WNT10B Functional Dualism: β -Catenin/Tcf-dependent Growth Promotion or Independent Suppression with Deregulated Expression in Cancer^D

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We found aberrant DNA methylation of the *WNT10B* promoter region in 46% of primary hepatocellular carcinoma (HCC) and 15% of colon cancer samples. Three of 10 HCC and one of two colon cancer cell lines demonstrated low or no expression, and 5-aza-2'-deoxycytidine reactivated *WNT10B* expression with the induction of demethylation, indicating that *WNT10B* is silenced by DNA methylation in some cancers, whereas *WNT10B* expression is up-regulated in seven of the 10 HCC cell lines and a colon cancer cell line. These results indicate that *WNT10B* can be deregulated by either overexpression or silencing in cancer. We found that *WNT10B* up-regulated β -catenin/Tcf activity. However, *WNT10B*-overexpressing cells demonstrated a reduced growth rate and anchorage-independent growth that is independent of the β -catenin/Tcf activation, because mutant β -catenin-transduced cells did not suppress growth, and dominant-negative hTcf-4 failed to alleviate the growth suppression by *WNT10B*. Although *WNT10B* expression alone inhibits cell growth, it acts synergistically with the fibroblast growth factor (FGF) to stimulate cell growth. *WNT10B* is bifunctional, one function of which is involved in β -catenin/Tcf activation, and the other function is related to the down-regulation of cell growth through a different mechanism. We suggest that FGF switches *WNT10B* from a negative to a positive cell growth regulator.

INTRODUCTION

The *WNT10B* gene is a member of the Wnt family (Lee *et al.*, 1995), which are conserved among diverse species and play crucial roles in normal development and neoplastic transformation (Nusse and Varmus, 1992; Moon *et al.*, 1997). Ectopic expression of Wnt1 induces embryonic axis duplication in *Xenopus* (McMahon and Moon, 1989), and it increases the number of mitogenic cells in the mouse spinal cord (Dickinson and McMahon, 1992). Wnt1 knockout mice show severe abnormalities in brain development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), whereas *Wnt3a*, *Wnt4*, and *Wnt7a* genes are required for somite and tailbud formation (Takada *et al.*, 1994), renal development (Stark *et al.*, 1994), and limb development (Parr and McMahon, 1995), respectively. Originally,

Wnt1 was identified in mice as a proto-oncogene (Nusse and Varmus, 1982). Similarly, *Wnt3* and *Wnt10b* are found in the mouse mammary tumor virus (MMTV) insertion sites where these genes are activated and cause mammary tumors (Roelink *et al.*, 1990; Lee *et al.*, 1995). The oncogenic activity of Wnt family genes also has been demonstrated in cultured cells. Wnt1, Wnt2, Wnt3, Wnt3a, Wnt5b, Wnt6, Wnt7a, and Wnt7b induce morphological transformation in mammary epithelial, fibroblast, and pheochromocytoma cell lines (Rijsewijk *et al.*, 1987; Bradley *et al.*, 1993; Ramakrishna and Brown, 1993; Shackleford *et al.*, 1993; Bradbury *et al.*, 1994; Shimizu *et al.*, 1997). These reports indicate that unscheduled expression of Wnt family genes is a significant event in oncogenesis. The expression of Wnt family genes is tightly regulated during normal mouse development (Buhler *et al.*, 1993; Weber-Hall *et al.*, 1994; Veltmaat *et al.*, 2004). In mammary glands, Wnt family genes are expressed in the embryonic stage, but they are not expressed in lactating mice (Gavin and McMahon, 1992). Wnt1 expression is found only in the testes of adult mice (Shackleford and Varmus, 1987). The abnormal expression of Wnt family genes has been reported in various types of cancer. Elevated expression levels of human *WNT2*, *WNT5A*, *WNT7B*, and *WNT10B* genes have been detected in proliferative lesions of human breast tissues (Huguet *et al.*, 1994; Lejeune *et al.*, 1995; Bui *et al.*, 1997). The *WNT2* gene is overexpressed in human colorectal carcinoma (Vider *et al.*, 1996). *WNT5A* is up-regulated in lung, colon, and prostate cancers, as well as melanomas (Iozzo *et al.*, 1995).

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Abbreviations used: 5Aza-dC, 5-aza-2'-deoxycytidine; COX-2, cyclooxygenase-2; FGF, fibroblast growth factor; HCC, hepatocellular carcinoma; MMTV, mouse mammary tumor virus; MSP, methylation-specific polymerase chain reaction; TSA, trichostatin A.

The prevalence of Wnt family activation in cancer has warranted functional analysis for a better understanding of the molecular interactions. Wnt family proteins are secreted glycoproteins that bind to the cell surface and extracellular matrix (Papkoff *et al.*, 1987; Bradley and Brown, 1990; Papkoff and Schryver, 1990), and they are thought to activate the Frizzled family of membrane receptors (Bhanot *et al.*, 1996). The activation suppresses the activity of the glycogen synthetase kinase 3 (GSK3) homologue, zw3 (Cook *et al.*, 1996). In turn, the catenin homologue, armadillo, is hypophosphorylated and accumulates in the cell (Peifer *et al.*, 1994). Stabilized β -catenin binds to the Tcf family of transcription factors to increase the expression of multiple genes (van de Wetering *et al.*, 1997). The protein level of β -catenin is regulated by several proteins. In mammals, β -catenin forms complexes with GSK3, adenomatous polyposis coli (APC), Axin, and Tcf (Willert and Nusse, 1998; Polakis, 2000). GSK3, a Ser/Thr protein kinase, phosphorylates the amino terminus of β -catenin, and it induces the degradation of β -catenin by the ubiquitin-proteasome pathway (Aberle *et al.*, 1997). APC is a major gene that is responsible for hereditary and sporadic colorectal carcinoma (Kinzler and Vogelstein, 1996), and APC inactivation leads to β -catenin stabilization (Korinek *et al.*, 1997; Morin *et al.*, 1997). β -Catenin mutations in GSK3 phosphorylation sites are found not only in colorectal carcinoma without APC mutations (Korinek *et al.*, 1997; Morin *et al.*, 1997), but also in melanoma (Rubinfeld *et al.*, 1997), medulloblastoma (Zurawel *et al.*, 1998), ovarian carcinoma (Palacios and Gamallo, 1998), endometrial carcinoma (Fukuchi *et al.*, 1998), hepatocellular carcinoma (HCC) (de La Coste *et al.*, 1998; Miyoshi *et al.*, 1998), hepatoblastoma (Koch *et al.*, 1999), prostatic carcinoma (Voeller *et al.*, 1998), and skin cancer (Chan *et al.*, 1999). Axin promotes GSK3-dependent phosphorylation of β -catenin through an interaction with the complex involving β -catenin, APC, and GSK3, resulting in the degradation of β -catenin (Hart *et al.*, 1998; Ikeda *et al.*, 1998). Axin mutations can be found in 9% of HCC (Satoh *et al.*, 2000). Activated β -catenin associates with hTcf-4, a member of Tcf transcription factor. Subsequently, the complex translocates into the nucleus (Behrens *et al.*, 1996; Korinek *et al.*, 1997) and transactivates target genes such as c-myc (He *et al.*, 1998), cyclin D1 (Tetsu and McCormick, 1999), cyclooxygenase-2 (COX-2) (Araki *et al.*, 2003), and NOS2 (Du *et al.*, 2006). These reports indicate that the β -catenin/Tcf pathway, which is downstream of the Wnt family gene, plays a crucial role in oncogenesis. Several of the Wnt family genes are known to regulate β -catenin. Wnt1 overexpression increases the steady-state levels of β -catenin in mouse mammary epithelial and mouse pituitary cell lines (Papkoff *et al.*, 1996). Wnt1, Wnt2, Wnt3, and Wnt3a are able to transform a mouse mammary epithelial cell line, demonstrating the accumulation of cytosolic β -catenin (Shimizu *et al.*, 1997). In a reporter assay, Wnt1 activates transcription from a promoter containing Tcf-binding elements (Young *et al.*, 1998). Together, the overexpression of Wnt family members is common in diverse types of cancer, and the oncogenic function of the Wnt family depends on the activation of β -catenin.

Int-2 transgenic mice produce mammary tumors in a focal manner. When the *int-2* transgenic mice are further infected with MMTV, multiple tumors develop in a mammary gland. Twenty-three percent (5 of 35) of the tumors have a MMTV-insertion at the *Wnt1* locus, and 6% (2 of 35 tumors) have it at the *Wnt10b* locus (Lee *et al.*, 1995). Similarly, *Wnt10b* transgenic mice produced mammary tumors in a solitary manner (Lane and Leder, 1997). These observations suggest that Wnt10b takes part in the development of mouse mammary tumors and that it requires other collaborating genes to develop cancer.

WNT10B is also overexpressed in human primary breast carcinomas, breast carcinoma cell lines, and neuroblastoma cell lines (Bui *et al.*, 1997; Yuza *et al.*, 2003). However, the precise roles of WNT10B in both development and oncogenesis are not well understood. *Wnt10b* is expressed in mouse embryonic yolk sac, fetal liver, and hematopoietic stem cells, suggesting that Wnt10b functions in hematopoiesis. Both mouse and human WNT10B induce the proliferation of hematopoietic stem cells as well as granulocyte macrophage progenitor cells (Austin *et al.*, 1997; Van Den Berg *et al.*, 1998), whereas WNT10B can suppress the proliferation of human erythroid progenitor cells (Van Den Berg *et al.*, 1998). This growth-suppressive effect is functionally dominant in that WNT10B overrides the growth stimulation by WNT2B and WNT5A (Van Den Berg *et al.*, 1998). WNT10B is also involved in adipogenesis by maintaining the preadipocyte in an undifferentiated state (Bennett *et al.*, 2003; Ross *et al.*, 2000). These imply that WNT10B has multiple functions, which are dependent on the cellular and microenvironmental context.

Recently, we have identified a candidate tumor suppressor, SOCS-1, in the structural and functional analyses of a gene identified by restriction landmark genomic scanning analysis (RLGS) (Yoshikawa *et al.*, 2001). Another aberrant NotI restriction DNA fragment that reduced the intensity in HCC samples compared with the normal counterparts has been found in the RLGS analysis (Nagai *et al.*, 1994). This DNA contained a part of the human *WNT10B* gene, and it was mapped to chromosome 12q13 (Yoshikawa *et al.*, 1997), where *WNT10B* is localized (Bui *et al.*, 1997). Because WNT10B has been minimally studied in human cancer, we analyzed promoter DNA methylation, expression, and functions with respect to tumor development.

MATERIALS AND METHODS

Cell Culture and Tissues

Cancer cell lines, SNU-182, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, PLC/PRF/5, H460, H209, H1155, SW480, RKO, and Raji were obtained from the American Type Culture Collection (Manassas, VA). HuH-1, HuH-4, and HuH-7 were from the Japanese Culture Collection (RIKEN BioResource Center, Saitama, Japan). These were grown in either DMEM or RPMI 1640 medium, supplemented with 10% fetal bovine serum. Normal DNA and RNA samples (liver, colon, and placenta) were purchased from Biochain Institute (Hayward, CA) and BD Biosciences (Palo Alto, CA). Primary HCC samples are as described previously (Yoshikawa *et al.*, 2001; Ye *et al.*, 2003). Colon cancer samples were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University).

Methylation-specific Polymerase Chain Reaction (MSP) Analysis

Genomic DNA was extracted using a standard method, and bisulfite modification of genomic DNA was performed as described previously (Herman *et al.*, 1996). The bisulfite-treated DNA was amplified either with a methylation-specific or unmethylation-specific primer set at 35 cycles at 95°C for 40 s, 58°C for 40 s, and 72°C for 40 s. The methylation-specific primer sequences for *WNT10B* were forward, AAAGTATAGAGTTTGTGTTTGTGTC and reverse, CTTCCTCAACGCCGCCG. These primers were designed from nucleotide (nt) 69 through nt 98 for the forward primer and from nt 174 through nt 158 for the reverse primer in U81787. The unmethylation-specific primer sequences for *WNT10B* were forward, GAGTAAAGTATAGAGTTTGTGTTTGTGTT, and reverse, TCACCACTTCCCCAACACCACCA. These primers were from nt 65 through nt 98 for the forward primer and from nt 180 through nt 158 for the reverse primer in U81787.

Bisulfite Sequencing Analysis

WNT10B noncoding exon1 was amplified from the bisulfite-treated DNA by using a primer set (GGTAGGGTGGGGAAGCCCCAGG and TGCTTTC-CCAGGTCTAATTACCTCCAG). The polymerase chain reaction (PCR) products were cloned, and 10 randomly selected clones for each sample were sequenced.

RNA Isolation and Reverse Transcription (RT)-PCR

Total cellular RNA, which was prepared using RNeasy Mini kit (QIAGEN, Valencia, CA), was treated with RNase-free DNase (RQ1; Promega, Madison,

WI) to eliminate contaminated DNA. cDNA was synthesized using a Superscript preamplification system (Invitrogen, Carlsbad, CA) from 3 μ g of total RNA. Semiquantitative PCR for *WNT10B* was performed using 2 μ l of cDNA, 2 μ M of each primer (TGGAGAATGCGGCTCTGA and CTCTCCAAAGTC-CATGTCATGG), 1.5 mM MgCl₂, 800 μ M dNTP mix, and 2.5 U of AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ) in a buffer supplied by the company. The condition was 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. An exponential amplification had been confirmed up to 38 cycles of the amplification (data not shown). A primer set for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (ACCACAGTC-CATGCCATCAC and TCCACCACCCTGTGCTGTA) was purchased from BD Biosciences. Semiquantitative PCR was performed as described in *WNT10B* amplification except that 1 μ l of cDNA was used, and 30 cycles. An exponential amplification had been confirmed up to 34 cycles of the amplification (data not shown). For a reactivation study, the cells were treated with 5-aza-2'-deoxycytidine (5Aza-dC) and trichostatin A (TSA) as described previously (Cameron *et al.*, 1999). Then, RT-PCR was performed as described above. Real-time PCR was performed with Taqman gene expression assay for *FGF-2* and *GAPDH* (Applied Biosystems, Foster City, CA) and an ABI PRISM 7000 sequence detection system (Applied Biosystems), by using the relative standard curve method. Values were normalized to the relative amounts of *GAPDH*.

Plasmids

Mutant *K-ras* plasmid (*pCGN K-ras 12V*) was a kind gift from Dr. Channing Der (University of North Carolina). β -Catenin/Tcf luciferase reporter plasmids (*pGL3/OT* and *pGL3/OF*), S 33 Y mutant β -catenin construct (*pCI-NEO- β -CATENINXL*), and the dominant-negative *hTcf-4* plasmid (*pcDNA/Myc-hTcf-4*) were from Dr. Bert Vogelstein. To construct the hygromycin-resistant vector carrying the dominant-negative *hTcf-4* gene, we transferred the N-terminal-deleted *Tcf-4* gene from *pcDNA/Myc-hTcf-4* into *pcDNA3.1/Hygro* (Invitrogen). Full-length *WNT10B* cDNA was amplified from human placenta RNA by using a primer set (TGGAGAATGCGGCTCTGAC and AGAGTGACCTTGGGAAGGAAATC). The PCR product was cloned into the *pT7blueT* vector (Novagen, Darmstadt, Germany). The recombinant DNA was propagated in *Epicurian coli* SCS110 (Stratagene, La Jolla, CA) to avoid Dam methylation. The full-length *WNT10B* was cut out from the recombinant with *XbaI* and *Clal*, and it was blunted with Klenow enzyme and then ligated into *EcoRV*-digested *pcDNA3.1/HisC* (Invitrogen). A clone, *pcDNA-WNT10B*, showed an in-frame ligation, sense orientation, and correct sequence to human *WNT10B* gene sequences of U81787 or X97057 (GenBank). The *WNT10B* insert in *pT7blueT* was also cloned into a *pCR3.1* vector (Invitrogen) by double digestion with *EcoRI* plus *XbaI* to generate another expression vector, *pCR-WNT10B*.

β -Catenin/Tcf Reporter Analysis

The β -catenin/Tcf luciferase reporter plasmid *pGL3/OT* contains a multiple β -catenin/Tcf motif, but *pGL3/OF* contains a multiple-mutated motif. To measure the activation of the β -catenin/Tcf reporter by exogenously expressed genes, cells (30×10^4) were plated and grown overnight in each well of six-well plates. Each 1 μ g of the reporter plasmid and an expression plasmid were transfected into the cells by using a Lipofectamine plus reagent (Invitrogen) according to the company's protocol. At 48 h after transfection, luciferase activity was measured using a reporter assay system (Promega). The luminescence was normalized to the relative protein concentration. To measure the steady-state level of reporter activities, cells (30×10^4) were plated and grown overnight in each well of six-well plates. One microgram of *pGL3/OT* with 1 ng of the reference plasmid, *pRL-CMV* (Promega), was transfected into these cells, and at 48 h posttransfection, luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega). The values of the β -catenin/Tcf reporter were normalized to those of the reference reporter.

RNA Interference

The target sequences used for *WNT10B* silencing were AAGGGUGGGAAGG-GAUAU (small interfering [si]RNA1), AAGCGCGGUUCCGUGUUU (siRNA2), and GAAUGCGAAUCCACACAA (siRNA3). Cells (20×10^4 in 60-mm dish) were transfected with *WNT10B*-specific siRNA or control siRNA (QIAGEN) at a concentration of 250 nM by using oligofectamine (Invitrogen). At 48 h posttransfection, cells were lysed, harvested, or incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent.

Establishment of Stably Transfected HuH-7 Clones

HuH-7 cells (30×10^4) were transfected with either the *WNT10B* expression or the backbone vector by using Lipofectamine Plus reagent (Invitrogen), and they were selected with 500 μ g/ml Geneticin (G-418; Invitrogen) for 4 wk. Because the *pcDNA3-WNT10B* generated *WNT10B* protein more efficiently than the *pCR-WNT10B* in an in vitro transcription-coupled translation experiment (data not shown), we established *WNT10B*-overexpressing clones with the *pcDNA-WNT10B*. Drug-resistant colonies were isolated, and expanded. A

mutant β -catenin-overexpressing HuH-7 clone also was generated using the S33Y mutant β -catenin expression vector.

Western Blot Analysis

At ~70% confluence, cells were lysed on ice in lysis buffer composed of 20 mM Tris, pH 8.0, 1% Nonidet P-40, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After 10-min incubation on ice, the cells were scraped into microfuge tubes and centrifuged at $15,000 \times g$ for 30 min. Supernatants (30 μ g) were boiled for 5 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, resolved by SDS-PAGE, and then electroblotted onto a nitrocellulose membrane. The blot was blocked with 5% skim milk in phosphate-buffered saline (PBS) for 1 h at room temperature (RT), and then it was incubated with an antibody for 1 h at RT. Anti-Wnt10B antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:1000 dilution, anti-actin antibody (Roche Diagnostics, Indianapolis, IN) was used at a 1:400 dilution, anti-cyclin D1 antibody (Upstate Biotechnology, Lake Placid, NY) was used at a 1:500 dilution, and anti-c-Myc antibody and anti-caspase-3 antibody (Santa Cruz Biotechnology) were used at a 1:250 dilution. After several washes, a 1:5000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) was added for 1 h at RT. After several washes, the blot was incubated with an enhanced chemiluminescent substrate and exposed to Hyperfilm (GE Healthcare, Arlington Heights, IL). To detect COX-2 protein, cells were transfected with mutant *K-ras* vector, and the lysate was analyzed as described previously (Araki *et al.*, 2003).

Flow Cytometry

Transfected cells were pelleted and washed with PBS. Ice-cold 80% ethanol was then added dropwise over the pellets with periodic vortexing to mix cells. After fixation, propidium iodide was added to 50 μ g/ml in PBS. The samples were then analyzed by flow cytometry.

Cell Proliferation Assay

Stably transfected HuH-7 cells (1×10^3) were plated in 96-well plates. At 24 and 48 h postplating, Cell Titer 96 Aqueous One Solution Reagent (Promega) was added into each well. After 4-h incubation, absorbance at 490 nm was recorded. The confluence was <70% under phase-contrast microscopy at 48 h postplating. For siRNA-treated cells, 100 μ l of the reagent was added to the medium (2 ml) at 48 h posttransfection. To examine the effects of fibroblast growth factor (FGF)-2 and FGF-7 on *WNT10B*-overexpressing clones, stably transfected HuH-7 clones (1×10^3 cells) were seeded in six-well plates. Cells were incubated with or without 5 ng/ml FGF-2 or FGF-7 (Chemicon International, Temecula, CA) for 2 wk. The medium was replaced every 4 d. Colony numbers were counted at 14 d postincubation.

Soft Agar Colony Formation Assay

Stably transfected HuH-7 cells (1×10^4) were suspended in RPMI 1640 medium containing 0.35% agar and 10% fetal bovine serum, and they were layered on 0.5% agar-containing RPMI 1640 medium and 10% fetal bovine serum in 100-mm tissue culture dishes. An additional 0.35% agar culture medium was overlaid every 5 d. The culture media were supplemented with 500 μ g/ml G-418. Colony formation was assessed at 21 d postincubation. To inactivate the β -catenin/Tcf complex, *WNT10B*-overexpressing cells (10×10^4) were transfected either with 1 μ g of dominant-negative *hTcf4* plasmid or the backbone *pcDNA3.1/Hygro* plasmid (Invitrogen) with Lipofectamine Plus reagent (Invitrogen). Cells were selected with 50 μ g/ml hygromycin (Invitrogen) for 3 wk in the 0.35% agarose containing RPMI 1640 medium and 10% fetal bovine serum.

Tumorigenicity in Athymic Nude Mice

Cells (1.25×10^6) of stably transfected HuH-7 clones were injected subcutaneously into each of the 10 athymic nu/nu female mice. Tumors were monitored weekly after 2 mo postinoculation, and they were examined pathologically, when the mice died.

RESULTS

Methylation and Expression Analysis of the *WNT10B* Gene in Cancer

We analyzed the DNA methylation status of the *WNT10B* gene by using MSP in primary human HCC and colon cancer samples, because we identified *WNT10B* by the RLGS analysis, in which the related NotI site was found aberrant in primary cancer compared with nontumorous samples. We designed MSP primers in noncoding exon1 where CpG density is relatively high and the NotI site is located (Figure

Figure 1. Aberrant methylation and expression of *WNT10B* in HCC. (A) Schematic representation of *WNT10B* promoter region. Arrowheads and arrows indicate positions of MSP and bisulfite sequencing primers, respectively. ATG represents the translation start site. CpG sequences are shown as vertical bars. (B) MSP analysis of *WNT10B* in primary HCC. Twenty-four HCC samples were analyzed. Visible bands in U lanes are unmethylated 106-base pair DNA products with unmethylation-specific primers, and those in M lanes are methylated 116-base pair DNA products with methylation-specific primers. (C) MSP analysis of *WNT10B* in primary colon cancer samples. Representative 18 of 46 samples were presented. The incomplete methylation can be explained by possible contamination of nontumorous cells in the tumor specimens, or by the heterogeneity in the methylation status in the tumor. (D) MSP analysis of six normal samples (1, 2, and 3 are normal liver samples and 4, 5, and 6 are normal colon samples). (E) MSP analysis of cancer cell lines. Three lung cancer (H460, H209, and H1155), two colon cancer (SW480 and RKO), and one leukemia (Raji) cell lines were analyzed. (F) Bisulfite sequencing analysis. Four cancer cell lines (RKO, Raji, HuH-7, and SW480) and two normal samples (liver and colon) were analyzed in the *WNT10B* noncoding exon1. ● and ○ represent methylation and unmethylation, respectively. Ten individual clones were sequenced for each sample. (G) RT-PCR analysis of *WNT10B*. One leukemia (Raji), two colon cancer (SW480 and RKO), 10 HCC cell lines (HuH-1, HuH-4, HuH-7, SNU-182, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, and PLC/PRF/5), and two normal samples were analyzed with *WNT10B* primers or *GAPDH* primers within cycles showing exponential amplification. Visible bands are 788- and 452-base pair DNA products in *WNT10B* and *GAPDH* amplification, respectively. (H) *WNT10B* reactivation by 5Aza-dC and TSA in HuH-7 and RKO. Cells were treated with 5Aza-dC for 3 d with or without TSA for the last 12 h and *WNT10B* expression was analyzed by RT-PCR. (I) Demethylation of *WNT10B* by 5Aza-dC. RKO cells were treated with 5Aza-dC for 3 d with or without TSA for the last 12 h. Demethylation was assessed by MSP. (J) Methylation and expression analysis in matched (DNA/RNA) primary HCC samples. Ten HCC and three nontumorous liver samples were analyzed by MSP (left) and RT-PCR (right). Numbers 1 through 10 are primary HCC samples, and 11 through 13 are nontumorous liver samples.

1A). Eleven of 24 (46%) HCC samples (Figure 1B) and seven of 46 (15%) colon cancer samples were methylated (Figure 1C). In contrast, no methylation was found in normal samples (Figure 1D). We further examined DNA methylation of *WNT10B* in cancer cell lines, including lung cancer, colon cancer, leukemia, and HCC. RKO and Raji cells were methylated (Figure 1E), whereas 10 HCC cell lines did not show any methylation in the region that we analyzed by MSP (data not shown). Bisulfite sequencing analysis demonstrated that RKO was densely methylated and 40% of Raji DNA was methylated in the noncoding exon1 (Figure 1F). We next examined *WNT10B* steady-state levels in 10 HCC, one leukemia and two colon cancer cell lines by RT-PCR. Seven of the 10 HCC and one of the two colon cancer cell lines expressed abundant *WNT10B* RNA; however, HuH-7, SNU-182, SNU-387, Raji, and RKO showed no detectable or minimal expression (Figure 1G). The *WNT10B* expression seemed a specific event to cancer, because normal liver and colon samples did not show detectable expression of

WNT10B. Despite the up-regulation of *WNT10B* expression in SW480 and the seven HCC cell lines, we observed aberrant DNA methylation in RKO and Raji cell lines as well as primary HCC and primary colon cancer samples. This finding lead us to determine whether DNA methylation of *WNT10B* was associated with its faint or lack of expression, which was observed in HuH-7, SNU-182, SNU-387, Raji, and RKO. We treated HuH-7 and RKO cells with a demethylating agent, 5Aza-dC, and we found the up-regulation of *WNT10B* expression. The combination treatment with 5Aza-dC and a histone deacetylase inhibitor, TSA, resulted in a more robust effect, as was shown in methylation-silenced genes (Figure 1H). We further examined 5Aza-dC-treated cells to confirm *WNT10B* reactivation by demethylation. MSP analysis revealed that unmethylated *WNT10B* DNA appeared in 5Aza-dC-treated cells, whereas that was not detectable in untreated cells (Figure 1I). This result was consistent with the reactivation of *WNT10B* expression in 5Aza-dC-treated cells. Interestingly, the addition of 1 mM

TSA did not increase demethylated *WNT10B* compared with 5Aza-dC treatment alone. We next examined DNA methylation and expression of *WNT10B* in primary HCC samples. We analyzed 10 primary HCC by using matched (DNA/RNA) samples. Three HCC samples (3, 4, and 6) were methylated in which *WNT10B* expression was not detectable, whereas four (1, 7, 9, and 10) of seven unmethylated samples expressed *WNT10B*. Three nontumorous liver samples (11, 12, and 13) showed neither methylation nor expression (Figure 1J). These results support *WNT10B* can be silenced by DNA methylation.

Regulation of the β -Catenin/Tcf Complex by *WNT10B* in HCC

Wnt1 regulated the β -catenin/Tcf complex (Papkoff *et al.*, 1996; Shimizu *et al.*, 1997), and activated β -catenin mutations were reported in HCC and hepatoblastoma (de La Coste *et al.*, 1998; Miyoshi *et al.*, 1998). Therefore, we studied exon3 of β -catenin where mutations were exclusively reported in diverse types of cancer (Korinek *et al.*, 1997; Morin *et al.*, 1997; Rubinfeld *et al.*, 1997; de La Coste *et al.*, 1998; Fukuchi *et al.*, 1998; Miyoshi *et al.*, 1998; Palacios and Gamallo, 1998; Voeller *et al.*, 1998; Zurawel *et al.*, 1998; Chan *et al.*, 1999; Koch *et al.*, 1999). We found no β -catenin mutations in any of the 10 HCC cell lines examined (data not shown). The up-regulation of *WNT10B* expression without β -catenin mutations suggested that *WNT10B* was able to activate the β -catenin/Tcf pathway in HCC. As was observed in normal colonic epithelium (Korinek *et al.*, 1997), *hTcf-4* was also expressed in normal liver (data not shown). Thus, we examined the β -catenin/Tcf reporter activity in *WNT10B* high- and low-producing cell lines (HuH-4 and HuH-7, respectively). HuH-4 cells demonstrated higher activity than that of HuH-7 cells (Figure 2A). Mutations in *AXIN1*, which was a negative regulator of β -catenin, were reported in 9% HCC (Satoh *et al.*, 2000); however, *AXIN1* was wild type in HuH-7 cells as well as β -catenin, indicating that these downstream proteins in WNT signaling were not defective in this cell line. To test the regulation of the β -catenin/Tcf complex by *WNT10B*, we cotransfected *WNT10B* expression and a reporter plasmid into the *WNT10B* low-producer cells (HuH-7). We observed elevated reporter activity by *WNT10B* (Figure 2B). The up-regulation was detected only in the reporter containing true β -catenin/Tcf binding motifs, but not in the mutated motifs. Therefore, *WNT10B* specifically stimulated the promoter for the β -catenin/Tcf complex. These findings suggested that the up-regulated *WNT10B* induced the activation of the β -catenin/Tcf pathway. Next, we established two *WNT10B* and a mutant β -catenin stably transfected clones. The R8 clone demonstrated an intermediate level, and the R9 clone showed a higher level of *WNT10B* protein (Figure 2C). The β -catenin/Tcf reporter activity was up-regulated in these *WNT10B*-overexpressing clones compared with the vector control. The *WNT10B* high-producer clone, R9, activated the reporter more than the intermediate-producer, R8. Similarly, the mutant β -catenin-overexpressing clone showed enhanced activity, which was higher than those of the *WNT10B*-overexpressing clones (Figure 2D). Then, we examined whether *WNT10B* transactivates β -catenin-responding genes (*Cyclin D1*, *c-MYC*, and *COX-2*). These genes contain the β -catenin/Tcf binding elements in their promoter regions, and they are reported to be transactivated by β -catenin (He *et al.*, 1998; Tetsu and McCormick, 1999; Araki *et al.*, 2003). We found that all three of these proteins were up-regulated in the *WNT10B* or mutant β -catenin-overexpressing clones. The R9 activated these target genes more than the R8, and the transactivations of *COX-2*, *Cyclin*

D1, and *c-MYC* genes in the mutant β -catenin-overexpressing clone were higher than those in the *WNT10B*-overexpressing clones (Figure 2E). These results demonstrated that the enhancement of β -catenin/Tcf activity by *WNT10B* was in line with the transduction by mutant β -catenin, although the up-regulation of *WNT10B* was less than that of mutant β -catenin.

WNT10B Inhibits Cell Growth through a β -Catenin-independent Mechanism

Next, we examined the effects of *WNT10B* expression on cell growth. *WNT10B*-overexpressing clones showed a reduced growth rate compared with the vector control and the mutant β -catenin-overexpressing clones (Figure 3A). Surprisingly, *WNT10B* acted differently from mutant β -catenin in this growth assay, despite the fact that these two proteins were in the same pathway (Figure 2, D and E). Therefore, we further investigated the mechanism of the growth-suppressive effects in these *WNT10B* clones. In a soft agar cloning experiment, *WNT10B*-overexpressing clones drastically reduced the formation of colonies compared with the control and the mutant β -catenin-overexpressing clones. The growth suppression efficiency between *WNT10B*-overexpressing clones was directly correlated with the amount of *WNT10B* expression (Figure 3B). The colon cancer cell line RKO in which *WNT10B* is inactivated with associated DNA methylation also demonstrated reduced colony formation, when *WNT10B* was transfected (data not shown). Although *WNT10B*-overexpressing clones showed the notable growth suppression in a soft agar culture, *c-MYC* was elevated in these cells, which might potentiate the growth suppression in some conditions (Evan *et al.*, 1992; Pelengaris *et al.*, 2000). Therefore, we overexpressed dominant-negative *hTcf-4* in the *WNT10B* high-producer clone, R9, to eliminate the activity of β -catenin (Morin *et al.*, 1997). Dominant-negative *hTcf-4* did not recover the growth of the *WNT10B*-overexpressing clone in soft agar (Figure 3B), indicating the growth suppression by *WNT10B* is not associated with the activity of the β -catenin/Tcf transcription complex. *WNT10B* overexpression suppressed the growth of HuH-7 cells, despite that *WNT10B* up-regulated cyclin D1 and *c-MYC*. We, therefore, tested if transient *WNT10B* overexpression induces apoptosis. *WNT10B*-transfected cells showed cleavage of caspase-3 by immunoblotting and increases of subG1 population by FACS analysis (Figure 3C), indicating that *WNT10B* was able to induce apoptosis when transiently overexpressed. We further studied the tumorigenicity of *WNT10B*-overexpressing clones in xenotransplanted athymic nude mice (Table 1). The mutant β -catenin-overexpressing clone showed an increased occurrence of tumors with similar latency and similar doubling time when compared with the vector control, whereas R9 showed a reduced occurrence of tumors, delayed latency, and extended doubling time compared with the vector control and the mutant β -catenin-overexpressing clone. R8 also had a decreased tumor occurrence compared with the mutant β -catenin-overexpressing clone. Interestingly, R8 had an increased tumor occurrence and maintained the latency and doubling time compared with the vector control. WNT family proteins are able to induce morphological changes (Young *et al.*, 1998). We observed *WNT10B* stably expressing clones under phase-contrast microscopy. *WNT10B*-overexpressing clones showed more of an ordered pattern compared with the control and β -catenin-overexpressing clones (Figure 3D).

Effects of *WNT10B* Inhibition by siRNA

Given the *WNT10B*-mediated activation of β -catenin and β -catenin-independent growth suppression by *WNT10B* overexpression, we next examined the effects of *WNT10B*

Figure 2. β -Catenin/Tcf reporter activity in HuH-4 and HuH-7 cell lines. (A) The β -catenin/Tcf reporter activity was measured by transient transfections with a β -catenin/Tcf reporter plasmid, *pGL3/OT*, and a reference plasmid, *pRL-CMV*, into a *WNT10B* high-producer cell line, HuH-4, and a low-producer cell line, HuH-7. The values of the β -catenin/Tcf reporter activity are normalized to those of the reference reporter, and they are the means of three replicates. Error bars represent standard deviations. The difference between HuH-4 and HuH-7 is statistically significant ($p < 0.05$). (B) The *WNT10B* low-producer cell line, HuH-7, was transiently transfected with the indicated vectors. *WNT10B* is the *pcDNA-WNT10B* expression construct. *pGL3/OT* and *pGL3/OF* contain true and mutated β -catenin/Tcf binding motifs, respectively. Luminescence was normalized to the respective protein concentration, and data are the mean of three replicates. Error bars represent standard deviations. The difference between the *WNT10B* and the vector control is statistically significant ($p < 0.05$). Another *WNT10B* expression plasmid, *pCR-WNT10B*, which was constructed in a *pCR3.1* vector, also showed a similar result (data not shown). (C) *WNT10B* stably overexpressing clones or the vector control was generated by transfections into HuH-7 cells with the *pcDNA-WNT10B* or the backbone plasmid, respectively. Thirty micrograms of protein were analyzed by SDS-PAGE and followed by immunoblotting with the anti-*Wnt10B* antibody or anti-actin antibody. (D) The β -catenin/Tcf reporter activity was measured by cotransfections with *pGL3/OT* and *pRL-CMV* into the *WNT10B* clones, mutant β -catenin clone, or vector control clone. The mutant β -catenin-overexpressing clone was established by selecting a single colony of HuH-7 cells transfected with the S33Ymutant β -catenin construct. The values of β -catenin/Tcf reporter activities are normalized to those of the reference reporter, and they are the means of three replicates. Error bars represent standard deviations. The differences in the activity among clones were all statistically significant ($p < 0.05$). (E) Western blot analysis of the downstream genes. Thirty micrograms of protein from the *WNT10B* stably overexpressing clones, mutant β -catenin clone, and vector control clone were analyzed by SDS-PAGE, followed by immunoblotting either with anti-cyclin D1, anti-c-Myc, or anti-actin antibody. For the COX-2 analysis, these stable clones were transfected with the mutant *K-ras* plasmid, and the protein was analyzed with the anti-COX-2 antibody. The intensities of cyclin D1, c-MYC, and COX-2 were normalized to those of actin, and the ratios against normalized intensities of the vector control were calculated. In cyclin D1, the *WNT10B* clone R8 was 1.2-fold, *WNT10B* clone R9 was 2.0-fold, and mutant β -catenin was 4.3-fold increased in intensity compared with the vector control. In the c-MYC, the *WNT10B* clone R8 was 1.2-fold, *WNT10B* clone R9 was 1.3-fold, and mutant β -catenin was 1.5-fold increased in intensity compared with the vector control. In the COX-2, the *WNT10B* clone R8 was 1.3-fold, *WNT10B* clone R9 was 1.8-fold, and mutant β -catenin was 2.1-fold increased in intensity compared with the vector control.

inhibition. Two of three *WNT10B*-specific siRNAs effectively inhibited *WNT10B* RNA expression in HuH-4 cells. We used siRNA3 in further experiments. Inhibition of *WNT10B* was confirmed by immunoblotting using siRNA3-transfected HuH-4 and SW480 cells (Figure 4A). In *WNT10B*-knockdown cells, cyclin D1 and c-MYC were down-regulated, as well as β -catenin (Figure 4B). We also evaluated the growth of *WNT10B*-inhibited cells. These cells showed reduced growth compared with the control siRNA-transfected cells (Figure 4C).

Synergy of *WNT10B* and FGF Family Proteins in Tumor Cells

Despite the up-regulation of β -catenin/Tcf reporter activity and the transactivation of target genes by *WNT10B*, *WNT10B*-overexpressing clones showed a reduction in growth rate and soft agar cloning efficiency. Tumorigenicity in athymic nude mice was also reduced in R9. Based on these results, we speculated that *WNT10B* requires some

factors for reversing its growth suppression effect. FGF family proteins were supposed to be candidates, because *Wnt10b* transgenic mice produced mammary tumors only in a solitary manner (Lane and Leder, 1997), and a member of FGF family, int-2, collaborated with *Wnt10b* to develop multiple mammary tumors (Lee *et al.*, 1995). We incubated stable HuH-7 clones with either FGF-2 or FGF-7. FGF-2 or FGF-7 enhanced the growth of *WNT10B*-overexpressing clones more than the vector control clone (Figure 5A), suggesting that *WNT10B* cooperated with FGF family proteins. R9 was more sensitive to FGF family proteins than R8. Significantly, FGF-2 or FGF-7 failed to enhance the growth of the mutant β -catenin clone. FGF-2 and FGF-7 did not affect the expression of *WNT10B* in R8 and R9 clones (Figure 5A). This finding again demonstrated that R8 and R9 clones were differently involved in cell growth from the mutant β -catenin clone. We further analyzed the expression of *WNT10B*

Figure 3. Induction of growth suppression by WNT10B. (A) MTS assay. Growth rates were calculated by the increase of absorbance between 24 and 48 h after plating. Vector control is a stable transfectant with an empty vector. The WNT10B clones R9 and R8 produce high and intermediate levels of WNT10B protein, respectively. Mutant β -catenin is the S33Y mutant β -catenin-overexpressing clone. The values are the means of three replicates. Error bars represent standard deviations. The differences between clone R9 and the vector control clone, and between clone R8 and the vector control clone, were statistically significant ($p < 0.05$). The difference between the vector control and the mutant β -catenin clones was not significant. The difference between clones R8 and R9 was not significant. (B) Soft agar cloning efficiency. Cells (vector control, mutant β -catenin, WNT10B-overexpressing clones R8 and R9) were grown in soft agar with G-418, and colony numbers were counted at 4 wk after seeding. The WNT10B-overexpressing clone R9 was either transfected with the dominant-negative *hTcf-4* expression construct (clone R9 DN Tcf-4) or backbone hygromycin-resistant plasmid (clone R9 hygro control), and then it was grown in soft agar with hygromycin. Hygromycin-resistant colonies were counted at 4 wk after seeding. The values are relative colony numbers to vector control (100%). The values are the means of three replicates. Error bars represent standard deviations. The difference between the WNT10B clones (R8 and R9) was statistically significant ($p < 0.05$). The differences between the mutant β -catenin clone and WNT10B clones (R8 and R9) were also statistically significant ($p < 0.05$), but the difference between the vector control and mutant β -catenin was not significant. The difference between clone R9 DN Tcf-4 and clone R9 hygro control was not significant. (C) WNT10B-mediated apoptosis. WNT10B was transiently transfected in HuH-7 and RKO cells. Cell lysates were analyzed by Western blotting using anti-caspase-3 and actin antibodies. Cleaved caspase-3 (20 kDa) was detected by anti-caspase-3 immunoblotting (left). Transfected cells were analyzed by fluorescence-activated cell sorting analysis. The sub-G1 populations

increased in WNT10B-transfected HuH-7 cells (16.35%) compared with the control vector-transfected cells (8.72%) and in WNT10B-transfected RKO cells (8.57%) compared with the control vector-transfected cells (1.93%) (right). (D) Microscopic observation of stably transfected clones. WNT10B (R8 and R9), mutant β -catenin, vector control, and parental HuH-7 cells were viewed by phase-contrast microscopy.

and *FGF-2* to examine expression patterns and tumor metastasis in surgically resected HCC samples. WNT10B expression was found in eight of the 22 samples (Figure 5B). Among the 22 samples, 14 samples are metastatic, and the remaining eight samples were not (Ye *et al.*, 2003). We quantitatively examined *FGF-2* expression by using real-time PCR, because *FGF-2* was expressed in normal liver. Ten of the 22 samples had increased *FGF-2* expression (see Supplemental Material). Double up-regulation of WNT10B and *FGF-2* expressions were found in five samples. Interestingly, four of the five WNT10B/*FGF-2* double up-regulated samples were metastatic cases (Table 2).

DISCUSSION

The Wnt family is involved in both development and oncogenesis, and a well-studied family member, Wnt1, was able

to transduce the β -catenin/Tcf-signaling pathway (Papkoff *et al.*, 1996; Young *et al.*, 1998). WNT10B is another family member that was isolated more than a decade later, and it has been poorly characterized. To better understand the structure and functions of WNT10B in cancer, we studied DNA methylation and the expression of WNT10B in HCC and colon cancer. Primary HCC and colon cancer showed aberrant DNA methylation in 46 and 15% of the samples, respectively. The DNA methylation is specific to the tumors because of the absence in normal samples. MSP analysis in cell lines showed full methylation in a colon cancer cell line, RKO, but no methylation was observed in 10 HCC cell lines. Bisulfite sequencing analysis revealed that the WNT10B CpG-rich region in noncoding exon1 where we designed MSP primers was densely methylated in RKO cells. RT-PCR analysis demonstrated that methylated RKO cells did not

Table 1. Tumorigenicity of WNT10B-overexpressing HuH-7 clones in nude mice

Cell line	Mice with tumor (n = 9 or 10)	Latency	Doubling time
Vector control	3/9	28.7 ± 2.3	2.9 ± 0.8
WNT10B clone, R8	6/10	34.7 ± 9.4	3.0 ± 1.1
WNT10B clone, R9	2/9	47.0 ± 7.0	4.5 ± 0.1
Mutant β -catenin	9/10	24.7 ± 6.1	3.0 ± 1.6

Stably transfected HuH7 clones were inoculated into each of the 10 athymic nude mice, and they were monitored up to 2 mo postinoculation. Palpable tumors were counted and measured. Vector control was the empty vector clone; WNT10B clone R9 produced high levels of WNT10B protein, clone R8 produced intermediate levels of WNT10B protein, and mutant β -catenin was the mutant β -catenin-expressing clone. Each mouse in the vector control and clone R9 groups that died before maturation was eliminated from the study. Latency and doubling time (means $\pm$ SD) are shown in days. For the latency data, the difference between vector control and R9 was statistically significant ($p < 0.05$). The differences between R9 and mutant β -catenin, and between R8 and mutant β -catenin, were significant ($p < 0.05$). The difference between R9 and R8 was also significant ($p < 0.05$). In the doubling time section, the differences between vector control and R9, and between R9 and mutant β -catenin, were statistically significant ($p < 0.05$).

express *WNT10B* in contrast with the abundant expression in unmethylated SW480 cells. In addition, *WNT10B* expression was not detectable in the methylated leukemia cell line (Raji). In primary HCC, methylated samples did not express *WNT10B*, whereas four of seven unmethylated samples did express *WNT10B*. Based on the aberrant DNA methylation and reduced expression observed, we suggest that DNA methylation is associated with reduced expression of *WNT10B* in cancer cells. We used a demethylating agent, 5Aza-dC, and a histone deacetylase inhibitor, TSA, to test methylation-associated silencing of *WNT10B* in HuH-7 and RKO cells. 5Aza-dC markedly up-regulated *WNT10B* expression in both cell lines. The combination treatment of 5Aza-dC and TSA demonstrated a more robust effect. Furthermore, 5Aza-dC treatment induced the demethylation of *WNT10B* in RKO cells. These results indicate that silenced expression of *WNT10B* is associated with DNA methylation. RKO is aberrantly methylated in the *WNT10B* promoter region, whereas HuH-7 did not show the methylation by the MSP analysis. This can be explained by the possible methylation in another DNA region such as enhancer. The expression analysis also revealed that *WNT10B* was up-regulated in seven of 10 HCC cell lines, one of two colon cancer cell lines, and four of 10 primary HCC samples. Together, the data indicate that *WNT10B* is up-regulated in some cancers, but observed DNA methylation and the reactivation of the expression by 5Aza-dC indicated that *WNT10B* is transcriptionally silenced in other cancers.

The seemingly paradoxical finding of the *WNT10B* activation and inactivation in cancer lead us to further investigate its biological activity. Activating mutations of β -catenin were reported in various cancers, including HCC. However, we did not find any β -catenin or *Axin* mutations in the 10 HCC cell lines examined. Therefore, we determined whether WNT10B transduced the β -catenin/Tcf pathway in those HCC cell lines. We compared the β -catenin/Tcf reporter activity by using endogenous β -catenin and hTcf-4 between *WNT10B* high-producer and low-producer cell lines. The higher activity in the *WNT10B* high-producer suggested that

Figure 4. Effects of WNT10B inhibition by siRNA. (A) Knocking WNT10B down by the specific siRNA. WNT10B-expressing cells were transfected with WNT10B-specific siRNAs. WNT10B expression was analyzed by RT-PCR (top) and immunoblotting (bottom). (B) Down-regulation of Cyclin D1, c-MYC, and β -catenin in WNT10B knockdown cells. siRNA-transfected HuH-4 and SW480 cells were lysed and immunoblotted by indicated antibodies. (C) Down-regulation of cell growth by WNT10B inhibition. HuH-4 and SW480 cells were transfected with WNT10B-specific or control siRNA. Cell growth was assessed by MTS assay at 48 h posttransfection.

WNT10B transduced the β -catenin/Tcf pathway. In addition, the reporter activity was enhanced by exogenously expressed *WNT10B*. Thus, we constructed stably transfected HuH-7 cells to investigate the function of WNT10B in more detail. We detected the elevation of the β -catenin/Tcf reporter activity in WNT10B stably overexpressing clones as in the mutant β -catenin clone. Furthermore, β -catenin/Tcf target genes, cyclin D1, c-MYC, and COX-2 (He *et al.*, 1998; Tetsu and McCormick, 1999; Araki *et al.*, 2003), were transactivated by WNT10B. Significantly, the WNT10B expression level was correlated with the level of β -catenin/Tcf activity. Consistent with WNT10B overexpression, inhibition of WNT10B by siRNA down-regulated cyclin D1 and c-MYC. Based on these results, we concluded that WNT10B is able to regulate the oncogenic β -catenin/Tcf pathway.

Wnt family members can promote the growth of rodent cells. Wnt1-, Wnt6-, or Wnt7b-transduced cells grew in a higher density (Bradbury *et al.*, 1994). Wnt1 induced serum-independent cellular proliferation (Young *et al.*, 1998) and enhanced tumorigenicity in nude mice (Rijsewijk *et al.*, 1987) and soft agar cloning efficiency by stimulating the β -catenin/Tcf pathway (Bafico *et al.*, 1998). Furthermore, as the downstream key factor of Wnt1, β -catenin also induced cellular transformation and enhanced the soft agar cloning efficiency (Orford *et al.*, 1999). These reports raise a possibility that WNT10B is an oncogenic protein involved in the β -catenin/Tcf pathway. Therefore, we studied whether WNT10B up-regulated cell growth in vitro and in vivo by

Figure 5. Synergy of WNT10B and FGF. (A) Effects of FGF family proteins on the WNT10B-overexpressing clones. Stably transfected HuH-7 clones were incubated with or without 5 ng/ml FGF-2 or FGF-7 for 2 wk, and then the colony numbers were counted. Black and white bars indicate -fold increases by FGF-2 and FGF-7, respectively. The values shown are the means of three replicates. Error bars represent standard deviations. The differences between WNT10B-overexpressing clones and the other clones were statistically significant ($p < 0.05$). The difference between clones R9 and R8 was also significant ($p < 0.05$), but the difference between the vector control and mutant β -catenin was not (top). WNT10B expression was analyzed by immunoblotting using FGF-2- or FGF-7-treated R8 and R9 cells (bottom). (B) WNT10B expression in primary HCC samples. Using RT-PCR as in Figure 1E, WNT10B and GAPDH expressions were analyzed in 22 primary HCC samples for which metastatic status and expression status of osteopontin were reported (Ye *et al.*, 2003).

Table 2. WNT10B and FGF-2 expressions in metastatic and nonmetastatic samples

Gene overexpression	HCC samples (n = 22)	HCC with metastasis (n = 14)	HCC without metastasis (n = 8)
WNT10B	8	5	3
FGF-2	10	6	4
Both WNT10B and FGF-2	5	4	1

WNT10B and FGF-2 overexpressions are classified with respect to tumor metastasis. The cut-off of WNT10B overexpression is 1.5 against the background level (see Figure 5B). The cut-off of FGF-2 expression is 1.5 against the normal liver (see Supplemental Material). The WNT10B and FGF-2 double overexpressions are not linked to metastasis statistically.

using WNT10B-overexpressing HuH-7 clones. Surprisingly, WNT10B-overexpressing clones suppressed cell growth, including the growth rate in a monolayer culture, soft agar cloning efficiency, and tumorigenicity in nude mice, except that the WNT10B intermediate clone increased the incidence of tumors in nude mice. In addition, growth suppression in the soft agar cloning efficiency was directly correlated with the amount of WNT10B expression. A same tendency was observed in the growth rate in a monolayer culture, although it was not statistically significant. A reported growth suppression by WNT10B in erythroid progenitor cells is consistent with our findings (Van Den Berg *et al.*, 1998). The up-regulation of c-MYC-induced apoptosis under certain conditions (Prendergast, 1999) lead us to investigate whether growth suppression by WNT10B is caused by increased c-MYC. However, the mutant β -catenin clone,

which activated c-MYC greater than the WNT10B clones, maintained the growth rate and soft agar cloning efficiency, and it increased tumorigenicity in nude mice. Dominant-negative hTcf-4, which can abrogate the transcriptional activity of the β -catenin/Tcf complex, failed to recover growth of the WNT10B-overexpressing clone. Therefore, c-MYC activation is not likely the cause of growth suppression by WNT10B. These findings indicate that WNT10B is involved in a growth suppression pathway independently of β -catenin/Tcf. Apoptosis may be one of the factors that induces WNT10B-mediated growth suppression, because we found the activation of caspase-3 in WNT10B transiently overexpressed cells. It seems that the balance between the up-regulating and down-regulating functions of WNT10B decide the outcome of cancer cell growth. This hypothesis might explain why the WNT10B intermediate producer showed a reduced growth rate and anchorage-independent growth, but increased tumorigenicity in nude mice. Given the growth suppression activity of WNT10B, we speculate that transcriptional silencing of *WNT10B* takes place to inhibit its growth suppression effect. Alternatively, the up-regulation of WNT10B is more favorable when its growth suppression activity is specifically alleviated. *Wnt10b* transgenic mice produce mammary solitary tumors (Lane and Leder, 1997), and transgenic mice with a member of the FGF family, *int-2*, produce multiple carcinomas only when MMTV activated *Wnt1* or *Wnt10b* (Lee *et al.*, 1995). These reports suggest that WNT10B activation is insufficient for malignant transformation. We postulated that WNT10B may cooperate with other growth factors in oncogenesis. Therefore, we incubated stable HuH-7 clones with two members of the FGF family proteins, and we found that FGF-2 or FGF-7 stimulated growth synergistically with WNT10B, but not with mutant β -catenin. In addition, the inhibition of WNT10B reduced the growth of HuH-4 or SW480 cells in which FGF-2 (data not shown) or FGF-20 (Kirikoshi *et al.*, 2000) was up-regulated, respectively. This suggests that WNT10B collaborates with FGF family proteins to promote oncogenesis. Expression analysis of *WNT10B* and *FGF-2* in primary HCC samples demonstrated interesting data. Four of five metastatic samples with up-regulated *WNT10B* showed increased *FGF-2* expression. Four of five *WNT10B*/*FGF-2* double up-regulated samples were metastatic cases, and metastasis-related osteopontin was not activated in one exceptional case (S49) (Ye *et al.*, 2003). The up-regulation of FGF family might promote the metastasis of tumor cells in *WNT10B*-expressing cancer cells. However, apparently *WNT10B*/*FGF-2* double up-regulation is not sufficient for metastasis, because one double up-regulated case was non-metastatic, and four cases with FGF-2 up-regulation alone were also nonmetastatic. Other metastasis-associated factors, including osteopontin, may also play a role in HCC (Ye *et al.*, 2003).

In general, WNT family proteins are thought to act as ligands to frizzled receptors. WNT10B seems to function in an autocrine or paracrine manner. We propose that WNT10B has dual functions, one function of which promotes oncogenesis through the β -catenin/Tcf pathway, and another function inhibits cell growth by a different mechanism. Our hypothesis is that autocrine or paracrine expression of FGF family proteins cooperates with WNT10B to switch its growth-suppressive effects to growth stimulatory. The release of growth regulatory factors, including FGF family members is an interesting mechanism in tumor growth and metastasis. Our current studies are identifying the mechanism of growth suppression by WNT10B.

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REFERENCES

- Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). β -catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* 16, 3797–3804.
- Araki, Y., Okamura, S., Hussain, S. P., Nagashima, M., He, P., Shiseki, M., Miura, K., and Harris, C. C. (2003). Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways. *Cancer Res.* 63, 728–734.
- Austin, T. W., Solar, G. P., Ziegler, F. C., Liem, L., and Matthews, W. (1997). A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. *Blood* 89, 3624–3635.
- Bafico, A., Gazit, A., Wu-Morgan, S. S., Yaniv, A., and Aaronson, S. A. (1998). Characterization of Wnt-1 and Wnt-2 induced growth alterations and signaling pathways in NIH3T3 fibroblasts. *Oncogene* 16, 2819–2825.
- Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of β -catenin with the transcription factor LEF-1. *Nature* 382, 638–642.
- Bennett, C. N., Hodge, C. L., MacDougald, O. A., and Schwartz, J. (2003). Role of Wnt10b and C/EBP α in spontaneous adipogenesis of 243 cells. *Biochem. Biophys. Res. Commun.* 302, 12–16.
- Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature* 382, 225–230.
- Bradbury, J. M., Niemeyer, C. C., Dale, T. C., and Edwards, P. A. (1994). Alterations of the growth characteristics of the fibroblast cell line C3H 10T1/2 by members of the Wnt gene family. *Oncogene* 9, 2597–2603.
- Bradley, R. S., and Brown, A. M. (1990). The proto-oncogene *int-1* encodes a secreted protein associated with the extracellular matrix. *EMBO J.* 9, 1569–1575.
- Bradley, R. S., Cowin, P., and Brown, A. M. (1993). Expression of Wnt-1 in PC12 cells results in modulation of plakoglobin and E-cadherin and increased cellular adhesion. *J. Cell Biol.* 123, 1857–1865.
- Buhler, T. A., Dale, T. C., Kieback, C., Humphreys, R. C., and Rosen, J. M. (1993). Localization and quantification of Wnt-2 gene expression in mouse mammary development. *Dev. Biol.* 155, 87–96.
- Bui, T. D., Rankin, J., Smith, K., Huguet, E. L., Ruben, S., Strachan, T., Harris, A. L., and Lindsay, S. (1997). A novel human Wnt gene, WNT10B, maps to 12q13 and is expressed in human breast carcinomas. *Oncogene* 14, 1249–1253.
- Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.* 21, 103–107.
- Chan, E. F., Gat, U., McNiff, J. M., and Fuchs, E. (1999). A common human skin tumour is caused by activating mutations in β -catenin. *Nat. Genet.* 21, 410–413.
- Cook, D., Fry, M. J., Hughes, K., Sumathipala, R., Woodgett, J. R., and Dale, T. C. (1996). Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J.* 15, 4526–4536.
- de La Coste, A., Romagnolo, B., Billaut, P., Renard, C. A., Buendia, M. A., Soubbrane, O., Fabre, M., Chelly, J., Beldjord, C., Kahn, A., and Perret, C. (1998). Somatic mutations of the β -catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc. Natl. Acad. Sci. USA* 95, 8847–8851.
- Dickinson, M. E., and McMahon, A. P. (1992). The role of Wnt genes in vertebrate development. *Curr. Opin. Genet. Dev.* 2, 562–566.
- Du, Q., Park, K. S., Guo, Z., He, P., Nagashima, M., Shao, L., Sahai, R., Geller, D. A., and Hussain, S. P. (2006). Regulation of human nitric oxide synthase 2 expression by Wnt β -catenin signaling. *Cancer Res.* 66, 7024–7031.
- Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69, 119–128.

- Fukuchi, T., Sakamoto, M., Tsuda, H., Maruyama, K., Nozawa, S., and Hirohashi, S. (1998). Beta-catenin mutation in carcinoma of the uterine endometrium. *Cancer Res.* 58, 3526–3528.
- Gavin, B. J., and McMahon, A. P. (1992). Differential regulation of the Wnt gene family during pregnancy and lactation suggests a role in postnatal development of the mammary gland. *Mol. Cell. Biol.* 12, 2418–2423.
- Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Curr. Biol.* 8, 573–581.
- He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* 281, 1509–1512.
- Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* 93, 9821–9826.
- Huguet, E. L., McMahon, J. A., McMahon, A. P., Bicknell, R., and Harris, A. L. (1994). Differential expression of human Wnt genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue. *Cancer Res.* 54, 2615–2621.
- Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. *EMBO J.* 17, 1371–1384.
- Iozzo, R. V., Eichstetter, I., and Danielson, K. G. (1995). Aberrant expression of the growth factor Wnt-5A in human malignancy. *Cancer Res.* 55, 3495–3499.
- Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* 87, 159–170.
- Kirikoshi, H., Sagara, N., Saitoh, T., Tanaka, K., Sekihara, H., Shiokawa, K., and Katoh, M. (2000). Molecular cloning and characterization of human FGF-20 on chromosome 8p21.3-p22. *Biochem. Biophys. Res. Commun.* 274, 337–343.
- Koch, A., Denkhaus, D., Albrecht, S., Leuschner, I., von Schweinitz, D., and Pietsch, T. (1999). Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. *Cancer Res.* 59, 269–273.
- Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 275, 1784–1787.
- Lane, T. F., and Leder, P. (1997). Wnt-10b directs hypermorphic development and transformation in mammary glands of male and female mice. *Oncogene* 15, 2133–2144.
- Lee, F. S., Lane, T. F., Kuo, A., Shackleford, G. M., and Leder, P. (1995). Insertional mutagenesis identifies a member of the Wnt gene family as a candidate oncogene in the mammary epithelium of int-2/Fgf-3 transgenic mice. *Proc. Natl. Acad. Sci. USA* 92, 2268–2272.
- Lejeune, S., Huguet, E. L., Hamby, A., Poulsom, R., and Harris, A. L. (1995). Wnt5a cloning, expression, and up-regulation in human primary breast cancers. *Clin. Cancer Res.* 1, 215–222.
- McMahon, A. P., and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 62, 1073–1085.
- McMahon, A. P., and Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* 58, 1075–1084.
- Miyoshi, Y., Iwao, K., Nagasawa, Y., Aihara, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., and Nakamura, Y. (1998). Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res.* 58, 2524–2527.
- Moon, R. T., Brown, J. D., and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* 13, 157–162.
- Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275, 1787–1790.
- Nagai, H., Ponglikitmongkol, M., Mita, E., Ohmachi, Y., Yoshikawa, H., Saeki, R., Yumoto, Y., Nakanishi, T., and Matsubara, K. (1994). Aberration of genomic DNA in association with human hepatocellular carcinomas detected by 2-dimensional gel analysis. *Cancer Res.* 54, 1545–1550.
- Nusse, R., and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31, 99–109.
- Nusse, R., and Varmus, H. E. (1992). Wnt genes. *Cell* 69, 1073–1087.
- Orford, K., Orford, C. C., and Byers, S. W. (1999). Exogenous expression of beta-catenin regulates contact inhibition, anchorage-independent growth, anoikis, and radiation-induced cell cycle arrest. *J. Cell Biol.* 146, 855–868.
- Palacios, J., and Gamallo, C. (1998). Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res.* 58, 1344–1347.
- Papkoff, J., Brown, A. M., and Varmus, H. E. (1987). The int-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway. *Mol. Cell. Biol.* 7, 3978–3984.
- Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996). Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol. Cell. Biol.* 16, 2128–2134.
- Papkoff, J., and Schryver, B. (1990). Secreted int-1 protein is associated with the cell surface. *Mol. Cell. Biol.* 10, 2723–2730.
- Parr, B. A., and McMahon, A. P. (1995). Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* 374, 350–353.
- Peifer, M., Pai, L. M., and Casey, M. (1994). Phosphorylation of the *Drosophila* adherens junction protein Armadillo: roles for wingless signal and zeste-white 3 kinase. *Dev. Biol.* 166, 543–556.
- Pelengaris, S., Rudolph, B., and Littlewood, T. (2000). Action of Myc in vivo—proliferation and apoptosis. *Curr. Opin. Genet. Dev.* 10, 100–105.
- Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev.* 14, 1837–1851.
- Prendergast, G. C. (1999). Mechanisms of apoptosis by c-Myc. *Oncogene* 18, 2967–2987.
- Ramakrishna, N. R., and Brown, A. M. (1993). Wingless, the *Drosophila* homolog of the proto-oncogene Wnt-1, can transform mouse mammary epithelial cells. *Dev. Suppl.*, 95–103.
- Rijsewijk, F., van Deemter, L., Wagenaar, E., Sonnenberg, A., and Nusse, R. (1987). Transfection of the int-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. *EMBO J.* 6, 127–131.
- Roelink, H., Wagenaar, E., Lopes da Silva, S., and Nusse, R. (1990). Wnt-3, a gene activated by proviral insertion in mouse mammary tumors, is homologous to int-1/Wnt-1 and is normally expressed in mouse embryos and adult brain. *Proc. Natl. Acad. Sci. USA* 87, 4519–4523.
- Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougald, O. A. (2000). Inhibition of adipogenesis by Wnt signaling. *Science* 289, 950–953.
- Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 275, 1790–1792.
- Satoh, S. *et al.* (2000). AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat. Genet.* 24, 245–250.
- Shackleford, G. M., and Varmus, H. E. (1987). Expression of the proto-oncogene int-1 is restricted to postmeiotic male germ cells and the neural tube of mid-gestational embryos. *Cell* 50, 89–95.
- Shackleford, G. M., Willert, K., Wang, J., and Varmus, H. E. (1993). The Wnt-1 proto-oncogene induces changes in morphology, gene expression, and growth factor responsiveness in PC12 cells. *Neuron* 11, 865–875.
- Shimizu, H., Julius, M. A., Giarre, M., Zheng, Z., Brown, A. M., and Kitajewski, J. (1997). Transformation by Wnt family proteins correlates with regulation of beta-catenin. *Cell Growth Differ.* 8, 1349–1358.
- Stark, K., Vainio, S., Vassileva, G., and McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 372, 679–683.
- Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., McMahon, J. A., and McMahon, A. P. (1994). Wnt-3a regulates somite and tailbud formation in the mouse embryo. *Genes Dev.* 8, 174–189.
- Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 398, 422–426.
- Thomas, K. R., and Capecchi, M. R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* 346, 847–850.
- van de Wetering, M. *et al.* (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene DTCF. *Cell* 88, 789–799.
- Van Den Berg, D. J., Sharma, A. K., Bruno, E., and Hoffman, R. (1998). Role of members of the Wnt gene family in human hematopoiesis. *Blood* 92, 3189–3202.

- Veltmaat, J. M., Van Veelen, W., Thiery, J. P., and Bellusci, S. (2004). Identification of the mammary line in mouse by Wnt10b expression. *Dev. Dyn.* 229, 349–356.
- Vider, B. Z., Zimmer, A., Chastre, E., Prevot, S., Gespach, C., Estlein, D., Wolloch, Y., Tronick, S. R., Gazit, A., and Yaniv, A. (1996). Evidence for the involvement of the Wnt 2 gene in human colorectal cancer. *Oncogene* 12, 153–158.
- Voeller, H. J., Truica, C. I., and Gelmann, E. P. (1998). Beta-catenin mutations in human prostate cancer. *Cancer Res.* 58, 2520–2523.
- Weber-Hall, S. J., Phippard, D. J., Niemeyer, C. C., and Dale, T. C. (1994). Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. *Differentiation* 57, 205–214.
- Willert, K., and Nusse, R. (1998). beta-Catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev.* 8, 95–102.
- Ye, Q. H. *et al.* (2003). Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat. Med.* 9, 416–423.
- Yoshikawa, H., Matsubara, K., Qian, G. S., Jackson, P., Groopman, J. D., Manning, J. E., Harris, C. C., and Herman, J. G. (2001). SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat. Genet.* 28, 29–35.
- Yoshikawa, H., Nagai, H., Oh, K. S., Tamai, S., Fujiyama, A., Nakanishi, T., Kajiyama, G., and Matsubara, K. (1997). Chromosome assignment of aberrant NotI restriction DNA fragments in primary hepatocellular carcinoma. *Gene* 197, 129–135.
- Young, C. S., Kitamura, M., Hardy, S., and Kitajewski, J. (1998). Wnt-1 induces growth, cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. *Mol. Cell. Biol.* 18, 2474–2485.
- Yuza, Y., Agawa, M., Matsuzaki, M., Yamada, H., and Urashima, M. (2003). Gene and protein expression profiling during differentiation of neuroblastoma cells triggered by 13-cis retinoic acid. *J. Pediatr. Hematol. Oncol.* 25, 715–720.
- Zurawel, R. H., Chiappa, S. A., Allen, C., and Raffel, C. (1998). Sporadic medulloblastomas contain oncogenic beta-catenin mutations. *Cancer Res.* 58, 896–899.