**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...
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 RPMI 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'-cagctttacaagccggcagc; and WNT5AR, 5'-cgcgctttgacctgcg; CCND1F, 5'-ttgtagagagagagaccct; and CCND1R, 5'-ggcgccggacagccc. 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 were: WNT5A-A, 5'-aatagttgtttgtgttttttttttt; or WNT5A-B, 5'-aatagttgtttgttttttta; or WNT5A-C, 5'-aatagttgtttgttttttttttta; or WNT5A-D, 5'-aatagttgtttgtttttttttttta; or WNT5A-E, 5'-aatagttgtttgttttttttttttttttttta; or WNT5A-F, 5'-aatagttgtttgtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Methylation of WNT5A in Colorectal Cancer

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 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.

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. Sh. 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).
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%; \( P = 0.025, \chi^2 \); 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 \( \beta \)-catenin degradation and down-regulates CCND1 expression.** The frequent activation of the Wnt/\( \beta \)-catenin pathway and epigenetic inactivation of WNT5A in CRC prompt us to examine whether WNT5A could counteract Wnt/\( \beta \)-catenin signaling. We determined the intracellular \( \beta \)-catenin levels before and after reexpression of WNT5A. In WNT5A-transfected cells, \( \beta \)-catenin protein levels were significantly decreased (to \( \sim 58\% \)), as compared with vector control or the nonfunctional short isoform–transfected cells (Fig. 3B). These results suggest that WNT5A directly affects the intracellular \( \beta \)-catenin level to interfere with Wnt/\( \beta \)-catenin signaling. In accordance with this down-regulation, the luciferase activity of TCF luciferase reporter construct TOPFLASH was significantly decreased (to \( \sim 36\% \)) in WNT5A-expressed, but not in control- or WNT5A short isoform–expressed cells (Fig. 3C). This result further confirmed that WNT5A directly antagonizes Wnt/\( \beta \)-catenin signaling in CRC cells. We also examined the effect of WNT5A reexpression on the expression of a \( \beta \)-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 \( \beta \)-catenin levels and the decrease of \( \beta \)-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 \( \beta \)-catenin degradation, and inhibits the clonogenicity of CRC cells. Our results are consistent with the recent findings that WNT5A expression leads to a significant
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 (Ca2+) flux, leading to the activation of Ca2+-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-NH2-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, 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 Ca2+ 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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References

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