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Suppression of β-Catenin Inhibits the Neoplastic Growth of *APC*-Mutant Colon Cancer Cells[1]

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ABSTRACT

Mutations involving the *adenomatous polyposis coli* (*APC*) tumor suppressor gene/β-catenin signaling pathway have been identified in the majority of colon carcinomas. However, the role of aberrant β-catenin signaling in the neoplastic growth of *APC*-mutant colon cancer cells has not been directly studied. To address this question, antisense oligonucleotides have been used to specifically down-regulate β-catenin expression in *APC*-mutant human colon carcinoma cells. Antisense-mediated suppression of β-catenin inhibits the *in vitro* proliferation, anchorage-independent growth, and cellular invasiveness of *APC*-mutant human colon carcinoma cells. The systemic administration of β-catenin antisense oligonucleotides down-regulates β-catenin expression *in vivo* in human colon cancer xenografts in nude mice. Such treatment inhibits the tumorigenic growth of colon cancer xenografts and can completely eradicate tumors in some treated animals. These studies formally demonstrate the critical role of β-catenin signaling in the neoplastic growth of *APC*-mutant colon cancer cells and suggest that strategies targeting β-catenin may be of use in the therapy of colon cancer.

INTRODUCTION

Molecular abnormalities involving the *APC*[3] tumor suppressor gene are among the most frequent mutations observed in human colorectal carcinomas (1, 2). A large body of indirect evidence suggests that the ability of the *APC* gene product to mediate the proteasomal degradation of β-catenin is critical to its function as a tumor suppressor (3, 4). Intracellular accumulation of β-catenin, resulting from inactivating mutations or deletions of the *APC* tumor suppressor gene, leads, in turn, to enhanced transcriptional activity of β-catenin/Tcf complexes in *APC*-mutant colon cancers (5). It has been suggested that such enhanced β-catenin/Tcf activity plays a critical role in colon carcinogenesis.

However, the APC protein interacts with multiple proteins distinct from β-catenin, and there are a number of studies which suggest that *APC* mutations may drive neoplastic growth by β-catenin-independent mechanisms. Effects of the APC protein on cell cycle progression may result from interactions with proteins distinct from β-catenin (6). Expression of an intact *APC* gene in *APC*-mutant human colon carcinoma cells induces apoptosis without altering β-catenin levels (7). The *APC* gene product appears to mediate certain effects on cell growth via alterations in γ-catenin rather than β-catenin (8). Furthermore, the expression of activated β-catenin alleles does not result in the neoplastic transformation of rodent fibroblasts (9, 10) or of transgenic mouse intestinal epithelium (11). Thus, despite substantial indirect evidence, a direct role for β-catenin activity in the neoplastic growth of *APC*-mutant colon cancer cells has not been formally demonstrated.

Antisense ODNs represent one experimental approach to the selective down-regulation of a particular target gene (12, 13). Antisense ODNs bind to complementary sequences of their target mRNA and catalyze the degradation of the mRNA molecule by the nuclease RNaseH. Whereas antisense studies have been plagued by a number of factors resulting in scientifically questionable or incorrect results (14), appropriately designed and selected ODNs can catalyze the degradation of a specific target mRNA (12–14). Such antisense-mediated down-regulation of gene expression has been documented both in cell culture systems and in experimental animals. In fact, antisense compounds have entered clinical trials in patients with a variety of diseases, most prominently cancer (15–17).

To further elucidate the function of β-catenin signaling in human colon carcinoma, we have used antisense ODNs to specifically down-regulate β-catenin mRNA expression and suppress β-catenin/Tcf signaling in *APC*-mutant colon carcinoma cells. Using this experimental approach, we have explored the role of β-catenin signaling in the neoplastic growth of colon carcinoma cells *in vitro* and in the tumorigenic growth of carcinoma xenografts implanted into nude mice.

MATERIALS AND METHODS

Cell Lines. Human cell lines used in this study included: SW480, Colo201, and DLD-1 (all APC-mutant colon carcinoma cell lines); BT474 and SKBR3 (*APC*-wild-type breast cancer cell lines); and WI-38 (diploid fibroblasts). All cell lines were obtained from ATCC (Rockville, MD) and were maintained in a 37°C incubator with 5% CO_2 humidified air in media recommended by ATCC.

ODNs and Cell Treatment Protocol. Phosphorothioate ODNs with the following sequences were obtained from Trilink Biotechnology (San Diego, CA):

β-catenin Antisense CA5 20mer TAAGAGCTTAACCACAACTG.
Mismatched β-catenin Antisense CA5:
1 base mismatch TAAGAGC*C*TAACCACAACTG
2 base mismatch TAAGAGC*C*TAACCACAA*T*TG
5 base mismatch T*G*AGAGC*C*TAAC*T*ACAA*T*T*A*
Scrambled Antisense Control 20mer CAGTAACTGAATAGCTACCA.

Lyophilized ODNs were reconstituted in sterile distilled water to 1 mM, and filter was sterilized and stored in aliquots at −20°C as stock solutions. The uptake of phosphorothioate ODNs by cells was facilitated using Lipofectin (Life Technologies, Inc., Gaithersburg, MD). Lipofectin was diluted to give a concentration of 100 μg/ml in 1/10 treatment volume of OPTIMEM I (Life Technologies, Inc.) and incubated at room temperature for 30 min. Phosphorothioate ODNs diluted to 10 times the final desired concentration in 1/10 treatment volume of OPTIMEM I were added, and the Lipofectin/DNA mixture was allowed to form a complex at room temperature for 15 min. The mixture was then warmed to 37°C for 5 min, and prewarmed OPTIMEM I was added to give final concentrations of 10 μg/ml Lipofectin and the desired ODN concentration (generally 1 μM). Cells were exposed to the mixture for 4 h after which time the ODN/Lipofectin containing media was replaced with standard culture medium.

Northern Blotting. Overnight culture of 5×10^5 cells/60-mm dish was treated with 10 μg/ml Lipofectin with or without ODNs for 4 h. Total RNA was prepared from cells 24 h after the initiation of ODN treatment using the phenol/guanidine isothiocyanate method with TRIzol Reagent (Life Technologies, Inc.). Total RNA (10 μg) per lane was electrophoresed on a formalde-

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[3] The abbreviations used are: *APC, adenomatous polyposis coli*; ODN, oligonucleotide; ATCC, American Type Culture Collection; GAPDH, glyceraldehyde phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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hyde containing 1% agarose gel, transferred onto a nylon membrane (Boehringer Mannheim, Indianapolis, IN), and hybridized with digoxigenin-labeled (Boehringer Mannheim) 1.1 kb human β-catenin cDNA probe (amplified from human β-catenin cDNA; ATCC). digoxigenin-labeled GAPDH probe (CLONTECH, Palo Alto, CA) was used as a loading and transfer control.

Western Blotting. Western blotting was performed as described (18) 48 h after ODN treatment. Antibodies specific for β-catenin (antibody C-18) and γ-catenin (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to cyclin D1 (AB2) were obtained from Oncogene Research (Cambridge, MA). An actin-specific monoclonal antibody (MSX) was obtained from Chemi-Con (Temecula, CA). All antibodies were used according to the supplier's recommendations.

Tcf Reporter Assay. SW480 colon cancer cells (2×10^5) were treated with 1 μM concentrations of corresponding ODN. After ODN treatment (18 h), cell culture dishes were transfected with 1 μg of either TOPFLASH or FOPFLASH (Upstate Biotechnology, Waltham, MA) reporter vector. After transfection with the reporter vector (30 h), cells were lysed in lysis buffer [20 mM Tris-PO_4 (pH 7.8), 2 mM DTT, 2 mM 1,2-cyclohexylenedinitrilotetraacetic acid, 10% Glycerol, and 1 mM Triton X-100]. Each lysate (20 μl) was used to detect luciferase activity using D-luciferin (PharMingen, San Diego, CA) dissolved in reaction buffer [20 nM Tricine, 1.07 nM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, and 530 μM ATP] as substrate, and relative light units were recorded in a luminometer (Monolight 3010). The ratio of relative light units generated by TOPFLASH transfection to FOPFLASH transfection represents Tcf activity (19).

MTT Cell Proliferation Assay. Cell proliferation was quantitated using an MTT (Roche Pharmaceuticals, Indianapolis, IN) assay as described (20). Results represent the means of triplicate samples and are presented as mean ± SE.

Anchorage-independent Growth Assay. Anchorage-independent growth capability was determined by assessing the colony-forming efficiency of cells suspended in soft agar as described (21). Cells were treated with ODNs (1 μM) 24 h before plating in soft agar and were fed weekly with 1 ml of culture medium. Colonies >0.25 mm were counted using an inverted phase microscope and a calibrated template. Colonies were counted after 28 days. Bar graphs represent mean ± SE of triplicate groups.

Boyden Chamber Invasion Assay. Cells (10^5) were treated with Lipofectin or 1 μM ODN, and 24 h after treatment, 10^4 cells were plated into top chambers of collagen-coated Millicell-CM assay plates (Millipore, Bedford, MA) in growth media containing 1% fetal bovine serum. The bottom chamber contained growth media with 10% fetal bovine serum to serve as a chemoattractant. After 36 h of incubation, the cells on top of the membrane were washed, and the membrane was fixed in 3% glutaraldehyde, and cells on the bottom side of the membrane were viewed after staining with crystal violet (Sigma Chemical Co., St. Louis, MO). In replicate experiments, cells from the top and bottom of the membrane were trypsinized and counted using a hemocytometer. Results are normalized for the fraction of viable cells in each population. Bar graphs represent mean ± SE of triplicate groups.

Tumor Cell Line Implantation and Measurement of Tumor Growth. SW480 tumor cells were released from tissue culture dishes and washed in serum-free medium. Tumor cells were diluted with HBSS to a concentration of 2×10^7 cells/ml and were injected into the mid-dorsum of BALB/c nude mice in a total volume of 0.1 ml. Animals were inspected daily for tumor development. Growing tumors were measured using vernier calipers, and tumor volume was calculated by the formula length $\times$ width$^2 \times 0.52$, which approximates the volume of an elliptical solid.

***In Vivo* Administration of Antisense ODNs.** Mice were weighed on the days of treatment. Stock ODNs were diluted with sterile saline to give a dose of 20 mg/kg in a volume of 0.5 ml. Tumor-bearing animals were injected i.p. with antisense or control ODN solutions in a total volume of 0.5 ml using a 27-gauge needle. Treatment was initiated on days 7 (Fig. 5*A*) or 1 (Fig. 5*B*) after tumor cell injection.

Analysis of β-Catenin Expression in Tumors. Tumors were surgically removed after sacrifice of the tumor-bearing mouse. Tumor tissue was homogenized in 10 volumes (w/v) homogenization buffer containing 20 mM HEPES (pH 7.2), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin. Tissue debris was removed by centrifugation at 2,000 *g* for 10 min. The supernatant fraction was transferred to a fresh tube and lysed by the addition of Triton X-100 to a final concentration of 1%. The sample was centrifuged at 30,000 *g* for an additional 30 min to remove insoluble material. Protein concentration was measured, and Western blotting with β-catenin and actin-specific antibodies was performed as described above.

RESULTS

Identification of an Antisense ODN Capable of Specifically Suppressing β-Catenin mRNA Expression. Antisense approaches to regulating gene expression have been limited, in part, by problems in identifying ODN sequences capable of potently down-regulating their mRNA target (12, 13). Antisense ODNs complementary to most regions of a particular target mRNA hybridize poorly because of tertiary structure considerations involving the mRNA target and, thus, will not catalyze its degradation (13). Trial and error approaches have thus far been the most successful method of identifying ODNs capable of binding to and down-regulating a particular mRNA target. To identify an antisense ODN capable of specifically down-regulating β-catenin mRNA, 11 15-base (15mer) ODNs complementary to distinct regions of the β-catenin mRNA sequence were synthesized. Phosphorothioate-modified ODNs were synthesized to enhance nuclease resistance, and Lipofectin was used to facilitate entry of ODNs into cells. Antisense sequences chosen were not complementary to regions of other known genes in the GenBank database and were selected to avoid ODNs that would contain GGGG or CG sequences, as inclusion of these sequences in ODNs may result in nonantisense effects (12–14).

Down-regulation of β-catenin mRNA in antisense-treated *APC*-mutant SW480 colon carcinoma cells was assessed by Northern hybridization. Blots were quantitated by densitometry, and results were normalized for effects on the unrelated gene *GAPDH* to control for nonspecific or toxic effects. Most of the antisense ODNs examined had little or no effect on β-catenin mRNA expression (Fig. 1*A*). However, one sequence, CA5, was able to potently down-regulate β-catenin expression and was chosen for additional study. To additionally enhance the potency of this β-catenin-specific antisense ODN, three 20-base β-catenin antisense molecules with sequences overlapping the CA5 15mer were synthesized and tested for their ability to suppress β-catenin mRNA levels. One 20-base molecule, termed CA5–20, was significantly more potent at down-regulating β-catenin mRNA expression than the CA5 15mer (data not shown) and has been used in subsequent experiments.

The effects of the CA5–20 β-catenin antisense ODN on β-catenin mRNA expression are dose-dependent and sequence-specific. The CA5–20 ODN inhibits β-catenin expression on Northern blots at submicromolar concentrations (Fig. 1*B*). There is no effect of a scrambled antisense (11-base mismatch) ODN sequence at any dose tested. Neither the CA5–20 ODN nor the scrambled control ODN has an effect on expression of mRNA encoding the unrelated gene *GAPDH* (Fig. 1*B*). The ability of a particular antisense ODN to down-regulate its target mRNA molecule reflects hybridization by Watson-Crick base-pairing. To confirm the sequence specificity of the CA5 ODN for its β-catenin target, similar ODNs mismatched at one, two, and five bases were synthesized, and their effects on β-catenin expression were compared with the effect of the intact CA5–20 ODN. As shown in Fig. 1*C,* the introduction of two or more mismatched bases largely abrogates the effect of the CA5–20 ODN on β-catenin mRNA expression. Thus, the ability of the CA5–20 antisense ODN to down-regulate β-catenin expression is sequence-specific and dose-dependent.

Antisense Treatment Down-Regulates the β-Catenin Protein and Inhibits Tcf-driven Gene Transcription. On the basis of the ability of the CA5–20 β-catenin antisense ODN to down-regulate total

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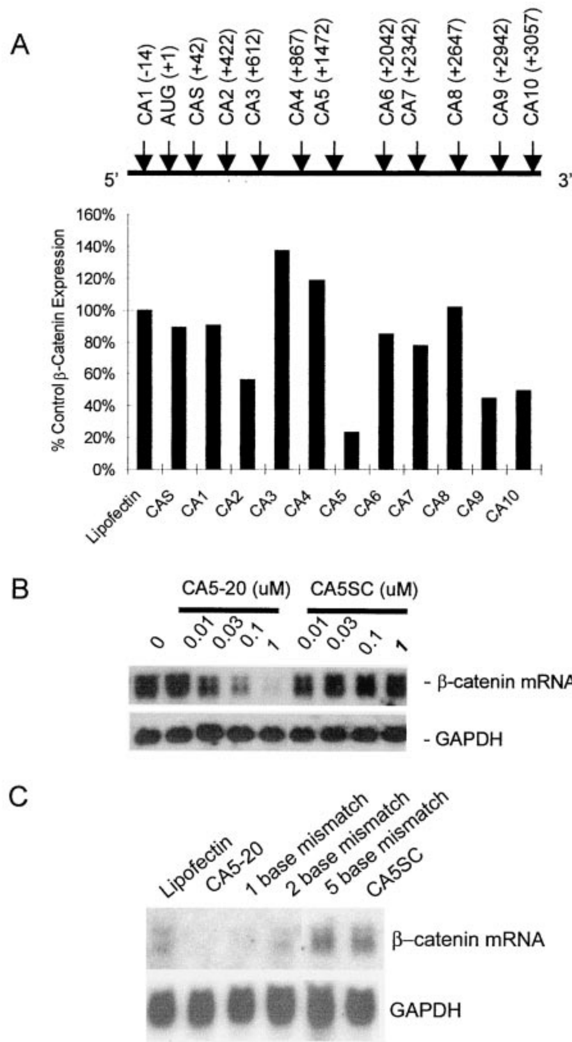

Fig. 1. Down-regulation of β-catenin mRNA by β-catenin antisense ODNs. *A,* screening of 15-base antisense sequences for down-regulation of β-catenin mRNA. Replicate cultures of SW480 colon carcinoma cells were treated with distinct 15mer antisense ODNs corresponding to regions of the β-catenin mRNA molecule indicated, at a 1 μM concentration in the presence of Lipofectin (10 μg/ml). β-catenin mRNA levels were quantitated by scanning densitometry and normalized to GAPDH mRNA levels. *B,* dose-dependent effects of CA5–20 antisense ODN treatment on β-catenin mRNA expression. *CA5–20,* β-catenin antisense ODN; *CA5SC,* scrambled sequence control ODN. *C,* sequence specificity of CA5–20 antisense ODN effects on β-catenin mRNA expression. Cultures were treated with ODNs as indicated at 1 μM. *CA5–20,* β-catenin antisense ODN; *CA5SC,* scrambled sequence control ODN.

cellular β-catenin mRNA expression, it seemed likely that levels of the β-catenin protein were also down-regulated. To confirm this hypothesis, the levels of β-catenin and the structurally related, *APC*-regulated protein γ-catenin were examined on Western blots. As shown in Fig. 2*A,* β-catenin antisense treatment selectively inhibits expression of the β-catenin protein but has little effect on the expression of γ-catenin. Cell fractionation experiments demonstrate parallel down-regulation of β-catenin in both nuclear and membrane fractions (data not shown).

Antisense-mediated down-regulation of β-catenin also inhibits the transcriptional activity of β-catenin-stimulated Tcf transcription factors. As shown in Fig. 2*B,* CA5–20 antisense treatment results in >70% inhibition of Tcf-regulated transcriptional activity. After antisense-mediated suppression of β-catenin/Tcf transcriptional activity, there is a corresponding decrease in the expression of *cyclin* D_1, a gene known to be transcriptionally regulated by β-catenin/Tcf complexes (Refs. 22 and 23; Fig. 2*C*). Thus, antisense-mediated suppression of β-catenin expression appears to functionally replace the activity of the *APC* gene in down-regulating β-catenin expression and transcriptional activity in an *APC*-mutant colon cancer cell line.

Down-Regulation of β-Catenin Inhibits the Proliferation of APC-Mutant Colorectal Cancer Cells. The potent activity of the CA5–20 antisense ODN in suppressing β-catenin expression and inhibiting β-catenin/Tcf-stimulated transcription has been used to examine the role of β-catenin signaling in *APC*-mutant colon cancer cell proliferation. As shown in Fig. 3*A,* β-catenin antisense treatment of SW480 colon cancer cells results in a dose-dependent inhibition of cell growth as determined by MTT assay. Similar results were obtained using direct cell counts (data not shown). This antiproliferative effect parallels the dose-dependent effects on β-catenin mRNA expression, as shown in Fig. 1*B*. In contrast to the effect of the CA5–20 ODN, the scrambled sequence control ODN has only minimal effects on SW480 cell proliferation at concentrations ≤1 μM. To avoid potentially confounding nonspecific toxic effects that occur at ODN concentrations >1 μM, all other experiments were conducted at ODN concentrations of ≤1 μM.

It has been demonstrated that the effect of the CA5–20 β-catenin antisense ODN in suppressing β-catenin mRNA levels is critically dependent on Watson-Crick base-pairing between the antisense molecule and its mRNA target (Fig. 1*C*). To additionally exclude the possibility that the inhibition of SW480 cell growth observed after β-catenin antisense treatment was the result of a nonantisense mech-

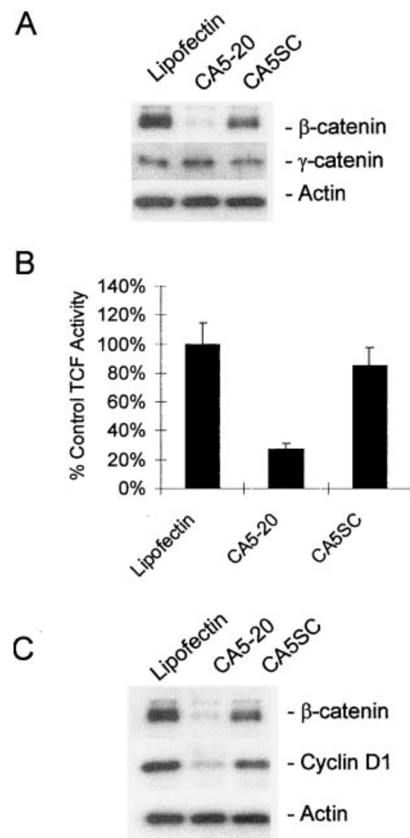

Fig. 2. Effects of β-catenin antisense treatment on β-catenin protein levels and Tcf transcriptional activity. SW480 cells were treated with Lipofectin alone, β-catenin antisense ODNs (*CA5–20*), or scrambled sequence control ODNs (*CA5SC*) at 1 μM as indicated. *A,* down-regulation of β-catenin but not γ-catenin after β-catenin antisense treatment, as determined by Western blotting. *B,* β-catenin antisense treatment inhibits Tcf transcriptional activity. Direct measurement of Tcf transcriptional activity, using the Topflash system, was performed as described in "Materials and Methods." Results represent the means ± SE of six experimental samples. *C,* effect of β-catenin antisense treatment on cyclin D_1 expression as determined by Western blotting.

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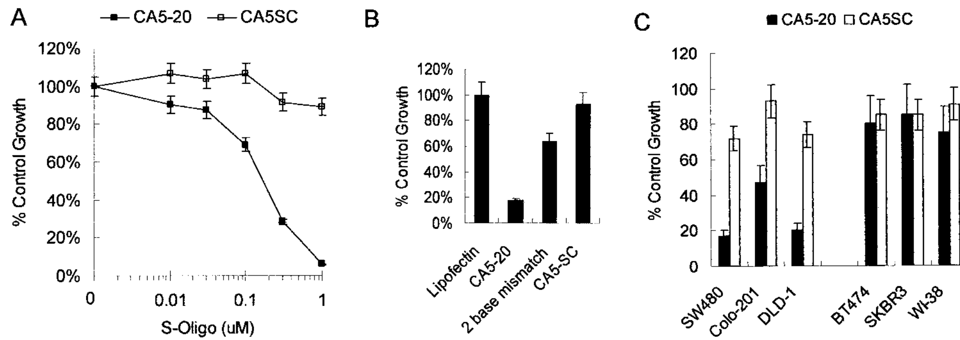

Fig. 3. Effect of β-catenin down-regulation on cell growth. Cultured cells were treated with Lipofectin alone (*Control Growth*), β-catenin antisense ODNs (*CA5–20*), or scrambled sequence control ODNs (*CA5SC*) at 1 μM. Cell growth was assayed by MTT assay. Results represent means ± SE of triplicate samples, expressed as a percentage of the MTT activity of cells treated with Lipofectin alone. *A,* dose-dependent effects of β-catenin antisense treatment on SW480 cell growth. *B,* sequence-specific effects of β-catenin antisense treatment on SW480 cell growth. *C,* effects of β-catenin antisense treatment on multiple cell lines. SW480, Colo201, and DLD-1 are *APC*-mutant colon cancer cell lines. BT474 and SKBR3 are breast cancer lines that express low levels of β-catenin and β-catenin-stimulated Tcf activity. WI-38 is a nonneoplastic diploid fibroblast line.

anism, the requirement for Watson-Crick base-pairing in such growth inhibition was examined. As shown in Fig. 3*B,* a CA5–20 ODN analogue that is mismatched at 2 of 20 bases has markedly less activity in inhibiting SW480 cancer cell growth than does the CA5–20 β-catenin antisense ODN itself. The loss of potency of the two-base mismatch ODN with regards to its effect on cell growth parallels its loss of potency in down-regulating β-catenin mRNA levels (Fig. 1*C*). This strongly suggests that the antiproliferative activity of the CA5–20 β-catenin antisense molecule is the result of its suppression of β-catenin mRNA.

β-catenin antisense treatment also inhibits the growth of *APC*-mutant colon cancer cell lines, distinct from SW480. As shown in Fig. 3*C,* the growth of two other colon carcinoma lines, Colo201 and DLD-1, which are known to contain *APC* mutations, is significantly inhibited after exposure to the CA5–20 antisense ODN. The proliferation of cancer cells containing wild-type *APC* and mutant β-catenin alleles, such as HCT116 (5), is also inhibited by β-catenin antisense treatment (data not shown). In contrast, there is little or no antiproliferative activity after CA5–20 antisense exposure in cancer cell lines which have both wild-type *APC* and wild-type β-catenin alleles, such as BT474 and SKBR3 (24, 25). It should be emphasized that antisense treatment does result in β-catenin down-regulation in such cells (data not shown), but the cells have undergone neoplastic transformation by a distinct molecular mechanism and are apparently not dependent on continued high levels of β-catenin expression to replicate. Similarly, the proliferation of the nontransformed diploid fibroblast line WI-38 is only minimally inhibited by β-catenin antisense treatment (Fig. 3*C*). In contrast, cells containing *APC* mutations are demonstrated to be dependent on continuously elevated β-catenin levels for cell proliferation.

Effects of β-Catenin Antisense Treatment on Anchorage-independent Growth and Cellular Invasiveness. Adherent cell growth *in vitro* is a relatively poor correlate of tumorigenic growth *in vivo*; the *in vitro* property of cancer cells which most accurately predicts *in vivo* tumorigenicity is anchorage-independent growth. Examination of the effect of β-catenin antisense ODN treatment on the anchorage-independent growth of SW480 cells suspended in soft agar demonstrates a significant inhibition of colony formation (Fig. 4*A*). There is no effect of the scrambled sequence control ODN on SW480 cell colony formation. Thus, down-regulation of β-catenin expression inhibits both adherent and anchorage-independent growth of *APC*-mutant colon carcinoma cells.

Cancer cell invasiveness, reflecting both cell migration and breakdown of the extracellular matrix by protease secretion, is a critical step in the development of invasive malignancies. Both cell migration and protease secretion have been linked to β-catenin expression (26). The effects of down-regulating β-catenin expression were therefore evaluated in an *in vitro* microinvasion assay using Boyden chambers. As shown in Fig. 4*B,* treatment with β-catenin antisense ODNs results in a substantial inhibition of SW480 colon carcinoma cell invasiveness. The scrambled sequence control ODN has no such effects. It should be noted that results are presented as the percentage of surviving viable cells that traverse the collagen membrane. This eliminates potentially confounding effects in interpreting the results of this assay

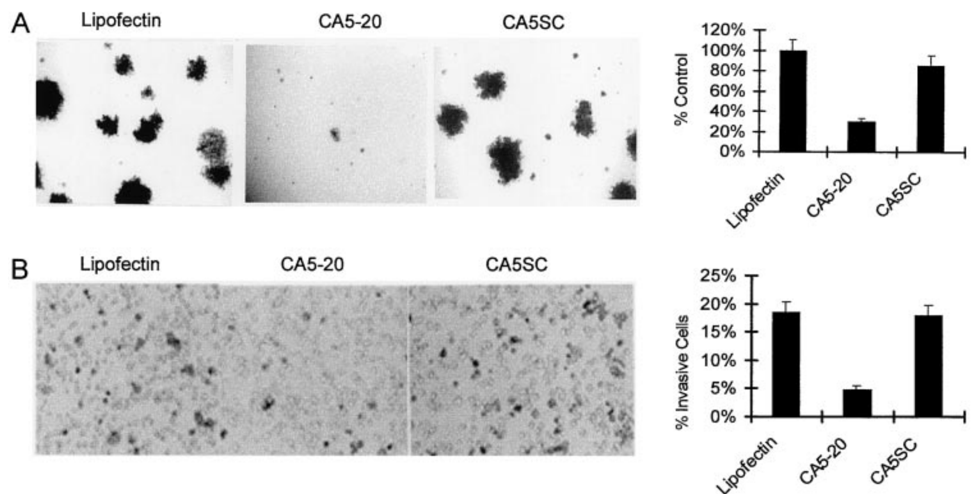

Fig. 4. Effects of β-catenin down-regulation on the transformed phenotype *in vitro*. SW480 cells were treated with Lipofectin, β-catenin antisense ODNs (*CA5–20*), or scrambled sequence control ODNs (*CA5SC*) at 1 μM. *A,* anchorage-independent growth. Soft agar assays were performed as described in "Materials and Methods." Results demonstrate representative regions of treated dishes and overall mean colony number ± SE of triplicate samples. *B,* cellular invasiveness assay in Boyden chambers. Invasiveness assays were performed as described in "Materials and Methods." Results demonstrate representative regions of chamber membranes; cells traversing the membrane are darkly stained. Overall mean number of invasiveness cells ± SE from triplicate experiments is shown in the bar graph.

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resulting from β-catenin antisense effects on cell viability or proliferation. Thus, in addition to effects on cancer cell growth, suppression of β-catenin expression results in inhibition of cancer cell invasiveness.

Effects of Systemic β-Catenin Antisense Treatment on Tumor Xenografts. Antisense ODNs have entered clinical trials for the treatment of a variety of disease states, most prominently, cancer (15–17). On the basis of the inhibitory effects of β-catenin antisense ODNs on the proliferation, viability, and invasiveness of *APC*-mutant colon cancer cells *in vitro,* it was of interest to: (*a*) determine whether antisense treatment of human colon cancer cells in a xenograft model could down-regulate β-catenin expression *in vivo*; and (*b*) examine the effects of such down-regulation on tumor growth. As shown in Fig. 5*A,* treatment of SW480 tumor-bearing nude mice with i.p. injections of the CA5–20 β-catenin antisense ODN is able to significantly reduce β-catenin protein levels in s.c. tumor nodules. Treatment of SW480 tumor-bearing mice with the scrambled sequence control ODN has no effect on tumor β-catenin levels. Thus, it is possible to down-regulate tumor β-catenin levels by the systemic administration of a β-catenin antisense ODN.

Antisense-mediated suppression of β-catenin exerts a significant inhibitory effect on the tumorigenic growth of SW480 cancer cells implanted into nude mice. As shown in Fig. 5*B,* tumor cells implanted into nude mice treated with i.p. injections of the CA5–20 antisense ODN at a dose of 20 mg/kg daily grow much more slowly than do tumors in mice treated with injections of saline, with three of five antisense-treated mice completely regressing their small tumor nodules. In contrast, tumors in mice injected with the scrambled sequence control ODN display a growth pattern not significantly different from the saline-treated mice; all of the animals in these two groups developed progressively growing lethal tumors. Similar results have been obtained in replicate experiments; some β-catenin antisense-treated mice demonstrate complete tumor regression, whereas all animals in saline and control ODN treatment groups developed progressively growing tumors. It should be noted that animals rendered tumor-free by β-catenin antisense treatment have been observed for an additional 120+ days (6 months total) without evidence of tumor recurrence. Thus, down-regulation of β-catenin expression *in vivo* can inhibit, and in some cases eradicate, the tumorigenic growth of *APC*-mutant colon cancer cells.

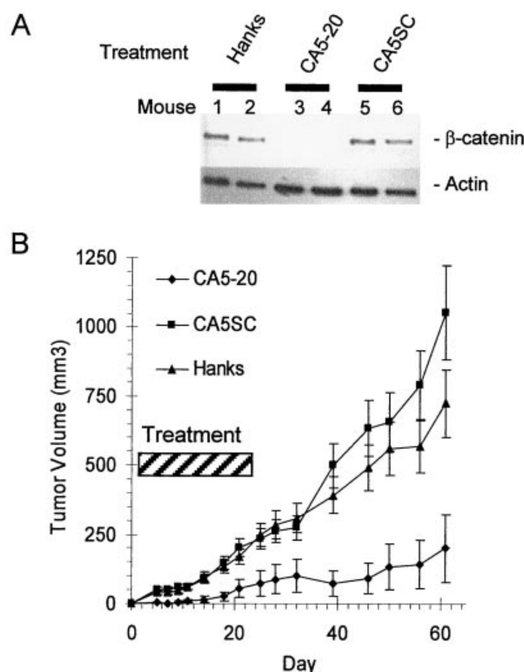

Fig. 5. *In vivo* effects of β-catenin antisense treatment. *A,* down-regulation of β-catenin expression in SW480 xenografts. A total of six nude mice were implanted s.c. with 2×10^6 SW480 cells. After 7 days, when small tumor nodules were present in all animals, they were randomized to groups of two and were treated for 5 days with i.p. injections of HBSS (*Hanks*), β-catenin antisense ODNs (*CA5–20,* 20 mg/kg), or scrambled sequence control ODNs (*CA5SC,* 20 mg/kg). After the last treatment (24 h), the animals were sacrificed, tumors were removed, and tumor lysates were analyzed for β-catenin expression by Western blotting. Blots were stained in parallel with an antiactin antibody to demonstrate equal protein loading. *B,* inhibition of SW480 tumor growth. Groups of five nude mice were injected s.c. with 2×10^6 SW480 tumor cells and were given daily i.p. injections with saline (▲), CA5–20 β-catenin antisense ODNs (20 mg/kg; ♦), or scrambled sequence control ODNs (20 mg/kg; ■) for 21 days. Tumor growth was measured as described in "Materials and Methods."

DISCUSSION

It has been demonstrated that antisense-mediated down-regulation of β-catenin expression can disrupt β-catenin-dependent Tcf transcriptional activity and inhibit the neoplastic growth of *APC*-mutant colon cancer cells *in vitro* and *in vivo*. Whereas in certain experimental systems antisense ODN effects on tumor cell proliferation have been shown to result from toxic effects of ODNs binding to cellular proteins rather than from true antisense effects on gene expression (12–14), the results presented here are unlikely to be attributable to such nonantisense mechanisms. The CA5–20 antisense sequence has been selected from a number of antisense ODNs for its potency in down-regulating β-catenin mRNA and does not contain sequences known to be associated with protein binding or nonspecific immune activation. The biological effects of this antisense molecule have been demonstrated to be dose-dependent, sequence-specific, target-specific, and occur at micromolar and submicromolar concentrations. β-catenin antisense treatment of cancer cell lines which do not have molecular abnormalities involving the *APC*/β-catenin pathway does not significantly inhibit their proliferation, providing additional evidence that the CA5–20 antisense treatment inhibits *APC*-mutant colon cancer cell growth by a β-catenin-specific mechanism.

Whereas the studies presented here demonstrate that targeted suppression of β-catenin inhibits the neoplastic growth of *APC*-mutant colon cancer cells, the mechanisms by which β-catenin contributes to the cancer phenotype remain to be elucidated. It is possible that alterations in β-catenin levels alter E-cadherin-mediated cell membrane properties, with resulting effects on cancer cell invasiveness and viability. It is also possible that the suppression of β-catenin/Tcf transcription factor activity resulting from antisense-mediated down-regulation of β-catenin inhibits cancer cell growth attributable to effects on the expression of downstream genes, such as cyclin D_1. Studies addressing these two nonmutually exclusive possibilities are in progress.

The availability of an antisense ODN capable of selectively disrupting β-catenin activity in cancer cells will be of use in additionally defining molecular mechanisms in colon carcinogenesis resulting from *APC* gene mutations. Studies using inducible *APC* constructs have begun to identify downstream molecules, the transcription of which is regulated by *APC* (27, 28). It has been suggested that these effects are all of the result of alterations in β-catenin signaling, but β-catenin may not be the only transcriptionally active molecule regulated by *APC* (8). It will be of interest to examine changes in gene expression after the direct inhibition of β-catenin activity and to compare these changes with those resulting from *APC* activity.

The antitumor effects observed as a result of β-catenin down-regulation are similar to the effects observed when *APC*-mutant colon cancer cells were treated with an adenoviral expression vector containing a β-catenin-binding segment of the *APC* gene (29). The latter construct, however, also contained γ-catenin and axin-binding do-

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mains of the *APC* protein, and, thus, antitumor effects could not be definitively linked to effects on β-catenin. In contrast, the β-catenin antisense ODN used in our studies appears to selectively target β-catenin. The results presented here thus formally establish β-catenin as a valid molecular target for additional developmental therapeutics.

The murine and human β-catenin genes are highly homologous, but the region of the human β-catenin mRNA targeted by the CA5–20 antisense ODN described here differs from the corresponding murine mRNA at 4 of 20 bases. There is no effect of CA5–20 ODN treatment on β-catenin expression in either murine cell lines or in other tissues of human tumor xenograft-bearing mice.[4] However, we have recently identified an antisense ODN with specific activity in suppressing murine β-catenin mRNA expression. Studies using this molecule to systemically suppress β-catenin in mice are ongoing; it appears that systemic suppression of β-catenin, using antisense ODN doses comparable with those shown to inhibit tumor growth here, is relatively well tolerated.[4] It may thus be possible to achieve antitumor effects using antisense ODNs to inhibit β-catenin expression in *APC*-mutant colon cancer cells without excessive toxicity to the tumor-bearing host.

The studies presented here demonstrate a novel approach to the targeted therapy of tumors containing tumor suppressor gene mutations: direct suppression of the molecular target normally regulated by the tumor suppressor gene product. This has a number of advantages compared with the more traditional gene therapy approach of attempting to replace the tumor suppressor gene, particularly in the areas of gene delivery and regulation. Successful delivery of a gene therapy construct into even a modest fraction of tumor cells in a treated animal remains an elusive goal. In contrast, antisense ODNs are widely distributed after *in vivo* administration (12) and are capable of selectively inhibiting their target in the majority of tumor cells, as demonstrated in the present work and in numerous other studies. The activity of antisense ODNs does not require transcriptional regulatory mechanisms and can be modulated pharmacologically by modifying the treatment dose. This approach clearly has activity in the *APC*/β-catenin system described here and might have utility in other tumor suppressor gene defects where the suppressor gene inhibits a well-defined downstream signaling pathway.

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[4] H. Roh *et al.,* unpublished observations.

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