# Exogenous Expression of β-Catenin Regulates Contact Inhibition, Anchorage-independent Growth, Anoikis, and Radiation-induced Cell Cycle Arrest

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*Abstract.* β-Catenin is an important regulator of cell–cell adhesion and embryonic development that associates with and regulates the function of the LEF/TCF family of transcription factors. Mutations of β-catenin and the tumor suppressor gene, adenomatous polyposis coli, occur in human cancers, but it is not known if, and by what mechanism, increased β-catenin causes cellular transformation. This study demonstrates that modest overexpression of β-catenin in a normal epithelial cell results in cellular transformation. These cells form colonies in soft agar, survive in suspension, and continue to proliferate at high cell density and following γ-irradiation. Endogenous cytoplasmic β-catenin levels and signaling activity were also found to oscillate during the cell cycle. Taken together, these data demonstrate that β-catenin functions as an oncogene by promoting the $G_1$ to S phase transition and protecting cells from suspension-induced apoptosis (anoikis).

Key words: β-catenin • oncogene • cell cycle • anoikis • apoptosis

β-Catenin is a 92–97-kD protein associated with the intracellular tail of the intercellular adhesion molecule E-cadherin (Ozawa et al., 1989). Through this association, β-catenin plays an important role in strong cell–cell adhesion as it links E-cadherin (and other members of the cadherin family) to the actin cytoskeleton through the protein α-catenin (Hirano et al., 1992; Kemler, 1993). One mechanism by which cell–cell adhesion can be negatively regulated is via the phosphorylation of β-catenin on tyrosine residues (Behrens et al., 1993). There are some indications that this may be an important event in the transition from a benign tumor to an invasive, metastatic cancer (Sommers et al., 1994).

β-Catenin is also a regulator of embryogenesis, a role that was first suspected when it was shown to be the mammalian homolog of the *Drosophila* segment polarity gene *Armadillo* (Peifer et al., 1992). Further studies in *Drosophila* and *Xenopus* have revealed that β-catenin is a component of the highly conserved Wnt/Wingless signal transduction pathway that regulates body patterning in both species (Peifer, 1995; Gumbiner, 1997).

The membrane-associated and cytoplasmic pools of β-catenin have disparate activities: adhesion and signaling, respectively. The accumulation of cytoplasmic β-catenin drives its interaction with members of the LEF/TCF family of nuclear transcription factors that results in altered gene expression, which is the transduction of the Wnt/Wg signal (Clevers and van de Wetering, 1997). This accumulation of cytoplasmic β-catenin is regulated at the level of its degradation (Peifer et al., 1994; Peifer, 1995; Papkoff et al., 1996). In the absence of the Wnt/Wg signal, phosphorylation of specific serine residues on β-catenin leads to its ubiquitination and degradation, removing it from the cytoplasm (Orford et al., 1997). Mutations of these serine residues inhibit the ubiquitination of β-catenin, which causes it to accumulate and signal constitutively (Morin et al., 1997; Orford et al., 1997).

Along with its position in a growth factor signaling pathway, the demonstration of an interaction between β-catenin and the product of the tumor suppressor gene, adenomatous polyposis coli (APC)[1], suggests that it is involved in oncogenesis (Rubinfeld et al., 1993; Peifer, 1997). Tumor cell lines with a loss of one copy of APC, and harboring mutations in the other allele, have high lev-

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1. *Abbreviations used in this paper:* APC, adenomatous polyposis coli; CON, control; EMT, epithelial to mesenchymal transition; FAK, focal adhesion kinase; HA, hemagglutinin epitope; ILK, integrin-linked kinase; PKC, protein kinase C; S37A, S37A mutant β-catenin plasmid; WT, wild-type β-catenin plasmid.

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els of cytoplasmic (signaling) β-catenin, which is markedly reduced when functional APC is reintroduced (Munemitsu et al., 1995). Importantly, all mutant forms of APC found in human cancers are unable to reduce β-catenin levels in these cells. The importance of elevated β-catenin in human cancer was further substantiated when mutations in the β-catenin gene were described in colon cancer and melanoma cell lines (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). At least one of these mutations results in a more stable form of the protein.

A retroviral insertion screen for oncogenes using the NIH-3T3 cell line also implicated β-catenin as a possible oncogene, as the insertion of the retrovirus resulted in the expression of a β-catenin protein that lacked the $NH_2$ terminus (Whitehead et al., 1995). In contrast, overexpression of a stabilized form of β-catenin is unable to mimic the morphological effects of Wnt-1 in fibroblasts (Young et al., 1998).

Although much is now known about this signaling system, the actual cellular processes in which β-catenin plays a regulatory role is unclear. As described above, it regulates cadherin-mediated cell–cell adhesion. Although it appears to regulate gene expression, few target genes have been demonstrated. Based on its relationship with Wnt and APC, it is possible that β-catenin may positively regulate cellular proliferation or inhibit apoptosis. It is also tempting to speculate that the adhesive and the putative oncogenic functions of β-catenin are related and that it may be, at least in part, the mechanistic link between cell–cell adhesion, contact inhibition, and/or apoptosis. However, no studies have directly tested the hypothesis that β-catenin is actually oncogenic.

This report utilizes the MDCK cell line to determine the impact of overexpressing wild-type or a stabilized mutant form of β-catenin in nontransformed epithelial cells. The data demonstrate that β-catenin alters cell cycle progression and confers enhanced growth in soft agar, a surrogate marker for tumorigenicity. In addition, β-catenin confers resistance to suspension-mediated apoptosis (anoikis), radiation-induced cell cycle arrest, and allows cells to continue cycling when cultured at confluence. In short, β-catenin functions as an oncogene in the MDCK normal epithelial cell line.

## Materials and Methods

### Cells, Plasmids, and Stable Transfections

MDCK cells are a canine kidney-derived nontransformed epithelial cell line that are maintained in DME (GIBCO BRL), supplemented with 5% FBS. A1N4 cells are a human mammary nontransformed epithelial cell line that are grown in IMEM, supplemented with 0.5% FBS, 0.5% hydrocortisone, 5 μg/ml insulin, and 10 ng/ml EGF (Stampfer and Bartley, 1988). These cells synchronize in $G_0$ in the absence of EGF. The wild-type (WT) and S37A mutant (S37A) β-catenin plasmids were described previously (Orford et al., 1997). The bacterial chloramphenicol acetyltransferase gene driven by the CMV promoter of the pcDNA 3 plasmid (Invitrogen Corp.) served as the negative control (CON). For stable transfections, 800,000 MDCK cells were plated per 100-mm tissue culture plate. The next day, 15 μg of the various plasmids were transfected using the lipofectamine PLUS method (GIBCO BRL): 32 μl lipofectamine and 45 μl PLUS reagent. All of the plasmids included the neomycin-resistance cassette for selection. 48 h later, the cells were split 1:20 and cultured for 2 wk in the presence of 500 μg/ml of Geneticin (GIBCO BRL). An approximately equal number of colonies grew up for each transfected plasmid. For each transfection, all of the colonies were trypsinized and combined to give stable cell pools.

### Immunoblotting

Whole cell and cytoplasmic lysates were made and immunoblotting performed as described previously (Orford et al., 1997).

### Immunofluorescence

Cells were grown to confluence in 4-well BIOCOAT chamber slides (Falcon Plastics). Cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Cells were then permeabilized in 0.2% Triton X-100, 4% paraformaldehyde in PBS for 10 min. After washing in PBS, cells were blocked in 3% ovalbumin for 1 h. The chambers were incubated with primary antibodies overnight at 4°C. After washing in PBS five times for 5 min each, fluorescein- or Texas red-conjugated secondary antibodies were added for 1 h. Primary and secondary antibodies were diluted in 6% normal goat serum. After removal of the secondary antibody, the chambers were washed five times for 5 min in PBS, and the chambers removed. The cells were mounted with Vectashield (Vector Labs, Inc.).

### Antibodies

The anti–β-catenin (C19220) and anti-p27 (K25020) mAbs were from Transduction Laboratories. The antihemagglutinin mAb (HA-11) was purchased from Berkeley Antibody Co., Inc. A second high affinity anti-HA mAb was purchased from Boehringer Mannheim Corp. (#186723). The anti–E-cadherin (SHE78-7) mAb was purchased from Zymed Labs, Inc. Peroxidase- and fluorescein-labeled secondary antibodies were purchased from Kirkegaard and Perry Laboratories, Inc. The Texas red-labeled secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc.

### β-Catenin–LEF/TCF Signaling Assays

In 12-well dishes, cells were transfected with 0.5 μg of the TOPFLASH LEF/TCF reporter plasmid (van de Wetering et al., 1997) and 0.005 μg of the constitutively expressed Renilla luciferase, as a normalization control. As a negative control, cells were transfected with the FOPFLASH reporter plasmid in which the LEF/TCF binding sites have been mutated. The cells were lysed and assayed for Firefly and Renilla luciferase activities using the STOP & GLO assay (Promega Corp.). All results are normalized to the Renilla luciferase activity.

### Soft Agar Growth Assay

For each cell pool, 150,000 cells were suspended in 3 ml DME + 5% FBS, and warmed to 37°C. 300 μl of a prewarmed (52°C) 3% agarose/PBS solution was mixed with the cell suspension and then layered into 3 wells of a 6-well plate (1 ml/well), which were previously coated with 1 ml of 0.6% agarose in DME. The agar was allowed to solidify at room temperature for 20 min before 3 ml of growth medium was added to each well. The medium was changed every three days. After 14 d, the colonies were counted by an Omnicon 3600 Colony Counter and photographed.

### Growth Curves

To have an equal number of cells plated at the first time point, 10,000 CON, and 5,000 WT and S37A cells were plated per well of 12-well plates. At each time point, the cells were washed once in PBS and trypsinized in 1 ml trypsin/versene (GIBCO BRL). The single cell suspension was counted on a Coulter Counter set at 10 μm min with 20-μm maximum diameter. Each data point was performed in triplicate.

### Plating Efficiency Assay

For each cell pool, 100 cells were plated onto each of three 100-mm tissue culture dishes in DME + 5% FBS. 4 d after plating, the colonies were photographed at 400×. After 8 d, the cells were washed with PBS, stained with crystal violet, and washed with water. The colonies were counted and then photographed. The plating efficiency is the mean number of colonies per dish/100 cells plated per dish.

### Quantification of Cell Shedding

Cells were cultured in 6-well plates 3 d after confluence. The cells were

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washed twice in PBS and 2 ml of fresh medium was added to each well. 24 h later, the shed cells were removed with medium and counted on a Coulter Counter, as described.

### Cell Cycle Analyses

Two flow cytometric assays were used.

*Vindelov Method.* Cells were washed in PBS and trypsinized. Cells were washed in PBS and pelleted. After removing the wash buffer, the pellet was vortexed and resuspended in 0.1 ml of citrate/DMSO buffer (250 mM sucrose, 40 mM $Na_3C_6H_5O_7$ $2H_2O$, 5% DMSO, pH 7.60). The pellets were then frozen at −80°C. The cells were then processed as in Vindelov et al. (1983).

*Ethanol Fixation Method.* Cells were washed once in PBS and trypsinized. Trypsinized cells were pelleted at 1000 *g* and washed in 5 ml cold PBS. After a second centrifugation, the cells were resuspended in 0.5 ml cold PBS and fixed by dripping in 1.5 ml cold 100% ethanol, while slowly vortexing the cell suspension. After at least 1 h at 4°C, the cells were stained with propidium iodide and DNA content was measured by flow cytometry. The ethanol fixation method was also used for the flow cytometric analysis of apoptosis.

### Cell Synchronization Experiments

*β-Catenin Protein Level.* A1N4 cells were plated in 100-mm tissue culture dishes and grown overnight to ∼40% confluency. The cells were washed three times in PBS and then maintained in the absence of EGF for 46–50 h. This synchronized >95% of the cells in the $G_0/G_1$ phase of the cell cycle. To stimulate reentry into the cell cycle, EGF-containing medium was added back to the cells. Parallel dishes were analyzed at each time point for β-catenin protein (whole cell or the cytoplasmic pool) and for the cell cycle distribution.

*β-Catenin–LEF/TCF Signaling.* 50,000 A1N4 cells were plated per well of 12-well dishes and transfected with 1 μg of the TOPFLASH reporter plasmid and 0.01 μg of the Renilla control plasmid by the calcium phosphate method. The cells were then synchronized by EGF starvation ($G_0/G_1$) or 1 μM nocodazole ($G_2$/M), or treated with the proteosomal inhibitor ALLN, which stabilizes β-catenin. The cells were collected and the luciferase measurements were made as described.

### Anoikis Assays

Confluent cells were trypsinized into a single cell suspension. 700,000 cells were plated in 150-mm tissue culture dishes coated with 0.8% agarose, to which they could not attach. At the various time points, the cells were collected, washed in PBS, and any cell aggregates were dispersed by trypsinization. Cells were then analyzed for apoptosis using three separate assays.

*DNA/Flow Cytometry.* Samples were analyzed by flow cytometry (see Cell Cycle Analyses, Ethanol Fixation). In this analysis, the hypodiploid peak constituted the apoptotic population.

*AnnexinV Labeling.* Samples were stained with fluorescein-labeled AnnexinV and propidium iodide (Trevigen) according to the manufacturer's protocol, and analyzed by flow cytometry. The two AnnexinV positive quadrants of the analysis were taken as the apoptotic fraction.

*Hoechst Staining.* Cells were fixed in 10% formalin for 10 min and stained with Hoechst #33258 (25 μg/ml in PBS) for 10 min at room temperature in the dark. Cells were placed on a glass slide and analyzed by fluorescence microscopy.

### γ-Irradiation

750,000 CON, and 500,000 WT and S37A cells were plated in T75 tissue culture dishes. 26 h later, the flasks were exposed to 5 Gy of γ-irradiation. Another group of flasks received a mock irradiation (0 Gy). At 8 and 24 h after irradiation, the cells were trypsinized and their cell cycle profile was determined.

## Results

### Expression of β-Catenin Transgenes in MDCK Cells

To investigate the effects of β-catenin on normal cellular function, MDCK cells were stably transfected with constitutively expressed β-catenin transgenes that have been engineered to contain a COOH-terminal HA tag. In addition to WT β-catenin, a construct harboring a previously described serine to alanine point mutation at residue 37 (S37A) was used, which encodes for a β-catenin protein largely resistant to ubiquitination (Orford et al., 1997). The cells used are pooled stable transfectants; that is, after selection with G418, all of the drug resistant colonies resulting from each transfection were combined. These will be referred to as cell pools. As a negative control, a cell pool expressing the bacterial chloramphenicol acetyl transferase gene was generated (CON). Stable cell pools were generated to avoid the phenotypic artifacts that can result from the selection and propagation of individual clones derived from single transfected cells. We found that MDCK cells are especially prone to clonal morphological variation.

When examined by immunoblotting, expression of the HA tag was detectable only in the cell pool expressing the more stable S37A mutant (Fig. 1, B–E). We believe that epitope inaccessibility and antibody insensitivity result in the poor detection of the HA-tagged β-catenin and, consequently, the HA tag was undetectable by immunoblotting in untreated WT cells. To demonstrate that the WT cells were capable of expressing HA-tagged β-catenin, all three cell pools were treated with the histone deacetylatase inhibitor sodium butyrate to nonspecifically increase gene expression. This treatment resulted in clearly detectable expression in the WT cells and very high expression in the S37A cells, whereas the CON cells lacked expression under both conditions. Sodium butyrate treatment was not used in any other experiments in this study. In untreated cells, a similar pattern was seen by immunofluorescence microscopy. Using an antibody specific for the HA tag and a fluorescein-labeled secondary antibody, staining was detectable in the S37A cell pool (Fig. 1 E), but was difficult to detect in the WT cells (data not shown). To demonstrate the HA tag in the WT cells, a high affinity anti-HA antibody (Boehringer Mannheim) and a Texas red-conjugated secondary antibody was used to increase the sensitivity of the assay. Under these conditions, expression of the HA-tagged protein was clearly demonstrable in most of the WT cells (Fig. 1 D), even in the absence of butyrate, whereas expression was not evident in the CON cells (Fig. 1 C). A β-catenin specific antibody revealed a normal staining pattern in all three cell pools (Fig. 1, F–H).

Whole cell lysates do not exhibit any significant increase in total β-catenin levels (data not shown) because MDCK cells express a large amount of endogenous β-catenin, most of which is complexed with E-cadherin at the cell membrane. However, it is the cytoplasmic pool that is involved in β-catenin signaling and an increase in this pool was evident in both WT and S37A expressing cells, as compared with the CON cell pool (Fig. 1 A).

To confirm that β-catenin was being functionally overexpressed in both the WT and S37A cell pools, LEF/TCF-dependent nuclear signaling was measured using the TOPFLASH reporter construct (van de Wetering et al., 1997). This reporter consists of four consensus LEF/TCF binding sites placed upstream of the cFos minimal promoter. As a negative control, a similar reporter construct (FOPFLASH), in which the LEF/TCF binding sites have been

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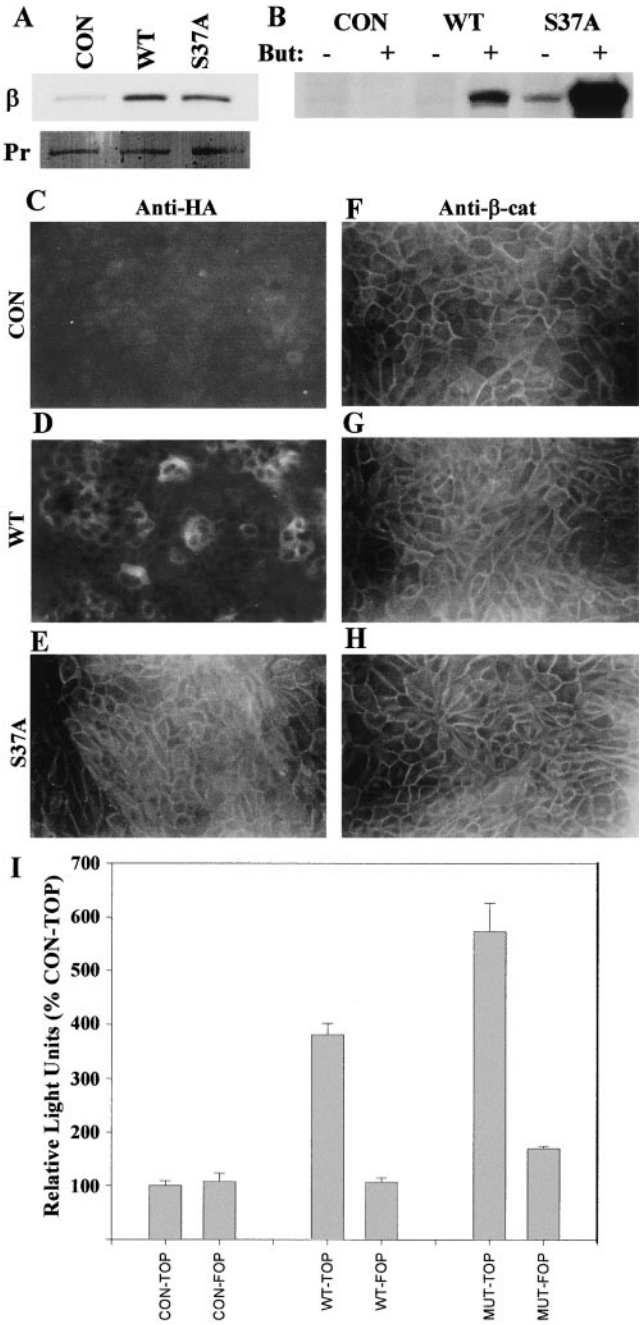


*Figure 1.* Expression of transgenes in MDCK stable cell pools. A, Equal protein from cytoplasmic extracts of CON, WT, and S37A cell pools was immunoblotted with an anti–β-catenin antibody. Amino black staining of nitrocellulose membrane demonstrates equal protein loading. B, Expression of HA-tagged β-catenin was determined by immunoblotting equal protein from whole cell lysates of the three cell pools cultured with and without sodium butyrate (But; to enhance gene expression) with anti-HA antibody (HA-11; BabCo). C–E, HA-tagged β-catenin can be detected in the WT (D) and S37A (E) cell pools by immunofluorescence. The HA-tag is absent in the CON cell pool (C). F–H, Expression of β-catenin in the same cell pools. I, β-catenin signaling activity was determined with the TOPFLASH LEF/TCF-responsive reporter construct. β-Catenin–LEF/TCF signaling is elevated above CON in both the WT and S37A cells. The negative control FOPFLASH reporter is essentially unaffected by β-catenin transfection.

mutated, was used. Even though the HA tag was not easily detected in the untreated WT cell pool, LEF/TCF signaling is elevated well above the control (Fig. 1 I, CON) in both the WT and S37A cell pools.

### *β-Catenin Overexpression Alters Cell Morphology*

Overexpression of β-catenin in MDCK cells previously was shown to alter cell morphology. The stable cell pools used in this report have essentially the same morphology as the MDCKs expressing an inducible form of $NH_2$ terminally truncated β-catenin (Barth et al., 1997). The WT and S37A cell pools are less efficient at forming tight colonies of cells, as compared with CON cells (Fig. 2). In addition, the cells along the edges of the WT and S37A colonies tend to extend projections more readily, giving them a more mesenchymal morphology. The morphology of these cell pools also varied at high density. In contrast to their appearance at lower density, the WT and S37A cells appeared to be more tightly adherent to each other (data not shown). This is supported by the fact that these cells are significantly slower to round up when trypsinized during normal cell passaging. To confirm that expression of the β-catenin transgenes did not prevent strong intercellular adhesion, the ability of the WT and S37A cells transepithelial resistance was measured in the presence and absence of $Ca^{2+}$. Both the WT and S37A cells formed a strong barrier in the presence of $Ca^{2+}$ (>1,000 ohms/chamber) that was completely diminished in the absence of $Ca^{2+}$. These results are consistent with what is seen in normal epithelial cell lines and confirms strong cadherin-mediated adhesion.

### *β-Catenin Stimulates Cell Proliferation*

To characterize the distribution of these cells in the cell cycle, DNA/flow cytometry analysis was performed on these cells during exponential growth phase. Both of the β-catenin overexpressing cell pools had a reduced proportion of $G_0/G_1$ cells and an increased proportion of S and $G_2$ cells, as compared with the control cells (Fig. 3 A). This suggests that either a greater proportion of the WT and S37A cells are cycling or the $G_1$ phase of the cycle is shorter in duration than it is in the CON cells.

Growth curves demonstrated a significant difference between the β-catenin overexpressing cells (WT and S37A) and the CON cells (Fig. 3 B). The curves depicting the growth of the WT and S37A cell lines diverged from that of the CON cells, demonstrating that the alterations in cell cycle distribution resulted in increased growth. Also, overexpression of β-catenin increased saturation density of these cells (Fig. 3 B, inset). Together with the demonstration that the WT and S37A cells proliferate more rapidly at confluence (Fig. 4), it is clear that β-catenin overexpression significantly diminishes the property of contact inhibition of growth.

Interestingly, in every replication of this experiment, the number of cells in the WT and S37A wells was elevated (up to 50%) above the CON cells at the first time point of the growth curve. To determine if a difference in plating efficiency might explain the discrepancy in the cell number on the first day of the growth curves, 100 cells were plated per 100-mm tissue culture dish in three dishes for each cell pool. The colony count provides a rough estimate of the

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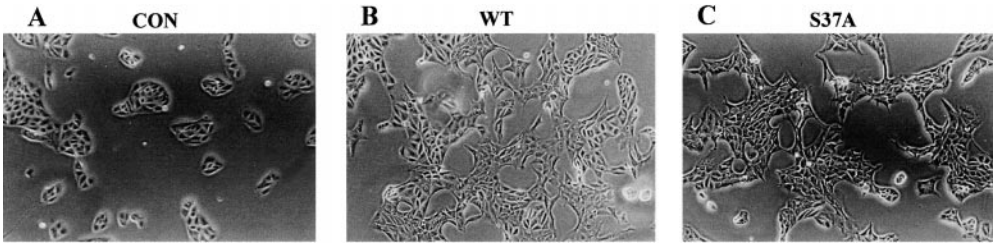


*Figure 2.* Exogenous β-catenin expression alters morphology of MDCK cells. Phase-contrast photographs of CON (A), WT (B), and S37A (C) cell pools demonstrate the effect of β-catenin overexpression on MDCK cell pools. β-Catenin-expressing cells show a more spindly, mesenchymal, less cell–cell adhesive morphology compared with the control cells.

plating efficiency of the cells. This experiment revealed a small (but not statistically significant) difference in plating efficiency that may contribute to the consistent differences in cell number, but does not explain them entirely (Fig. 3 C). We believe that the combination of increased plating efficiency and elevated proliferation rate account for the differences seen at the first time point.

An obvious increase in the rate of colony growth in the β-catenin overexpressing cells was more dramatic. The colonies from the WT and S37A cells were many fold larger than those from the CON cells. The morphology of these clones provides one explanation for the difference in colony size (Fig. 3, D, E, and F). Whereas the CON cells formed tightly adhesive, epithelioid colonies (Fig. 3 G), the WT and S37A cells formed a large number of colonies containing a more scattered, mesenchymal phenotype (Fig. 3 H). The morphological changes suggest that enhanced motility may contribute to this dramatic increase in colony size, but this is speculative. Also, the reduced adhesiveness in the WT and S37A cells may promote large colony formation by avoiding the contact inhibitory effect of tight cell–cell adhesion. In addition, other data suggest that the WT and S37A cells have an increased proliferative rate, even in the presence of strong intercellular adhesion (Fig. 4).

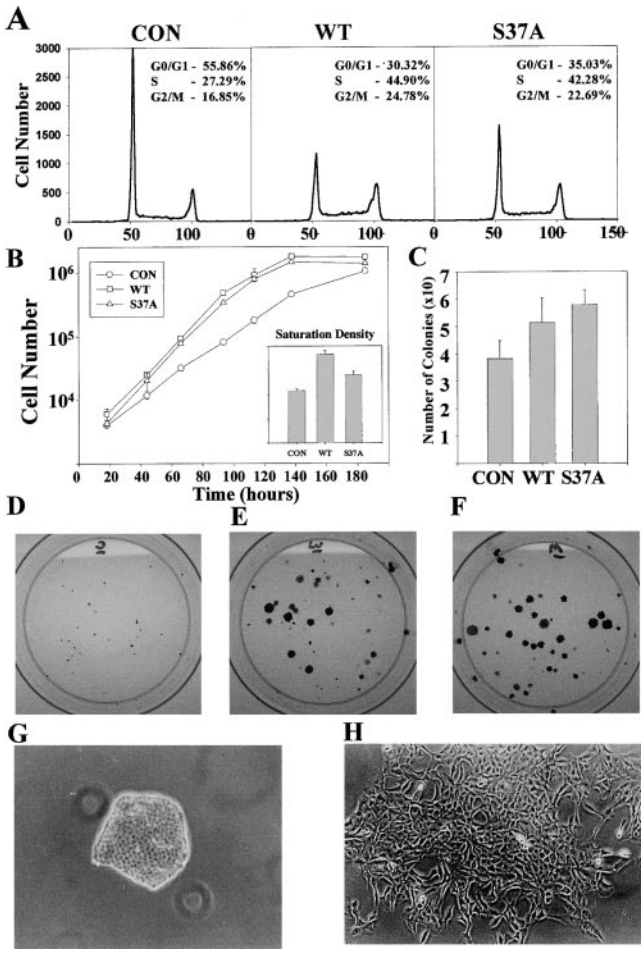


*Figure 3.* β-Catenin overexpression alters proliferation, plating efficiency, and colony morphology. A, DNA/flow cytometric analysis of the three cell pools during exponential growth demonstrates that the WT and S37A cell pools have a significantly lower percentage of cells in the $G_0/G_1$ phase of the cycle and a higher percentage in both S and $G_2$ phases of the cell cycle. B, Growth curves reveal that WT and S37A cells proliferate more rapidly than CON cells. To have approximately equal numbers of cells at time 0, 10,000 CON, 5,000 WT, and 5,000 S37A cells were plated per well in 12-well tissue culture plates. Each time point was done in triplicate. Graphing and SD calculations were performed with Sigmaplot. Error bars are hidden by symbols at several time points. Inset, saturation density of the three cell pools. Cells were counted at absolute confluence in 12-well plates. Each measurement is the mean of the cell counts from at least six wells. Graphing and SD calculation was performed with Sigmaplot. C–H, Plating efficiency assay reveals changes in colony morphology. 100 cells from each of the cell pools were plated in 100-mm dishes. After eight days, the colonies were stained with crystal violet, counted, and photographed. C, Number of colonies counted for each of the three cell pools. D–F, Photographs of crystal violet stained CON (D), WT (E), and S37A (F) colonies. G and H, Phase-contrast photographs of representative colonies from the CON (G) and WT (H) cell pools at four days. S37A colonies looked identical to the WT colony pictured. All experiments were performed at least three times with consistent and repeatable results.

## *β-Catenin Promotes Proliferation at High Cell Density*

The reduction in proliferative rate that nontransformed cells experience at high cell density has been termed contact inhibition of growth. Although this is a widely recognized phenomenon, the signaling mechanisms involved remain unknown. To address this, the MDCK cell pools

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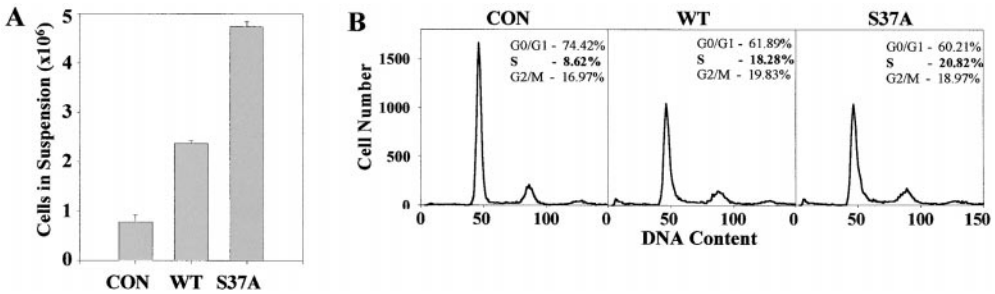


*Figure 4.* β-Catenin overexpression promotes proliferation at high cell density. A, Cells were cultured three days after confluence. After washing, cell shedding was measured over a 24 h period by counting the number of cells suspended in the medium. Graphing and SD calculation were performed using Sigmaplot. B, Cell cycle profiles of cells grown three days after confluence. After cells were washed twice with PBS, cell cycle analysis was performed on the adherent cells. The S phase percentage is increased 2–2.5-fold in WT and S37A cells relative to CON. All experiments were performed at least three times with consistent and repeatable results.

were grown to confluence and cell cycle parameters were monitored. Pilot experiments revealed that the WT and S37A cells shed more cells into the medium than CON cells. To quantify this effect, cells that were two to three days after confluence were washed twice with PBS, and fresh medium was added. The medium was collected from the wells on the next day and the suspended cells were counted. The number of shed cells was markedly elevated in the WT and S37A cells, as compared with the CON cells (Fig. 4 A). In these experiments, shedding of the S37A cell pool was consistently higher than in the WT cell pool.

The hypothesis that a higher proliferative rate was responsible for the difference in cell shedding was tested by performing cell cycle analysis of these cells grown three days after confluence. This analysis demonstrated that the WT and S37A cells had a higher proportion of S phase and $G_2$ phase, and a lower percentage of $G_0/G_1$ phase, as compared with the CON cells (Fig. 4 B). This cell cycle profile is precisely what would be expected if the WT and S37A cells were proliferating more rapidly than the CON cells, and is consistent with other experiments in which the $G_1$/S checkpoint control regulates contact inhibition (Dietrich et al., 1997; Kato et al., 1997). Presumably, in the absence of additional space to attach to the culture dish, the newly formed cells are shed into the medium.

### *β-Catenin Attenuates the Radiation-induced $G_1$/S Cell Cycle Block*

One important aspect of cell cycle regulation is cell cycle blockade after DNA damage. These blocks, which occur at the $G_1$/S and $G_2$/M transitions, presumably allow the cell to repair its DNA before the damage-induced errors become permanent (Weinert, 1998). We postulated that β-catenin overexpression might alter the DNA damage-induced late $G_1$ block of the cell cycle in the MDCK cells. The three cell pools were γ-irradiated with 0 or 5 Gy. Eight hours after irradiation, all of the cell pools show some $G_1$/S and $G_2$/M cell cycle blockade (Fig. 5). However, while CON had very few S-phase cells (5.96%), the WT and S37A cells retained a significant number of cells in S phase (15.26 and 14.99%). 24 h after irradiation, 25.2 and 21.4% of the WT and S37A cells, respectively, were in S phase, compared with 0.77% of CON cells. These data demonstrate that the radiation-induced $G_1$/S block is strongly attenuated by the overexpression of β-catenin and indicates that elevated β-catenin might lead to the accumulation of DNA damage and increased incidence of other mutations.

### *β-Catenin Expression Fluctuates throughout the Cell Cycle*

The previously described block of $G_1$/S progression by APC in normal cells points to a role of endogenous β-catenin in the regulation of cell cycle progression in nontransformed cells (Baeg et al., 1995). Together, with our demonstration that even the modest elevations of β-catenin described in this study can regulate cell cycle progression,

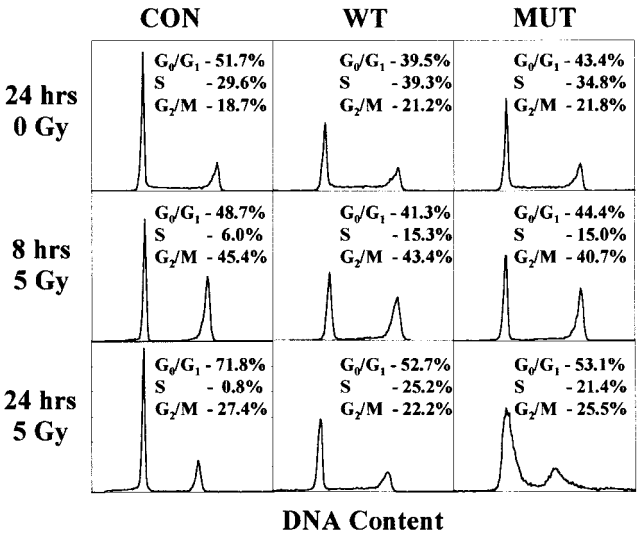


*Figure 5.* β-Catenin overexpression attenuates the γ-irradiation $G_1$ cell cycle block. Cells were γ-irradiated with 0 or 5 Gy. 8 and 24 h later, the cell cycle distribution was determined. The unirradiated cells all had a similar profile with the characteristic differences in $G_0/G_1$ and S phases (see Fig. 3). The S phase population of CON cells is significantly reduced at 8 h and absent at 24 h. At 24 h, the CON cells were blocked entirely in the $G_0/G_1$ or $G_2$ phases of the cell cycle. A slight decline in the proportion of WT and S37A cells in S phase occurs at 8 h after irradiation, but this is much less than that which occurs in CON cells. In contrast to CON cells, at 24 h the S phase proportions of the WT and S37A cells have partially recovered. All experiments were performed at least three times with consistent and repeatable results.

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this led us to investigate its level of expression throughout the cell cycle. Preliminary experiments were performed with parental MDCK cells that were partially synchronized in early $G_1$ by serum starvation. Parallel wells of cells were collected at various time points after release from $G_0$ by the addition of serum to make whole cell or cytoplasmic lysates for analysis of β-catenin protein levels. Although total β-catenin protein did not vary appreciably during the cell cycle, cytoplasmic β-catenin levels increased significantly from $G_1$ to S phase (data not shown). The increase began in late $G_1$ and continued through S phase. These pilot experiments led us to examine this phenomenon in the A1N4 cell line, which is easily synchronized in early $G_1$ by the removal of EGF from the growth medium. Like MDCK cells, cytoplasmic levels of β-catenin protein increased in late $G_1$ and continued to rise in S phase (Fig. 6 A), whereas total cell β-catenin did not vary (data not shown). Densimetric scanning revealed a 23-fold increase in cytoplasmic levels from early $G_1/G_0$ to S phase (Fig. 6 B). As a control, the blot was reprobed for cyclin dependent kinase inhibitor, p27 (Fig. 6 A). As expected, variations in p27 were inversely related to β-catenin. To determine if this oscillation in cytoplasmic β-catenin led to fluctuations in β-catenin–LEF/TCF signaling, A1N4 cells were assayed for TOPFLASH activity after being synchronized in $G_1$ phase or $G_2$/M phase of the cell cycle. The level of β-catenin–LEF/TCF signaling corresponded with the levels of cytoplasmic β-catenin measured by Western blotting (Fig. 6 C). The elevation in signaling at $G_2$/M was greater than that induced by treatment with the proteosomal inhibitor, ALLN. These data indicate that oscillations in β-catenin signaling may be involved in the normal regulation of cell cycle progression.

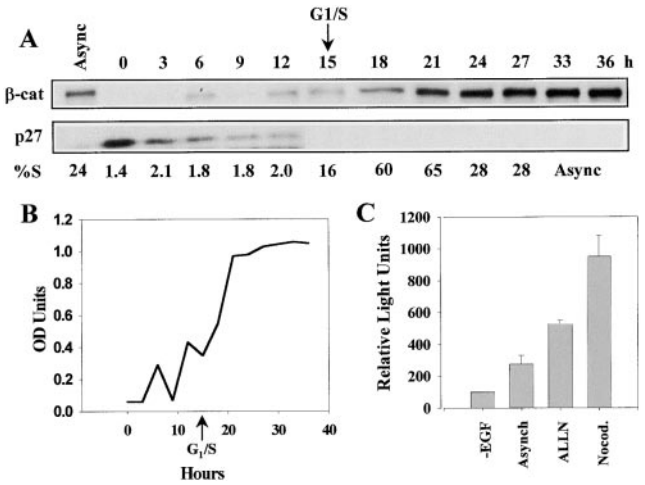


*Figure 6.* Cytoplasmic β-catenin oscillates during the cell cycle. A, A1N4 cells were synchronized in $G_0/G_1$ by EGF starvation. After releasing the cells into the cell cycle by the addition of EGF, cytoplasmic lysates were made every 3 h and assayed for β-catenin and p27 protein by immunoblotting. The distribution of cells in the cell cycle was determined at each time point by analyzing parallel cell cultures by flow cytometry. The percentage of S phase cells (%S) is provided. B, The level of expression was determined at each time point by densitometry and the results plotted against time after EGF addition. C, β-catenin–LEF/TCF signaling was measured in cells that were blocked in $G_0/G_1$ by EGF starvation (−EGF), growing asynchronously (Asynch), blocked near the S/$G_2$ transition by the proteosomal inhibitor ALLN, or blocked at $G_2$/M with nocodazole (Nocod). The results are expressed relative to the $G_0/G_1$ synchronized samples. Experiments represented in A and B were performed three times with consistent and repeatable results. Experiments represented in C were performed twice with consistent and repeatable results.

## *β-Catenin Promotes Colony Formation in Soft Agar*

The ability of cells to proliferate in the absence of attachment to a solid substrate correlates well with the transformed, tumorigenic phenotype. To assess the oncogenic capacity of β-catenin in vitro, cells were suspended in 0.3% agar and allowed to grow for two weeks. The ability of the WT and S37A cells to form colonies in soft agar was clearly enhanced relative to the CON cells (Fig. 7, A–C). Although the CON cells do exhibit a background level of colony formation, expression of the β-catenin transgenes resulted in a 10–20-fold increase in the number of colonies and an obvious increase in colony size (Fig. 7 D). Multiple experiments did not demonstrate a significant difference between the WT and S37A cell pools. This is the first demonstration that full-length β-catenin, WT and S37A mutant, has transforming capacity.

## *β-Catenin Inhibits Anoikis*

When nontransformed epithelial cells are deprived of attachment to an extracellular matrix for an extended period of time they undergo apoptosis (Frisch and Francis, 1994;

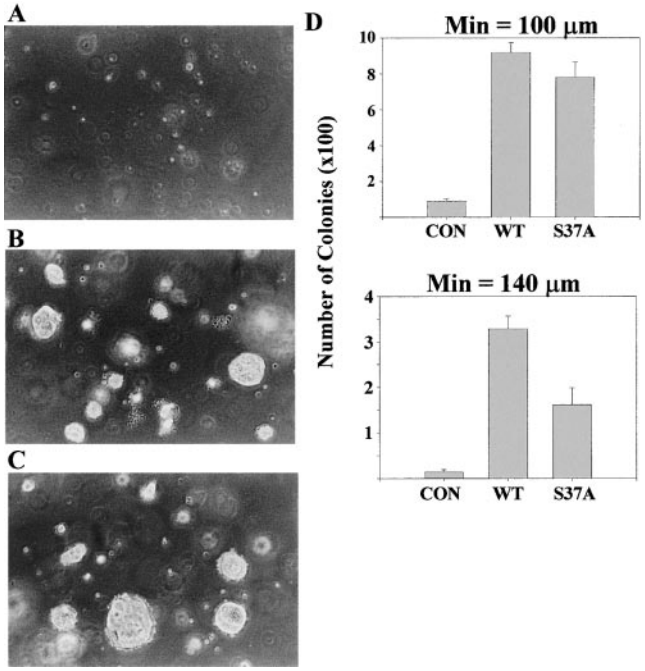


*Figure 7.* β-Catenin overexpression regulates soft agar colony formation. A–C, Phase-contrast photographs of colonies formed by the CON (A), WT (B), and S37A (C) cell pools after 14 d in soft agar. D, The number of colonies per 35-mm dish quantified by the Omnicon 3600 colony counter, using either 100 or 140 μm as the threshold for colony diameter. Experiments were repeated three times with consistent and repeatable results.

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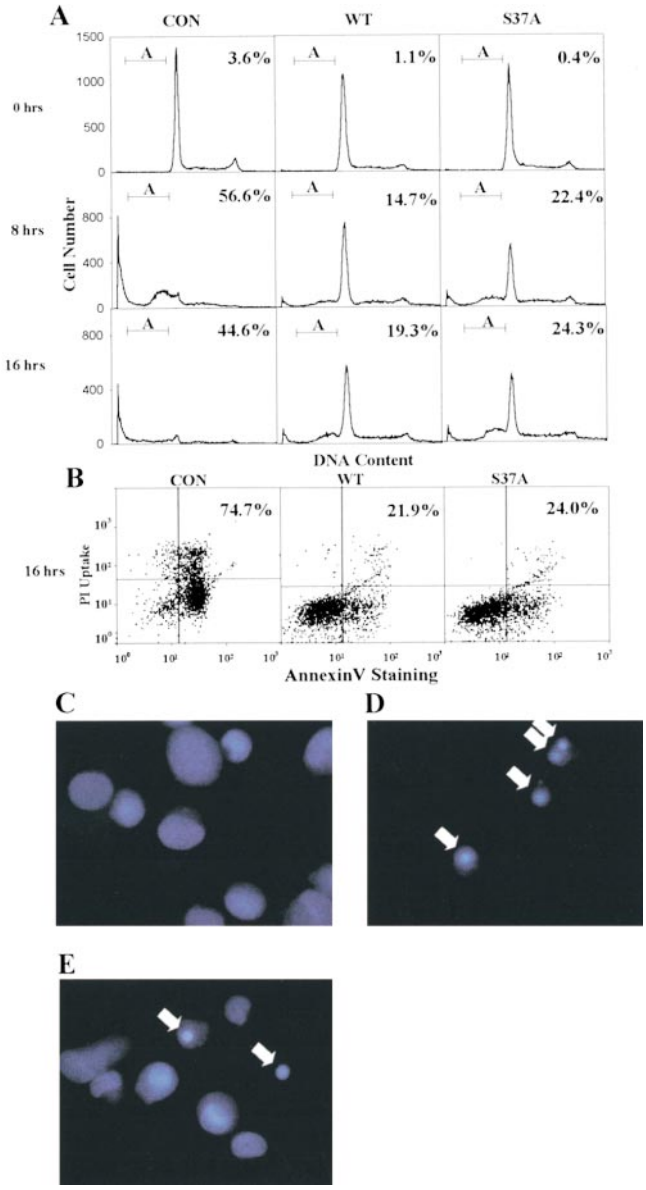


*Figure 8.* β-Catenin expression prevents anoikis. A, DNA/flow cytometric analysis of CON, WT, and S37A cells after incubation in suspension for 0, 8, or 16 h. The hypodiploid population corresponds to the apoptotic cells. The percentage in each panel represents the hypodiploid fraction. B, AnnexinV and propidium iodide staining of the same cells at 16 h also demonstrates a significant protection by β-catenin. C, Hoechst staining demonstrates nuclear morphology of CON cells before suspension. All nuclei look normal. WT and S37A cells looked similar. D, Hoechst staining of CON cells after 16 h in suspension. Most nuclei have a shrunken apoptotic morphology (arrows). E, S37A cells after 16 h in suspension. Most cells had the normal nuclear morphology, but a significant fraction (∼25%) were shrunken apoptotic (arrows). Experiments represented in A and B were performed at least three times with consistent and repeatable results. Experiments represented in C–E were performed twice with consistent and repeatable results.

Frisch and Ruoslahti, 1997). This suspension-induced apoptosis has been termed anoikis. In the soft agar growth experiments, it appeared that most CON cells die when suspended in soft agar. However, the remaining cells did contribute to a background rate of colony formation. To investigate the possibility that β-catenin increases the colony-forming capacity of MDCK cells by preventing anoikis, cells were cultured on a cushion of 0.8% agar in normal growth medium, collected at eight hour intervals over a 24-h period, and assayed for apoptosis. Microscopic examination of the cells after 16- and 24-h incubations revealed that the majority of the WT and S37A cells were larger and more refractile to light than the CON cells (data not shown), suggesting that the CON cells were preferentially undergoing apoptosis. These preliminary results were confirmed by DNA/flow cytometry and AnnexinV staining of cells that had been kept in suspension for 0, 8, or 16 h (Fig. 8, A and B). Both methods showed that anoikis was significantly inhibited by β-catenin overexpression.

The results of further analysis of the flow cytometry and AnnexinV data for the percentage of hypodiploid and AnnexinV-positive cells, respectively, are compiled in Table I. The DNA/flow cytometry data revealed that the percentage of hypodiploid cells was markedly and consistently lower in the WT and S37A cells relative to the CON cells. However, these data significantly underestimate the percentage of apoptotic cells in the CON samples at the 16 h time point, as the disintegrating apoptotic cells were lost from the analysis. The AnnexinV assays appeared to retain these cells and probably give a more accurate estimate at 16 h.

As a third independent method of measuring apoptosis, nuclear morphology of cells before and after suspension was analyzed by Hoechst staining. In contrast to the nonsuspended cells, which all had normal nuclear morphology (Fig. 8 C), most of the suspended CON cells displayed characteristically shrunken apoptotic nuclei (Fig. 8 D). In contrast, the nuclei of the majority of WT and S37A cells displayed a normal morphology (Fig. 8 E). A fraction of the cells (∼1/4) were apoptotic, which is consistent with the AnnexinV and flow cytometry results. Interestingly, a minority of CON cells were found to be associated with clumps of five or more cells. Most of these cells displayed normal nuclear morphology. This was a clear demonstration that cell–cell adhesion can prevent apoptosis induced by suspension, and this probably caused us to underestimate the percentage of apoptosis among the suspended CON cells by the AnnexinV and flow cytometric methodologies.

These data demonstrate that β-catenin overexpression may promote soft agar colony formation of MDCK cells by the promotion of cell cycle progression and the inhibition of anoikis.

*Table I. β-Catenin Prevents Anoikis, as Measured by DNA/Flow Cytometry and AnnexinV Labeling*

| | Hypodiploid cells | | | AnnexinV positive | | |
|---|---|---|---|---|---|---|
| | 0 h | 8 h | 16 h | 0 h | 8 h | 16 h |
| | | % | | | % | |
| CON | 3.6 | **56.6** | 44.6 | 1.7 | 50.4 | **74.7** |
| WT | 1.1 | **14.7** | 19.1 | 2.4 | 18.0 | **21.9** |
| MUT | 0.4 | **22.4** | 24.3 | 0.7 | 31.0 | **24.0** |

The percentage of apoptotic cells in the three cell pools after different periods of suspension, as measured by flow cytometry (hypodiploid) or AnnexinV labeling (AnnexinV positive). The percentages in **bold** demonstrate the most notable effects.

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## Discussion

It is suspected that the cadherin-associated protein β-catenin promotes the process of carcinogenesis (Peifer, 1997). The data that support this hypothesis include the following observations: it associates with and is downregulated by the tumor suppressor APC; it transduces (at least partly) the oncogenic Wnt growth factor signal to the nucleus; it is mutated in a significant number of human cancers; and, overexpression of an $NH_2$ terminally truncated form of β-catenin in the epidermis of transgenic mice produced well-differentiated hair tumors (Rubinfeld et al., 1993; Su et al., 1993; Cadigan and Nusse, 1997; Ilyas et al., 1997; Fukuchi et al., 1998; Gat et al., 1998; Miyoshi et al., 1998; Palacios and Gamallo, 1998; Voeller et al., 1998). However, no studies provide direct evidence for the transforming potential of full-length β-catenin. In addition, no investigations have addressed the question of which cellular processes β-catenin may regulate to effect cellular transformation.

### *β-Catenin Transforms the Epithelial MDCK Cell Line*

This report characterizes phenotypic alterations that result from β-catenin overexpression in a nontransformed epithelial cell line. Effects are seen in the regulation of three important cellular activities/properties: proliferation, apoptosis, and morphology. It demonstrates that modest β-catenin overexpression significantly enhances the ability of these cells to proliferate, especially in situations that would normally inhibit the cell cycle at the $G_1$/S transition. Most striking is the demonstration that it promotes growth in soft agar, a phenotype closely correlated with tumorigenicity. Most nontransformed cells require adhesion through integrin receptors to extracellular matrix components to transit through the $G_1$ phase of the cell cycle (Mehta et al., 1986; Polyak et al., 1994). In addition, suspension of normal, attachment-dependent cells blocks them late in $G_1$ phase.

β-Catenin overexpression also resulted in increased proliferation of cells at high cell density. The mechanism by which high cell density inhibits proliferation is unknown, but also involves a block in late $G_1$. The presence of cell–cell adhesion, the reduction of cell-substrate adhesion, and the depletion of growth factors have all been implicated (Chen et al., 1997). β-Catenin's dual activities as a regulator of cadherin-mediated cell–cell adhesion and as the transducer of a mitogenic signal implicate it in this regulatory process. Both cadherin and α-catenin can inhibit β-catenin signaling in other experimental systems (Fagotto et al., 1996; Simcha et al., 1998). Together, with the results of the present study, these data support the hypothesis that cell–cell adhesion promotes the formation of cadherin/β-catenin/α-catenin complexes and that these complexes negatively regulate β-catenin signaling, which discourages cell cycle progression. However, the fact that proliferation is reduced at high cell density, as compared with sparsely plated cells, even in the WT and S37A cells, suggests that other mechanisms are also involved (for example, cell shape; Chen et al., 1997).

The cell cycle analyses and growth curves in this study demonstrate that β-catenin overexpression can significantly alter the proliferative rate of these cells. The distribution of the WT and S37A cells is weighted heavily toward S phase and away from $G_1$. When considered along with the other cell cycle data, it appears that β-catenin overexpression expedites the $G_1$/S transition in MDCK cells. The easing of the barrier to $G_1$/S transition manifests as a difference in cell growth on plastic, as growth curves of the β-catenin overexpressing cells diverged significantly from the control cells.

β-Catenin overexpression also has a notable effect on cell morphology. The MDCK cell line is a nontransformed epithelial line that has very strong intercellular adhesion and extends cell membrane extensions only to a limited degree. β-Catenin overexpression converts MDCKs into a more mesenchymal cell type (Barth et al., 1997; and the present study). At low density, cell–cell adhesion is reduced and the cells take on a more spindly, stretched shape. This change in morphology is reminiscent of an epithelial to mesenchymal transition (EMT; Huber et al., 1996). EMTs are developmentally important cellular conversions, especially during gastrulation, the point in development at which β-catenin knockout mouse embryos are aborted. Also, an EMT has been suggested to underlie the progression from benign tumor to metastatic carcinoma (Sommers et al., 1991; Birchmeier et al., 1996). Indeed, it previously has been suggested that β-catenin signaling may regulate this process (Sommers et al., 1994; Huber et al., 1996).

The absence of anoikis is another characteristic of transformed cells. The present study and others have shown that MDCK cells are very dependent on attachment to the extracellular matrix for survival (Frisch and Francis, 1994; Frisch et al., 1996a,b). After 16 h in suspension, the majority of CON cells were apoptotic, as measured by three independent methods. The expression of the WT and S37A β-catenin transgenes markedly retards this process, allowing ~75% of the single cells to survive. This is a vigorous inhibition of anoikis. Taken together, the proliferation, anoikis, and morphology data demonstrate that these cells are clearly transformed by β-catenin.

These in vitro results suggest that overexpression of full-length β-catenin should promote tumorigenesis in vivo. Two separate studies have demonstrated the effect of tissue-specific overexpression of an $NH_2$ terminally truncated form of β-catenin. Expression of the truncated form of β-catenin in the epidermis of transgenic mice by Gat et al. (1998) resulted in the formation of two types of hair follicle-related tumors. Taken together with the present study, these results strongly suggest that full-length forms of β-catenin are important mediators of oncogenesis in vivo. Interestingly, a study by Wong et al. (1998), in which an $NH_2$ terminally truncated form of β-catenin was overexpressed in the intestinal epithelium of transgenic mice, produced conflicting results. Proliferation of the intestinal epithelial cells in these animals was stimulated 1.5–3-fold, in accordance with the results of the present study. However, the elevated proliferation rate was balanced by an increase in apoptosis, the net result being no change in intestinal villus height. To explain the discrepancy between these results and our own, we suggest that β-catenin overexpression can protect cells only from certain apoptotic signals. It is possible that the compensatory mechanism by which the authors suggested that the transgenic mice might have maintained their cell census in the face of in-

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creased proliferation is mediated through the stimulation of β-catenin–insensitive apoptosis. It is also possible that full-length β-catenin has signaling capacities that are lost when its $NH_2$ terminus is removed.

The results presented in the present study also differ from those published previously by Young et al. (1998). They reported that overexpression of the Wnt-1 growth factor transformed Rat-1 fibroblasts while expression of the S37A mutant form of β-catenin we described previously had no effect. Two differences between the two studies may explain the conflicting results. First, the morphological effects we describe may only be detectable in an epithelial cell type. Second, the studies of Young et al. (1998) were carried out without serum, whereas the present ones were done with serum. It is possible that Wnt-1 activates parallel signaling pathways (in addition to β-catenin signaling) that may circumvent the need for serum to stimulate proliferation. β-Catenin's position lower in the pathway may preclude the activation of such parallel pathways and, therefore, it is unable to stimulate proliferation of Rat-1 fibroblasts in the absence of serum.

### *β-Catenin Attenuates the Cell's Response to γ-Irradiation*

The cell cycle blocks that characterize the response of cells to DNA damage are important for the maintenance of genomic integrity. To prevent the permanent incorporation of mutations induced by various DNA damaging stimuli, the cell cycle can pause at the $G_1$/S and the $G_2$/M transitions (Weinert, 1998). During these delays, the cell assesses the damage to its DNA and either repairs the damage or destroys itself. Premature reentry into the cell cycle may result in the accumulation of mutations to oncogenes and tumor suppressor genes, which would increase the likelihood of cellular transformation and cancer. The data from this study suggest that β-catenin overexpression may result in the premature reentry of cells into the cell cycle after γ-irradiation–induced DNA damage, and thereby promote the accumulation of oncogene mutations and carcinogenesis.

### *β-Catenin Overexpression Inhibits Anoikis*

An association between apoptosis and the APC/β-catenin axis has been suggested previously. Reexpression of the APC gene in a tumor cell line that lacks WT APC resulted in the induction of apoptosis within 24 h (Morin et al., 1996). Since one of the functions of APC is to downregulate β-catenin, it is possible that β-catenin itself is a regulator of apoptosis. Our demonstration that β-catenin alone significantly protects cells from anoikis strongly implies that it can be a potent inhibitor of apoptosis. Also, during the process of apoptosis, caspase-3 can cleave β-catenin protein (Brancolini et al., 1997). One purpose of this cleavage may be to destroy the antiapoptotic β-catenin signal within the cell and thereby hasten the completion of the apoptotic process. The caspase-mediated cleavage of focal adhesion kinase (FAK) is thought to function in this manner (Wen et al., 1997).

It has been postulated that the induction of apoptosis by the loss of appropriate extracellular matrix attachment (i.e., anoikis) is a means of protecting the organism from improper cell growth (Frisch and Ruoslahti, 1997). Anoikis is prevented by integrin-mediated signaling. Several enzymes have been implicated as being downstream of integrins in this signal transduction pathway. These include FAK, phosphoinositide-3-kinase, protein kinase B/Akt, and integrin-linked kinase (ILK; Clark and Brugge, 1995; Giancotti, 1997; Wu et al., 1998). The present report suggests that β-catenin may also lie downstream of integrins. Several integrin-stimulated signaling pathways might lead to the induction of β-catenin signaling. One possible connection between integrins and β-catenin is the integrin-activated, antiapoptotic kinase PKB/Akt. PKB is known to inhibit the activity of glycogen synthase kinase 3-β, a serine kinase that functions directly to reduce β-catenin protein and signaling (Siegfried et al., 1992; Cook et al., 1996; Cadigan and Nusse, 1997). It is possible that the result of these two inhibitory interactions is that activation of PKB by integrin signaling functions to positively activate β-catenin signaling.

The data presented in this report describing the effects of β-catenin overexpression are similar to previous reports describing the effects of ILK (Novak et al., 1998; Wu et al., 1998). ILK is a 59-kD serine kinase that was first described as a $\beta_1$-integrin-associated kinase. ILK overexpression causes cells to undergo an EMT and promotes their growth in soft agar. This is associated with an increase in LEF-1 protein levels. As a result of increased LEF-1, β-catenin becomes completely localized to the nucleus and β-cat-LEF/TCF signaling increases significantly. In addition, loss of cell attachment to the underlying ECM was shown to result in a dramatic reduction in LEF protein. In a separate study, ILK directly phosphorylated and inhibited the activity of GSK-3β. This may constitute another mechanism by which integrin signaling may result in increased β-catenin–LEF/TCF signaling.

Anoikis results from the interruption of integrin-mediated signaling (Frisch and Ruoslahti, 1997). In addition to ILK, the integrin-associated nonreceptor tyrosine kinase FAK may also be involved in the transduction of these signals because FAK signaling suppresses p53-dependent apoptosis (Ilic et al., 1998). Ilic et al. (1998) also demonstrated that an atypical protein kinase C isoform (PKCλ/ι) is required for this p53-dependent apoptotic pathway, since inhibition with both chemical PKC inhibitors and a dominant–negative construct protect FAK-defective cells from apoptosis. Previously, we reported that an atypical PKC isoform was involved in regulating β-catenin degradation (Orford et al., 1997). Inhibiting atypical PKC activity using the same chemical PKC inhibitors used by Ilic et al. (1998) resulted in the inhibition of the ubiquitination and degradation of β-catenin. In addition, treatment of cells with these PKC inhibitors increases β-catenin–LEF/TCF signaling (unpublished results). Taken together with the present study, it is possible that the inhibition of PKCλ/ι or another atypical PKC may increase β-catenin stability and signaling, leading to the suppression of p53-mediated apoptosis (Fig. 9 A).

### *β-Catenin Oscillations during the Cell Cycle May Regulate Normal Cellular Proliferation*

The c-myc promoter is also regulated by the APC/β-cate-

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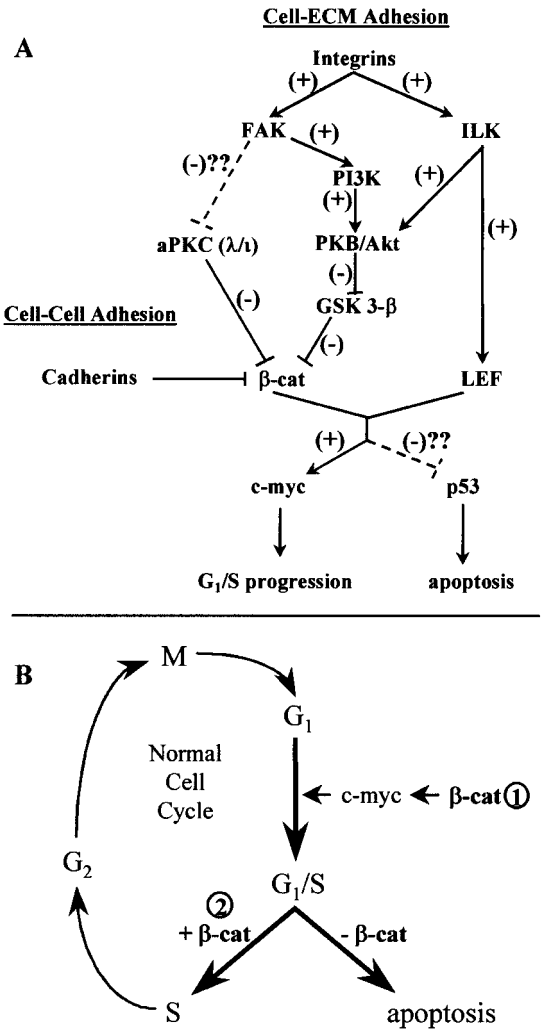


*Figure 9.* Hypothetical signaling pathways by which β-catenin might integrate cell adhesion, cell cycle, and apoptosis. A, The individual regulatory relationships depicted by unbroken arrows and blockades have been demonstrated in various published reports. However, these signaling pathways have never been demonstrated in their entirety within a single experimental system. The broken blockades are hypothetical regulatory events suggested in the present report. Integrin-activated FAK activity may regulate β-catenin signaling by two different pathways. In both cases, two sequential negative regulatory interactions downstream of FAK may result in the activation of β-catenin signaling. By a parallel pathway, ILK can regulate the activities of PKB and GSK-3β, as well as upregulate the expression of the transcription factor LEF-1. Together, β-catenin and LEF-1 might stimulate the $G_1$/S transition in the cell cycle (possibly via c-myc) and inhibit p53-mediated apoptosis. The inhibition of apoptosis may be through direct modulation of p53 action or through a parallel antiapoptotic pathway. The role of p53 in β-catenin–mediated signaling is speculative. B, β-catenin may regulate the cell cycle by two separate mechanisms: 1, β-catenin can stimulate the expression of c-myc, which is a strong stimulator of cell cycle progression; 2, the $G_1$/S transition represents an important decision-point for the cell. It is known that this transition requires the presence of survival factors. In their absence, the cell chooses apoptosis over proliferation. β-Catenin may regulate the $G_1$/S transition as a survival factor functioning to permit cell cycle progression by preventing apoptosis.

nin signaling pathway (He et al., 1998). The upregulation of c-myc by β-catenin may constitute one mechanistic link between β-catenin and tumor formation. c-myc is potent oncogene that regulates cell cycle progression. However, c-myc overexpression cannot induce cellular transformation on its own. In fact, when overexpressed alone, c-myc markedly increases the susceptibility of cells to apoptosis (Desbarats et al., 1996; Steiner et al., 1996; Thompson, 1998). To transform cells, c-myc requires an accompanying survival signal to prevent cells from undergoing apoptosis. Advancement through the $G_1$ phase of the cell cycle can result in either progression into S phase or apoptosis, depending on the presence or absence of certain survival signals, for example, IGF-1 (Evan et al., 1995). In addition to stimulating c-myc, β-catenin may transduce the requisite antiapoptotic signal that would permit cell cycle progression. The increase of cytoplasmic β-catenin protein before S phase during the cell cycle may serve this purpose in normal cells (Fig. 6). Additionally, β-catenin would protect against anoikis if overexpressed in epithelial cells.

Our data do not demonstrate any reproducible phenotypic difference between the WT and S37A expressing cells, except in the measurement of protein expression and in cell shedding at confluence. It is important to note that in both the WT and S37A cell pools, the level of cytoplasmic β-catenin protein and β-catenin–LEF/TCF signaling is elevated relative to the CON cells. This implies that a modest increase of cytoplasmic β-catenin can result in significant changes in signaling and cellular transformation and that overexpression of the wild-type gene alone is sufficient. This may also explain how the relatively small increase in endogenous cytoplasmic β-catenin that occurs before the onset of S phase may regulate the $G_1$/S transition in the normal cell cycle (Fig. 9 B). However, it is interesting to note that the increase in signaling above CON levels and the difference between the WT and S37A cells are relatively small when compared with other published results (Morin et al., 1997; Porfiri et al., 1997; Young et al., 1998). It is possible that the fact that this study was performed with cells that stably express a constitutively active transgene is responsible for both phenomena. We believe that the very high levels of β-catenin expression and signaling that can be achieved in nontransformed cells by transient transfection is not conducive to their survival and propagation. If true, selection pressures against very high expression would: result in the production of stable cells expressing only moderately elevated β-catenin protein and signaling; and, limit the extent to which the S37A mutation could stimulate signaling above WT β-catenin. In addition, some studies have used different β-catenin mutants, which may be more active.

It is plausible that some of the phenotypic alterations induced by β-catenin overexpression could be the result of altered cadherin function and independent of β-catenin signaling. However, the fact that these cells display strong intercellular adhesion at high density and retain the ability to generate tight junctions (as measured by electrical resistance across the monolayer in culture) demonstrates that E-cadherin function remains intact.

The APC/β-catenin signaling pathway has been implicated in a large number of epithelial cancers (Munemitsu et al., 1995; Inomata et al., 1996; Ilyas et al., 1997; Korinek

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et al., 1997; Mareel et al., 1997; Morin et al., 1997; Peifer, 1997; Rubinfeld et al., 1997; Palacios and Gamallo, 1998; Voeller et al., 1998). In most cases, mutations in either APC or β-catenin result in stabilization of β-catenin protein and elevated β-catenin–LEF/TCF signaling. However, it is not clear what role this pathway has in normal cells. In this study, we demonstrate that β-catenin is a potent oncogene. All of the major phenomena that characterize cellular transformation, that is, soft agar growth, altered morphology, inhibition of apoptosis, and stimulation of cell cycle progression, can be induced by the modest overexpression of β-catenin in a nontransformed epithelial cell line. This clearly indicates that β-catenin can play a direct role in the process of carcinogenesis and that a major component of APC function is its downregulation. These data suggest that, as an early event in the progression of colorectal cancer, activation of β-catenin signaling promotes adenoma formation by promoting proliferation and survival of epithelial cells in the abnormal tissue architecture of a tumor mass. In addition, it may also promote the accumulation of mutations and cancer progression by attenuating the DNA damage-induced $G_1$ cell cycle block.

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