or only few CpG sites (demethylated HCT116-DKO cell line) were detected. Thus, BGS results further confirmed our MSP analyses.

Pharmacologic or genetic demethylation reactivated WNT5A expression. We next analyzed whether *WNT5A* methylation directly mediates its silencing. Fully methylated and silenced HCT116 was treated with a demethylating agent 5-aza-2′-deoxycytidine and assessed. Undetectable before 5-aza-2′-deoxycytidine treatment, *WNT5A* expression was dramatically induced after the treatment (Fig. 1E). This reactivation was associated with an increase of unmethylated alleles of the *WNT5A* promoter, as assessed by MSP. Similarly, *WNT5A* could be activated in HCT116 by genetic demethylation through double knockout of both *DNMT1* and *DNMT3B* (DKO cell line), but not in single knockout of either *DNMT1* or *DNMT3B* (1KO or 3BKO cell line; Fig. 1E). Concomitantly, complete demethylation of the *WNT5A* promoter was detected in DKO cells, but not in *DNMT1* or *DNMT3B* single knockout cells. Further BGS methylation analysis confirmed *WNT5A* demethylation in HCT116-DKO cells (Fig. 1F). Taken together, these results indicate that CpG methylation of the *WNT5A* promoter directly mediates its transcriptional repression in CRC cells, and the maintenance of *WNT5A* methylation is mediated by DNMT1 and DNMT3B together, like other functional tumor suppressor genes that we and others have

previously examined (25, 27, 30, 31). Our results show that, for the first time, *WNT5A* expression is epigenetically regulated and repressed by promoter methylation in CRC cells.

Frequent methylation of WNT5A in primary tumors. Subsequently, *WNT5A* methylation was examined in primary CRC tumors using MSP (Fig. 2A). Aberrant methylation was detected in 14 of 29 (48%) tumors, significantly more frequently than the paired normal colon samples (2 of 15, 13%; $P = 0.025$, χ^2; Fig. 2B). Direct sequencing of MSP products confirmed the methylation (Fig. 2C). Further BGS analysis showed densely methylated promoter alleles in tumors, and only rarely methylated CpG sites in paired normal tissues (Fig. 2D). Thus, promoter methylation of *WNT5A* is frequent and tumor-specific in CRC.

Ectopic expression of WNT5A inhibits tumor cell clonogenicity. To evaluate whether *WNT5A* functions as a tumor suppressor in CRC cells, we transfected HCT116, in which *WNT5A* was fully silenced by methylation, with vector alone or *WNT5A*-expressing vectors. After G418 selection, we compared vector- or *WNT5A*-transfected cells for their colony-forming abilities. Ectopic expression of *WNT5A* substantially inhibited tumor cell colony formation ($P < 0.01$). In contrast, reexpression of the short isoform of *WNT5A* with a deleted WNT domain showed no tumor suppression (Fig. 3A).

WNT5A expression promotes β-catenin degradation and down-regulates CCND1 expression. The frequent activation of the Wnt/β-catenin pathway and epigenetic inactivation of *WNT5A* in CRC prompt us to examine whether *WNT5A* could counteract Wnt/β-catenin signaling. We determined the intracellular β-catenin levels before and after reexpression of *WNT5A*. In *WNT5A*-transfected cells, β-catenin protein levels were significantly decreased (to ~58%), as compared with vector control or the nonfunctional short isoform–transfected cells (Fig. 3B). These results suggest that *WNT5A* directly affects the intracellular β-catenin level to interfere with Wnt/β-catenin signaling. In accordance with this down-regulation, the luciferase activity of TCF luciferase reporter construct TOPFLASH was significantly decreased (to ~36%) in *WNT5A*-expressed, but not in control- or *WNT5A* short isoform–expressed cells (Fig. 3C). This result further confirmed that *WNT5A* directly antagonizes Wnt/β-catenin signaling in CRC cells. We also examined the effect of *WNT5A* reexpression on the expression of a β-catenin target gene *CCND1/cyclin D1*, using semiquantitative RT-PCR. Results showed that the expression of *CCND1* RNA was down-regulated in HCT116 cells transfected with *WNT5A*-expressing vector (Fig. 3D), which is consistent with the down-regulation of intracellular β-catenin levels and the decrease of β-catenin activity observed above.

Discussion

In this study, we show for the first time, that *WNT5A* is frequently silenced by methylation in CRC cell lines and primary tumors but seldom in normal colon tissues. *WNT5A* restoration in silenced cells antagonizes Wnt signaling by promoting intracellular β-catenin degradation, and inhibits the clonogenicity of CRC cells. Our results are consistent with the recent findings that *WNT5A* expression leads to a significant

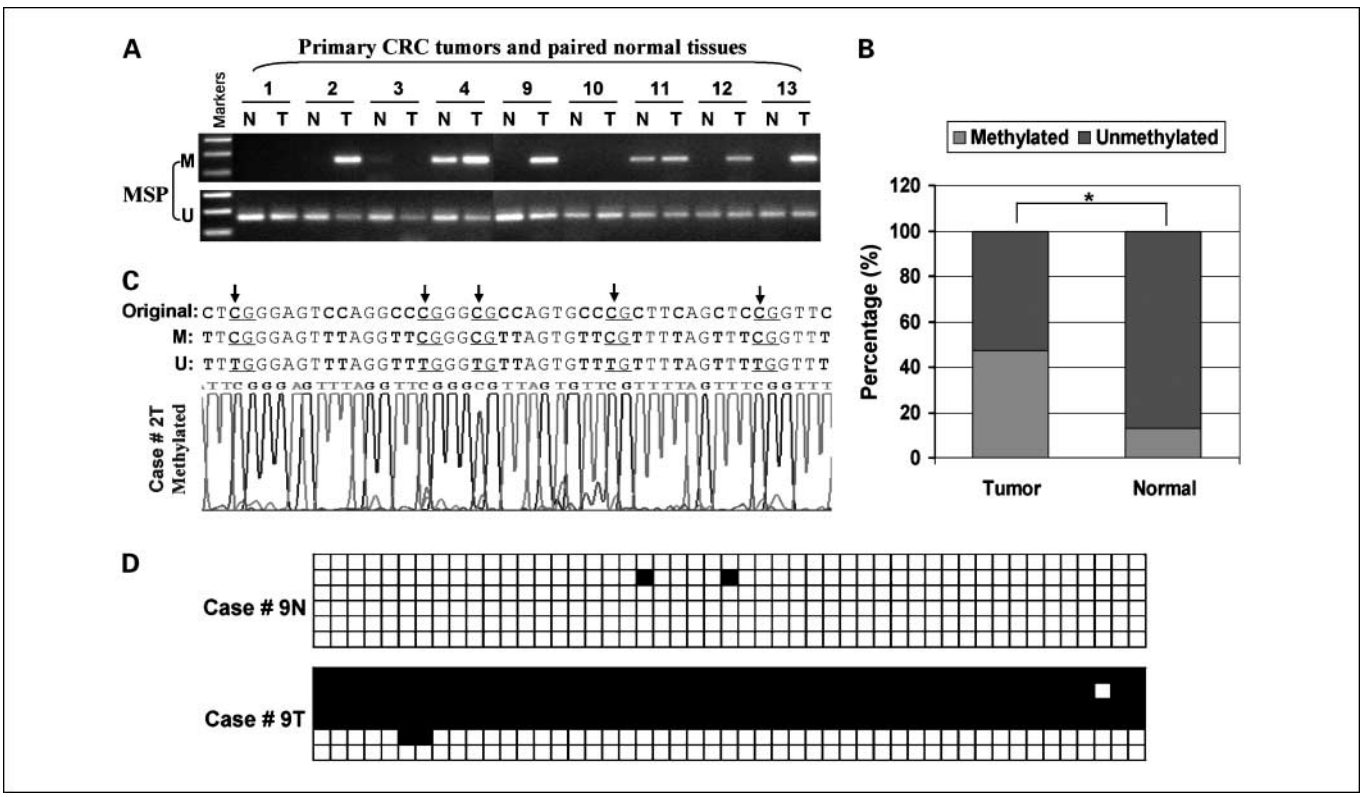

Fig. 2. Frequent methylation of *WNT5A* in primary CRC tumors. *A,* representative MSP results showing frequent *WNT5A* methylation in primary carcinomas (*T*), but rarely in paired normal tissues (*N*). M, methylated; U, unmethylated. *B,* percentage of methylated and unmethylated samples in CRC and paired normal tissues (*, $P < 0.05$). *C,* MSP products were further confirmed by direct sequencing. *D,* high-resolution methylation mapping of individual CpG sites in the *WNT5A* CGI by BGS in CRC tumors and normal tissues.

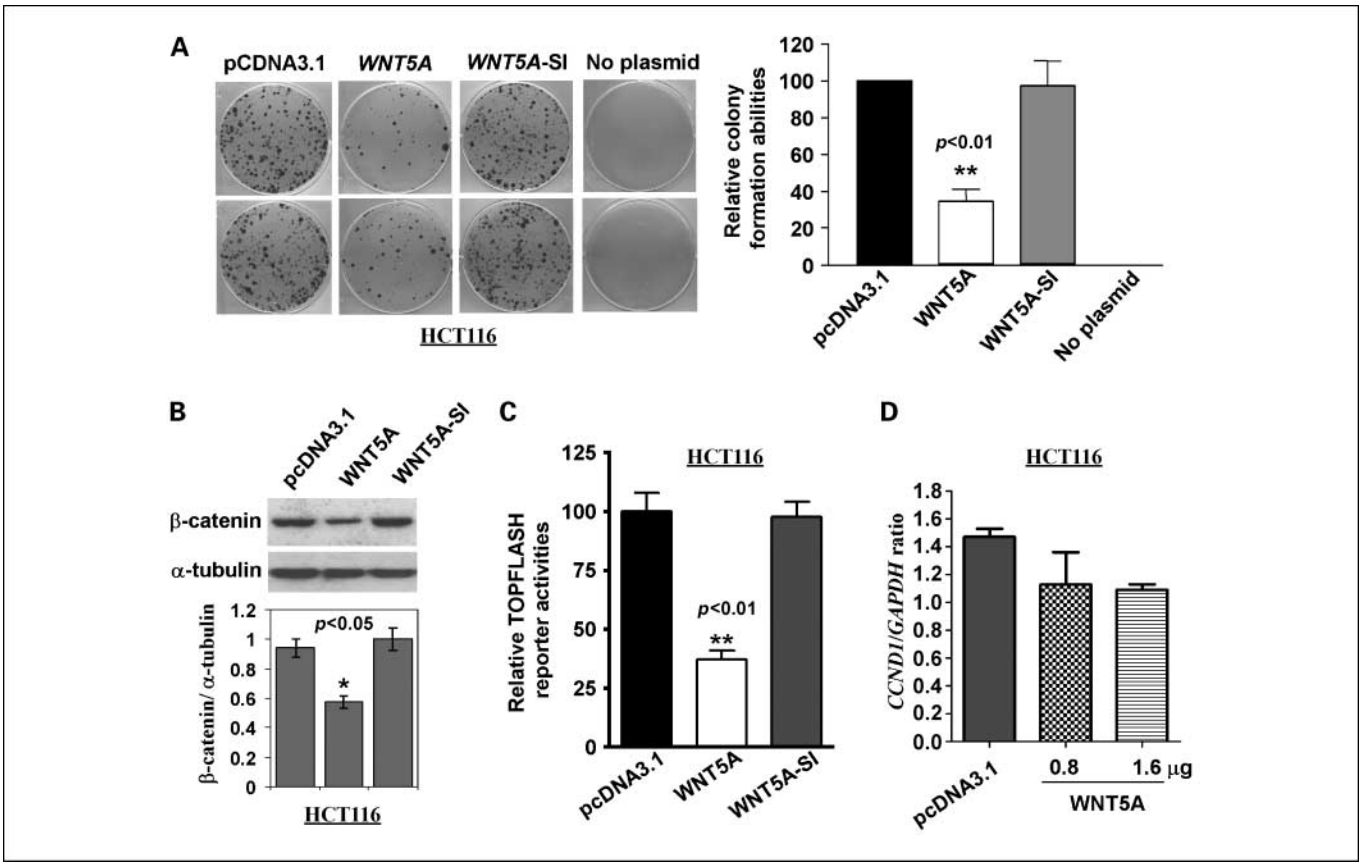

Fig. 3. Ectopic *WNT5A* expression suppressed tumor cell clonogenicity and inhibited β-catenin signaling activity in HCT116 cells. *A,* representative inhibition of colony formation by *WNT5A* through monolayer culture assays. HCT116 cells were transfected with pcDNA3.1 (+) *WNT5A*, pcDNA3.1 (+) *WNT5A*-SI (short isoform) or vector control, and selected with G418. Quantitative analyses of colony numbers (*right*). The number of G418-resistant colonies in each vector-transfected cell line were set to 100%. Columns, mean of at least three independent experiments; bars, SD (**, $P < 0.01$). *B,* ectopic expression of *WNT5A*, but not its short isoform (*WNT5A-SI*), reduced the intracellular β-catenin protein level in HCT116 cells (*, $P < 0.05$). *C, WNT5A* expression inhibits TOPFLASH TCF-reporter construct activity in HCT116 cells (**, $P < 0.01$). *D,* semiquantitative RT-PCR analyses of total RNA from HCT116 cells transfected with empty vector or WNT5A-expressing vector (amount of transfected plasmid DNA μg/well as indicated). Columns, mean of relative expression ratios of *CCND1* to *GAPDH*; bars, SD.

decrease of total β-catenin protein levels in HEK293 cells, colon, and thyroid carcinoma cells (16, 17, 32, 33), and that transfection of antisense *WNT5A* causes cell transformation, similar to the effect induced by the activation of the Wnt/β-catenin pathway (34). Our results further support the notion that WNT5A could serve as an antagonist to Wnt signaling, with tumor suppressor activities in certain tumors including CRC. *WNT5A* might thus be a potential epigenetic biomarker or therapeutic target for CRC.

A recent study also reported that *WNT5A* could be suppressed at the posttranscriptional level in breast cancer (35), mediated by the embryonic lethal abnormal vision–like protein HuR through its binding to the highly conserved AU-rich sequence in the 3′-untranslated region of *WNT5A* mRNA and thus inhibiting translation. The lack of *WNT5A* protein in some invasive breast tumors with high or normal levels of *WNT5A* mRNA could be due to this suppression of translation. Whether a similar suppression exists in CRC needs further investigation.

For Wnt members of the canonical signaling pathway (such as Wnt1 and Wnt3a), their expression leads to β-catenin accumulation in the cell nucleus without ubiquitination and degradation, which further activates the expression of β-catenin target genes such as *CCND1* and *c-myc* to transform cells (3, 4). Noncanonical Wnts, including Wnt4, 5a, and 11, are not thought to be involved in β-catenin/TCF-mediated transcriptional regulation, and thus, with no transforming activity (3, 4). WNT5A stimulates intracellular calcium (Ca^{2+}) flux, leading to the activation of Ca^{2+}-dependent effectors such as calcium/calmodulin-dependent kinase II, nuclear factor associated with T cells, and protein kinase C (4, 36). WNT5A could also activate other noncanonical pathways through c-*Jun*-NH_2-kinase and small Rho-GTPases (5, 6). Depending on the receptor context, *WNT5A* could either activate or inhibit β-catenin/TCF signaling (37, 38). Previous evidence suggests that *WNT5A* has growth and metastasis-enhancing properties in certain tumor types (8–10, 12, 39, 40), associated with proliferation and invasion (9). On the other hand, Wnt-5a antagonizes the canonical Wnt pathway by promoting β-catenin degradation in HEK293 cells (33). *WNT5A* expression predicts longer disease-free survival of patients with CRC (18), prevents the metastasis of invasive breast carcinoma, and its loss is associated with early relapse (19). *WNT5A*-heterozygous mice develop myeloid leukemia and B-cell lymphomas, suggesting that *WNT5A* serves as a tumor suppressor in certain circumstances (15). As shown here, *WNT5A* expression leads to the decrease of intracellular β-catenin protein levels, acting as a tumor suppressor in CRC cells (Fig. 4).

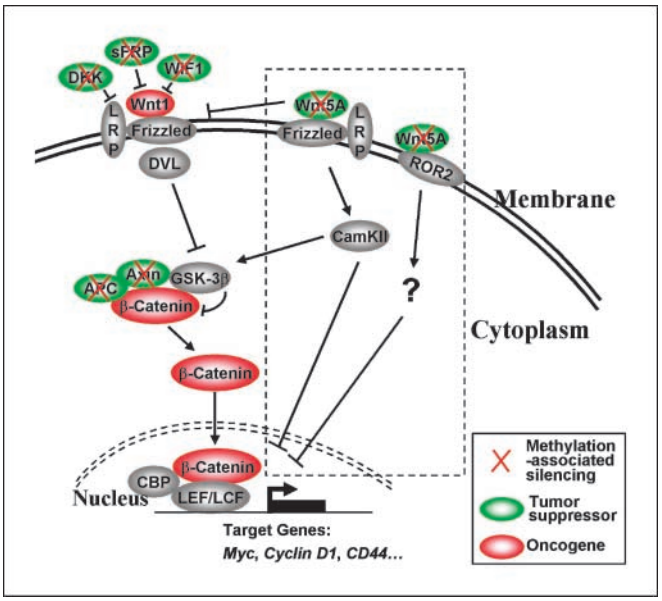

Fig. 4. Proposed model on the role of epigenetic inactivation of *WNT5A* in the activation of Wnt/β-catenin signaling pathway in CRC. CamKII, calcium/calmodulin-dependent kinase II. Dash-lined square, the work presented in this study.

Aberrant activation of Wnt/β-catenin signaling through both genetic and epigenetic mechanisms occurs in most CRC. *APC* mutation is the most frequent genetic event (6), whereas somatic mutations of β-catenin exon 3 causing protein stabilization (41, 42) and *SFRP1* mutations occur in a minority of CRCs (43). Meanwhile, epigenetic silencing of Wnt pathway components including *APC*, *SFRP*, *DKK*, and *WIF1* have frequently been reported in tumors (22, 44–46). These genetic or epigenetic changes lead to the activation of canonical Wnt signaling (Fig. 4). The noncanonical Wnt proteins (such as *WNT5A*) inhibit β-catenin stabilization (33) by activating alternative signaling pathways, or induce Ca^{2+} flux to block downstream canonical signaling by inhibiting TCF-mediated transcription (47), playing important roles in antagonizing inappropriate Wnt signaling (Fig. 4). Recently, Mikels and Nusse (38) showed that *WNT5A* inhibits Wnt3a-induced canonical Wnt signaling in a dose-dependent manner, mediated by the orphan tyrosine kinase ROR2 (Fig. 4). Thus, our results indicate a possible new way, through epigenetic inactivation of *WNT5A*, to activate the canonical and noncanonical Wnt signaling in CRC cells (Fig. 4). Meanwhile, we also detected methylation-mediated silencing of *WNT5A* in other tumors (nasopharyngeal carcinoma and lymphomas; data not shown; Ying et al., Blood, 2007, In press). In parallel, epigenetic inactivation of another Wnt member, *WNT7A*, by methylation has recently been reported in 71% of exocrine pancreatic tumors as well (48).

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