

# Restoration of Epithelial Cell Polarity in a Colorectal Cancer Cell Line by Suppression of $\beta$ -catenin/T-Cell Factor 4-mediated Gene Transactivation<sup>1</sup>

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## ABSTRACT

$\beta$ -Catenin acts as a transcriptional coactivator by forming a complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) DNA-binding proteins. Aberrant transactivation of a certain set of target genes by  $\beta$ -catenin and TCF4 complexes has been implicated in familial and sporadic colorectal tumorigenesis. A colorectal cancer cell line, DLD-1, becomes irregularly multilayered, when maintained confluent for 2–3 weeks, and forms numerous dome-like polypoid foci piled-up over the surface of cell sheets. By the use of a strict tetracycline-regulation system, we found that the continuous suppression of  $\beta$ -catenin/TCF4-mediated gene transactivation by dominant-negative TCF4B ( $\Delta$ N30) reduced these piled-up foci and restored a simple monolayer of polarized columnar cells resembling normal intestinal epithelium. The restoration of epithelial cell polarity was evident in two ways: (a) the formation of microvilli over the apical surface; and (b) the distribution of a tight junction protein, ZO-1, to the lateral plasma membrane. Retroviral expression of stabilized  $\beta$ -catenin ( $\Delta$ N89) induced the formation of similar piled-up foci in untransformed IEC6 intestinal epithelial cells. Sulindac, a nonsteroidal anti-inflammatory drug effective against colorectal tumorigenesis in familial adenomatous polyposis syndrome, suppressed the formation of foci. The loss of epithelial cell polarity may be a critical cellular event driving  $\beta$ -catenin/TCF4-mediated intestinal tumorigenesis.

## INTRODUCTION

An inherited mutation of a tumor suppressor gene, APC<sup>3</sup>, is causative for FAP syndrome (1, 2). A murine model of FAP syndrome, Min mouse, also carries a mutation in the *Apc* gene (3). Over 80% of sporadic colorectal tumors harbor mutations of APC (4). Mutation of the APC gene is observed even in small adenomas of the colon and rectum (5). The mutational inactivation of the APC gene occurs earlier than any other genetic event in the adenoma-carcinoma sequence. On the basis of these observations, the APC gene has been recognized as a key “gatekeeper” of colorectal carcinogenesis (6). Indeed, conditional knock-out of the *Apc* gene by the Cre/loxP recombination induces rapid formation of adenomatous polyps in the murine intestine (7).

The majority of mutations seen in the APC gene cause the premature termination of the protein (8, 9). Truncated APC proteins often lose some of the 20-amino acid repeats and SAMP repeats, which are necessary for degradation of  $\beta$ -catenin by the ubiquitin-proteasome pathway (10–12). We previously demonstrated the cytoplasmic and

nuclear accumulation of  $\beta$ -catenin protein in adenoma and carcinoma cells of FAP patients (13). One-half of sporadic colorectal cancers with wild-type APC harbor mutually exclusive mutations in the GSK3 $\beta$  phosphorylation site of  $\beta$ -catenin, which make  $\beta$ -catenin itself resistant to proteolysis (14–17). Intestinal neoplasms induced by chemical carcinogens frequently carry mutations in  $\beta$ -catenin, but not in APC (18, 19). The accumulation of  $\beta$ -catenin and subsequent cellular modifications seem to allow adenomatous proliferation of intestinal epithelium. The Cre/loxP-mediated expression of stabilized  $\beta$ -catenin in the murine intestine resulted in the formation of adenomatous polyps, emphasizing the crucial role of  $\beta$ -catenin in intestinal tumorigenesis (20).

$\beta$ -Catenin was originally identified as an intracellular protein associated with cadherin cell adhesion molecules (21, 22). As well as its involvement in cell adhesion,  $\beta$ -catenin acts as a transcriptional coactivator by forming a complex with TCF/LEF DNA-binding proteins (23–25). Aberrant transactivation of a certain set of target genes by  $\beta$ -catenin and TCF/LEF complexes has been identified in human colorectal cancer cell lines and implicated in colorectal tumorigenesis (26). Thus far, a limited number of genes, including *c-myc*, *cyclin-D1*, *matrilysin*, *TCF1*, *PPAR $\delta$* , and *MDR1*, have been reported to be the direct targets of  $\beta$ -catenin and TCF/LEF-mediated transactivation in colorectal cancer cells (27–34). However, the whole picture of gene expression profiles and cellular mechanisms governing colorectal tumorigenesis still remains obscure.

A TCF4 construct lacking a 30-amino acid  $\beta$ -catenin-binding site in its NH<sub>2</sub>-terminus specifically suppresses transactivation of target genes by the  $\beta$ -catenin/TCF4 complex in a DN manner (26). We previously established colorectal carcinoma cell lines, DLD-1 Tet-ON TCF4B $\Delta$ N30, capable of inducing a DN TCF4 protein under the strict control of the tetracycline-regulatory system (34). We anticipated that these cell lines would allow precise pinpointing of the molecular and cellular changes that occur after the inactivation of the  $\beta$ -catenin/TCF4 complex. In fact, using these cell lines, we successfully identified the *MDR1* gene as a target of the  $\beta$ -catenin/TCF4 transcriptional complex (34). In this study, we focused on the cellular changes caused by the induction of DN TCF4 to define the biological mechanisms underlying  $\beta$ -catenin/TCF4-mediated early colorectal tumorigenesis.

## MATERIALS AND METHODS

**Chemicals.** Sulindac (Sigma) was dissolved in 1 M Tris-HCl (pH 7.5) to a stock concentration of 400 mM, and the pH was adjusted to 7.2 with 4 M HCl. Sulindac was added to culture media at a final concentration of 200  $\mu$ M. Dox (Sigma), a derivative of tetracycline, was dissolved in deionized water to a stock concentration of 1 mg/ml. Dox was added to culture media at a final concentration of 0.5  $\mu$ g/ml.

**Cell Culture.** Three stable clones, DLD-1 Tet-ON TCF4B $\Delta$ N30–1, –5, and –7, all of which were capable of inducing the AU1-tagged TCF4B protein lacking a 30-amino acid  $\beta$ -catenin binding site in its NH<sub>2</sub>-terminus in the presence of Dox, and three mock clones carrying empty pTRE, DLD-1 Tet-ON control-A, –B, and –D, were established from a single parental clone, DLD-1 Tet-ON (Fig. 1a), as described previously (34). DLD-1 cells were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% Tet-system-approved FBS (Clontech Laboratories), which does not contain a detectable

Received 9/28/00; accepted 1/17/01.

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<sup>1</sup> Supported in part by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan. Y. N. and A. S. T. are recipients of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

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<sup>3</sup> The abbreviations used are: APC, adenomatous polyposis coli; TCF, T-cell factor; LEF, lymphoid enhancer factor; FAP, familial adenomatous polyposis; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; DN, dominant-negative; Dox, doxycycline; ZO-1, zonula occludens-1; NSAID, nonsteroidal anti-inflammatory drug; Cox, cyclooxygenase; FBS, fetal bovine serum; RIPA, radioimmunoprecipitation assay (buffer).

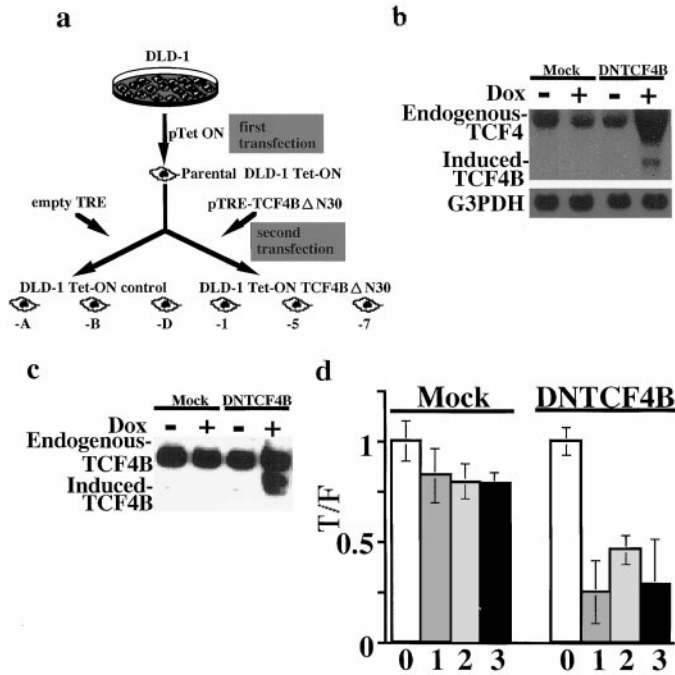


Fig. 1. Stable induction of DN TCF4 mRNA and protein in DLD-1 Tet-ON TCF4 $\Delta$ N30 cells. *a*, establishment of DLD-1 Tet-ON TCF4 $\Delta$ N30 cells. DLD-1 was double-transfected sequentially with regulatory pTet-ON and then with responsive empty pTRE (Clontech) or pTRE-TCF4 $\Delta$ N30 plasmids, as described previously (34). Three stable clones, DLD-1 Tet-ON TCF4 $\Delta$ N30-1, -5, -7, and three mock clones, DLD-1 Tet-ON control-A, -B, and -D, were isolated from one parental DLD-1 Tet-ON clone. *b*, Northern blot analysis. Total RNA (15  $\mu$ g) extracted from DLD-1 Tet-ON TCF4 $\Delta$ N30-7 cells, untreated or treated with 0.5  $\mu$ g/ml Dox for 3 weeks, and DLD-1 Tet-ON control-A cells, untreated or treated with 0.5  $\mu$ g/ml Dox for 3 weeks, was fractionated and hybridized with TCF4B (*top*) or G3PDH (*bottom*) cDNA. *c*, Western blot analysis. Nuclear extracts (50  $\mu$ g/lane) from DLD-1 Tet-ON TCF4 $\Delta$ N30-7 cells, untreated or treated with 0.5  $\mu$ g/ml Dox (*Dox*) and DLD-1 Tet-ON control-A cells, untreated or treated with 0.5  $\mu$ g/ml Dox (*Dox*) for 3 weeks, were subjected to immunoblotting using anti-TCF4B polyclonal antibody. *d*, luciferase reporter assay. DLD-1 Tet-ON TCF4 $\Delta$ N30-7 cells (*DNTCF4B*, right) and DLD-1 Tet-ON control-A cells (*Mock*, left) were transiently transfected with reporter constructs containing trimerized optimal TCF/LEF motifs (TOP-FLASH) or mutant motifs (FOP-FLASH). Dox (0.5  $\mu$ g/ml) was added 1 (*column 1*), 2 (*column 2*), or 3 (*column 3*) weeks prior to luciferase assays, or not (*column 0*). *Columns*, the mean TOP:FLASH ratio normalized to *Renilla* luciferase activity. Luciferase activity is expressed as a ratio to that of untreated cells, taken as 1. *Error bars*, SD.

level of tetracycline or its derivatives, in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. An immortalized rat small intestinal epithelial cell line, IEC6 (35), was obtained from the Riken Cell Bank and cultivated in Dulbecco's MEM containing 5% FBS and 4  $\mu$ g/ml insulin (Life Technologies, Inc.). An embryonal kidney cell line, 293, was obtained from the Riken Cell Bank and cultivated in Dulbecco's MEM supplemented with 10% FBS.

**Characterization of Cell Growth and Morphology.** Cells were suspended in culture media containing 10% FBS or 2% FBS with or without 0.5  $\mu$ g/ml Dox. Cell numbers were counted with a hemocytometer after trypsinization every 48 h for up to 15 days or once a week for up to 3 weeks. At least three independent experiments were performed to confirm the consistency of the results.

For morphological analyses, 2  $\times$  10<sup>6</sup> cells were seeded in 100-mm tissue culture dishes, and culture media were changed every 3 days thereafter. After maintaining the confluent culture for 3 weeks, three randomly selected low-power fields ( $\times$ 40) were photographed under a phase-contrast microscope. Piled-up areas were circumscribed by the NIH Image software program and the ratio of the piled-up areas to the total area was calculated. Data were expressed as mean  $\pm$  SD. Differences between groups were analyzed using the *t* test and were considered significant when the *P* was less than 0.05.

To visualize cross-sections, cells were cultured on PetriPERM dishes (Heraeus) for 3 weeks. The membrane-bases of the PetriPERM dishes were cut with scalpels, fixed in 3.7% paraformaldehyde in PBS and embedded in paraffin. Thin sections (3  $\mu$ m) were stained with standard H&E techniques.

**Soft Agar Colony-forming Assay.** To evaluate anchorage-independent growth, 5  $\times$  10<sup>3</sup> cells were suspended in 1 ml of 0.3% molten top agarose (Life Technologies, Inc.) with or without 0.5  $\mu$ g/ml of Dox and were overlaid onto 1 ml of 0.5% solid bottom agarose in each well of 6-well tissue culture clusters. After solidification, the top agarose was covered with 1 ml of culture medium with or without 0.5  $\mu$ g/ml Dox. The clusters were maintained at 37°C and the culture media were changed every 3 days thereafter. Ten days after plating, colonies were photographed under a phase-contrast microscope and their images were captured using the NIH Image software program. The average area of at least 50 colonies was calculated. Data were expressed as mean  $\pm$  SD. Differences between groups were analyzed using the Student *t* test and were considered significant when the *P* was less than 0.05.

**Northern Blot Analysis.** Total RNA (15  $\mu$ g/lane) was fractionated by electrophoresis and transferred to Hybond N (Amersham Pharmacia Biotech). Hybridization was performed by using <sup>32</sup>P-radiolabeled cloned cDNA fragments of TCF4B (nucleotides 546-2015), as described previously (34). The quality and quantity of electrophoresed RNA was determined by hybridization with G3PDH cDNA (Clontech).

**Western Blot Analysis.** Anti-TCF4B rabbit polyclonal antibody was raised against a KLH-conjugated synthetic peptide, CYKVKAAASAH-PLQMEAY. Anti- $\beta$ -catenin and anti-E-cadherin monoclonal antibodies were purchased from Transduction Laboratories, and anti-ZO-1 monoclonal antibody from Zymed Laboratories. After washing them with PBS, cells were extracted using RIPA buffer [150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)] containing protease inhibitor cocktail (Roche Molecular Biochemicals) at 4°C. Nuclear extracts were prepared as described previously (26). Samples were fractionated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Blots were detected by an enhanced chemiluminescence (ECL) method (Amersham), as described previously (36).

**Immunofluorescence Microscopy.** For immunofluorescence microscopy, cells were grown on collagen I-coated coverslips (Asahi Technoglass). Cells were fixed with 3.7% paraformaldehyde for 10 min and then permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After blocking with 10% normal swine serum (Vector Laboratories) for 30 min at room temperature, anti-ZO-1 polyclonal antibody (Zymed) diluted 1:250 in 10% swine serum was applied for 16 h at 4°C. After incubation with biotinylated antirabbit IgG and then with Texas red-conjugated avidin D (Vector; Ref. 36), coverslips were mounted in Vectashield mounting medium (Vector) and observed with an immunofluorescence microscope (Nikon).

**Scanning Electron Microscopy.** Cells grown in confluence on 2-mm<sup>2</sup> glasses were washed twice with PBS, and fixed with 2.5% glutaraldehyde for 16 h at 4°C. After being washed 3 times with PBS, cells were postfixed with 2% osmium tetroxide at 4°C for 30 min and dehydrated in an ascending ethanol series (50–70–80–90–100%) for 30 min at each concentration. After critical-point drying using liquid CO<sub>2</sub>, the specimens were coated with gold-palladium and examined under a JSM T-300 electron microscope (JEOL) with a 15-kV accelerating voltage.

**Luciferase Reporter Assay.** To evaluate the TCF/LEF transcriptional activity, we used a pair of luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate Biotechnology). TOP-FLASH contains three copies of the TCF/LEF binding site (AAGATCAAAGGGGGT) upstream of the thymidine kinase minimal promoter, and FOP-FLASH contains a mutated TCF/LEF binding site (AAGGCCAAAGGGGGT). Cells were transiently transfected by one of these luciferase reporters and pRL-TK (Promega) by using FuGENE 6 transfection reagent (Roche) in triplicate, as instructed by the suppliers. Luciferase activity was measured with the Dual-luciferase reporter assay system (Promega), with the *Renilla* luciferase activity as an internal control, 48 h after transfection.

**Retroviral Expression of  $\beta$ -Catenin $\Delta$ N89 Protein.** With the use of the primers 5'-TACGTCGACGCCACCACCTACAGGTACATC-CGAGCTCAGAGGGTACGAGCT-3' and 5'-GGCATCGATGGCAAATC-CATTGTATTGTTACTCC-3', a fragment of  $\beta$ -catenin cDNA (nucleotides 482-2584 in X87838) was amplified from pBlueScript plasmid DNA containing a full-length  $\beta$ -catenin cDNA (generous gift from Dr. E. Ezoe, ERATO Hirohashi Cell Configuration Project). After digestion with the restriction enzymes *Sall* and *Clal*, cDNA fragments were cloned into the relevant site of pLNCX2 (Clontech), resulting a construct encoding AU1-tagged  $\beta$ -catenin lacking the NH<sub>2</sub>-terminal 89 amino acids (pLNCX2- $\beta$ -catenin $\Delta$ N89, Fig. 9a).

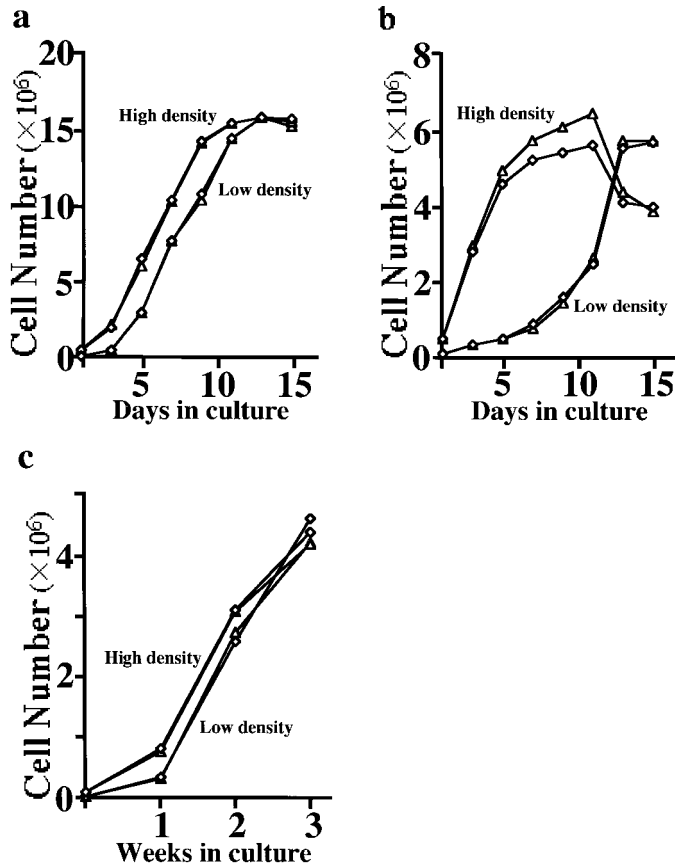


Fig. 2. Growth curves of DLD-1 Tet-ON TCF4B $\Delta$ N30-7 cells. *a*,  $1 \times 10^5$  (low density) or  $5 \times 10^5$  (high density) DLD-1 Tet-ON TCF4B $\Delta$ N30-7 cells were cultured in wells of 6-well plates for the indicated number of days with 10% FBS in the presence ( $\Delta$ ) or absence ( $\diamond$ ) of Dox. Cell numbers were counted every 48 h with a hemocytometer. *b*,  $1 \times 10^5$  (low density) or  $5 \times 10^5$  (high density) DLD-1 Tet-ON TCF4B $\Delta$ N30-7 cells were cultured in wells of 6-well plates for the indicated number of days with 2% FBS in the presence ( $\Delta$ ) or absence ( $\diamond$ ) of Dox. *c*,  $2 \times 10^4$  (low density) or  $1 \times 10^5$  (high density) DLD-1 Tet-ON TCF4B $\Delta$ N30-7 cells were cultured in wells of 24-well plates for the indicated number of days with 10% FBS in the presence ( $\Delta$ ) or absence ( $\diamond$ ) of Dox.

The composition of the construct was confirmed by restriction endonuclease digestion and by sequencing.

293 cells were cotransfected with pCL-Eco (Imgenex; Ref. 37) and one of either pLNCX2- $\beta$ -catenin $\Delta$ N89 or control empty pLNCX2 using FuGENE 6 transfection reagent, and after 48 h, conditioned media containing retroviral particles were recovered and filtered. IEC6 cells were infected for 24 h in the presence of 8  $\mu$ g/ml polybrene (Sigma).

## RESULTS

**Stable Induction of DN TCF4B in a Colorectal Cancer Cell Line.** The human colorectal adenocarcinoma cell line DLD-1 has a mutation at codon 1416 of the *APC* gene and loss of the other allele, resulting in constitutively active TCF/LEF activity. Using a tetracycline-regulation system (38), we established three clones (DLD-1 Tet-ON TCF4B $\Delta$ N30-1, -5, and -7) that were capable of inducing DN TCF4B, and three mock clones (DLD-1 Tet-ON control-A, -B, and -D) from one parental DLD-1 Tet-ON clone (Fig. 1*a*). DLD-1 Tet-ON TCF4B $\Delta$ N30-1, -5, -7 cells induced truncated TCF4B within 12 h after the addition of Dox, as described previously (34). Even after culturing with Dox for 3 weeks, DLD-1 Tet-ON TCF4B $\Delta$ N30 clones maintained expression of TCF4B $\Delta$ N30 mRNA (Fig. 1*b*) and protein (Fig. 1*c*).

Transient transfection and luciferase reporter assays revealed that the TCF/LEF activity (as determined by the TOP:FOP ratio) of the

three DLD-1 Tet-ON TCF4B $\Delta$ N30 clones was maintained suppressed in accordance with the expression of DNTCF4B (Fig. 1*d*, on right) for the 3-week culture period. On the other hand, the TCF:LEF ratio was not affected in the three mock clones by the addition of Dox (Fig. 1*d*, left).

**Effects of DN TCF4B on Cell Growth.** The growth kinetics of DLD-1 Tet-ON TCF4B $\Delta$ N30 clones in media with or without Dox were compared (Fig. 2). DLD-1 Tet-ON TCF4B $\Delta$ N30 clones proliferated at the same rate regardless of the presence or absence of 0.5  $\mu$ g/ml Dox. We repeatedly examined various culture conditions [low or high cell density, low (2%; Fig. 2*b*) or ordinary (10%; Fig. 2*a*) concentration of FBS]; however, there seemed to be no significant effects of the suppression of TCF4 activity on cell growth, except for marginal variations in saturation density. Even in confluent cultures sustained for up to 3 weeks, DLD-1 Tet-ON TCF4B $\Delta$ N30 clones did not show any difference in overall cell number in the presence or absence of Dox (Fig. 2*c*).

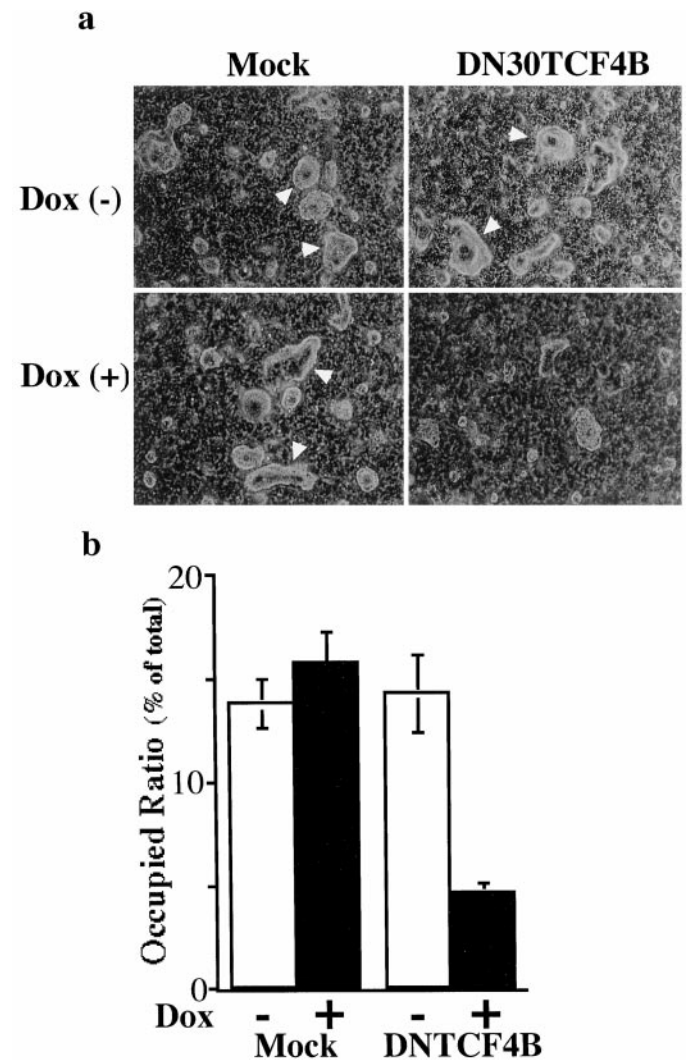


Fig. 3. The formation of piled-up foci in confluent cultures. *a*, phase-contrast microscopy. DLD-1 Tet-ON TCF4B $\Delta$ N30-7 cells cultured without Dox (right top) and DLD-1 Tet-ON control-A cells cultured with 0.5  $\mu$ g/ml Dox (left bottom) or without Dox (left top) for 3 weeks, formed numerous dome-like foci (arrowheads) piled-up over the flat surface of cell sheets. On the other hand, DLD-1 Tet-ON TCF4B $\Delta$ N30-7 cells cultured with 0.5  $\mu$ g/ml Dox (right bottom) showed a few small foci ( $\times 40$ ). *b*, the occupancy ratio of polypoid foci. The images of polypoid areas were captured with the NIH Image software program and the ratios to the total were calculated. DLD-1 Tet-ON TCF4B $\Delta$ N30-1 (right, DNTCF4B) and DLD-1 Tet-ON control-A (left, Mock) cells were cultured without Dox (□, Dox (-)) or with Dox (■, Dox (+)) for 3 weeks. Bars and error bars, the average  $\pm$  SD.

**Morphological Changes Caused by DN TCF4B.** When  $2 \times 10^6$  cells were seeded into 100-mm cell culture dishes, DLD-1 Tet-ON TCF4B $\Delta$ N30 clones and mock clones reached confluence and formed similar tight monolayers within 72 h. There was no significant morphological difference in these short culture periods among the DLD-1 Tet-ON TCF4B $\Delta$ N30 clones and mock clones, regardless of the presence or absence of Dox (not shown).

However, when the cell cultures were sustained in confluence for 2–3 weeks, morphological differences became apparent (Fig. 3a). In the absence of Dox (active  $\beta$ -catenin/TCF4), DLD-1 Tet-ON TCF4B $\Delta$ N30 clones formed numerous dome-like polypoid foci piled up over the flat surface of cell sheets. The polypoid foci of DLD-1 Tet-ON TCF4B $\Delta$ N30–1 reached 14.3% of the total culture area after 3 weeks. In sharp contrast, in the presence of Dox (inactive  $\beta$ -catenin/TCF4), the occupancy rate of foci was significantly suppressed to 4.6% (Fig. 3b). Similar effects were observed in two other DLD-1 Tet-ON TCF4B $\Delta$ N30 clones, -5 and -7, but Dox did not affect the formation of foci in any mock clones.

To visualize these polypoid foci in vertical cross-sections, cells were cultured on PetriPERM membranes for 3 weeks under similar conditions. DLD-1 Tet-ON TCF4B $\Delta$ N30 clones without Dox treatment (active  $\beta$ -catenin/TCF4) revealed numerous polypoid foci piled up over the free surfaces throughout the specimens. In addition, cross-sections revealed that these cells were irregularly multilayered and that the polarity of individual cells was completely lost (Fig. 4b). Cells were detached from the bottom of the culture dishes and were irregularly mounded up. In contrast, in the presence of Dox (inactive  $\beta$ -catenin/TCF4), the piled-up foci were distributed sparsely, cells adhered tightly to the culture dishes, and cross-sections manifested a simple monolayer of polarized columnar cells resembling normal intestinal epithelium (Fig. 4d). Again, such restoration of epithelial architecture was never observed in any mock clones regardless of the presence or absence of Dox (Fig. 4, a and c).

Scanning electron microscopy revealed that DLD-1 Tet-ON TCF4B $\Delta$ N30 cells treated with Dox (inactive  $\beta$ -catenin/TCF4)

showed numerous microvilli over the apical surface and distinct cell borders (Fig. 5, g and h). On the other hand, DLD-1 Tet-ON TCF4B $\Delta$ N30 cells untreated with Dox and mock cells (active  $\beta$ -catenin/TCF4) showed sparse microvilli, and individual cells could not be distinguished (Fig. 5, a–f).

**Redistribution of ZO-1 to the Lateral Plasma Membrane.** The assembly of tight junctions occurs with the establishment of apical-basolateral polarity (39). In fully polarized epithelial cells such as colonocytes, the ZO-1 protein is sorted into tight junctions (40–42). DLD-1 Tet-ON TCF4B $\Delta$ N30 cells were grown to confluence for 5 days in the presence or absence of Dox, and the subcellular localization of ZO-1 was visualized by immunofluorescence microscopy (Fig. 6a). In DLD-1 Tet-ON TCF4B $\Delta$ N30 cells treated with Dox (inactive  $\beta$ -catenin/TCF4), ZO-1 was localized at the lateral plasma membrane with a tight cell-cell contact (right bottom, Fig. 6a) and in the cytoplasm. In contrast, in DLD-1 Tet-ON TCF4B $\Delta$ N30 clones untreated with Dox (active  $\beta$ -catenin/TCF4), ZO-1 was no longer localized at the lateral plasma membrane but was distributed diffusely in the perinuclear cytoplasm (right top, Fig. 6a). Western (Fig. 6b) and Northern (not shown) blot analyses, however, showed no apparent alteration in the expression level of the ZO-1 protein or mRNA.

**Additive Effects of NSAID and DN TCF4B.** Sulindac, a NSAID, has been shown to possess chemopreventive efficacy against colorectal adenomatous polyp formation in FAP patients (43, 44). To investigate whether the piling-up *in vitro* is a phenomenon simulating the polyp formation of intestinal epithelia, cells were cultured for 3 weeks in the presence or absence of sulindac. The addition of sulindac to culture media significantly reduced the formation of piled-up foci both in DLD-1 Tet-ON TCF4B $\Delta$ N30 clones and in mock clones (Fig. 7). The addition of Dox further decreased the piling up only in the three DLD-1 Tet-ON TCF4B $\Delta$ N30 clones, but not in any mock clones, which indicated that the induction of DN TCF4B has additive effects with sulindac.

**DN TCF4B Suppresses Colony Formation in Soft Agar.** The formation of polypoid foci in sustained confluent cultures seems to be

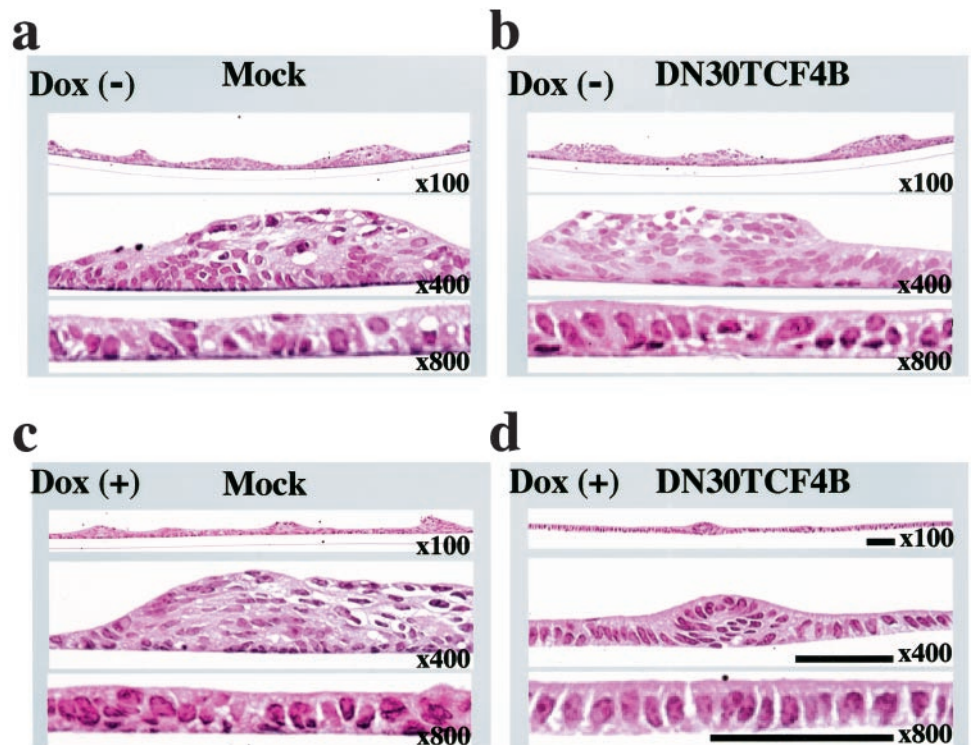


Fig. 4. Vertical cross-sections of polypoid foci. Five  $\times 10^5$  DLD-1 Tet-ON TCF4B $\Delta$ N30–7 cells (b and d, DN30TCF4B) and DLD-1 Tet-ON control-A cells (a and c, Mock) were cultured without [a and b, Dox (–)] or with 0.5  $\mu$ g/ml Dox [c and d, Dox (+)] on PetriPERM dishes. The membrane bases of dishes were cut and stained with standard H&E techniques [  $\times 100$  (top);  $\times 400$  (middle);  $\times 800$  (bottom)]. Bars, 0.1 mm.

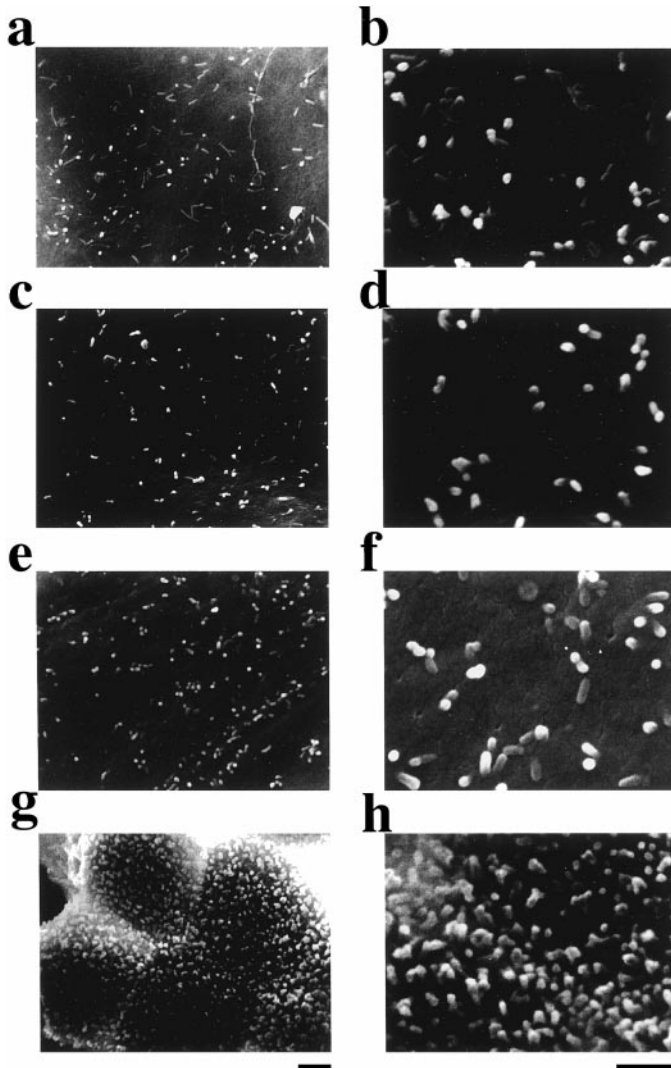


Fig. 5. Scanning electron microscopy. DLD-1 Tet-ON TCF4 $\Delta$ N30-7 cells (e-h) and DLD-1 Tet-ON control-A cells (a-d) were cultured without (a, b, e, f) or with (c, d, g, h) 0.5  $\mu$ g/ml Dox on cover glasses in wells of 6-well plates for 3 weeks. The cell surface was photographed under a scanning electron microscope [ $\times 15,000$  (a, c, e, g);  $\times 26,000$  (b, d, f, h)]. Bars, 1  $\mu$ m.

a phenotype of mixed biological events, including the losses of contact inhibition, anchorage-dependency, and polarity. Because piled-up cells have to survive and proliferate without attachment to culture dishes, we examined the effects of suppression of  $\beta$ -catenin/TCF4-mediated gene transactivation on the anchorage-independent growth of DLD-1 cells (Fig. 8). The addition of Dox (inactive  $\beta$ -catenin/TCF4) significantly decreased the size, but not the number, of colonies of all DLD-1 Tet-ON TCF4 $\Delta$ N30 clones in soft agar. Similar treatment had no effect in any mock clones.

**Retroviral Expression of Stabilized  $\beta$ -Catenin in Intestinal Epithelial Cells.**  $\beta$ -Catenin lacking the entire GSK3 $\beta$ -phosphorylation site in the NH<sub>2</sub> terminus ( $\Delta$ N89; Fig. 9a) is resistant to degradation by the ubiquitin-proteasome pathway (45, 46).  $\beta$ -Catenin $\Delta$ N89 was expressed retrovirally in an immortal but untransformed rat intestinal epithelial cell line, IEC6, as revealed by immunoblotting with a monoclonal antibody against the COOH terminus of  $\beta$ -catenin (Fig. 9b). IEC6 cells expressing the truncated  $\beta$ -catenin protein developed numerous piled-up foci when kept confluent for 2-3 weeks, in a similar way to DLD-1 cells (right top and left bottom, Fig. 9c). IEC6 cells infected with control viral particles maintained a flat monolayer even after the parallel 3-week

confluent culture (left top, Fig. 9c). The addition of 200  $\mu$ M sulindac to the culture media completely suppressed the formation of polypoid foci of IEC6 cells expressing the  $\beta$ -catenin $\Delta$ N89 protein (right bottom, Fig. 9c). The distribution of ZO-1 to the lateral plasma membrane (right, Fig. 9d) was significantly disturbed on the expression of stabilized  $\beta$ -catenin $\Delta$ N89 (left, Fig. 9d).

## DISCUSSION

We explored in depth the biological effects of the inactivation of  $\beta$ -catenin/TCF4-mediated transcription on a colorectal cancer cell line because we believe that a better understanding of the biological effects caused by the accumulation of  $\beta$ -catenin will provide a clue for new therapeutic strategies against intestinal carcinogenesis. We previously established colorectal cancer transfectants with manipulable TCF/LEF activity by using the strict tetracycline-regulatory system (34). Mock transfectants carrying an empty pTRE plasmid were also included in every experiment as controls (Fig. 1a) to exclude any unexpected effects of Dox.

The DN form of TCF4 lacking the  $\beta$ -catenin-binding site suppressed the transcriptional activity of TCF/LEF (Fig. 1d) and altered the multicellular architecture of a colon carcinoma cell line, DLD-1 (Figs. 3-5). DLD-1 Tet-ON cells formed numerous polypoid foci during a 3-week culture period without the induction of DN TCF4B (active status of  $\beta$ -catenin/TCF4-mediated gene transcription; Fig. 3a). CaCo2, another colorectal cancer cell line with active TCF/LEF transcription, was reported to form dome-like structures in long-term culture (47), similar to those observed with DLD-1 in this study. The induction of DN TCF4B significantly suppressed the formation of piled-up foci (Fig. 3). Although the biological mechanisms underlying these morphological alterations still remain obscure, they are not likely to be caused by changes in overall cell proliferation, at least in our system, because at no time did we observe any decrease of cell number during the course of cell culture in the presence of Dox (Fig. 2). Retroviral expression of Wnt-1, an upstream effector of  $\beta$ -catenin,

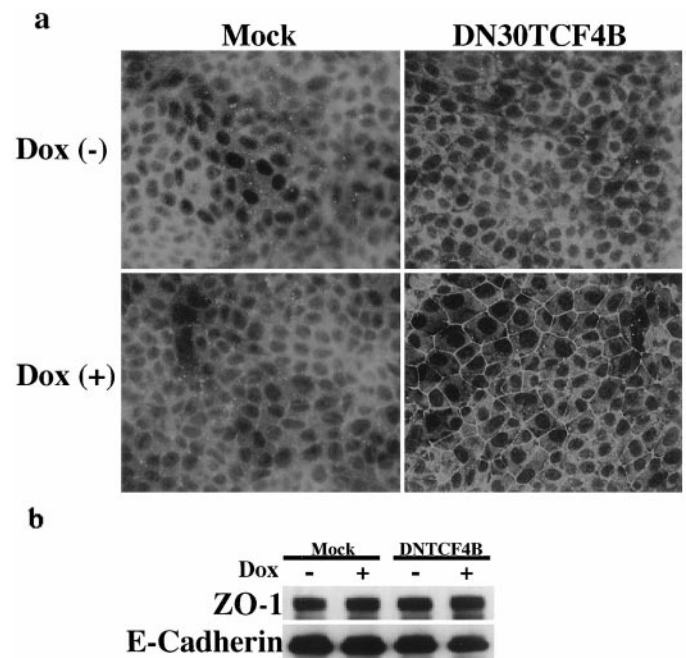


Fig. 6. Subcellular localization of ZO-1. a, immunofluorescence microscopy showing the localization of ZO-1 (red). DLD Tet-ON control-A (left, Mock) and DLD-1 Tet-ON TCF4 $\Delta$ N30-7 (right, DN30TCF4B) cells were cultured without [top, Dox (-)] or with Dox [bottom, Dox (+)] for 5 days. b, immunoblot analysis. The ZO-1 (top) and E-cadherin (bottom) proteins were detected in RIPA-extracted cell lysates.

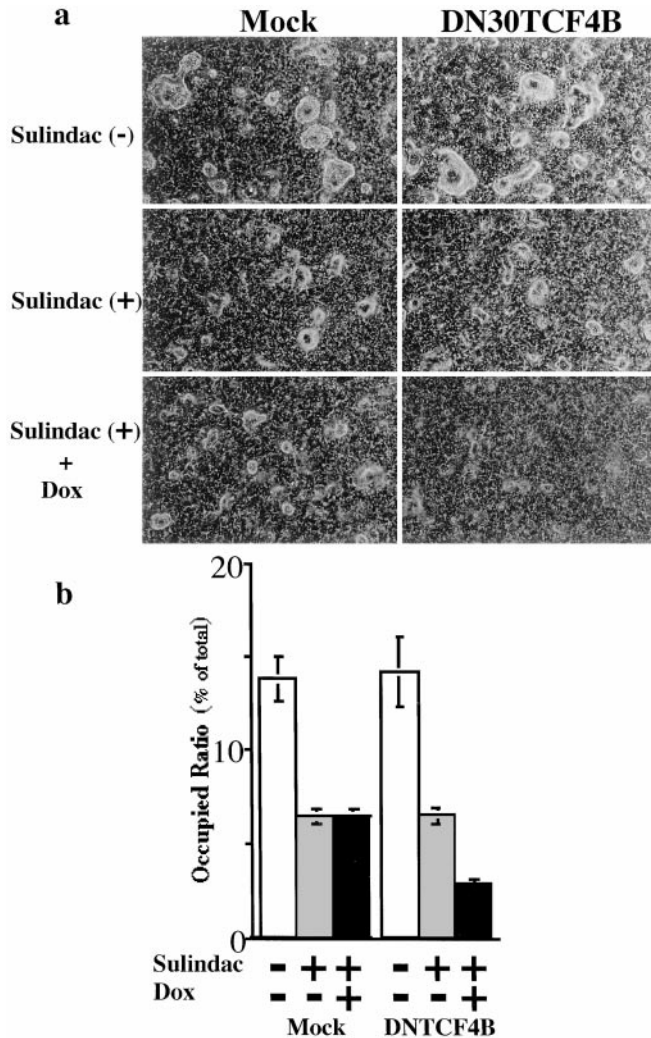


Fig. 7. Effects of sulindac on the formation of polypoid foci. *a*, phase-contrast microscopy. DLD-1 Tet-ON control-A (left, *Mock*) and DLD-1 Tet-ON TCF4 $\Delta$ N30-7 (right, *DN30TCF4B*) cells were untreated (top), treated with 200  $\mu$ M of sulindac alone (middle), or treated with 200  $\mu$ M of sulindac and 0.5  $\mu$ g/ml Dox (bottom) for 3 weeks ( $\times 40$ ). *b*, the occupancy ratio of polypoid foci. DLD Tet-ON control-A (left, *Mock*) and DLD-1 Tet-ON TCF4 $\Delta$ N30-7 (right, *DN30TCF4B*) cells were untreated ( $\square$ ), treated with 200  $\mu$ M of sulindac alone ( $\square$ ), or treated with 200  $\mu$ M of sulindac and 0.5  $\mu$ g/ml Dox ( $\blacksquare$ ) for 3 weeks. Bars and error bars, the average ratio to the total  $\pm$  SD.

did not alter the cell number of preadipocytes (48). On the other hand, the accumulation of mutant  $\beta$ -catenin was described as altering the cellular morphology of murine L cells, inducing an increase of the saturation density, and reducing the serum dependency (49).

The suppression of formation of polypoid foci in phase contrast microscopy (Fig. 3) seems to be tightly associated with the restoration of epithelial cell polarity (Fig. 4). The continuous suppression of  $\beta$ -catenin/TCF4-mediated gene transactivation by the induction of DN TCF4B changed a multilayer to a simple columnar monolayer resembling the normal intestinal epithelium (lower right, Fig. 4). Individual cells in the columnar monolayer seem to regain their epithelial cell polarity, characterized by distinct apical, lateral, and basal cell surfaces. The evidence for this is: (*a*) the formation of numerous microvilli over the apical surface (Fig. 5); and (*b*) the distribution of a tight junction protein, ZO-1, to the lateral plasma membrane (Fig. 6*a*). We did not observe any apparent difference in the distribution of adherens junction proteins, E-cadherin and catenins (not shown). There is a report showing the down-regulation of ZO-1 mRNA by transfection of  $\beta$ -catenin into colorectal cell lines (42).

Although we observed dramatic changes in the subcellular distribution of the ZO-1 protein on the induction of DN TCF4B (Fig. 6*a*), Western (Fig. 6*b*) and Northern (not shown) blot analyses showed no significant change in the expression levels of ZO-1 protein or mRNA, which suggested posttranslational modifications of the protein. Additional studies are required to resolve this issue.

The ability of cells to proliferate without attachment to solid substrates has been recognized as a hallmark of transformed and tumorigenic cells and seems to be crucial for multilayered cell growth (46). Although anchorage dependency and cell polarity may be mutually dependent phenomena, we speculate that the suppression of formation of piled-up foci by DN TCF4 requires the acquisition of anchorage dependency in addition to the restoration of epithelial cell polarity because multilayered cells have to survive and proliferate without attachment to culture dishes. The overexpression of  $\beta$ -catenin was reported to promote colony formation in soft agar (50). The induction of DN TCF4B significantly reduced the size (Fig. 8), but not the

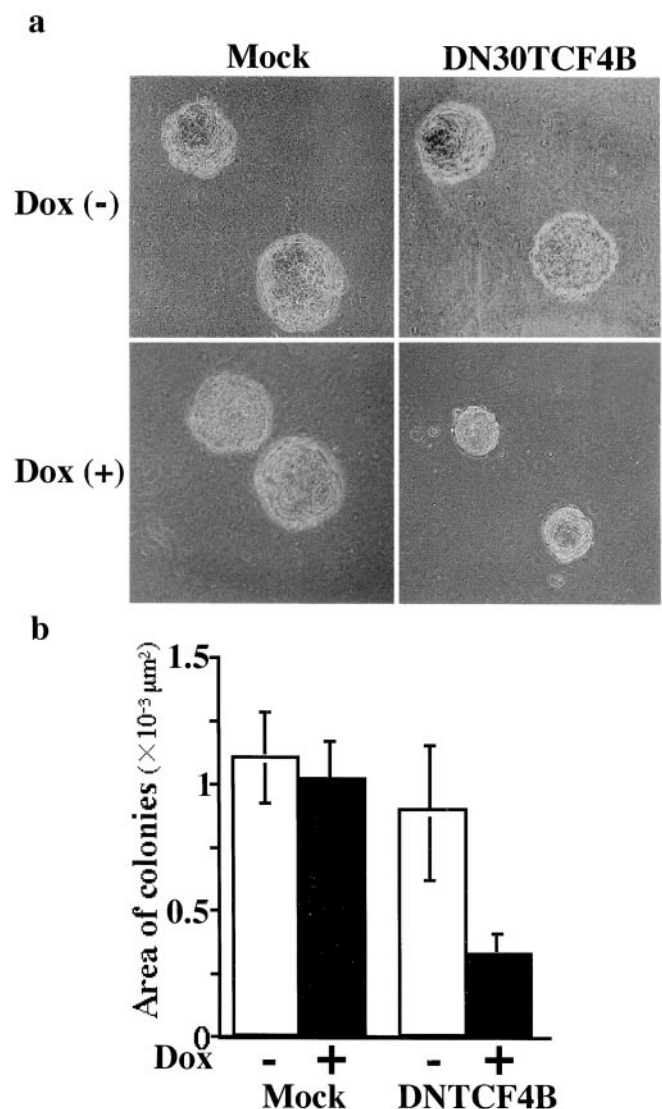


Fig. 8. Colony formation in soft agar. *a*, phase-contrast microscopy. DLD-1 Tet-ON control-A cells (left, *Mock*) and DLD-1 Tet-ON TCF4 $\Delta$ N30-7 cells (right, *DN30TCF4B*) were cultured in 1 ml of 0.3% molten agar for 10 days without [top, *Dox* (-)] or with Dox [bottom, *Dox* (+)] ( $\times 40$ ). *b*, measurement of the area of colonies. DLD-1 Tet-ON TCF4 control-A (left, *Mock*) and DLD-1 Tet-ON TCF4 $\Delta$ N30-7 cells (right, *DN30TCF4B*) were cultured in the absence ( $\square$ , *Dox* (-)) or presence of Dox ( $\blacksquare$ , *Dox* (+)). The areas of colonies were captured by the NIH Image software and the occupancy ratios of colonies to the total were calculated. Bars and error bars, the average  $\pm$  SD.

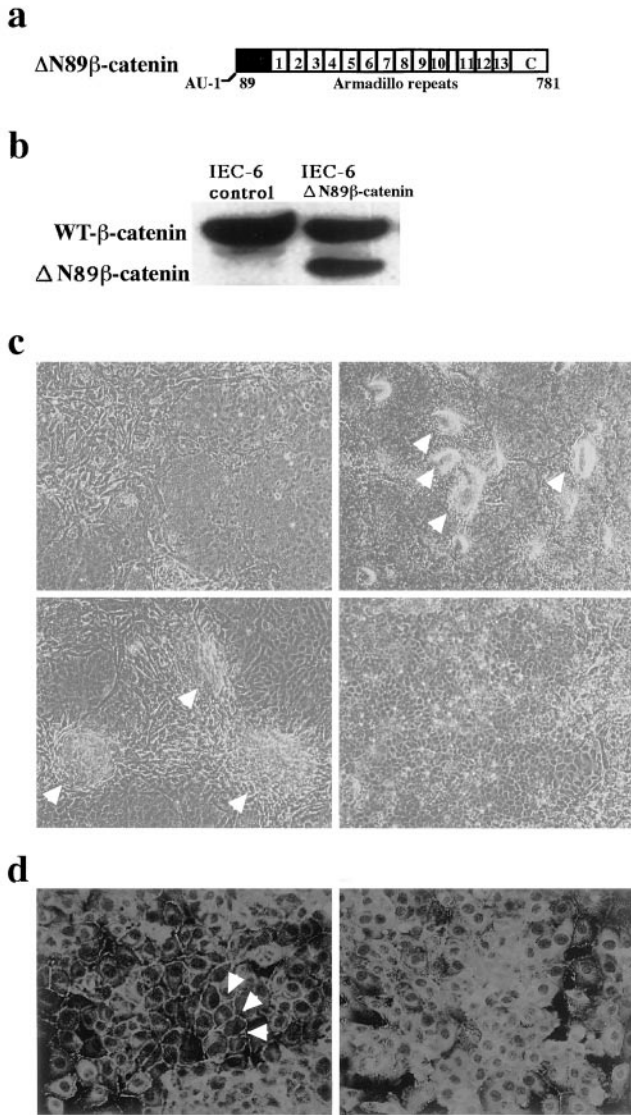


Fig. 9. Retroviral expression of  $\beta$ -catenin $\Delta$ N89. *a*, a schematic presentation of the construct encoding truncated  $\beta$ -catenin ( $\Delta$ N89). This construct lacks the entire GSK3 $\beta$ -phosphorylation sites of  $\beta$ -catenin. A Kozak sequence and an AU1-tag were introduced into the new NH<sub>2</sub> terminus of the protein. *b*, Western blot analysis. IEC6 cells were infected with retroviral particles produced by control pLNCX2 (*left*) or pLNCX2- $\beta$ -catenin $\Delta$ N89 (*right*). Cell lysates were extracted 4 days after infection, fractionated by SDS-PAGE, and blotted with anti- $\beta$ -catenin monoclonal antibody. *c*, phase-contrast microscopy. IEC6 cells infected with retroviral particles produced by control pLNCX2 (*left top*,  $\times 100$ ), pLNCX2- $\beta$ -catenin $\Delta$ N89 (*right top*,  $\times 100$ ; *left bottom*,  $\times 200$ ; *right bottom*,  $\times 100$ ) were cultured for 3 weeks in confluence and photographed. Sulindac (200  $\mu$ M) was added to the medium of IEC6 cells expressing  $\beta$ -catenin $\Delta$ N89 (*right bottom*). *d*, immunofluorescence microscopy showing the subcellular localization of ZO-1 in IEC6 cells infected with retroviral particles produced by control pLNCX2 (*left*) or pLNCX2- $\beta$ -catenin $\Delta$ N89 (*right*;  $\times 200$ ).

number, of colonies of DLD-1 cells in soft agar, revealing that partial restoration of anchorage-dependency is caused by the suppression of  $\beta$ -catenin/TCF4-mediated gene transactivation.

NSAIDs, including sulindac, suppress intestinal tumorigenesis in laboratory animals and FAP patients (43, 44, 51, 52), probably by inhibiting Cox-2 (53, 54). We showed that sulindac can suppress the piling up of DLD-1 Tet-ON cells (Fig. 7) and of IEC6 cells expressing the stabilized  $\beta$ -catenin protein (Fig. 9). The induction of DN TCF4B had additive effects with those of sulindac against the formation of polypoid foci (Fig. 7), which suggested that the  $\beta$ -catenin/TCF4-mediated pathway and the Cox-2 pathway are not completely overlapped, and that the suppression of  $\beta$ -catenin/TCF4 activity may enhance the

efficacy of NSAIDs against colorectal tumorigenesis. The piling up *in vitro* may reflect adenomatous polyp formation in the intestine *in vivo*. The culture assay using DLD-1 cells may become a simple alternative method for screening candidate chemopreventive drugs against intestinal tumorigenesis, eliminating the use of experimental animals.

Finally, disturbed cellular polarity is one of the histological characteristics of adenomatous polyps of the colon and rectum. The biological and molecular mechanisms underlying this disturbed polarity have been a long-standing question for surgical pathologists. Our observations in this study may give a clue to understanding the formation of the microarchitecture of colorectal adenoma (Fig. 10). The intestinal crypts are lined by simple columnar epithelium. The intestinal stem cells anchored near the bottom of crypts continuously proliferate and give rise to a variety of committed cells. To maintain the monolayer of aligned columnar epithelium, committed cells must migrate upwards one after another as though they are on a conveyor belt (Fig. 10*a*). The mutational inactivation of APC in stem cells and the subsequent activation of TCF4 disturbs the polarity of individual cells. The loss of polarity in components of the "conveyor belt" is likely to interfere with proper migration (Fig. 10*b*). The failure of upward migration may cause an inward movement and give rise to outpocketing pouch or nascent microadenoma, as described in APC-knockout mice (55; Fig. 10*c*). However, the relationship between the loss of epithelial cell polarity and disturbances in  $\beta$ -catenin-mediated transcription still seems to be distant. To gain insight into the molecular mechanisms involved, large-scale profiling of genes transactivated by the  $\beta$ -catenin/TCF4 complex is necessary.

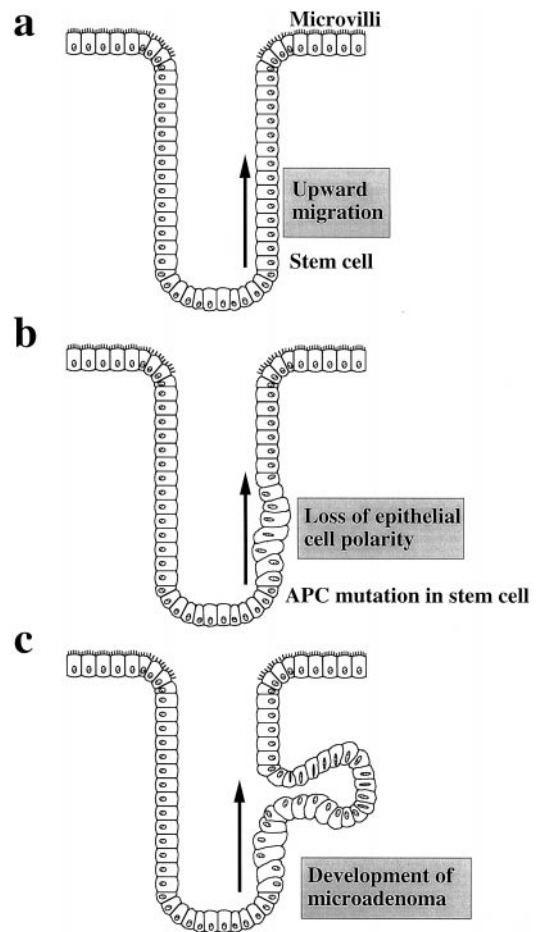


Fig. 10. Schematic presentation of a model of adenoma formation in colorectal epithelia by disturbed cell polarity. See the text in "Discussion" for details.

## ACKNOWLEDGMENTS

We thank Dr. J. Yokota for useful suggestions about a tetracycline-regulatory system, Dr. E. Ezeo for providing  $\beta$ -catenin cDNA, and Y. Yamauchi for her excellent technical assistance.

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## Restoration of Epithelial Cell Polarity in a Colorectal Cancer Cell Line by Suppression of $\beta$ -catenin/T-Cell Factor 4-mediated Gene Transactivation

Yasuyoshi Naishiro, Tesshi Yamada, Asako S. Takaoka, et al.

*Cancer Res* 2001;61:2751-2758.

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