Advances in Brief

Small Interfering RNAs Directed against β-Catenin Inhibit the *in Vitro* and *in Vivo* Growth of Colon Cancer Cells[1]

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Abstract

The β-*catenin* and *APC* genes are key components of the Wnt signaling pathway. Mutation of these genes results in increased levels of the β-catenin protein, which is associated with enhanced cellular proliferation and the development of both colon polyps and colon cancer. Recently, a technique known as RNA interference has been successfully adapted to mammalian cells so that it is now possible to specifically decrease the expression of cellular genes after transfection of annealed small interfering 21-mer RNAs. In the current study, we used small interfering RNA (siRNA) directed against β-catenin to determine the effects of decreasing the high constitutive levels of this protein in colon cancer cell lines with mutations in either β-catenin or APC. Our studies demonstrate that siRNA directed against β-catenin markedly decreased β-catenin-dependent gene expression and inhibited cellular proliferation as reflected in the reduced growth of these colon cancer cells both in soft agar and in nude mice. These results indicate that siRNA can target specific factors whose expression is altered in malignancy and may have the potential as a therapeutic modality to treat human cancer.

Introduction

Colon cancer is one of the most common human malignancies, occurring in approximately 6% of the population in the United States (1). A frequent genetic abnormality seen in both hereditary and sporadic forms of polyps and cancer of the colon is mutation of the *APC* and β-*catenin* genes (2–8). These genes are key regulators of the Wnt pathway, which plays a critical role in the control of cellular proliferation.

The current model of Wnt signaling indicates that the binding of Wnt proteins to their receptor, frizzled, stabilizes β-catenin by inhibiting the activity of the serine/threonine kinase GSK-3β.[4] GSK-3β is associated with β-catenin in a multiprotein complex that also includes the adenomatous polyposis coli tumor suppressor protein APC, axin or conductin, protein phosphatase 2A, and disheveled (5). GSK-3β phosphorylation of sites in the NH_2 terminus of β-catenin induces its degradation via the ubiquitin-proteasome pathway (9). After Wnt signaling, β-catenin associates with members of the TCF/lymphocyte-enhancer factor family and migrates to the nucleus, where this complex functions as a transcriptional activator (10). TCF/LEF in conjunction with β-catenin can activate the transcription of a variety of target genes including c-*myc* (11) and *cyclin D1* (12, 13).

A major regulator of β-catenin protein levels is APC (7, 14). Mutations in APC are frequently seen in both hereditary and sporadic colorectal cancers (6, 15), leading to the accumulation of β-catenin and increased levels of β-catenin/TCF-regulated transcription (4, 6, 8, 11). Some colon tumors that do not contain mutations in APC have increased levels of β-catenin as a result of mutations in the NH_2 terminus of β-catenin that prevent GSK-3β phosphorylation and subsequent degradation by ubiquitin-dependent proteolysis (9). Mutations in β-catenin and APC account for the majority of defects seen in tumors that have increased β-catenin levels (3–6).

Although the role of β-catenin in regulating cellular function has been extensively analyzed in other systems (2, 5), the ability to specifically reduce its levels by genetic means in established colon cancer cell lines is important for better defining its role in maintaining the malignant phenotype. Thus, we investigated whether specifically reducing the levels of β-catenin protein in established colon cancer cell lines, in which this protein was overexpressed, might result in decreased *in vitro* and *in vivo* proliferation. For this analysis, RNAi with siRNAs directed against β-catenin was used. RNAi is an evolutionary conserved mechanism that is operative in insects, nematodes, plants, and mammalian cells (16–18). In this process, sequence-specific posttranscriptional silencing is initiated by the introduction into cells of double-stranded annealed sense and antisense RNAs that are homologous to the sequence of the silenced gene (16). The ultimate mediators of RNAi-mediated degradation are 21-mer siRNAs that are generated by RNase III cleavage of double-stranded RNAs that have been introduced into cells and may extend up to several hundred nucleotides. For adaptation of

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[4] The abbreviations used are: GSK, glycogen synthase kinase; RNAi, RNA interference; TCF, T-cell factor; siRNA, small interfering RNA; NF-κB, nuclear factor κB; RSV, Rous sarcoma virus; TFIIB, transcription factor IIB; DEVD, Asp-Glu-Val-Asp; AMC, 7-amino-4-methylcoumarin; siRNAi, small interfering RNA interference; LEF, lymphoid enhancer factor; Tax, HTLV-I transactivator; CDK, cyclin dependent kinase; SPT, suppressor of transcription.

RNAi to mammalian cells, 21-mer sense and antisense RNA oligonucleotides that correspond to a portion of the gene of interest are synthesized and annealed (16–18). The annealed 21-mer RNAs are introduced into cells by transfection, where they bind specifically to the cellular mRNA of interest and activate a RNA degradation process that leads to 80–90% decreases in the corresponding protein levels. The use of 21-mer RNAs in mammalian cells, rather than longer RNAs that are used in other species, avoids the activation of the double-stranded dependent protein kinase, PKR, and nonspecific RNases that nonspecifically silence gene expression.

The studies reported here demonstrate that RNAi provides a simple, reproducible, and highly efficient means to determine the role of β-catenin on the growth of colon cancer cells both in culture and in nude mice. In addition, these data indicate that RNAi provides a useful methodology with which to study the role of regulatory genes that control the proliferation of cancer cells. Finally, our *in vivo* studies suggest that RNAi may have therapeutic potential in the treatment of cancer.

Materials and Methods

Cell Lines. The colon cancer adenocarcinoma cell lines SW480 (4, 14) and HCT116 (3, 4), with mutations in APC or β-catenin, respectively, were obtained from the American Type Culture Collection (Manassas, VA). These cells were propagated and maintained in McCoy's 5A and Leibovitz L-15 media (Life Technologies, Inc., Rockville, MD), respectively, and supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The HCT116 cells were maintained with 5% CO_2, whereas the SW480 cells were maintained without CO_2.

RNA Oligonucleotides. siRNA oligonucleotides with two thymidine residues (dTdT) at the 3′-end of the sequence were designed to β-catenin-1 (sense, 5′-AGCUGAUAUUGAUGGACAG-3′), which extends between amino acids 79 and 85 of β-catenin, β-catenin-2 (sense, 5′-CAGUUGUGGUUAAGCUCUUdAdC-3′), which extends between amino acids 491 and 498 of β-catenin, APC (sense, 5′-GCAACAGAAGCAGAGAGGU-3′), which extends between amino acid 238 and 245 of APC, the NF-κB p65 subunit (sense, 5′-GCCCUAUCCCUUUACGUCA-3′), which extends between amino acids 347 and 353 of p65; and HTLV-1 Tax (sense, 5′-GAUGGACGCGUUAUCGGCU-3′), which extends between amino acids 60 and 66 of Tax, along with their corresponding antisense oligonucleotides, as described previously (Ref. 18; Dharmacon Research Inc., Lafayette, CO). These RNAs were dissolved in TE [10 mM Tris-Cl (pH 8.0) and 1 mM EDTA] as 200 μM solutions. Double-stranded siRNAs were generated by mixing the corresponding pair of sense and antisense RNA oligonucleotides at 20 μM concentration in annealing buffer [30 mM HEPES-KOH (pH 7.9), 100 mM potassium acetate, and 2 mM magnesium acetate]. The reaction mixture was heated to 95°C for 2 min, allowed to come to room temperature over 30 min, and then aliquoted and stored at –20°C.

Transfection of RNA Oligonucleotides. Approximately, 1×10^6 cells were plated per 6-well plate in media containing 10% fetal bovine serum to give 30–50% confluence, and transfection of the RNA oligonucleotides was performed using Oligofectamine (Invitrogen, Carlsbad, CA) to result in a final RNA concentration of 50 nM. The cells were harvested at different time points and lysed in PD buffer [40 mM Tris-HCl (pH 8.0), 500 mM sodium chloride, 0.1% NP40, 6 mM EDTA, 6 mM EGTA, 10% glycerol, 10 mM sodium fluoride, and 1 mM sodium orthovanadate] for Western blot analysis.

To determine the effects of siRNA on β-catenin reporter constructs, the cells were transfected using 4 μl of GeneJuice (Novagen, Madison, WI) at 24 h after siRNA transfection with either TOPFLASH or FOPFLASH (0.5 μg) luciferase and a RSV-β-galactosidase reporter (0.1 μg; Ref. 8). The TOPFLASH and FOPFLASH reporters contain three wild-type or mutant β-catenin and TCF/LEF binding sites respectively inserted upstream of a minimal c-*fos* promoter. The cells were harvested after an additional 24 h, lysed in buffer (Promega, Madison, WI), and analyzed for luciferase and β-galactosidase activity using specific assays (Promega).

Western Blot Analysis. Cells were prepared in PD buffer, and Western blot analysis was performed as described previously (19). The antibodies and dilutions used included anti-APC (Ab-1; 1:1000; Oncogene, San Diego, CA), anti-β-catenin (1:1000; Transduction Laboratories, San Diego, CA), anti-c-*myc* (9E-10; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin D1 (1:500; Santa Cruz Biotechnology), anti-actin (1:1000; Santa Cruz Biotechnology), anti-CDK9 (1:1000; Santa Cruz Biotechnology), anti-p65 (1:1000; Santa Cruz Biotechnology), anti-SPT5 (1:1000; Ref. 19), or anti-TFIIB (1:1000; Transduction Laboratories). After extensive washing, the membranes were incubated with antimouse or antirabbit IgG-horseradish peroxidase conjugate antibody (Amersham, Piscataway, NJ) at a 1:2000 dilution for 1 h at room temperature and developed using enhanced chemiluminescence (Amersham).

Immunofluorescence Microscopy. HCT116 cells were grown on coverslips to 30–40% confluence and transfected with the various RNA oligonucleotides. At 72 h, the cells were processed for immunofluorescence as described previously (20) and analyzed on a Zeiss Axioskop 2 microscope at ×63 magnification. Antibodies against β-catenin and lamin B (Santa Cruz Biotechnology) were used at a dilution of 1:200, whereas the respective FITC- or Rhodamine Red-X-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1:400 dilutions.

Cellular Proliferation Assays. HCT116 and SW480 cells transfected with the indicated RNA oligonucleotides were cultured in 96-cell well plates in replicates of 12. The cells were then incubated with 200 μl of tissue culture medium and pulsed with 1 μCi/well [^{3}H]thymidine (Perkin-Elmer, Boston, MA) for 12 h at 37°C. Extracts were prepared using a semiautomated cell harvester, and the [^{3}H]thymidine-labeled DNA was extracted and attached to glass fiber filter paper. The filter discs were air dried, transferred to a scintillation vial containing 3 ml of scintillation fluid, and counted on a Beckman LS 6000 liquid scintillation beta counter.

Caspase-3 Assays. Induction of apoptosis after siRNA transfection was assessed by detecting caspase activity in cell lysates 48 h after siRNA transfection. The activity of DEVD-specific proteases was measured using an EnzCheck Caspase-3 Assay Kit (Molecular Probes, Eugene, OR) as suggested by the

manufacturer. At 48 h after siRNA transfection, the cells were harvested, and 1×10^6 cells were frozen at −80°C until the time of assay. The frozen cells were resuspended in 50 μl of lysis buffer and incubated on ice for 30 min, and cellular debris was pelleted. Lysates (50 μl) were transferred to 96-well plates and incubated at room temperature for 50 min with substrate (Z-DEVD-AMC) in 2× reaction buffer at a 100 μM final concentration. Cell lysate alone with the caspase inhibitor Ac-DEVD-CHO at a 100 μM final concentration was also included. Fluorescence was measured by a fluorometer at an excitation wavelength of 350 nm and a detection wavelength of 450 nm.

Soft Agar Colony Assays. At 24 h after siRNA transfection, the cells were mixed with tissue culture media containing 0.6% agar to result in a final agar concentration of 0.4%. Then 1 ml of this cell suspension was immediately plated in 6-well plates coated with 0.6% agar in tissue culture media (1 ml/well), and the colonies were counted 10 days after plating. The cultures were analyzed in triplicate, and the number of colonies/10^5 cells was calculated.

Murine Xenograft Model. Institutional guidelines and a Animal Research Committee-approved protocol were followed for mouse studies. For these studies, 4–6-week-old female nude$^{nu/nu}$ mice were obtained from Charles River (Wilmington, MA) and housed in clean specific pathogen-free rooms in groups of 5 and cages containing microisolator tops. At either 6 or 24 h after transfection of HCT116 cells with siRNA directed against either β-catenin (experimental group) or Tax (control group), the cells were harvested, washed twice in ice-cold serum-free McCoy's 5A media, counted for viable cells by trypan blue exclusion, and resuspended in the same media. The cells were resuspended in this same medium and used to inject each group of mice s.c. in the right flank with 2.5×10^6 cells in a volume of 0.25 ml. The tumors were measured in 3 axes from day 7 onward, and the tumor volume was calculated from these measurements. The survival was determined from the day of tumor injection to the day of euthanasia. As per institutional requirements, the mice were euthanized once tumors grew to greater than 2 cm.

In another experiment, 2×10^6 untreated HCT116 cells were injected into nude mice by i.p. injection. These mice were randomly divided into two groups of nine mice each. One group of mice was treated with siRNA directed against β-catenin, whereas the other group of mice was treated with siRNA directed against Tax. These mice were given i.p. injections containing 250 pmol of each of the siRNAs complexed with Oligofectamine and diluted into 0.5 ml of serum-free media. These treatments consisted of i.p. injections of the siRNAs administered once a day every 4 days for four doses starting 6 h after injection of the HCT116 cells. Afterward, the siRNAs were injected weekly for 3 additional weeks. Mice were carefully observed for survival, which was measured from day of injection of the HCT116 cells to either the day of spontaneous death or the day of euthanasia in the case of moribund mice.

Assessment of Expression β-Catenin in *in Vivo* Growing Tumor Samples. s.c. tumors were induced in nude mice by s.c. injection of 2.5×10^6 unmanipulated HCT116 cells, as described above. Seven days after induction of tumor, the mice were given 250 pmol of siRNA diluted in 250 μl of sterile PBS by i.v. injection in the lateral tail vein. The control group received Tax siRNA, whereas the experimental group was given β-catenin siRNA. Tissue samples were obtained 48–72 h after the i.v. dose of oligonucleotides, by core biopsy. The core biopsy was performed on tumors under anesthesia. Tissue samples were collected in PD buffer and incubated on ice for 30 min with intermittent mixing. After solubilization, the supernatant was collected by centrifugation at 14,000 rpm at 4°C for 20 min. The protein concentration was measured in each sample, and an equal amount of protein was used to analyze β-catenin levels by Western blot.

Statistical Analysis. Sigma Plot (SPSS Inc., Chicago, IL) was used to analyze the data and plot curves. Two-tailed unpaired *t* test was used to compare the statistical significance of the differences in data from two groups, where appropriate. Kaplan-Meier analysis was used to plot survival curves for mice injected with HCT116 cells after β-catenin or Tax siRNAi treatment.

Results

siRNA Directed against β-Catenin Reduces Its Expression in Colon Cancer Cell Lines with Mutations in Either APC or β-Catenin. In the majority of colon cancers, mutation in either the *APC* or β-*catenin* gene leads to increased levels of β-catenin (3, 4, 6, 8). The increased levels of β-catenin lead to the enhanced expression of β-catenin/TCF-regulated genes such as c-*myc* and *cyclin D1* (11–13), resulting in increased cellular proliferation (21–23). Two colon cancer cell lines, SW480 (4, 14) and HCT116 (3, 4), with mutations in either the *APC* or β-*catenin* gene, respectively, result in increased β-catenin levels (3, 4, 8, 14). In this study, RNAi was used in an attempt to decrease β-catenin expression in these cell lines to address whether its overexpression may have a role in regulating the proliferation of established colon cancer cells.

For these studies, annealed 21-mer sense and antisense RNA oligonucleotides directed against a portion of the β-*catenin* gene were used. In addition, RNA oligonucleotides directed against either the *APC* or the human T-cell leukemia virus *Tax* gene were used as controls. Each of these annealed RNAs was transfected into SW480 and HCT116, and their effects on β-catenin protein levels were compared with mock-transfected cells by Western blot analysis at 48 and 72 h posttransfection (Fig. 1*A*).

siRNA directed against β-catenin reduced the levels of β-catenin in both SW480 and HCT116 cells but did not affect the levels of actin (Fig. 1*A*). Interestingly, siRNA directed against β-catenin altered the mobility but not the level of APC, whereas siRNA directed against APC reduced the levels of this protein but had only minimal effects on the levels of β-catenin (Fig. 1*A*). SiRNA directed against Tax or mock transfection did not alter either β-catenin or APC levels (Fig. 1*A*). Similar results with β-catenin siRNAs were also seen in other cell lines including HeLa and 293 (data not shown). These results indicate that siRNA can markedly reduce β-catenin levels in colon cancer cell lines that have elevated levels of this protein as a consequence of mutations in either β-catenin or APC.

It was important to address whether siRNA directed against β-catenin altered either its level or cellular distribution when assayed by immunofluorescence. siRNA directed against either

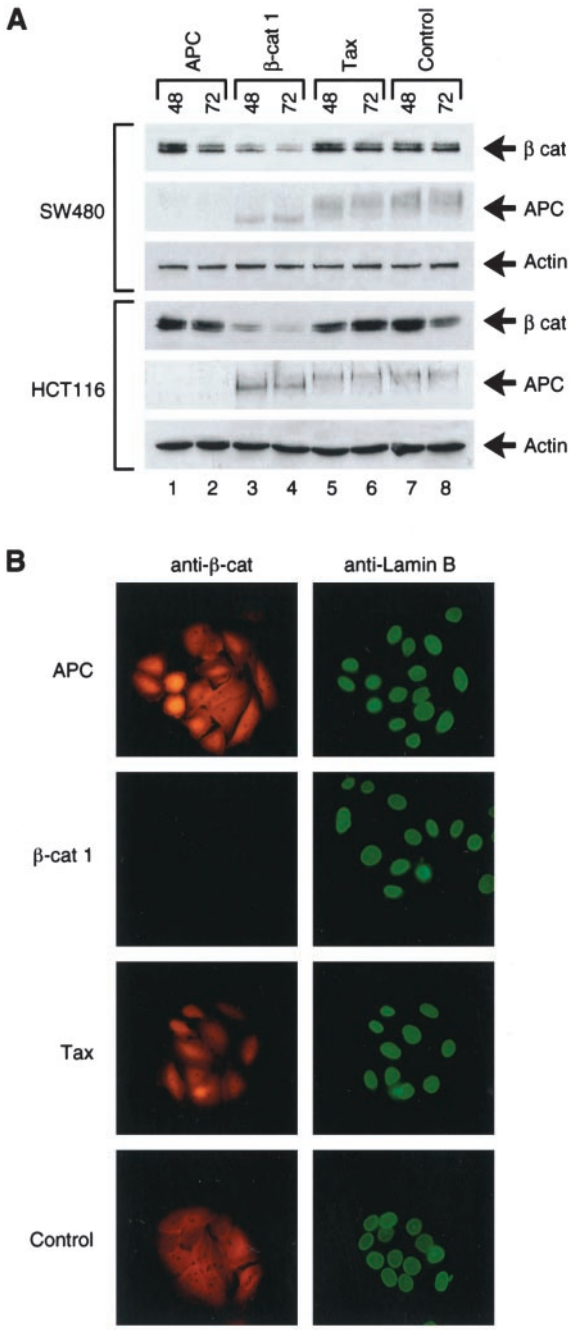


Fig. 1 siRNA directed against β-catenin specifically inhibits its expression. *A*, SW480 and HCT116 cells were transfected with 50 nM annealed sense and antisense 21-mer RNA oligonucleotides directed against either APC, β-catenin, or Tax containing the sequences listed in "Materials and Methods" or mock-transfected. Cells were harvested at either 48 or 72 h posttransfection, and Western blot analysis was performed. *B*, HCT116 cells grown on coverslips were transfected with the same annealed 21-mer RNA oligonucleotides used in *A* and stained with β-catenin or lamin B primary antibodies and Rhodamine Red-X- or FITC-conjugated secondary antibodies. The cells were then subjected to analysis with confocal microscopy.

APC, β-catenin, Tax, or mock transfection of HCT116 cells was performed, and these cells were then stained with antibody directed against either β-catenin or lamin B (Fig. 1*B*). In contrast to the membrane and nuclear staining of β-catenin that has been previously noted (20), siRNA directed against β-catenin reduced its levels such that it was not detected with an antibody directed against β-catenin (Fig. 1*B*). There was readily detectable nuclear staining of lamin B in these same cells. siRNA directed against APC resulted in slightly increased nuclear staining of β-catenin, whereas siRNA directed against Tax resulted in similar immunostaining of β-catenin as seen with control cells (Fig. 1*B*). Because APC has been demonstrated to enhance the nuclear export of β-catenin, siRNA-mediated decreases in APC can potentially result in increased nuclear levels of β-catenin and β-catenin-dependent gene expression (24). Thus, siRNA directed against β-catenin reduced its levels using both Western blot and immunofluorescence assays.

Time Course for siRNA-mediated Reductions in β-Catenin Protein. Next we addressed the duration of siRNA-mediated decreases in the expression of the β-catenin protein in both HCT116 and SW480 cells. In addition, we addressed whether siRNAs directed against another region of β-catenin could also reduce β-catenin levels and determined the specificity of β-catenin siRNA by assaying its effects on the expression of a variety of other cellular proteins. To address these points, siRNAs directed against either of two distinct regions of β-catenin (regions 1 and 2), APC, the p65 subunit of the NF-κB protein, or Tax were transfected into HCT116 and SW480 cells. The siRNA directed against the p65 NF-κB subunit was included in these studies to further assess the specificity of these siRNAs. Cells were harvested at 2, 3, 7, and 10 days after siRNA transfection, and extracts were prepared and analyzed by Western blot analysis for the expression of β-catenin and other transcriptional regulators including p65, SPT5, and CDK9, in addition to actin (Fig. 2).

This analysis revealed that β-catenin protein levels were reduced in both SW480 (Fig. 2*A*) and HCT116 (Fig. 2*B*) cells by siRNAs directed against two separate regions of β-catenin, but not by siRNAs directed against either Tax, APC, or p65 (Fig. 2). The levels of β-catenin were decreased at 48 h after siRNA transfection and remained reduced in both SW480 and HCT116 cells for 10 days after siRNA transfection (Fig. 2). A similar degree of inhibition over this time frame was seen with siRNA directed against the p65 subunit of NF-κB (Fig. 2). Western blot analysis demonstrated that the β-catenin siRNAs decreased β-catenin protein levels but not the levels of p65, SPT5, CDK9, or actin. Similarly, the p65 siRNA decreased the expression of p65 but not the levels of β-catenin, SPT5, CDK9, or actin. These results indicate that siRNAs have a high degree of specificity and resulted in prolonged decreases in specific cellular gene expression without marked effects on the expression of other cellular proteins.

β-Catenin-dependent Gene Expression Is Reduced by siRNA. When β-catenin is present in the nucleus, it is able to bind in conjunction with TCF/LEF to elements found in the promoters of a variety of cellular genes (10). A luciferase reporter with a minimal c-*fos* promoter and multiple binding sites for the TCF/LEF and β-catenin complex known as TOPFLASH and another luciferase reporter that contains mutated TCF/LEF binding sites

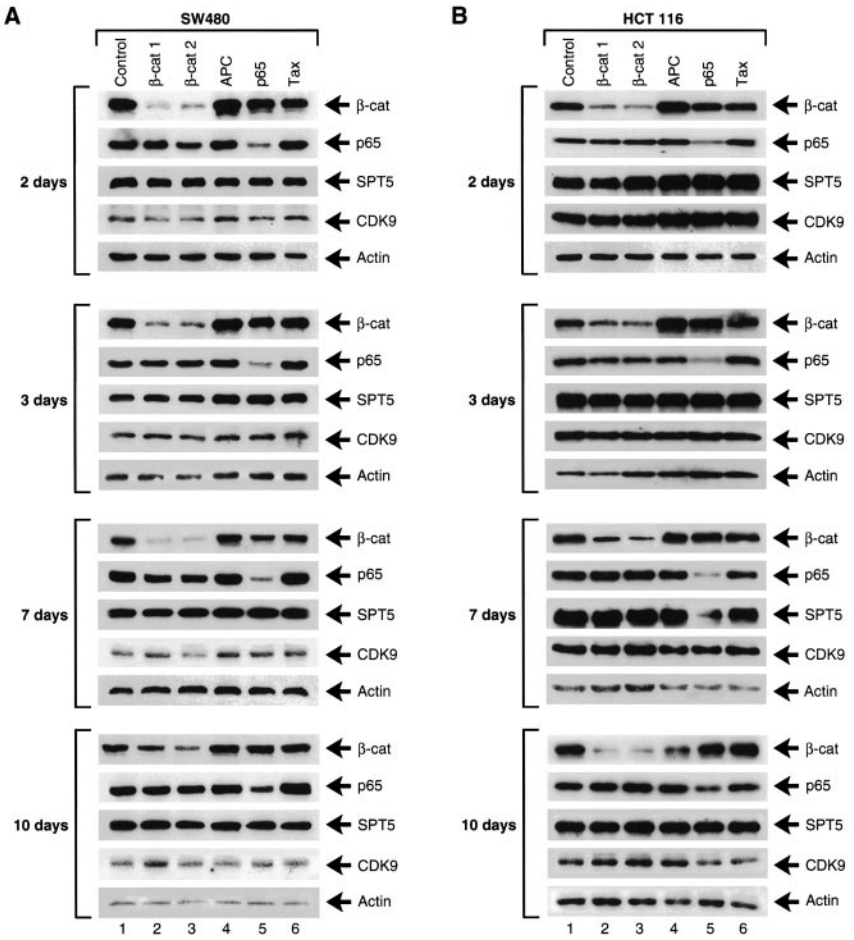


Fig. 2 Time course of siRNA reduction in β-catenin protein levels. (*A*) SW480 or (*B*) HCT116 cells were transfected with 21-mer annealed RNA oligonucleotides (50 nM) directed against either of two regions of β-catenin (1 and 2), the NF-κB p65 subunit, APC, or Tax or mock (control)-transfected. Extracts were prepared from each set of these cells at 2, 3, 7, or 10 days posttransfection and analyzed by Western blot analysis for the expression of β-catenin, p65, SPT5, CDK9, and actin.

known as FOPFLASH have been widely used to characterize β-catenin-dependent gene expression (8). These reporter constructs were transfected into siRNA-treated SW480 and HCT116 cells, and luciferase activity was determined.

siRNA directed against β-catenin, but not siRNA directed against APC or Tax, reduced TOPFLASH activity between 6- and 8-fold in SW480 cells (Fig. 3*A*). SiRNA directed against β-catenin also resulted in a 5–6-fold decrease in TOPFLASH activity in HCT116 cells (Fig. 3*B*). There was little effect of siRNA directed against β-catenin on the FOPFLASH reporter (Fig. 3, *C* and *D*). HCT116 cells contain a wild-type *APC* gene that can reduce the nuclear levels of β-catenin via its ability to export β-catenin to the cytoplasm (4). siRNA directed against APC markedly stimulated β-catenin-dependent gene expression in HCT116 cells (Fig. 3*B*). This result was consistent with the increased nuclear levels of β-catenin seen in these cells when analyzed by immunofluorescence (Fig. 1*B*). Finally, we addressed whether siRNA directed against β-catenin reduced the expression of two cellular genes known to be regulated by the TCF/LEF and β-catenin. The endogenous expression of c-*myc* and cyclin D1, but not the transcription factor TFIIB, was reduced by transfection of siRNA directed against β-catenin, but not APC or Tax (Fig. 3*E*). These results indicate that siRNA can specifically decrease the expression of β-catenin-dependent genes.

Role of β-Catenin on Regulating Cellular Proliferation. Previous studies have demonstrated that β-catenin increases cellular proliferation (22, 23), likely due to its ability to increase the expression of specific cellular genes (11–13). Increased β-catenin levels may play a role in the proliferative advantage seen in colon polyps and other premalignant lesions (5). Whether increased levels of β-catenin also regulate the proliferation of established cancer cell lines remains unclear.

First, SW480 and HCT116 cells were either mock-transfected or transfected with siRNA directed against either APC, β-catenin, or Tax. The number of SW480 (Fig. 4*A*) and HCT116 (Fig. 4*B*) cells was then determined at both 24 and 72 h posttransfection. These studies indicated that siRNA directed against β-catenin slightly but reproducibly decreased the growth of SW480 cells at 72 h posttransfection (Fig. 4*A*). There was a much greater effect of the siRNA directed against β-catenin on the growth of HCT116 cells, with a 40–50% reduction in the growth of these cells seen at 72 h posttransfection in four separate experiments (Fig. 4*B*). In addition, siRNAi directed against β-catenin was also used to determine its effects on [^{3}H]thymidine incorporation in both SW480 and HCT116 cells.

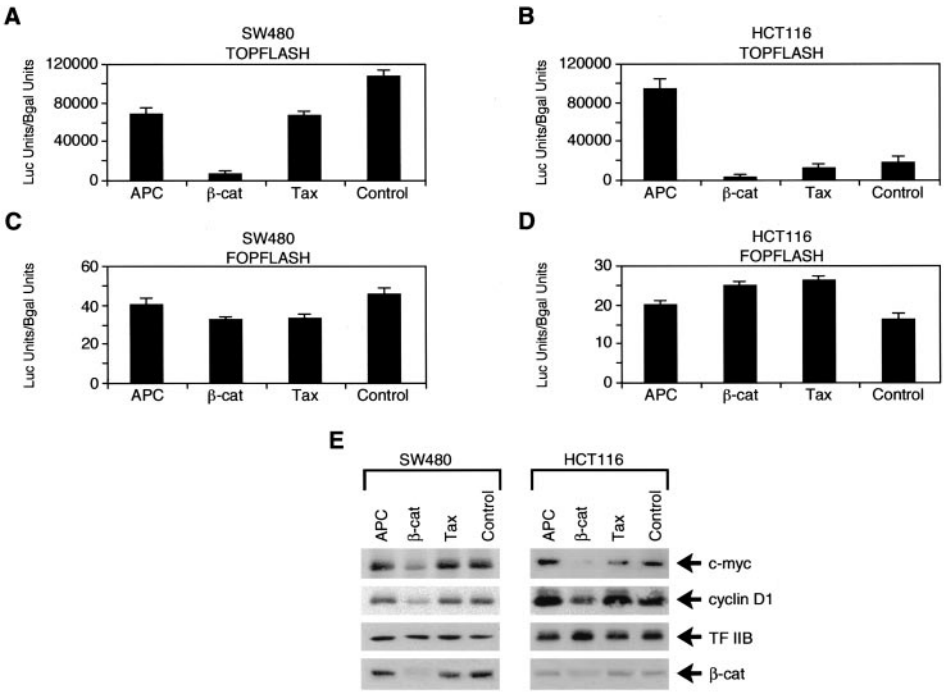


Fig. 3 siRNA directed against β-catenin reduces β-catenin-dependent gene expression. (*A* and *C*) SW480 and (*B* and *D*) HCT116 cells were transfected with 21-mer annealed RNA oligonucleotides (50 nM) directed against either APC, β-catenin, or Tax or mock-transfected. At 24 h posttransfection, the cells were transfected with either (*A* and *B*) TOPFLASH or (*C* and *D*) FOPFLASH reporters and a RSV β-galactosidase reporter. Luciferase and β-galactosidase activity were determined 48 h later. All transfections were normalized using RSV-β-galactosidase expression. The luciferase assays were performed in triplicate and repeated in three separate experiments. *E*, Western blot analysis was performed on extracts prepared from SW480 and HCT116 cells transfected for 72 h with RNA oligonucleotides directed against APC, β-catenin, or Tax or mock-transfected using antibodies directed against either β-catenin, c-myc, cyclin D1, or TFIIB.

RNAi directed against β-catenin resulted in a 20–30% decrease in the incorporation of [^{3}H]thymidine in both SW480 and HCT116 cells (Fig. 4, *C* and *D*). In contrast, RNAi directed against either APC or Tax did not result in significant decreases in [^{3}H]thymidine incorporation.

Finally, we wanted to address whether the decrease in growth or [^{3}H]thymidine incorporation seen in the β-catenin siRNA-transfected cells was due to increased levels of apoptosis. Caspase-3 activity in these siRNA-transfected cells was assayed in both the absence and presence of the caspase-3 inhibitor DEVD (Fig. 4, *E* and *F*). This analysis revealed that there was no increase in the level of apoptosis in either SW480 or HCT116 cells in the presence of the β-catenin siRNA. Western blot analysis of these samples confirmed that the β-catenin siRNAi resulted in decreased β-catenin levels (data not shown). These experiments, which were repeated on three separate occasions with similar results, suggested that β-catenin is important in regulating the proliferation of established colon cancer cell lines but does not lead to increased levels of apoptosis.

Reductions in β-Catenin Protein Inhibit Colony Formation. Given the effects of β-catenin siRNA on cellular proliferation, we next tested whether siRNA-mediated reductions in β-catenin levels decreased the ability of SW480 and HCT116 cells to form colonies in soft agar. To test this point, each of these cell lines was transfected with siRNA directed against either APC, β-catenin, or Tax or mock-transfected (Fig. 5, *A* and *B*). At 24 h posttransfection, the cells were placed into media with soft agar, and colony formation was assayed after 10 days. siRNA directed against β-catenin resulted in a significant decrease in colony formation in both SW480 (Fig. 5*A*) and HCT116 cells (Fig. 5*B*). There was a 3–4-fold decrease in colony formation seen in both SW480 and HCT116 cells transfected with siRNA directed against β-catenin, but not APC or Tax. These results suggested that reductions in β-catenin levels decreased the ability of colon cancer cells to form colonies in soft agar.

siRNA Directed against β-Catenin Reduces Tumor Growth *in Vivo*. Next, we addressed whether the transient exposure of HCT116 cells in culture to siRNA directed against β-catenin altered the ability of these cells to proliferate after injection into nude mice. In addition, we asked whether the *in vivo* administration of siRNA directed against β-catenin into nude mice after the i.p. injection of HCT116 cells could inhibit the growth of these cells and result in increased survival of these mice. Thus, we could address the potential *in vivo* effects of siRNA on inhibiting the proliferation of colon cancer cells.

In the first series of experiments, HCT116 cells were first transfected with siRNA directed against either β-catenin, Tax, or APC for either 6 or 24 h. Western blot analysis at 24 h posttransfection demonstrated a small reduction in β-catenin levels in the HCT116 cells transfected with β-catenin siRNA but not in those transfected with APC or Tax siRNAs (Fig. 6*A*). [^{3}H]Thymidine labeling of a portion of these HCT116 cells between 16 and 24 h after siRNA transfection revealed that there was little difference in the thymidine incorporation in these cells before their injection into nude mice (Fig. 6*B*). A similar number of these HCT116 cells that were transfected for either 6 (Fig. 6*C*) or 24 h (Fig. 6*D*) with siRNA directed against either β-catenin or Tax were then injected into two groups of five nude mice. At 4 weeks after injection of these siRNA-transfected HCT116 cells, the size of the tumors was determined.

As seen in Fig. 6, *C* and *D*, there was more than a 3-fold decrease in the average size of the HCT116 tumors derived from

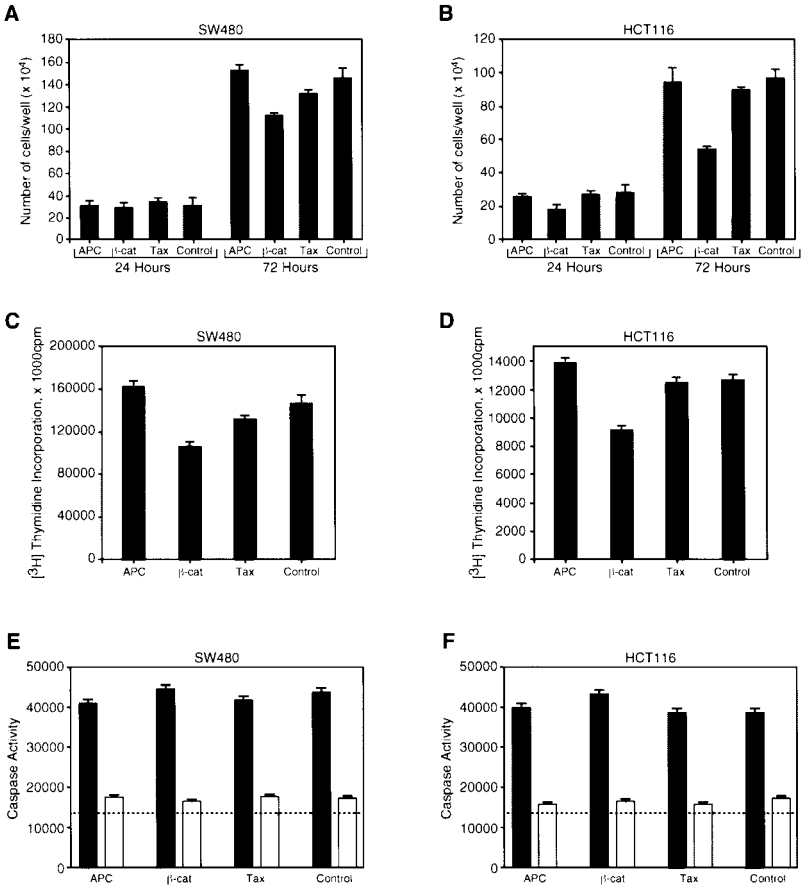
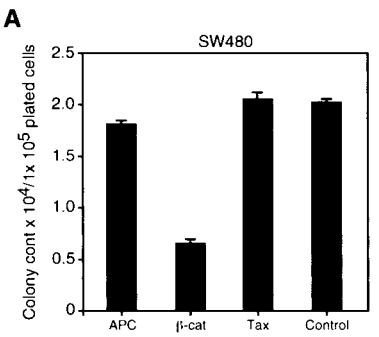


Fig. 4 siRNA directed against β-catenin leads to reduced cellular proliferation. (*A*, *C*, and *E*) SW480 and (*B*, *D*, and *E*) HCT116 cells were transfected with annealed 21-mer RNA oligonucleotides (50 nM) directed against either APC, β-catenin, or Tax or mock-transfected. *A* and *B*, after 24 h, the cells were trypsinized, replated in triplicate, and counted 24 and 72 h later by trypan blue exclusion with the SE indicated. *C* and *D*, HCT116 and SW480 cells were transfected with 50 nM concentrations of annealed 21-mer RNA oligonucleotides directed against APC, β-catenin, or Tax or mock-transfected (*Control*). At 60 h posttransfection, the cells were labeled with [^{3}H]thymidine for 12 h, and the amount of incorporation was determined for the same number of cells as described in "Materials and Methods." *E* and *F*, caspase-3 activity was assayed in HCT116 and SW480 cells transfected with siRNAs directed against APC, β-catenin, or Tax or mock-transfected. The activity of caspase-3 and related DEVD-specific proteases was assessed by fluoroscopic measurement of proteolytic cleavage of Z-DEVD-AMC to the fluorescent molecule AMC. The specificity of this reaction was assayed by measuring fluorescence in samples in the presence of the DEVD-specific protease inhibitor Ac-DEVD-CHO. Caspase activity in arbitrary units as measured by fluorescence of samples after incubation with substrate (■) and in presence of inhibitor (□) is shown. The *horizontal line* indicates baseline fluorescence.



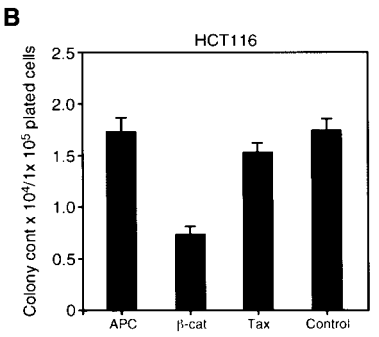


Fig. 5 siRNA directed against β-catenin reduces colony formation in soft agar. (*A*) SW480 and (*B*) HCT116 cells were transfected with annealed 21-mer RNA oligonucleotides (50 nM) directed against APC, β-catenin, or Tax or mock-transfected and plated in media containing soft agar at 24 h posttransfection. At 10 days posttransfection, the colonies were counted in three different plates, and the averages were plotted. The reduced number of colonies in the β-catenin group as compared with the control was statistically significant ($P < 0.01$).

cells transfected with siRNA directed against β-catenin as compared with HCT116 cells transfected with siRNA directed against Tax. These results were statistically significant with a P of <0.001 and <0.05, respectively. A Kaplan-Meier survival curve from an additional experiment using these siRNA-transfected HCT116 cells is shown in Fig. 6*E*. Of the five mice that received the HCT116 cells transfected with siRNA directed against β-catenin, two mice were still alive at 100 days after injection. In contrast, none of the mice who received the HCT116 cells transfected with siRNA directed against Tax were alive at this time.

Next, we addressed whether the i.p. injection of siRNAs directed against β-catenin as compared with Tax could alter the growth of HCT116 cells in nude mice (Fig. 6*F*). Approximately 2×10^6 HCT116 cells were injected i.p. into two groups of nine mice. These mice then received i.p. injection with 250 pmol of either Tax or β-catenin siRNAs, with the first injection performed 6 h after installation of the cells, and three additional injections were administered at 4-day intervals and then weekly for 3 weeks. There was one early death in the β-catenin siRNA group in the second week of the study due to unrelated causes because this mouse had no evidence of tumor at necropsy. Of the remaining mice, which were followed for a period of 70 days, none of the nine mice injected with Tax siRNA were alive,

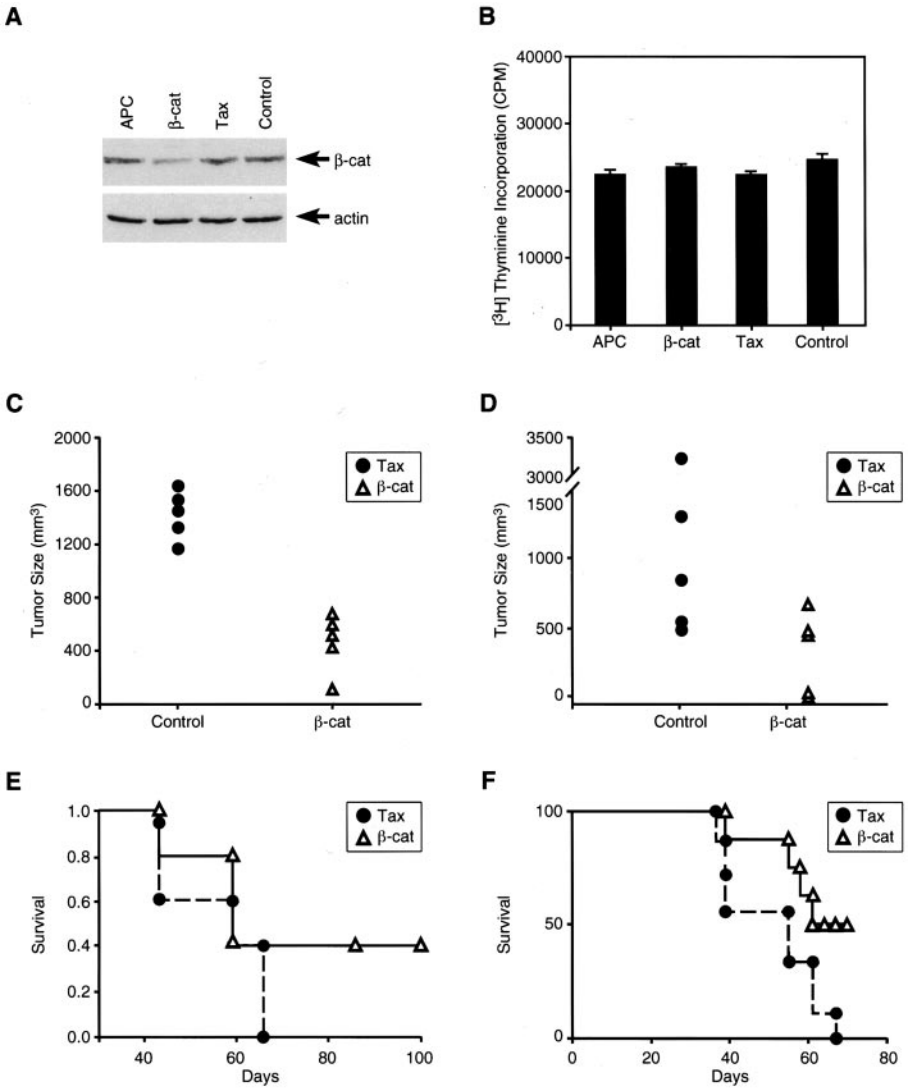


Fig. 6 siRNA directed against β-catenin reduces HCT116 tumor growth in nude mice. *A*, HCT116 cells were transfected with RNA oligonucleotides directed against APC, β-catenin, or Tax or mock-transfected. At 24 h posttransfection, extracts were prepared and analyzed by Western blot analysis using antibodies directed against β-catenin and actin. *B*, a portion of the HCT116 cells was also pulsed with [^{3}H]thymidine between 16 and 24 h after siRNA transfection, and [^{3}H]thymidine incorporation was determined. The HCT116 cells (2.5×10^6) used in *A* and *B* that were transfected with siRNAs directed against either Tax or β-catenin for either (*C*) 6 or (*D*) 24 h were injected into five nude mice each, as described in "Materials and Methods." Tumors were measured twice weekly, and their size at 4 weeks after injection is indicated. The *P* for difference between the growth of the tumors in the presence of Tax and β-catenin siRNA was $P < 0.05$ and $P < 0.001$, respectively. *E*, the survival of nude mice that received injection with 1×10^6 HCT116 cells transfected with siRNAs directed against either β-catenin or Tax was determined over a 100-day time period, and Kaplan-Meier analysis is shown. Mice were sacrificed when the tumor size reached 2.0 cm. *F*, two groups of nine nude mice each received i.p. injection with 2.5×10^6 HCT116 cells and then received i.p. injection with 250 pmol of siRNA directed against either β-catenin or Tax as described in "Materials and Methods." The survival of these mice was measured from the day of the initial tumor injection, and Kaplan-Meier survival probability was plotted. The survival of β-catenin siRNA-injected mice as compared with Tax siRNA-injected mice is shown ($P < 0.05$ by log-rank test).

whereas four of eight mice injected with β-catenin siRNA remained alive. Analysis of tumors from mice treated with either Tax or β-catenin siRNA revealed similar histology with no changes in the state of differentiation of the HCT116 cells. A Kaplan-Meier curve indicated that these survival differences were statistically significant with a $P < 0.05$ (Fig. 6*F*).

Finally, we addressed whether the administration of β-catenin siRNAs specifically reduced β-catenin levels after the i.v. administration of these siRNA. Two groups of five mice, each bearing HCT116 tumors that had grown for 1 week, received i.v. injection with 250 pmol of β-catenin or Tax siRNAs, and the tumors were biopsied 72 h later. The β-catenin siRNA significantly reduced β-catenin levels as compared with the Tax siRNA (Fig. 7). Thus, siRNA appears to be effective in reducing β-catenin levels and the *in vivo* growth of HCT116 cells in nude mice when administered by either transfection or after i.v. and i.p. injections.

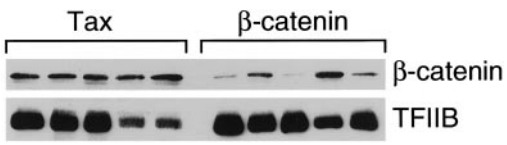


Fig. 7 Decreased expression of β-catenin in tumors of mice treated with siRNA to β-catenin. As outlined in "Materials and Methods," tumor samples were collected from mice treated with 250 mol of siRNA directed against either β-catenin or Tax, solubilized in PD buffer, and subjected to Western blot analysis. Immunoblots were analyzed using anti-β-catenin or anti-TFIIB (control) antibodies at 72 h postinjection.

Discussion

RNAi is an important technique with which to determine how decreases in the expression of specific regulatory genes alter the growth of both normal and cancer cells. In the current study, we determined how decreases in the high constitutive

levels of β-catenin in established colon cancer cells altered their *in vitro* and *in vivo* growth. Mutations in multiple components of the Wnt pathway including APC, β-catenin, and axin can result in increased levels of β-catenin (2, 5). The increased levels of β-catenin frequently found in both premalignant and malignant cells are associated with increased rates of cellular proliferation (5, 23). Moreover, modest overexpression of β-catenin in epithelial cells leads to increased proliferation and can result in transformation (21–23).

In our study, the functional consequences of RNAi-mediated decreases of β-catenin in established colon cancer cells were determined using assays of β-catenin-dependent gene expression, cellular proliferation, and *in vitro* and *in vivo* assays of tumor growth. In addition to mutations of β-*catenin* and *APC,* the cell lines used in this study have mutations in a variety of other cellular regulatory genes such as mismatch repair genes in the case of HCT116 and *p53* in the case of SW480 (25). Because mutations in the Wnt pathway leading to elevated levels of β-catenin frequently occur at an early stage of the neoplastic process, it was not clear whether reducing its expression at later stages of this process would decrease either cellular proliferation or tumor formation. Our analysis indicates that decreases in the levels of β-catenin in established colon cancer cell lines decrease proliferation by a mechanism that does not depend on increased apoptosis. Although it has been reported that down-regulation of signaling by β-catenin and TCF/LEF correlates with differentiation of Caco-2 cells in cell culture (26), histological analysis of the HCT116 xenografts did not demonstrate that siRNA directed against β-catenin resulted in increased differentiation of these cells.[5] These results suggest that reductions in TCF/LEF signaling may be associated with cell type-specific effects. In summary, our results demonstrated that reductions in β-catenin levels in established colon cancer cell lines decrease colony formation in soft agar and result in prolonged decreases in the growth of these cells when implanted into nude mice. These studies support a role for a β-catenin as an important factor in increasing the proliferation of established colon cancer cells and as a potential target to inhibit the growth of these cells.

Recent work suggests that after Wnt signaling, the dephosphorylated form of β-catenin is preferentially increased to result in higher levels of β-catenin-dependent gene expression (27). These data suggest that the β-catenin mutants in NH_2-terminal phosphorylation sites may have properties that differ from wild-type β-catenin. Targeted disruption of wild-type or mutant β-*catenin* genes in HCT116 cells was used to further characterize these alleles (28). This analysis revealed that the mutant β-catenin protein, as compared with the wild-type protein, exhibited decreased association with E-cadherin, had increased nuclear localization and transcriptional activity, and resulted in enhanced growth of cells when plated at low densities. However, disruption of the mutant β-catenin allele alone did not prevent the growth of HCT116 cells either in culture or in nude mice. Thus, disruption of the mutant β-*catenin* gene reduced the ability of HCT116 cells to form colonies in cell culture under specific conditions, although it was not essential for the growth of this established colon cancer cell line. It is important to note that in our studies, transfection of siRNAs reduced the amounts of both the wild-type and mutant β-catenin proteins and thus likely resulted in more severe decreases in β-catenin protein levels with greater effects on cellular proliferation than seen in targeted disruption of these individual alleles.

Because mutation of different components of the Wnt pathway including β-catenin, APC, axin, and protein phosphatase 2A can lead to β-catenin overexpression in cancer cells (3–6, 14), siRNA can be used to decrease β-catenin levels that result from mutations in different components of the Wnt pathway. For example, the elevated levels of β-catenin in SW480 cells resulting from mutations in the *APC* gene could also be targeted by siRNA directed against β-catenin. It was also interesting to note that siRNA directed against β-catenin resulted in alteration in the mobility of APC when analyzed by Western blot analysis. It seems likely that β-catenin binding to APC may either directly or indirectly alter APC phosphorylation or other posttranslational modifications as has been noted previously (29).

Antisense oligonucleotides have also been shown to inhibit β-catenin-dependent gene expression (30). Thus, questions arise about the relative efficacy of RNAi as compared with antisense oligonucleotides to decrease β-catenin-dependent gene expression. In this previous study, only 1 of 12 phosphorothioate oligonucleotides directed against β-catenin was able to decrease its protein expression by 80% (30). In contrast, siRNA targeting against multiple regions of β-catenin and a variety of other proteins demonstrated that approximately half of these siRNAs could result in 80–90% decreases in the level of this protein.[6] Furthermore, we found that a 2 μM concentration of phosphorothioate oligonucleotides was needed to significantly reduce the level of β-catenin, whereas the transfection of only 20 nM siRNAs directed against the same sequences in the β-*catenin* gene resulted in similar reductions in the level of this protein.[6] These results suggest potential advantages of siRNA as compared with phosphorothioate oligonucleotides for cell-based studies to reduce gene expression.

Our *in vivo* data suggest that siRNAs may have therapeutic potential for inhibiting the expression of genes that enhance the growth of tumors. Both transfection and *in vivo* administration of siRNAs directed against β-catenin led to reduced proliferation of HCT116 cells in nude mice. The ability of siRNAs directed against β-catenin to result in prolonged suppression of β-catenin levels in cell culture and reduce the *in vivo* growth of HCT116 cells when transfected for as little as 6 h suggests that RNA oligonucleotides are likely relatively stable in the cell. A variety of other genes such as HER-2/*neu*, c-*myc*, *bcl-2*, and specific components of the NF-κB pathway that are overexpressed in cancer cells may also be targeted by RNAi in an attempt to reduce their levels and thus decrease the proliferation of tumor cells (31). The ability to modify RNA oligonucleotides so that they are more stable *in vivo* will likely be necessary before adopting this technique for true *in vivo* therapy. In

[5] U. N. Verma and R. B. Gaynor, unpublished observations.

[6] R. M. Surabhi and R. B. Gaynor, unpublished observations.

summary, RNAi has been used in this study to demonstrate that reductions in β-catenin levels can reduce tumor growth under both *in vitro* and *in vivo* conditions. Given its specificity and the lower concentrations needed to inhibit gene expression as compared with those required for antisense oligonucleotides, RNAi may have potential therapeutic utility in a variety of disease states.

Acknowledgments

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