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Frequent epigenetic inactivation of *DICKKOPF* family genes in human gastrointestinal tumors

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Activation of Wnt signaling has been implicated in tumorigenesis, and epigenetic silencing of Wnt antagonist genes has been detected in various cancers. In the present study, we examined the expression and methylation of *DICKKOPF* (*DKK*) family genes in gastrointestinal cancer cell lines. We found that all known *DKK* genes were frequently silenced in colorectal cancer (CRC) cells (*DKK1*, 3/9, 33%; *DKK2*, 8/9, 89%; *DKK3*, 5/9, 56% and *DKK4*, 5/9, 56%), but not in normal colon mucosa. *DKK1*, *-2* and *-3* have 5′ CpG islands, and show an inverse relation between expression and methylation. *DKK* methylation also was frequently observed in gastric cancer (GC) cell lines (*DKK1*, 6/16, 38%; *DKK2*, 15/16, 94% and *DKK3*, 10/16, 63%), but was seen less frequently in hepatocellular carcinoma and pancreatic cancer cell lines. *DKK*s also were frequently methylated in primary CRCs (*DKK1*, 7/58, 12%; *DKK2*, 45/58, 78% and *DKK3*, 12/58, 21%) and GCs (*DKK1*, 15/31, 48%; *DKK2*, 26/31, 84% and *DKK3*, 12/31, 39%). Against a background of *CTNNB1* or *APC* mutations, Dickkopfs (Dkks) were less effective inhibitors of Wnt signaling than secreted frizzled-related proteins, though over-expression of Dkks suppressed colony formation of CRC cells with such mutations. Our results demonstrate that *DKK*s are frequent targets of epigenetic silencing in gastrointestinal tumors, and that loss of *DKK*s may facilitate tumorigenesis through β-catenin/T-cell factor-independent mechanisms.

Introduction

The Wnt signaling pathway, which plays important roles in embryogenesis, development and homeostasis, is also closely linked to carcinogenesis (1,2). The Wnt ligands bind to Frizzled receptors and low-density lipoprotein receptor-related protein (Lrp) 5/6 co-receptors located at the plasma membrane and activate both canonical and non-canonical pathways. Activation of the canonical Wnt pathway leads to stabilization of β-catenin and its accumulation in the cytoplasm. This is followed by translocation to the nucleus, where β-catenin associates with T-cell factor (TCF)/lymphocyte enhancer factor family transcription factors to stimulate the expression of target genes. Constitutive activation of the canonical Wnt pathway resulting from mutation of *APC*, *AXIN1* and *CTNNB1* (β-catenin) has been observed in various human cancers (2). The non-canonical Wnt pathways are known to transduce signals independent of β-catenin and to include the planar cell polarity, Wnt/Ca^{2+} and c-jun N-terminal kinase pathways, yet much about their roles in cancer remains unknown (3).

Several classes of secreted Wnt antagonists are known, including the Cerberus, Wnt inhibitory factor 1 (WIF1), secreted frizzled-related protein (SFRP) and Dickkopf (Dkk) families (4). Vertebrates express four Dkk proteins (Dkk1, -2, -3 and -4) and a unique Dkk3-related protein termed Soggy (5). Dkk1 binds to Lrp5/6 and another transmembrane protein, Kremen, to selectively inhibit the canonical Wnt pathway by preventing Wnt and Frizzled from forming a ternary complex with Lrp5/6. During vertebrate development, Dkks are crucially involved in head induction, limb formation, osteoblast differentiation and eye formation (5). And in mouse small intestine and colon, forced Dkk1 expression inhibits intestinal epithelial cell proliferation (6,7).

Human *DICKKOPF* (*DKK*) genes have been implicated in tumorigenesis. For instance, ectopic expression of *DKK1* was reported to suppress HeLa cell transformation (8). *DKK1* is a target of β-catenin/TCF transcription factors (9,10), and moreover, *DKK1* was reported to be epigenetically inactivated in colorectal cancer (CRC) (11). In addition, *DKK3*, which is also known as reduced expression in immortalized cells (REIC) (12), is down-regulated in various cancers (5), and ectopic expression of *DKK3* inhibits proliferation of osteosarcoma cells (13), HeLa and hepatocellular carcinoma (HCC) cells (14) and prostate cancer cells (15,16). It also increases cell–cell adhesion and decreases the invasiveness of osteosarcoma (17) and malignant melanoma cells (18). Epigenetic inactivation of *DKK3* has been seen in various cancers, including acute lymphoblastic leukemia (19), prostate cancer (15), bladder cancer (20) and renal cell carcinoma (21).

We showed previously that silencing *SFRP* genes through methylation is frequently the mechanism by which Wnt signaling is activated in CRC and gastric cancer (GC) (22–24). Our aim in the present study was to characterize the epigenetic alteration of *DKK*s in gastrointestinal tumors and assess its functional effect. We found that all four *DKK* genes are potential targets of epigenetic silencing in CRC, though methylation of *DKK2* was particularly frequent. We also found that Dkks may inhibit tumor cell proliferation via β-catenin/TCF-independent mechanisms.

Materials and methods

Cell lines and tumor specimens

DNA was prepared from nine CRC cell lines (CaCO2, Colo320, DLD1, HCT116, HT29, LoVo, RKO, SW48 and SW480), 16 GC cell lines (MKN1, MKN7, MKN28, MKN45, MKN74, SH101, SNU1, SNU638, KatoIII, JRST, AZ521, NUGC3, NUGC4, AGS, NCI-N87 and SNU16), 10 HCC cell lines (HT17, PLC/PRF/5, Li-7, huH-1, HuH-7, HepG2, Hep3B, HLF, HLE and JHH-4) and nine pancreatic cancer cell lines (PxPC3, Capan2, CFPAC, KLM1, KP1NL, MIAPaCa, Panc-1, PK-8 and PK-9). HCT116 cells with genetic disruptions within the *DNMT1* and *DNMT3B* loci (DKO2) have been described previously (23). To analyze restoration of *DKK* genes, CRC cells were treated with 2.0 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St Louis, MO) for 72 h, replacing the drug and medium every 24 h. Isolation of specimens from 58 primary CRCs and 29 colorectal adenomas was as described previously (25). Isolation of specimens from 31 primary GCs also was as described (24). In addition, 11 CRC xenografts established in our laboratory were used to examine *DKK*s methylation and expression (26). Briefly, CRC specimens were resected and transplanted into the dorsum of severe

Abbreviations: CRC, colorectal cancer; DKK, DICKKOPF; Dkk, Dickkopf; GC, gastric cancer; HCC, hepatocellular carcinoma; Lrp, lipoprotein receptor-related protein; MSP, methylation-specific polymerase chain reaction; PCR, polymerase chain reaction; RT, reverse transcriptase; SFRP, secreted frizzled-related protein; TCF, T-cell factor; WIF1, Wnt inhibitory factor 1; 5-aza-dC, 5-aza-2′-deoxycytidine.

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combined immunedeficient mice without *in vitro* culture. When the xenografts reached ~1 cm in diameter, they were harvested and stored at –80°C until use. Informed consent was obtained from all patients before collection of the specimens. Genomic DNA was extracted using the standard phenol–chloroform procedure. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) and then treated with a DNA-free kit (Ambion, Austin, TX). Total RNA from normal colon mucosa from a healthy individual was purchased from BioChain (Hayward, CA).

Reverse transcriptase–polymerase chain reaction

Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). The integrity of the cDNA was confirmed by amplifying glyceraldehydes-3-phosphate dehydrogenase. The polymerase chain reaction (PCR) protocol entailed 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C; and a 7 min final extension at 72°C. Reverse transcriptase (RT)–PCR primer sequences for *DKK1* were 5′-CTTTCTCCCTCTTGAGTCCTTCTG-3′ (forward) and 5′-CATAGCGTGACGCATGCAGCGTT-3′ (reverse); primers for *DKK2* were 5′-GCAGTGATAAGGAGTGTGAAGTT-3′ (forward) and 5′-AATGCAGTCTGATGATCGTAGGC-3′ (reverse); primer sequences for *DKK3* were 5′-AGGCAGAAGAAGCTGCTGCTAA-3′ (forwad) and 5′-AGCTGGTCTCCACAGCACTCACT-3′ (reverse); primers for *DKK4* were 5′-ACGGACTGCAATACCAGAAAGTT-3′ (forward) and 5′-CAAAGTCCAGGGCCACAGTCAA-3′ (reverse). RT–PCR primers for glyceraldehydes-3-phosphate dehydrogenase were as described previously (24).

Real-time RT–PCR

Real-time RT–PCR was carried out using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. SDS2.2.2 software (Applied Biosystems) was used to perform comparative delta Ct analysis. glyceraldehydes-3-phosphate dehydrogenase served as endogenous controls.

Methylation analysis

Bisulfite treatment of genomic DNA was performed as described (22). Methylation was analyzed by using methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing. PCR was run in a volume of 25 µl containing 50 ng bisulfite-treated DNA, 1× MSP buffer [67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$ and 10 mM 2-mercaptoethanol], 1.25 mM deoxynucleoside triphosphate, 0.4 µM each primer and 0.5 U of JumpStart REDTaq DNA polymerase (Sigma). The PCR protocol for MSP entailed as follows: 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C and a 7 min final extension at 72°C. MSP primers for unmethylated *DKK1* were 5′-TTAAGGGGTTGGAATGTTTTGGGTTTGT-3′ (forward) and 5′-AAACCTAAATCCCCACAAAACCATACCA-3′ (reverse); MSP primers for methylated *DKK1* were 5′-AGGGGTCGGAATGTTTCGGGTTCGC-3′ (forward) and 5′-CCTAAATCCCCACGAAACCGTACCG-3′ (reverse); MSP primers for unmethylated *DKK2* were 5′-TGTTTTTTAGGTATTGTTGTGTTGGTAGT-3′ (forward) and 5′-ATAAAAAATCAAAAAACATCCCCAAACCA-3′ (reverse); MSP primers for methylated *DKK2* were 5′-TTTTTAGGTATCGTTGCGTTGGTAGC-3′ (forward) and 5′-AAAATCAAAAAACGTCCCCGAACCG-3′ (reverse); MSP primers for unmethylated *DKK3* were 5′-GAGTGAGTAGATTTAGTTTGGTTTGTAGT-3′ (forward) and 5′-CCCCTTAACATCAAATCCTACTCAAACA-3′ (reverse); MSP primers for methylated *DKK3* were 5′-CGAGTAGATTTAGTTCGGTTCGTAGC-3′ (forward) and 5′-CTTAACGTCGAATCCTACTCGAACG-3′ (reverse). The PCR protocol for bisulfite sequencing entailed as follows: 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C and a 7 min final extension at 72°C. Bisulfite-sequencing PCR primers for *DKK1* were 5′-GYGGGGTGAAGAGTGTTAAAGGTTT-3′ (forward) and 5′-GTCACTTTACAAACCTAAATCCCCAC-3′ (reverse); primers for *DKK2* were 5′-TGGGAAGTTATAAAAAGTAAAGAGGAT-3′ (forward) and 5′-ACAATACTCCTTTTCAAAATTAACAAAC-3′ (reverse); primers for *DKK3* were 5′-GGGGGYGGAGAGGGAGTTTGGTG-3′ (forward) and 5′-CCTAAAATAAACCAAACACAAATCAAC-3′ (reverse); primers for *DKK4* were 5′-ATAGATTTGAAGGGATTTGTTGAAGTTT-3′ (forward) and 5′-CAAAACCAACTCAACCCCAACAAAAC-3′ (reverse). Amplified bisulfite-sequencing PCR products were cloned into pCR2.1-TOPO vector (Invitrogen) and 10–12 clones for each sample were sequenced by using an ABI3100 automated sequencer (Applied Biosystems).

Promoter reporter assay

Upstream regions of *DKK1* and *DKK2* were PCR amplified by using genomic DNA from HEK293 cells as templates. PCR was run in a volume of 50 µl containing 50 ng DNA, 1× PCRx Amplification buffer (Invitrogen), 0.2 mM deoxynucleoside triphosphate, 1.5 mM $MgSO_4$, 0.2 µM each primer, 1× PCRx Enhancer Solution (Invitrogen) and 2.5 U of Platinum *Taq* DNA polymerase (Invitrogen). Primer sequences for *DKK1* promoter region 1 were 5′-CCGCTCGAGGGTTTCTTATTTCTCCACATT-3′ (forward) 5′-CCCAAGCTTTTGATGGGAAGTTTAGAGAGG-3′ (reverse); primers for *DKK1* promoter region 2 were 5′-CCGCTCGAGGGTTTCTTATTTCTCCACATT-3′ (forward) 5′-CCCAAGCTTAGCAGGAACTCTGGGAACTTG-3′ (reverse); primers for *DKK1* promoter region 3 were 5′-CCGCTCGAGGGTTTCTTATTTCTCCACATT-3′ (forward) 5′-CCCAAGCTTTGACCGTCACTTTGCAAGCCT-3′ (reverse); primers for *DKK1* promoter region 4 were 5′-CCGCTCGAGCCTCTCTAAACTTCCCATCAA-3′ (forward) 5′-CCCAAGCTTAGCAGGAACTCTGGGAACTTG-3′ (reverse); primers for *DKK1* promoter region 5 were 5′-CCGCTCGAGCCTCTCTAAACTTCCCATCAA-3′ (forward) 5′-CCCAAGCTTTGACCGTCACTTTGCAAGCCT-3′ (reverse); primers for *DKK1* promoter region 6 were 5′-CCGCTCGAGCAAGTTCCCAGAGTTCCTGCT-3′ (forward) 5′-CCCAAGCTTTGACCGTCACTTTGCAAGCCT-3′ (reverse); primers for *DKK2* promoter region 1 were 5′-GGGGTACCAAGACCCAAATCGAACAGGCT-3′ (forward) 5′-CGACGCGTCTAATTCCTGACAACAACTCC-3′ (reverse); primers for *DKK2* promoter region 2 were 5′-GGGGTACCAAGACCCAAATCGAACAGGCT-3′ (forward) 5′-CGACGCGTCACAAGAGACAGCGAATCGCT-3′ (reverse); primers for *DKK2* promoter region 3 were 5′-GGGGTACCAAGACCCAAATCGAACAGGCT-3′ (forward) 5′-CGACGCGTAGGTGCAAGAAAACCCAGCCC-3′ (reverse); primers for *DKK2* promoter region 4 were 5′-GGGGTACCGGAGTTGTTGTCAGGAATTAG-3′ (forward) 5′-CGACGCGTCACAAGAGACAGCGAATCGCT-3′ (reverse); primers for *DKK2* promoter region 5 were 5′-GGGGTACCAGCGATTCGCTGTCTCTTGTG-3′ (forward) 5′-CGACGCGTAGGTGCAAGAAAACCCAGCCC-3′ (reverse). Amplified PCR products were cloned into pCR2.1-TOPO (Invitrogen). Each forward PCR primer for *DKK1* carried a 5′ overhang that contained an XhoI recognition site, whereas the reverse primer contained a HindIII site. Each forward PCR primer for *DKK2* carried a 5′ overhang that contained a KpnI recognition site, whereas the reverse primer contained a MluI site. After verifying the sequences, *DKK1* promoter fragments were cut using XhoI and HindIII and *DKK2* fragments were cut using KpnI and MluI and were ligated into pGL3-Basic (Promega, Madison, WI). Cells (5×10^4 cells per well in 24-well plates) were then transfected with 100 ng of one of the reporter plasmids and 2 ng of pRL-TK (Promega) using Lipofectamine 2000 (Invitrogen). A pGL3-Basic vector without an insert served as a negative control. Luciferase activities were measured 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Expression vectors

Full-length *DKK* cDNAs were PCR amplified by using cDNA derived from HEK293 cells as templates. PCR was run in a volume of 50 µl containing 1× PCRx Amplification buffer (Invitrogen), 0.2 mM deoxynucleoside triphosphate, 1.5 mM $MgSO_4$, 0.2 µM each primer, 1× PCRx Enhancer Solution (Invitrogen) and 2.5 U of Platinum *Taq* DNA polymerase (Invitrogen). Primer sequences for *DKK1* were 5′-TGATGATGGCTCTGGGCGCAGCG-3′ (forward) and 5′-TAGCTGGTTTAGTGTCTCTGACAA-3′ (reverse); primers for *DKK2* were 5′-TGATGGCCGCGTTGATGCGGAG-3′ (forward) and 5′-CCTCAATGGTGATCAAATTTTCTG-3′ (reverse); primers for *DKK3* were 5′-TGATGCAGCGGCTTGGGGCCAC-3′ (forward) and 5′-CCTGGTCCAGATCTAAATCTCTTC-3′ (reverse). Amplified PCR products were cloned into pCR2.1-TOPO (Invitrogen), and after the sequences were verified, fragments were cut using EcoRI and ligated into EcoRI-digested pcDNA3.1/HisA (Invitrogen). Expression constructs of SFRPs, Wnt1, β-catenin, β-cateninΔ45, a mutant form of β-catenin which lacks Ser 45, were prepared as described (23).

β-Catenin/TCF reporter assay

Reporter assays with pGL3-OT (a β-catenin/TCF-responsive luciferase reporter plasmid) and pGL3-OF (a negative control plasmid) were carried out as described previously (23). Briefly, HEK293 cells (5×10^4 cells per well in 24-well plates) were transfected with 100 ng of pGL3-OT or pGL3-OF; 50 ng of pcDNA3.1-Wnt1, pcDNA3.1-β-catenin, pcDNA3.1-β-catenin Δ45 or an empty vector; 50 ng of one of the Dkk or SFRP expression vectors or an empty vector and 2 ng of pRL-CMV using Lipofectamine 2000 (Invitrogen). Firefly and renilla luciferase activities were measured 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Colony formation assay

Colony formation assay was performed as described (26). Briefly, cells (2×10^6 cells) were transfected with 5 µg of one of the pcDNA3.1His-Dkk vectors or with an empty vector using a Cell Line Nucleofector kit V (Amaxa, Cologne, Germany) and a Nucleofector I electroporation device (Amaxa) according to the manufacturer's instructions. Cells were then plated on 60 mm culture dishes and selected for 14 days with 0.6 mg/ml G418. Colonies were

stained with Giemsa and counted using National Institute of Health IMAGE software.

Western blotting

Cells were transfected with one of the Dkk expression vectors or with an empty vector as described above. Cells were harvested 48 h after transfection, and samples (20 μg) of the cell lysate were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, after which the resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were then blocked with 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline and probed with anti-Xpress antibody (Invitrogen), after which the blots were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Immunofluorescence microscopy

Cells were transfected with Dkk expression vectors, as described above, and cultured on chamber slides for 48 h. Cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde, after which they were incubated with anti-Xpress antibody (Invitrogen) and stained with anti-mouse IgG Alexa Fluor 488 (Invitrogen). Cells were examined using an FV300-IX71 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Flow cytometry

Fluorescence-activated cell sorting analysis was carried out as described previously (26). Briefly, cells were transfected with one of the Dkk or SFRP vectors or with an empty vector as described above. Cells were harvested 48 h after transfection, fixed with methanol, rehydrated with phosphate-buffered saline, treated with 2 μg/ml RNase for 30 min at 37°C, stained in propidium iodide solution (50 μg/ml) and analyzed using a FACSCalibur instrument (Becton Dickinson, San Jose, CA).

Results

Analysis of DKK expression in CRC cell lines

To test whether *DKK* family genes are epigenetically silenced in CRC, we first performed RT–PCR analysis with a set of CRC cell lines. We found that expression of *DKK1* mRNA was completely absent in three of nine CRC cell lines (DLD1, RKO and SW48) and is down-regulated in Colo320 (Figure 1). *DKK2* mRNA was absent or barely detectable in eight cell lines (CaCO2, DLD1, HCT116, HT29, LoVo, RKO, SW48 and SW480) (Figure 1). *DKK3* was absent or significantly down-regulated in five cell lines (DLD1, HCT116, HT29, RKO and SW48), and *DKK4* was absent in five cell lines (Colo320, DLD1, LoVo, RKO and SW48) (Figure 1). In many of the cells, in which *DKK*s were down-regulated, treatment with the DNA methyltransferase inhibitor 5-aza-dC rapidly restored mRNA expression, which is indicative of epigenetic silencing of the genes through methylation (Figure 1). We also analyzed HCT116 cells in which the DNA methyltransferase genes *DNMT1* and *DNMT3B* were genetically disrupted (DKO2 cells), and thus DNA methylation was abrogated (23). Those cells showed substantially greater *DKK2* and *-3* expression than the parental HCT116 cells (Figure 1). In contrast to the cancer cells, all of the *DKK* genes were expressed in normal colon mucosa from a healthy individual (Figure 1). These results strongly suggest that in CRC, *DKK* genes are frequent targets of epigenetic silencing through methylation.

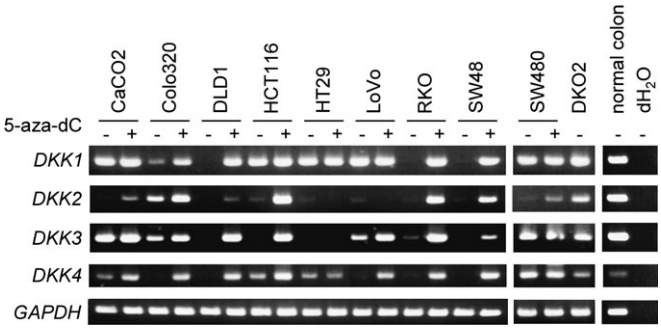

Fig. 1. RT–PCR analysis of *DKK1*, *-2*, *-3* and *-4* expression in the indicated CRC cell lines, with or without treatment with 5-aza-dC, and in normal colon mucosa. RT–PCR with glyceraldehydes-3-phosphate dehydrogenase was carried out for all samples to ensure the cDNA quality; dH_2O indicates no RNA added.

Analysis of DKK methylation in cancer cell lines

Of the four *DKK* genes, *DKK1*, *-2* and *-3* contain CpG islands in the region around their transcription start sites. We therefore designed MSP primers and bisulfite-sequencing primers for these genes (supplementary Figure 1 is available at *Carcinogenesis* Online). MSP analysis revealed that *DKK1* is completely methylated in three CRC cell lines (DLD1, RKO and SW48) that did not show *DKK1* expression unless treated with 5-aza-dC (Figures 1 and 2A); that *DKK2* was methylated in all of the CRC cell lines tested, even the Colo320 cell line, which showed basal expression that was up-regulated by 5-aza-dC (Figures 1 and 2A); and that *DKK3* is methylated in five cell lines (DLD1, HCT116, HT29, RKO and SW48) that showed little or no expression of the gene (Figures 1 and 2A). Thus, for three *DKK* genes there was an inverse relation between expression and methylation. DKO2 cells, which served as a negative control for DNA methylation, did not show methylation of any of the *DKK* genes (Figure 2A).

In selected samples, we verified the MSP results using bisulfite sequencing. Sequencing analysis revealed that the CpG islands of *DKK1*, *-2* and *-3* are extensively methylated in CRC cell lines in which methylation was detected by MSP (Figure 2B–D). In contrast, no *DKK1* CpG methylation was detected in HCT116 cells, which expressed *DKK1* mRNA and showed no methylation with MSP (Figure 2B). The CpG islands of *DKK2* and *-3* were extensively methylated in HCT116 cells, but almost no methylation was seen in DKO2 cells (Figure 2C and D).

Mapping *DKK4* mRNA (Acc. No. NM_014420) to a human genome database (http://genome.ucsc.edu) revealed no CpG island associated with the gene. Thus, we designed a bisulfite-sequencing primer set to analyze a region encompassing the transcription start site of *DKK4*, which did not satisfy the criteria for a CpG island (i.e. C + G content >60% and CpG-to-GpC ratio >0.6), and analyzed three CRC cell lines that showed different basal levels of *DKK4* expression: DLD1 and HCT116 cells expressed little or no *DKK4* mRNA, whereas SW480 cells showed substantial basal expression (Figure 1). Bisulfite sequencing revealed that the region around the transcription start site was methylated in all of the CRC cell lines tested, regardless of the expression status. This prompted us to conclude that methylation around the transcription start site of *DKK4* is most probably not related to transcriptional silencing.

To further investigate methylation of *DKK* genes in cancers derived from other tissue types, we used MSP to test 16 GC, 10 HCC and nine pancreatic cancer cell lines. We detected *DKK1* methylation in six GC cancer cell lines and one pancreatic cancer cell line; *DKK2* methylation in 15 GC, three HCC and four pancreatic cancer cell lines; and *DKK3* methylation in 10 GC, one HCC and two pancreatic cancer cell lines (representative results in Figure 2A; Table I; supplementary Table 1 is available at *Carcinogenesis* Online). Collectively, these results show that *DKK* methylation occurs more frequently in gastrointestinal cancers than in HCC or pancreatic cancers and that, among the *DKK* genes, *DKK2* is the most frequently methylated in all of the cancer types analyzed (Table I).

Analysis of DKK promoters

One possible explanation for the observed tumor-specific absence of *DKK* mRNA expression is the inability of the cells to support *DKK* transcription due to the loss of one or more critical transactivation factors or the expression of repressor factors. To assess this possibility, we constructed a set of luciferase reporter vectors that contained various portions of regions upstream of *DKK1* and *-2* (Figure 3A and C) and then carried out luciferase assays in two CRC cell lines (DLD1 and HCT116) in which one or both of the genes was silenced and methylated.

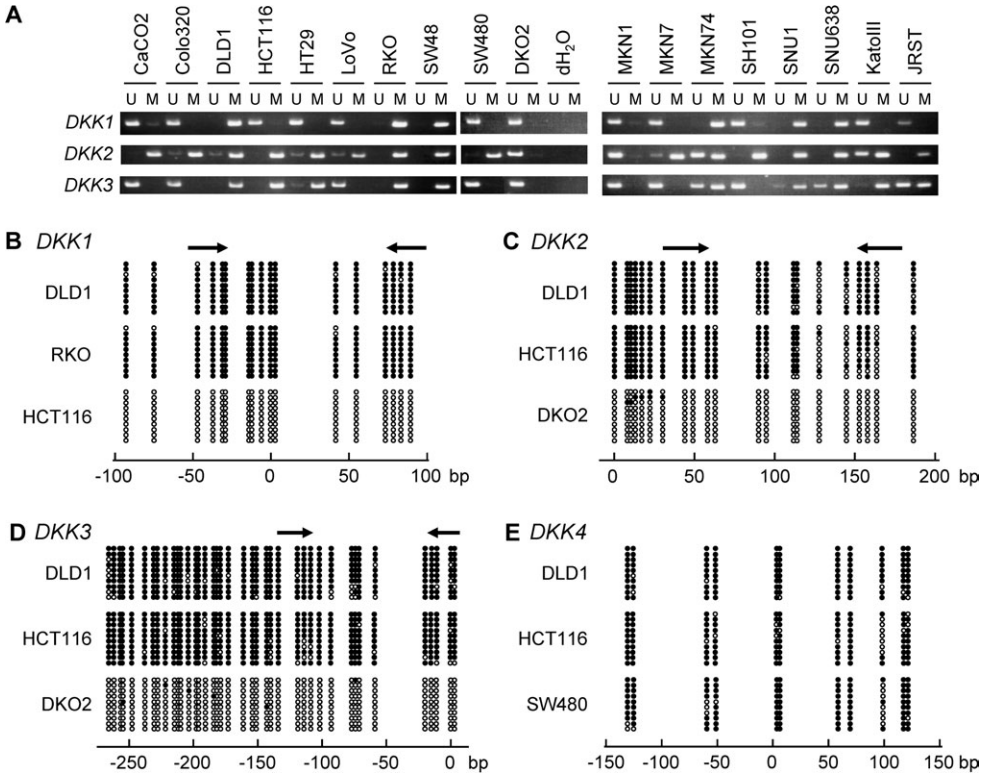

Fig. 2. Analysis of *DKK*s methylation in CRC and GC cell lines. (**A**) Results of MSP analysis of the indicated CRC and GC cell lines. Bands in the 'M' lanes are PCR products obtained with methylation-specific primers; those in the 'U' lanes are products obtained with unmethylated-specific primers; dH_2O indicates no DNA added. (**B**) Bisulfite sequencing of *DKK1* in the indicated CRC cell lines. Open and filled circles represent unmethylated and methylated CpG sites and gray circles represent partially methylated CpG sites. The locations of MSP primer sites are shown by arrows on the top. The location of each CpG site relative to the transcription start site is shown below. (**C**–**E**) Bisulfite sequencing of *DKK2* (C), *-3* (D) and *-4* (E).

Table I. Summary of *DKK* methylation in cancer cell lines and primary tumors

	DKK1 (%)	*DKK2* (%)	*DKK3* (%)
Cell lines			
Colorectal	3/9 (33)	9/9 (100)	5/9 (56)
Stomach	6/16 (38)	15/16 (94)	10/16 (63)
Hepatocellular	0/10 (0)	3/10 (30)	1/10 (10)
Pancreatic	1/9 (11)	4/9 (44)	2/9 (22)
Primary tumors			
CRC	7/58 (12)	45/58 (78)	12/58 (21)
Colorectal adenoma	1/29 (3)	24/29 (83)	7/29 (24)
GC	15/31 (48)	26/31 (84)	12/31 (39)

Earlier studies have shown that the *DKK1* promoter contains multiple TCF-binding elements and that *DKK1* is a target of β-catenin/TCF (9,10) (Figure 3A). For our reporter assay, we selected DLD1 and HCT116 cells, in which Wnt signaling is constitutively activated due to mutation of *CTNNB1* or *APC*, respectively. *DKK1* is methylated and silenced in DLD1 cells, but is unmethylated and expressed in HCT116 cells. Following transient transfection of the two cell lines, we observed high levels of luciferase activity with the pGL3-DKK1-P3, -P5 and -P6 vectors, which contain a high-density CpG region and two TCF-binding elements near the transcription start site (Figure 3B). Similarly, luciferase vectors containing *DKK2* promoter sequences encompassing the region of the transcription start site showed strong promoter activity (Figure 3D). We conclude from these findings that CRC cells retain the ability to support promoter-driven *DKK* transcription and that the absence of *DKK* expression is strongly associated with DNA methylation within the promoter-associated CpG islands, not with the loss of critical transactivation factors or the expression of repressor factors.

Analysis of DKK methylation in primary tumors

As we frequently found *DKK* methylation in cultured CRC and GC cell lines, we next attempted to determine the extent to which *DKK*s are aberrantly methylated in primary CRC and GC. Using MSP, we examined specimens from 58 primary CRCs, 29 colorectal adenomas and 31 primary GCs, as well as adjacent specimens of normal colon mucosa from 34 of 58 CRC patients (Table I; representative results in Figure 4A). Methylation of *DKK1*, *-2* and *-3* was detected in seven (12%), 45 (78%) and 12 (21%) of the primary CRCs, respectively. *DKK*s were methylated in a tumor-specific manner or tumor-predominant manner, and little or no methylation was detected in adjacent normal colon tissues (Figure 4). In colorectal adenomas, *DKK2* and *-3* were methylated at frequencies similar to those in primary CRCs (*DKK2*, 24/29, 83% and *DKK3*, 7/29, 24%), suggesting that methylation of these genes is an early event in colorectal carcinogenesis. In contrast, *DKK1* was methylated in only one of 29 (3%) adenomas. We also found significant frequencies of *DKK*s methylation in primary GCs (*DKK1*, 15/31, 48%; *DKK2*, 26/31, 84%; *DKK3*, 12/31, 39%).

We next compared the methylation status of each *DKK* gene and the clinicopathological features of primary CRCs, but there were no statistically significant correlations between the methylation status and age, tumor location, pT status, pN status, pM satus and tumor stage (data not shown). We also compared *DKK* methylation and clinicopathological features of primary GCs, but there were no significant correlations between the methylation status and age, macroscopic type, histology, pT status, pN status, pM satus and tumor stage (data not shown).

We carried out bisulfite sequencing in a selected pair of CRC tumor specimen and corresponding normal colon mucosal tissue to examine

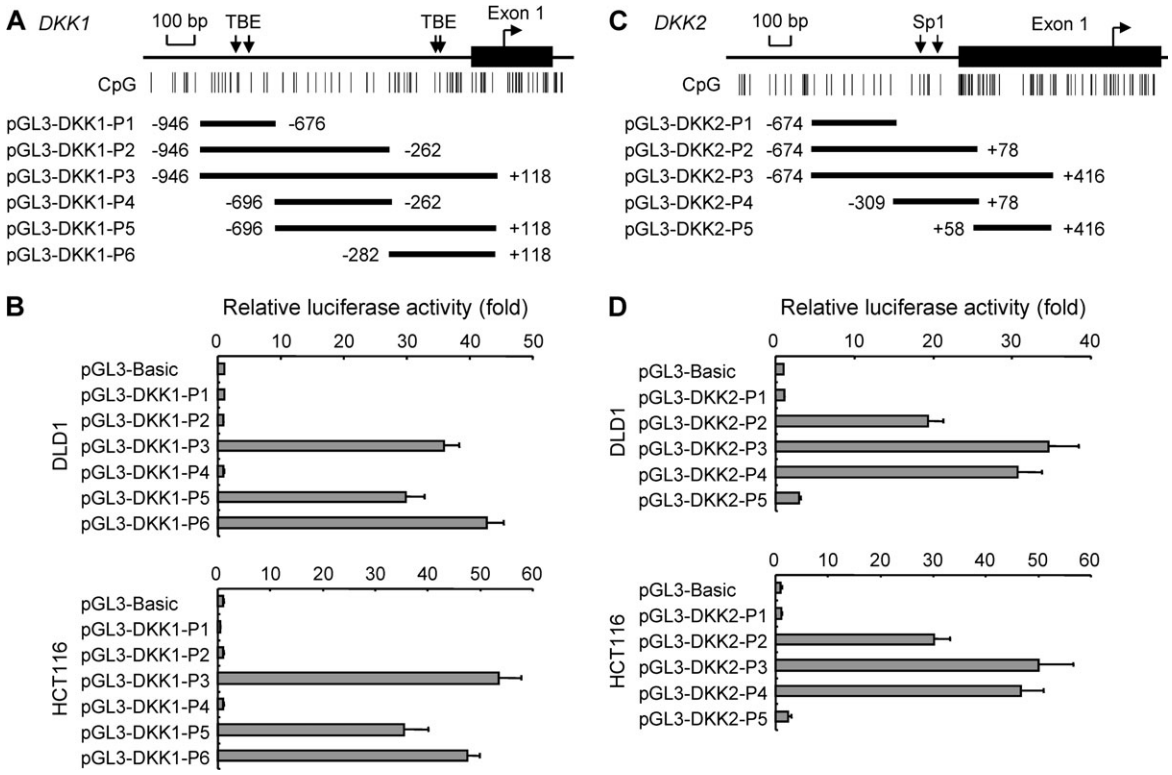

Fig. 3. Analysis of *DKK1* and *DKK2* promoter activity in CRC cells. (**A**) Diagram of the 5′ CpG island of *DKK1*. Various portions of the *DKK1* 5′-flanking region shown as bars below the CpG sites were amplified by PCR and cloned into pGL3-basic vector. Locations of TCF-/lymphocyte enhancer factor-binding elements (TBEs) are also shown. (**B**) The indicated reporter vectors were transiently transfected into DLD1 and HCT116 cells. Luciferase activity is shown for each construct expressed relative to the activity of the pGL3-basic vector after correction for transfection efficiency using the renilla luciferase activity. Shown are the means of four replications; the error bars represent standard deviations. Cell lines are indicated on the left. (**C**) Diagram of the 5′ CpG island of *DKK2* and portions of the *DKK2* promoter that were cloned as in (A). Locations of Sp1-binding sites are also shown. (**D**) Results of luciferase assays carried out using *DKK2* promoter reporter constructs in DLD1 and HCT116 cells.

the methylation status of *DKK*s in more detail. In the normal colon tissue, a small number of alleles showed some spotty methylation, while the majority of alleles were completely unmethylated (Figure 4B). The tumor tissue showed a mixture of entirely methylated alleles and partially and unmethylated alleles, probably due to contamination by normal cells (Figure 4B).

Because the primary tumors we analyzed were not microdissected, unmethylated alleles derived from contaminating normal colon mucosa were also present. Therefore, we used CRC xenografts, which are relatively pure, to additionally examine the role of *DKK* methylation in gene silencing in tumor tissues. Xenografts that showed no or only partial methylation of *DKK*s expressed their mRNA to various degrees (supplementary Figure 2 is available at *Carcinogenesis* Online). In contrast, expression of *DKK*s was significantly decreased or undetectable by real-time RT–PCR in xenografts that showed complete methylation of the genes (supplementary Figure 2 is available at *Carcinogenesis* Online). Thus, these results confirm that the methylation-dependent silencing of *DKK* genes is not a cell line-specific event.

Functional analysis of Dkks

To examine the effect of Dkks on β-catenin/TCF transcriptional activity, we used a β-catenin/TCF-responsive reporter (pGL3-OT) to analyze the functional interaction between Wnt1 or β-catenin and Dkks. In HEK293 cells, over-expression of Wnt 1 increased pGL3-OT reporter activity ∼8-fold (Figure 5A). The signaling induced by Wnt1 was suppressed to near basal levels by co-transfection of SFRP1, SFRP2 or SFRP5 (Figure 5A), which is consistent with our earlier findings (23), and it was also inhibited by Dkk1, -2 and, to a lesser extent, Dkk3 (Figure 5A). We then examined the interaction between wild-type or mutant β-catenin and Wnt antagonists in HEK293 cells. Exogenous wild-type β-catenin induced stronger reporter activity than Wnt1 (Figure 5B), but the activity was suppressed by both SFRPs and Dkks (Figure 6B). In contrast, although SFRPs also inhibited the activity induced by mutant β-catenin (β-cateninΔ45), Dkks were less effective (Figure 5C). Moreover, Dkks could not inhibit β-catenin/TCF-induced reporter activity in CRC cells showing constitutive Wnt signaling due to *APC* mutations (DLD1 and SW480) (data not shown).

Finally, to test whether over-expression of Dkk would suppress CRC cell growth, we carried out colony formation assays after transfecting each Dkk vector into DLD1 cells, which respectively harbor *APC* mutation, and RKO cells, in which both *APC* and *CTNNB1* are intact. Expression of exogenous Dkks was verified by western blot analysis and fluorescent immunocytochemistry (supplementary Figure 3 is available at *Carcinogenesis* Online). After selection with G418 for 2 weeks, we found that introduction of Dkks markedly suppressed colony formation in the cell lines tested (Figure 5D and E), which suggests Dkks may inhibit cell growth through β-catenin/TCF-independent mechanisms. We also observed that ectopic expression of Dkk2 and Dkk3 suppressed colony formation of HCT116 cells, in which *CTNNB1* is mutated (data not shown). Fluorescence-activated cell sorting analysis confirmed induction of apoptosis in DLD1 cells with ectopic Dkks expression, although incidences of apoptosis were lower than that in cells transfected with SFRP2 (Figure 5F).

Discussion

There is increasing evidence that the epigenetic silencing of genes encoding secreted Wnt antagonists plays an important role in the

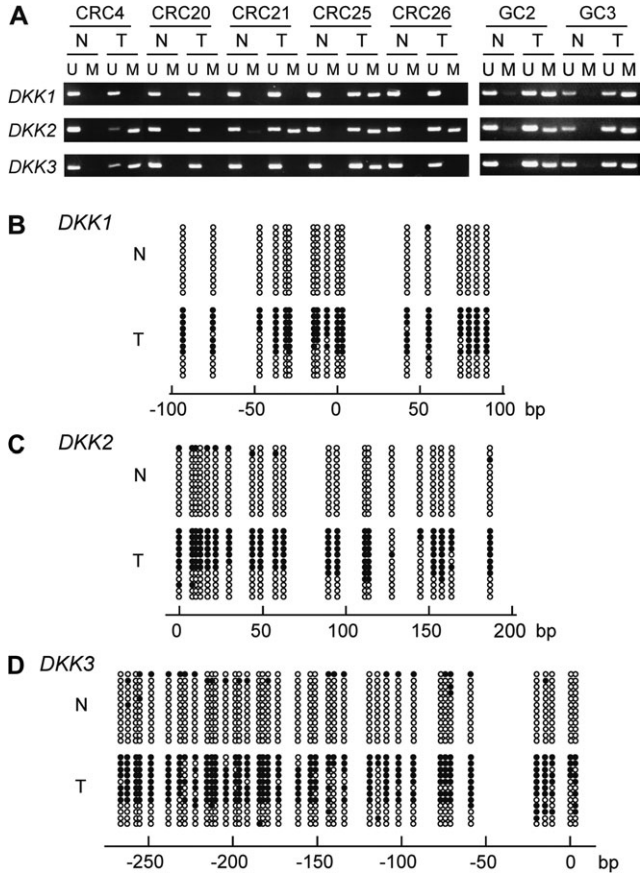

Fig. 4. Analysis of *DKK* methylation in primary CRCs and GCs. (**A**) Representative results of MSP analysis of *DKK1*, *-2* and *-3* in primary CRC and GC tissues (T) and adjacent non-tumorous colon or gastric mucosa from the same patients (N). (**B**–**D**) Results of bisulfite sequencing of *DKK1* (B), *-2* (C) and *-3* (D) in a CRC tumor (CRC25-T) and the corresponding adjacent non-tumorous mucosa tissue (CRC25-N).

development and progression of various cancers. In particular, *SFRPs* and *WIF1* are frequently silenced by methylation in gastrointestinal cancers (22,27). Moreover, restoration of SFRP1, SFRP2 and SFRP5 expression in CRC and GC cells attenuates Wnt signaling, even in the presence of downstream mutations (23,24). We now report that all four *DKK* genes are potential targets of epigenetic silencing in gastrointestinal cancers. Recently, Aguilera *et al.* (11) reported that *DKK1* is methylated at a relatively low frequency (17%) in CRC. Interestingly, although *SFRPs* and *WIF1* are methylated in a wide spectrum of CRCs, *DKK1* was found to be methylated only in advanced tumors (11). In the present study, we obtained similar results for *DKK1*, which was methylated in 12% of primary CRCs (7/58) and was rarely methylated in colorectal adenomas (1/29, 3%). *DKK2*, *-3* and *-4* were silenced more frequently than *DKK1* in CRC cell lines, and inverse relations between expression and methylation of 5′ CpG islands were seen in all of the *DKK*s except *DKK4*, which lacked a typical CpG island in its 5′ region. *DKK2* methylation was particularly frequent in both cultured and primary CRCs; in fact, *DKK2* was most the commonly methylated of *DKK*s in all tumor types. We also found that *DKK2* and *-3* were methylated in colorectal adenomas as frequently as in primary CRCs, suggesting that methylation of these genes is an early event during colorectal carcinogenesis.

In addition, we detected *DKK* methylation in GC, HCC and pancreatic cancer cell lines and in primary GC tumors. In particular, we found frequent *DKK* methylation in both cultured (*DKK1*, 6/16, 38%; *DKK2*, 15/16, 94% and *DKK3*, 10/16, 63%) and primary GCs (*DKK1*, 15/31, 48%; *DKK2*, 26/31, 84% and *DKK3*, 12/31, 39%). That *SFRPs* and *WIF1* also are methylated in approximately 80–90% of CRC and GC tumors (22,24,27) means the majority of gastrointestinal tumors probably harbor simultaneous methylation of all three families of secreted Wnt antagonists, which is indicative of the essential role played by Wnt signaling in gastrointestinal tumorigenesis.

Our RT–PCR results indicate that *DKK4* is epigenetically silenced in cancer cells, even though the gene does not have a 5′ CpG island. Similar results have been observed for other genes as well. Using microarray analysis, we showed previously that treating cancer cells with 5-aza-dC and Trichostatin A leads to up-regulation of a number of genes, including some without a 5′ CpG island (22). There are several possible explanations for this. First, an unidentified transcriptional regulatory region may be located further upstream. Second, instead of DNA methylation, histone modifications, such as H3-K9 and H3-K27 methylation, may be responsible for the gene silencing (28). Third, the up-regulation induced by 5-aza-dC may be a secondary effect of induction of transcription factors that are epigenetically silenced in cancer cells. This third possibility is supported by the finding of simultaneous epigenetic silencing of the transcription factor genes *GATA4* and *-5* and their target genes in CRC and GC (29).

The reason for the variation in the frequency with which *DKK*s are methylated is unclear, but it may reflect the functional differences among Dkk proteins. Earlier studies showed that Dkk1 is a pure inhibitor of Wnt signaling, whereas Dkk2 can both activate and inhibit the pathway (5). And in contrast to Dkk1 and -2, Dkk3 neither binds Lrp6 nor significantly affects Wnt signaling (5). Kawano *et al.* (16) showed that Dkk3 does not affect the levels of β-catenin protein or inhibit β-catenin/TCF-induced transcription in prostate cancer cells. On the other hand, there are conflicting results regarding the inhibitory effect of Dkk3 on Wnt signaling. Caricasole *et al.* (30) reported that Dkk3 inhibits Wnt7a-induced canonical signaling in PC12 cells, while Hoang *et al.* (17) reported that Dkk3 reduces cytoplasmic accumulation of β-catenin in Saos2 cells. We found that whereas Dkk1 and Dkk2 can inhibit β-catenin/TCF transcription induced by Wnt1 in HEK293 cells, Dkk3 is a weaker inhibitor. Nonetheless, when we, respectively, co-transfected cells with a wild-type β-catenin expression vector and each Dkk construct, we found that all of the Dkks exerted inhibitory effects. Thus, Dkk3 may suppress β-catenin via a different mechanism than Dkk1 and -2. In comparison with SFRPs, however, Dkks had only limited effect against mutant β-catenin, and ectopic expression of Dkks did not inhibit transcription mediated by β-catenin/TCF in cancer cells with downstream mutations. The reason for this difference is unclear, though one possible explanation is that inhibition by SFRPs depends on their ability to directly inhibit the binding of Wnt to Frizzled, which cannot be mimicked by Dkks (11). Still, ectopic expression of any of the Dkks suppressed colony formation of CRC cells.

Recent findings suggest that the tumor-suppressive function of Dkks and other Wnt antagonists may be, at least in part, independent of the Wnt/β-catenin pathway. For instance, Mikheev *et al.* (8) reported that Dkk1 suppressed the tumorgenicity of HeLa cells and that the effect did not reflect inhibition of β-catenin/TCF transcription. And in β-catenin-deficient mesothelioma cells, Dkk1 suppressed cell growth and induced apoptosis that was suppressed by a c-jun N-terminal kinase inhibitor (31). The same group also demonstrated that inhibition of Wnt1 or disheveled in mesothelioma cells using short interfering RNA also induced apoptosis that could be suppressed by a c-jun N-terminal kinase inhibitor (32). In addition, He *et al.* (33) showed that *SFRP4* is silenced in the same mesothelioma cells and re-expression of the gene induced apoptosis and suppressed cell growth. Similarly, Lodygin *et al.* (15) showed that ectopic expression of SFRP1 or Dkk3 inhibited colony formation of prostate cancer cells, in which both of genes were methylated, but canonical Wnt signaling was not activated. They also demonstrated that Dkk3 down-regulated the phosphorylation of extracellular signal-regulated kinase 1 and extracellular signal-regulated kinase 2 in the prostate cancer cells. These results strongly suggest that non-canonical Wnt signaling pathways also play critical roles in tumorigenesis.

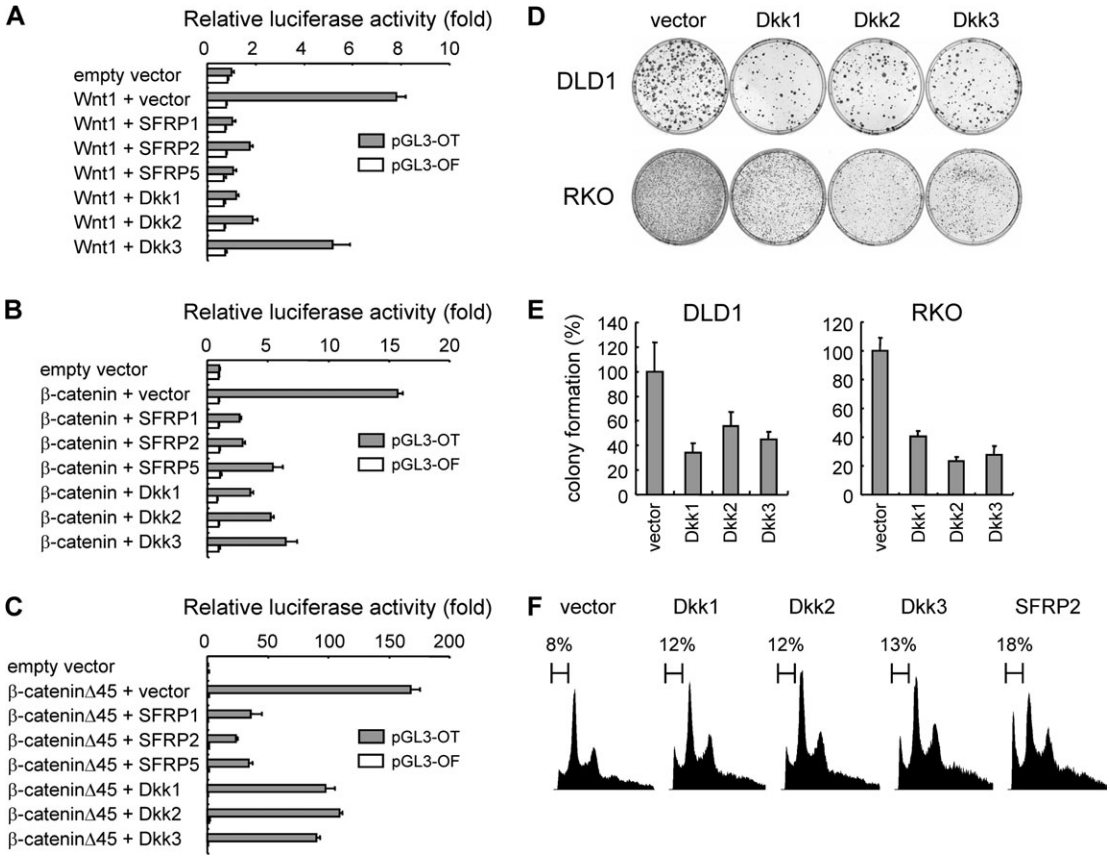

Fig. 5. Functional analysis of Dkks. (**A**) Relative luciferase activity obtained using a β-catenin/TCF-responsive reporter (pGL3-OT) and a negative control (pGL3-OF) in HEK293 cells transfected with a Wnt1 expression vector and the indicated SFRP or Dkk expression construct. Results are shown relative to a value of 1 (assigned to cells transfected with an empty vector) after correction for transfection efficiency using renilla luciferase activity. Shown are means of four replications; error bars represent standard deviations. (**B**) Results in HEK293 cells transfected with a wild-type β-catenin expression vector and the indicated SFRP or Dkk expression construct. (**C**) Results in HEK293 cells transfected with a mutant β-catenin (β-cateninΔ45) expression vector and the indicated SFRP or Dkk expression construct. (**D**) Representative results from a colony formation assay carried out using the indicated CRC cell lines. (**E**) Relative colony formation efficiencies of CRC cells transfected with Dkk or control plasmid (vector). Shown are means of three replications; error bars represent standard deviations. (**F**) Representative results of fluorescence-activated cell sorting analysis in DLD1 cells transfected with Dkk or control plasmid (vector). SFRP2 served as a positive control. Cells were harvested and analyzed 48 h after transfection. Apoptotic cells are indicated as the sub-G1 fraction and percentages are shown on the top.

In summary, we have shown that epigenetic silencing of *DKK*s is a common event in gastrointestinal tumors. The tumor suppressor function of the Dkks and other Wnt antagonists may reflect inhibition of both canonical and non-canonical Wnt signaling pathway. Wnt ligands should be one of the most promising targets for cancer treatment, though further analysis will be necessary to clarify the mechanisms how Wnt antagonists exert their antitumor effects.

Supplementary material

Supplementary Table 1 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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