Effects of Forced Expression of an NH$_2$-terminal Truncated β-Catenin on Mouse Intestinal Epithelial Homeostasis

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Abstract. β-Catenin functions as a downstream component of the Wnt/Wingless signal transduction pathway and as an effector of cell–cell adhesion through its association with cadherins. To explore the in vivo effects of β-catenin on proliferation, cell fate specification, adhesion, and migration in a mammalian epithelium, a human NH$_2$-terminal truncation mutant (ΔN89β-catenin) was expressed in the 129/Sv embryonic stem cell–derived component of the small intestine of adult C57Bl/6–ROSA26ΔN89β-catenin chimeric mice. ΔN89β-Catenin was chosen because mutants of this type are more stable than the wild-type protein, and phenocopy activation of the Wnt/Wingless signaling pathway in Xenopus and Drosophila. ΔN89β-Catenin had several effects. Cell division was stimulated fourfold in undifferentiated cells located in the proliferative compartment of the intestine (crypts of Lieberkühn). The proliferative response was not associated with any discernible changes in cell fate specification but was accompanied by a three- to fourfold increase in crypt apoptosis. There was a marked augmentation of E-cadherin at the adherens junctions and basolateral surfaces of 129/Sv (ΔN89β-catenin) intestinal epithelial cells and an accompanying slowing of cellular migration along crypt-villus units. 1–2% of 129/Sv (ΔN89β-catenin) villi exhibited an abnormal branched architecture. Forced expression of ΔN89β-catenin expression did not perturb the level or intracellular distribution of the tumor suppressor adenomatous polyposis coli (APC). The ability of ΔN89β-catenin to interact with normal cellular pools of APC and/or augmented pools of E-cadherin may have helped prevent the 129/Sv gut epithelium from undergoing neoplastic transformation during the 10-mo period that animals were studied. Together, these in vivo studies emphasize the importance of β-catenin in regulating normal adhesive and signaling functions within this epithelium.

β-Catenin plays important roles in cell adhesion and cell signaling (for review see Miller and Moon, 1996; Nusse, 1997). The protein influences adhesion by providing a functional bridge between cadherins and the actin cytoskeleton. Calcium-dependent homotypic interactions between the extracellular domains of cadherins result in the formation of adhesion “zippers” between adjacent cells (Overduin et al., 1995; Shapiro et al., 1995). Although these interactions help define the specificity of cellular interactions, they are not sufficient for productive adhesion. Productive adhesion at the adherens junction is accomplished by the binding of β-catenin to the conserved cytoplasmic domains of cadherins and to the cytoplasmic protein α-catenin. α-Catenin, in turn, is linked to the cytoskeleton via its interactions with other proteins (e.g., actinin; Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990; Aberle et al., 1994; Hinck et al., 1994; Jou et al., 1995; Rimm et al., 1995).

β-Catenin is also a critical downstream component of the Wnt signal transduction pathway in vertebrates. In the absence of a Wnt signal, serine/threonine phosphorylation by glycogen synthase kinase-3 (GSK-3) leads to rapid degradation of cytoplasmic pools of β-catenin through a ubiquitin–proteosome pathway (Miller and Moon, 1996; 1)

1. Abbreviations used in this paper: β-gal, β-galactosidase; ΔN89β-catenin, NH$_2$-terminal truncation mutant of human β-catenin lacking amino acid residues 1–89; APC, adenomatous polyposis coli protein or gene; BrdU, 5′-bromo-2′-deoxyuridine; Cy3, indocarboxyamine; DBA, Dolichos biflorus agglutinin; ES cell, embryonic stem cell; Fabp1, fatty acid binding protein gene; GSK-3, glycogen synthase kinase-3; hGH, human growth hormone gene; LEF-1, lymphocyte enhancing factor-1; PLP, periodate-lysine-paraformaldehyde; RT, reverse transcriptase; TAg, T antigen; Tcf, T-cell factor; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.
Munemitsu et al., 1996; Yost et al., 1996; Aberle et al., 1997; Cadigan and Nusse, 1997). In contrast, stimulation of the Wnt pathway leads to repression of GSK-3 (Noordermeer et al., 1994; Cook et al., 1996), decreased β-catenin phosphorylation, and enhanced protein stability. The resulting augmentation of β-catenin pools facilitates formation of complexes between β-catenin and high mobility group box transcription factors (T-cell factor [Tcf] and lymphocyte enhancing factor-1 [LEF-1]; Behrens et al., 1996; Huber et al., 1996). In the nucleus, β-catenin functions to coactivate transcription of largely unspecified gene targets (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996; Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997).

Studies in genetically manipulatable nonvertebrate species as well as nonmammalian vertebrate organisms have shown that β-catenin–mediated signaling affects axis formation and cell fate specification (McCrea et al., 1993; Heasman et al., 1994; Funayama et al., 1995; Cox et al., 1996). Members of the Paneth cell lineage differentiate as they move downward to the base of the crypt where they are removed after an ~20-d residence (Cheng, 1974b). E-Cadherin, the principal intestinal epithelial cadherin, β-catenin, α-catenin, and APC are expressed in cells distributed along the length of crypt-villus units (Hermiston et al., 1996; Näthke et al., 1996; Wong et al., 1996). The importance of cadherins and their related proteins to normal intestinal epithelial homeostasis was established when a dominant-negative cadherin was expressed in the 129/Sv component of C57Bl/6→129/Sv chimeric mouse gut. Cell–cell and cell–substratum interactions were disrupted leading to alterations in migration rates, diminished cell survival, breakdown of mucosal barrier function, inflammatory bowel disease, and adenoma formation (Hermiston et al., 1995a,b).

In the present report, we have examined the effects of forced expression of a human β-catenin lacking its NH2-terminal 89 residues (ΔN89β-catenin) on the small intestinal epithelium of C57Bl/6→129/Sv chimeric mice. ΔN89β-Catenin was chosen for several reasons. Studies in cultured cell lines indicated that this mutant, which lacks putative GSK-3 phosphorylation sites (Yost et al., 1996), has a longer half-life than the wild-type protein, leading to its accumulation both as a stable monomer and as a complex with APC (Munemitsu et al., 1996; Barth et al., 1997; Rubinfeld et al., 1997). The NH2-terminal truncation does not appear to affect binding to E-cadherin, does not involve the domains involved in binding α-catenin or Tcf (Munemitsu et al., 1996; Orsuic and Peifer, 1996), and preserves all of the protein’s structurally and functionally important armadillo repeats (Huber et al., 1997). Finally, studies in Drosophila and Xenopus had shown that NH2-terminal truncation mutants of β-catenin promote signaling (Yost et al., 1996; Pai et al., 1997), whereas expression in cultured mammalian epithelial cells affects cell migration (Pollack et al., 1997). Thus, we anticipated that ΔN89β-catenin would allow us to examine the effects of β-catenin on both signaling and adhesion/migration in the mouse intestine.

Materials and Methods

Generation of Chimeric-Transgenic Mice

pl596hGHPNeoΔB2 (Hermiston et al., 1996) contains nucleotides -596 to +21 of the rat fatty acid binding protein gene (Fabpl) upstream of nucleotides +3 to +2150 of the human growth hormone gene (hGH). A phosphoglycerate kinase (pgk) neomycin resistance selection cassette is located just downstream of Fabpl-hGH. pl596hGHPNeoΔB2 was cleaved at its unique BamHI site located at nucleotide +3 of hGH, and a double-stranded oligonucleotide containing flanking BamHI sites and internal KpnI and XbaI sites (5′-GATCCGGTTACCATGAGGTCTCTAGAG-3′) was inserted. The resulting DNA, pLhGHPNeoK contains a unique KpnI site for subcloning cDNAs into exon 1 of hGH. pCABβ-cateninΔN20-d2 was inserted. The resulting DNA, pLFhGHpNeoK contains a unique KpnI site for subcloning cDNAs into exon 1 of hGH. pCABβ-cateninΔN20-d2 was inserted.
cDNA was generated from DNA-free intestinal RNA. Four separate the RNeasy kit (QIAGEN Inc., Santa Clarita, CA). Oligo dT-primed were used to generate B6-ROSA26

b

in chimeras was established using a PCR protocol (The Jackson Labora-

lines were analyzed at 6 wk, 6 mo, and 10 mo of life. Their 129/Sv contribu-

nin) chimeric-transgenic mice. Ch imeras generated from three of the cell

light cycle and fed a standard chow diet ad libitum (Pico Lab Rodent Diet

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k

A segment was taken from the middle of the small intes-

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C. The samples were washed with PBS (three cycles of 5 min each), in-

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KpnI/XbaI fragment from pCAN

D3 129/Sv ES cells (Hermiston et al., 1995

D

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1,000; Chemicon International Inc.); (3) rabbit anti-

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Chemicon International, Inc., Temecula, CA); (3) rabbit anti-mouse collagen type

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1,000; PharMingen, San Diego, CA); (2) monoclonal rat antibody to E-cadherin (1:1000; Sigma Chemical Co., Hermiston et al., 1995a); (4) rat anti-ZO-1 (polyclonal antibodies, 1:50; Chemicon International, Inc., Temecula, CA); (b) rabbit anti-laminin (1: 1,000; Chemicon International Inc.); (i) rabbit anti-mouse fibronectin (1: 1,000; Chemicon International Inc.); (j) rabbit anti-mouse collagen type IV (1:1,000; Chemicon International Inc.); (k) rat anti-mouse β1 integrin (1:500; Pharmingen, San Diego, CA); (l) rat anti-mouse β2 integrin (1:500;
PharMingen); (m) rat anti-mouse β6 integrin (1:500; PharMingen); (n) rat anti-mouse α6 integrin (1:500; PharMingen); (o) goat anti-BrdU (1:1,000; Cohn et al., 1992); (p) rabbit anti-serotonin (1:1,000, a marker of the predominant enteroendocrine subpopulation in the adult mouse intestine; Incstar, Stillwater, MN); (q) rabbit anti-chromagranin A (1:1,000, a general marker of enteroendocrine cells; Incstar); and (r) rabbit anti-liver fatty acid binding protein (1:1,000, an enteroendocrine lineage marker; Sweeter et al., 1988).

Antigen-antibody complexes were detected with indocarbocyanine (Cy3)- or FITC-conjugated donkey anti-rabbit, anti-rat, or anti–goat Ig (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Analysis of the components of the diffuse gut–associated lymphoid tissue was performed on PLP-fixed frozen sections of jejunum. Antigen–antibody complexes were visualized by tyramide signal amplification as described previously (Garabedian et al., 1997).

PLP-fixed and X-Gal–stained sections of paraffin-embedded whole mounts were also incubated with a series of lectins (all used at a final concentration of 5 μg/ml PBS blocking buffer and detected according to Falk et al., 1994): (a) Ulex europaeus agglutinin 1 (carbohydrate specificity = Fucα1,2Gal epitopes; lineage specificity = Paneth, goblet, and enteroendocrine cells; Sigma Chemical Co.); (b) Peanut (Arachis hypogaea) agglutinin (Galβ3GalNAc epitopes; all four epithelial lineages; Sigma Chemical Co.); (c) Dolichos biflorus agglutinin (GalNAcα3GalNAc and GalNAcα3Gal epitopes; Paneth and goblet cells plus enterocytes; Sigma Chemical Co.); (d) Helix pomatia agglutinin (α-GalNAc; GalNAcβ4Gal epitopes; Paneth and goblet cells; Sigma Chemical Co.); (e) Jacalin-1 (Arctocarpus integrifolia) agglutinin; Gaiα6Gal; Galβ3GalNAc epitopes; enterocytes and goblet cells; E.Y. Laboratories, Inc., San Mateo, CA).

Stained sections were viewed with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and/or model 2001 confocal microscope (Molecular Dynamics, Inc., Sunnyvale, CA). Scans in the confocal microscope were performed at 1-μm intervals.

**Results**

**Generation of B6-ROSA26→129/Sv(ΔN89β-Catenin) Chimeric Mice**

129/Sv ES cells were stably transfectected with a recombinant DNA consisting of nucleotides -596 to +21 of Fabpl (Sweetser et al., 1988) positioned upstream of an open reading frame encoding ΔN89β-catenin with an NH2-terminal myc tag. The Fabpl transcriptional regulatory elements were selected because previous studies in neonatal and adult transgenic mice had shown that they could be used to direct expression of a variety of proteins to the region of small intestinal crypt containing the multipotent stem cell, and to all four of the stem cell’s descendant cell lineages throughout the course of their differentiation (Trahair et al., 1989; Kim et al., 1993; Simon et al., 1993; Hermiston et al., 1996). Moreover, Fabpl-reporter transgene expression commences coincidently with initial cytodifferentiation of the intestinal epithelium in late fetal life and is sustained throughout adulthood, with highest levels of expression occurring in the middle third of the small bowel (jejunum).

Individual clones of stably transfected, Fabpl–N89β-catenin ES cells, or control nontransfected ES cells, were injected into C57Bl/6-ROSA26 (B6-ROSA26) blastocysts (Friedrich and Soriano, 1991; Wong et al., 1996). There are several reasons why the resulting chimeras are well-suited for studying the effects of transgene expression on the gut. First, intestinal epithelial cells of each genotype are separated into anatomically distinct units. By the time gut morphogenesis is completed, all crypts in B6-ROSA26→129/Sv chimeras are monoclonal: they contain either B6-ROSA26 or 129/Sv epithelial cells, but not a mixture of both (Wong et al., 1996). Several crypts surround the villus base and supply epithelial cells to each villus. Thus, the chimeric small intestine will contain patches of transgenic 129/Sv crypt-villus units and adjacent patches of normal (nontransgenic) B6-ROSA26 crypt-villus units. A villus located at the border of a patch of B6-ROSA26 crypts and a patch of 129/Sv crypts will be polyclonal, containing a vertical coherent column of transgenic epithelial cells emanating from a monoclonal 129/Sv crypt and an adjacent column of normal epithelial cells emanating from a monoclonal B6 crypt (Fig. 1, A and B). Second, genotyping is simple. All undifferentiated and differentiated epithelial cells in B6-ROSA26 patches produce E. coli β-gal (Wong et al., 1996). Therefore, the B6-ROSA26 component can be identified by staining the opened chimeric small intestine with X-Gal (see blue-stained villi in Fig. 1 A). The 129/Sv epithelium does not produce β-gal and can be identified, even in chi-
meras with a low percentage 129/Sv contribution, by its white appearance. Third, regions of nontransgenic B6 epithelium serve as a critical internal control for defining the effects of the transgene’s product. Since the small intestine is characterized by complex cephalocaudal variations in cell production and differentiation, it is critical that the control epithelial population be represented in a similar location as the genetically manipulated population. In the case of a polyclonal villus, the effect of the transgene can be defined by comparing 129/Sv and B6 cells located at a given cell stratum of a single villus positioned at a unique location along duodenal-ileal axis of an individual animal (Fig. 1B).

Four independent Fabpl–ΔN89β-catenin ES cell lines were injected into B6-ROSA26 blastocysts to generate B6-ROSA26–129/Sv(ΔN89β-catenin) chimeric-transgenic mice. Total RNA was isolated from the jejunum of 6-wk-old animals with 20–80% 129/Sv contribution (defined by coat color). Reverse transcriptase (RT)-PCR analysis revealed the expected sized product from the Fabpl–ΔN89β-catenin transgene in all four chimeric-transgenic lines (Fig. 2A). The RT-PCR product was not present in RNA prepared from the jejunum of normal control B6-ROSA26–129/Sv chimeras produced from nontransfected ES cells, or in RNA isolated from the skeletal muscle of chimeric-transgenic animals (Fig. 2A).

Transgene expression was independently confirmed by immunoblot analysis of total jejunal proteins isolated from 6-wk-old chimeric-transgenic and normal control chimeric mice with equivalent 129/Sv contributions. When the immunoblots were probed with antibodies that recognize the myc tag, an 80-kD protein corresponding to the predicted mass of myc–ΔN89β-catenin was detected in the jejunums of chimeric-transgenic but not control chimeric animals (Fig. 2B). Duplicate blots were probed with polyclonal antibodies raised against a peptide derived from an absolutely conserved region of human and mouse β-catenin. The steady-state level of immunoreactive β-catenin species was severalfold higher in the jejunums of chimeric-transgenic mice compared with control chimeras (Fig. 2B), consistent with successful forced expression of ΔN89β-catenin.

Figure 2. Evidence for expression of the Fabpl–ΔN89β-catenin transgene. (A) RT-PCR analysis of RNAs isolated from the jejunum and skeletal muscle of a 6-wk-old-chimeric-transgenic mouse (ΔN89β-catenin) and from the jejunum of a 6-wk-old chimera generated using nontransfected ES cells (nl chimera). The arrow points to the expected size of the product generated from ΔN89β-catenin mRNA. (B) Duplicate immunoblots are shown each containing total cellular proteins isolated from the jejunum of a 6-wk-old normal control B6-ROSA26–129/Sv chimeric mouse and a 6-wk-old B6-ROSA26–129/Sv(ΔN89β-catenin) chimeric-transgenic animal (both with 30–40% 129/Sv contribution based on coat color). The blot on the left of the panel was probed with antibodies that recognize the myc epitope tag present at the NH2 terminus of the 80-kD ΔN89β-catenin mutant (arrow). The blot on the right of the panel was probed with antibodies raised against the COOH terminus of β-catenin (molecular mass of wild-type β-catenin = 91 kD). (C) PLP-fixed frozen section of a polyclonal villus from a B6-ROSA26–129/Sv(ΔN89β-catenin) mouse. The section was incubated with affinity-purified rabbit antibodies to the myc tag, Cy3-tagged donkey anti-rabbit Ig, and the nuclear stain bis-benzimide (dark blue). Myc-tagged ΔN89β-catenin (magenta) is prominently represented in 129/Sv but not B6-ROSA26 villus epithelial cells. (Genotyping was accomplished by staining an adjacent serial section with antibodies to β-gal.) (D) Section of a polyclonal villus from a normal chimeric mouse stained with the same reagents as in C. 129/Sv epithelial cells lack the transgene, and therefore do not contain any myc-tagged protein. Bars: (C and D) 25 μm.
A final confirmation of transgene expression was provided by immunohistochemistry. Serial sections of chimeric-transgenic and normal chimeric jejunums were stained with antibodies raised against the myc tag and β-gal. In chimeric-transgenic mice, immunoreactive myc was present in β-gal-negative 129/Sv epithelial cells, but not in β-gal-positive B6-ROSA26 epithelial cells (Fig. 2 C). In normal control chimeras, the myc tag was not detectable in either 129/Sv or B6-ROSA26 epithelium (Fig. 2 D).

**Villus Branching in 129/Sv(ΔN89β-Catenin) Epithelium**

Chimeric-transgenic animals generated from three of the four ES cell lines were characterized further. All had similar phenotypes. There were no statistically significant differences between the growth rates or adult body weights of chimeric-transgenic and normal chimeric mice (n = 72).

Surveys of X-Gal–stained intestinal wholemounts from 1.5-, 6-, and 10-mo-old chimeric-transgenic mice (n = 1–8 animals/line per time point) revealed that the β-gal–negative 129/Sv(ΔN89β-catenin) epithelium contained branched villi. Typically, there were two branches per villus, each of equivalent height. Branched villi were similar in height to surrounding unbranched villi (Fig. 3, A and B). When polyclonal villi were encountered with branching, there was always a wholly 129/Sv(ΔN89β-catenin) branch (Fig. 3 C).

The frequency of villus branching was quantitated by examining serial sections of X-Gal–stained jejunal wholemounts from 6-mo-old chimeric-transgenic and normal chimeric mice. An average of 3,400 villi were scored per animal using the criteria described in Materials and Methods (n = two or three animals/line). The percentage of branched villi detected in 129/Sv(ΔN89β-catenin) jejunal epithelium ranged from 0.5 to 2%, depending upon the Fabpl-ΔN89β-catenin ES cell line used to produce the chimeras (Fig. 4). In contrast, the frequency of villus branching in their B6-ROSA26 jejunal epithelium was <0.01%. This difference was statistically significant (P < 0.05). Branched villi were extremely rare (<0.01%) in both the 129/Sv and B6-ROSA26 components of jejunum harvested from aged-matched normal control chimeras (Fig. 4). Villus branching remained confined to the 129/Sv epithelium of 10-mo-old chimeric-transgenic mice (n = 20). The frequency of branching was similar to that observed in 6-mo-old mice.

**ΔN89β-Catenin Expression Is Associated with Alterations in Proliferation, Apoptosis, and Migration but Not in Cell Fate Specification or Differentiation**

Histochemical stains, plus a panel of antibodies and lectins (refer to Materials and Methods), were used to determine whether the epithelium overlying 129/Sv(ΔN89β-catenin) crypt-villus units exhibited any changes in differentiation. Epithelial cells were compared in adjacent 129/Sv and B6-ROSA26 jejunal crypt-villus units, or within a single polyclonal villus, in both chimeric-transgenic and normal control chimeric mice. Expression of ΔN89β-catenin was not associated with any detectable perturbations in cell fate specification or in the terminal differentiation programs of the enterocytic, enteroendocrine, goblet, or Paneth cell lineages. Cell polarity appeared unaffected in the enterocytic lineage, as judged by the intracellular distribution of actin or by the distribution of apical- and Golgi membrane–associated glycoconjugates (Fig. 5 A, for example). The subcellular and crypt-villus distributions of the tight junction protein ZO-1 were similar in the 129/Sv and B6 components of polyclonal villi (Fig. 5 C). In addition, there were no discernible changes in the levels or crypt-villus distributions of α6, β1, β4, or β7 integrin subunits, laminin, fibronectin, or type IV collagen (Fig. 5, B and D; data not shown).
To determine the effects of ΔN89β-catenin on cellular proliferation, M-phase cells were scored in jejunal crypt-villus units of 6-mo-old chimeric-transgenic or normal chimeric mice. The ratio of M-phase cells in adjacent patches of 129/Sv and B6-ROSA26 crypts was calculated for each mouse in each line. Values obtained from all animals in a given line were then averaged (n = 400–600 crypts scored/mouse per line; n = two or three mice/line). Fig. 6 A shows there was a statistically significant two- to fourfold increase in the mitotic ratio (index) in chimeric-transgenic mice compared with age-matched normal chimeras (P < 0.05). This was due to an increase in the number of M-phase cells per 129/Sv(ΔN89β-catenin) crypt section. There were no statistically significant differences in the number of M-phase cells in the B6-ROSA26 crypts of chimeric-transgenic and normal chimeric mice (data not shown). The proliferative abnormality produced by ΔN89β-catenin was confined to the crypt epithelium; no M-phase cells were noted on villi with normal morphology or with a branched phenotype.

A crypt apoptotic index was also defined in these animals. Like the mitotic index, this index was expressed as the ratio of apoptotic cells in adjacent patches of 129/Sv and B6-ROSA26 crypts. An increase in apoptosis, equivalent to the increase in proliferation, was observed in 129/Sv(ΔN89β-catenin) jejunal crypts (Fig. 6 B). There were no statistically significant differences in the number of apoptotic cells between the jejunal B6-ROSA26 crypts of chimeric-transgenic and normal control chimeric animals (data not shown).

The effect of ΔN89β-catenin expression on cell migration and differentiation of goblet cells (open arrows) is equivalent in the 129/Sv and B6-ROSA26 components of the polyclonal villus. (B) The base of a polyclonal villus with its crypt-villus junction indicated by closed arrows. Three crypts are seen (open arrows at their base): the one on the left is supplying cells to another villus. The section was incubated with rat anti-β4 integrin subunit, Cy3 donkey anti-rat Ig, rabbit anti-β-gal, FITC donkey anti-rabbit Ig, and bis-benzimide. Nuclei (blue); the β4 integrin subunit (orange); β-gal (green-brown). The location of β4 integrin at the base of epithelial cells and its distribution along the crypt-villus unit are unaffected by ΔN89β-catenin. (C) Villi sectioned perpendicular to their crypt-villus axis. The tight junction protein ZO-1 (orange) was detected with rat anti-ZO-1 and Cy3 donkey anti-rat Ig. β-Gal (green) was visualized with the same reagents used in the preceding sections. The levels and location of ZO-1 in the 129/Sv(ΔN89β-catenin) and B6-ROSA26 components of polyclonal villi are similar (e.g., open arrows). (D) Villi sectioned perpendicular to their crypt-villus axis as in C. The section was incubated with rat anti-β, integrin, Cy3-donkey anti-rat Ig, rabbit anti-laminin, rabbit anti-β-gal, FITC donkey anti-rabbit Ig, and bis-benzimide. B6-ROSA26 cells exhibit diffuse staining of their cytoplasm due to the presence of β-gal (green). β integrin is confined to intraepithelial lymphocytes (orange). Comparable numbers of these cells are seen in the B6-ROSA26 and 129/Sv(ΔN89β-catenin) components of polyclonal villi and in wholly 129/Sv(ΔN89β-catenin) villi. Laminin appears as linear green immunoreactivity underlying 129/Sv and B6-ROSA26 epithelium (e.g., closed arrows). The intensity of staining is similar under cells of both genotypes. Bars, 25 μm.
Wholemount preparations of chimeric-transgenic and normal control chimeric mice were injected with BrdU to label crypt epithelial cells in S phase. Animals were killed 60 h later (n = two mice/line). Serial sections were prepared from their jejunums and then the sections were stained with antibodies to β-gal and BrdU. Analysis of polyclonal villi present in chimeric-transgenic mice revealed a marked difference in migration between 129/Sv(ΔN89β-catenin) and B6-ROSA26 epithelial cells. 60 h after pulse labeling, the leading edge of BrdU-positive B6 cells had moved from the crypt to the upper quarter of the villus, whereas the leading edge of BrdU-positive 129/Sv(ΔN89β-catenin) cells had only reached the middle portion of the villus (Fig. 7). This effect was related to forced expression of ΔN89β-catenin: control studies revealed that there were no detectable differences in migration between 129/Sv and B6-ROSA26 epithelial cells located in the polyclonal villi of normal chimeras (for illustration see Hermiston et al., 1996). Based on previous determinations of the time it takes BrdU-tagged cells to move from the crypt to the villus tip (Hermiston et al., 1996), the observed difference represents a slowing of 129/Sv(ΔN89β-catenin) cell migration by ~12–24 h.

Comparisons of polyclonal jejunal villi present in the wholemount preparations of chimeric-transgenic and normal control chimeric mice (refer to Fig. 1 A and Fig. 3, A and C) established that forced expression of ΔN89β-catenin did not affect the orderliness of migration. The borders between adjacent columns of B6 and 129/Sv(ΔN89β-catenin) epithelium were sharp. There was no sign of infiltration of 129/Sv(ΔN89β-catenin) epithelial cells into adjacent B6-ROSA26 cellular columns, as was seen when the same Fabpl transcriptional regulatory elements were used to force expression of wild-type human APC (Wong et al., 1996). Hematoxylin- and eosin-stained sections failed to disclose any piling up of cells at the junctions of 129/Sv(ΔN89β-catenin) crypts and their associated villi. Similarly, there was no aberrant accumulation of cells at the villus tip where cell extrusion normally occurs.

The slowing of migration was not accompanied by any detectable perturbations in contacts between epithelial cells. There was no evidence of disrupted mucosal barrier function: (a) surveys of hematoxylin- and eosin-stained jejunal sections from 6-mo-old chimeric-transgenic mice failed to show any signs of inflammatory bowel disease; and (b) immunohistochemical analyses indicated that the number and crypt-villus distributions of several components of the diffuse gut–associated lymphoid tissue were unperturbed (e.g., β7-positive intraepithelial lymphocytes,
CD4$^+$ T cells, and CD8$^+$ T cells; refer to Fig. 5 D plus data not shown).

Because aberrant β-catenin signaling has been implicated in the pathogenesis of intestinal neoplasia (refer to Introduction), we carefully surveyed X-Gal-stained wholemounts and serial sections of the small intestine from chimeric-transgenic animals for evidence of dysplasia, adenoma formation, or adenocarcinoma. None of the mice had any evidence of these pathologic changes ($n=72$; ages 1.5–10 mo).

**Mechanistic Analyses: ΔN89β-Catenin Expression Is Associated with an Augmentation in Cellular Pools of E-Cadherin**

Conventional light microscopic and confocal microscopic surveys of jejunal sections, prepared from chimeric-transgenic mice and stained with antibodies against the myc tag or β-catenin, failed to disclose ΔN89β-catenin and endogenous β-catenin in the nuclei of transgenic 129/Sv or normal B6-ROSA26 epithelial cells (refer to Fig. 2 C and see Fig. 8 A), β-Catenin was present at the adherens junctions and basolateral surfaces of epithelial cells (Fig. 8 A).

We examined whether forced expression of ΔN89β-catenin affected the intracellular distribution or levels of its known partners APC, E-cadherin, or α-catenin. To do so, jejunal sections were incubated with antibodies to each protein. Surveys of adjacent 129/Sv (ΔN89β-catenin) and B6-ROSA26 crypt-villus units, as well as polyclonal villi, failed to disclose any appreciable differences in APC or α-catenin localization or levels. α-Catenin remained associated with adherens junctions and the basolateral surfaces of 129/Sv (ΔN89β-catenin) epithelial cells (data not shown), whereas APC was prominent at the periphery of villus epithelial cells where it appeared to form small granular aggregates (Fig. 8 B).

In contrast, the steady-state level of E-cadherin was markedly increased in ΔN89β-catenin–producing epithelial cells (Fig. 8, C and D). The augmented concentration of E-cadherin was not accompanied by detectable changes in its intracellular location (adherens junctions and basolateral surfaces). Analysis of polyclonal jejunal villi from normal chimeras established that the increased level of E-cadherin was not simply due to genotypic differences between 129/Sv and B6-ROSA26 epithelium (Fig. 8, E and F).

Previous studies, using the same Fabpl transcriptional regulatory elements used in this report, had shown that forced expression of wild-type E-cadherin in the jejunal crypt-villus units of chimeric-transgenic mice produced a slowing of epithelial migration equivalent to that observed with ΔN89β-catenin (Hermiston et al., 1996). To determine whether augmented levels of E-cadherin were responsible for the other phenotypic changes observed B6-ROSA26→129/Sv (ΔN89β-catenin) chimeras, we defined the jejunal crypt mitotic and apoptotic indices in 6-mo-old chimeric-transgenic mice generated using ES cells stably transfected with Fabpl mouse E-cadherin DNA (Hermiston et al., 1996). The increase in total M-phase cells/crypt section associated with ΔN89β-catenin expression was not evident in E-cadherin overexpressing crypts (refer to Fig. 6 A), indicating that this feature was directly related to ΔN89β-catenin production rather than to a sec-

![Figure 8](image-url)

Figure 8. ΔN89β-Catenin expression results in augmented cellular pools of E-cadherin. Frozen sections were prepared from PLP-fixed jejenum recovered from a 6-mo-old B6-ROSA26→129/Sv (ΔN89β-catenin) chimeric-transgenic animal (A–D) and a 6-mo-old normal B6-ROSA26→129/Sv chimera (E and F). (A) Polyclonal villus incubated with rabbit anti–β-catenin, Cy3 donkey anti-rabbit Ig, and bis-benzimide. Prominent β-catenin staining (red) is evident at the adherens junctions and basolateral surfaces of epithelial cells. No immunoreactive protein is detectable in 129/Sv or B6-ROSA26 nuclei. (B) Polyclonal villus incubated with rabbit anti-APC, Cy3 donkey anti-rabbit Ig, and bis-benzimide. The levels and intracellular distribution of APC (red) are similar in B6-ROSA26 and 129/Sv (ΔN89β-catenin) epithelial cells. (C and D) Villi from the chimeric-transgenic mouse that have been sectioned perpendicular their crypt-villus axis. (C) Section stained with rat anti-E-cadherin and Cy3-donkey anti-rat Ig. (D) Dual exposure of the same section after incubation with rabbit anti–β-gal and FITC donkey anti-rabbit Ig. Steady-state levels of E-cadherin are markedly increased in the 129/Sv (ΔN89β-catenin) component of polyclonal villi. (E and F) Villi from a control normal chimera processed as in (C and D). E-Cadherin levels are comparable in the B6-ROSA26 and 129/Sv villus epithelium. Bars, 25 μm.
ondary effect of elevated E-cadherin concentrations. We could not make such a statement in the case of the apoptotic response, because similar elevations in crypt apoptosis were associated with forced expression of wild-type E-cadherin and ΔN89β-catenin (Fig. 6 B). Forced expression of E-cadherin did not produce villus branching (data not shown).

Discussion

Expression of ΔN89β-catenin, which lacks sites for GSK-3 phosphorylation, can be viewed as simulating at least one effect of active Wnt signaling: generation of augmented cellular pools of stable, hypophosphorylated β-catenin. Comparable NH2-terminal truncation mutants are known to phenocopy activation of the Wnt signaling pathway in Xenopus and Drosophila. In these nonmammalian systems, the consequences include axis duplication and changes in cell fate specification (Yost et al., 1996; Pai et al., 1997). Moreover, components in the Wnt signaling pathway play critical roles in specifying endoderm and gut differentiation during early Caenorhabditis elegans development (Han, 1997; Rocheleau et al., 1997; Thorpe et al., 1997). ΔN89β-Catenin production in the small intestine of chimeric-transgenic mice was not only designed as an in vivo test of the role of Wnt signaling in establishing and maintaining a self-renewing mammalian epithelium, but also as a test of the effects of β-catenin on a system with complex adhesive requirements (i.e., preservation of cell–cell contacts in a mucosal barrier, orderly yet rapid cell migration, and exfoliation at a defined point in the cellular life cycle).

ΔN89β-Catenin Expression Results in a Proliferative Response Restricted to Crypts

Forced expression of Wnt-1 causes mammary epithelial hyperplasia and tumor formation (Brown et al., 1986; Tsukamoto et al., 1988). In addition, ectopic expression of Wnt-1 enhances proliferation in the developing mammalian central nervous system (Dickinson et al., 1994). The mitogenic response of crypt epithelial cells to ΔN89β-catenin is consistent with the observed mitogenic effects of active Wnt signaling in these other lineages. Surprisingly, this proliferative response was observed in the absence of detectable myc-tagged ΔN89β-catenin (or endogenous β-catenin) within the nucleus of crypt epithelial cells. Forced expression of wild-type β-catenin or ΔN89β-catenin in Drosophila, Xenopus, two-cell mouse embryos, and cultured mammalian cells has been reported to result in redistribution of β-catenin to the cytoplasm and nucleus (Funayama et al., 1995; Huber et al., 1996; Munemitsu et al., 1996; Yost et al., 1996; Pai et al., 1997). We assume that the amount of immunoreactive β-catenin in the nuclei of crypt epithelial cells was below the limits of detection of our staining techniques.

The proliferative response did not involve villus epithelial cells despite sustained expression of ΔN89β-catenin from the crypt to the villus tip. Studies of nontransgenic mice have shown that these short-lived postmitotic cells retain many critical regulators that allow entry into S phase (e.g., cyclin E and cdk4; Chandrasekaran et al., 1996). Moreover, the villus microenvironment does not impose an absolute prohibition on crossing the G1/S boundary: e.g., villus enterocytes can be induced to undergo a pRB-dependent reentry into the cell cycle by forced expression of SV40 large T antigen (TAG; Chandrasekaran et al., 1996; Coopersmith et al., 1997). The reason why ΔN89β-catenin fails to evoke a proliferative response in villus cells remains unresolved. However, terminal differentiation of these cells may change the availability or function of downstream effectors of the Wnt pathway, such as high motility group box transcription factors.

Primary Versus Secondary Responses: Stimulation of Apoptosis in ΔN89β-Catenin–Producing Crypts and Villus Branching

The intestinal epithelium has a great capacity to initiate compensatory responses that preserve the steady-state cellular census when that census is threatened by changes in proliferative status or cell survival (Hermiston et al., 1995a; Coopersmith et al., 1997). As a consequence, it is often difficult to distinguish between primary responses to an applied stimulus and responses that compensate for an effect produced by that stimulus. At this point, we cannot determine whether the apoptosis reflects a direct effect of augmented cytosolic β-catenin pools (and signaling), or a secondary compensatory response to augmented crypt proliferation. Whatever the underlying mechanism, given the lack of discernible effects on villus height, we conclude that the observed changes in cell death are generally able to compensate for the observed changes in cell production.

A quandary exists regarding the interpretation of villus branching. There is little information available about the determinants of villus geometry (Totafurno et al., 1990). For example, are the determinants of morphology primarily epithelial-based or do they also involve mesenchymal signals? The normal variation in villus height observed along the duodenal-ileal axis of the adult mouse intestine can be correlated with the number of crypts that surround the base of each villus (Wright and Irwin, 1982). Totafurno et al. (1987) examined rare branched villi in normal mice and found that the number of crypts that surrounded their base was approximately twice the normal number, suggesting that villus branching may be a compensatory response to increased epithelial cell input. Increased cell production may not have to come from crypts: SV40 TAg expression in villus enterocytes leads to reentry into the cell cycle and branching in a subset of villi (Coopersmith et al., 1997).

We do not view branching as analogous to the axis duplication observed in Xenopus when the Wnt pathway is stimulated. It is unclear how a branched shape can be maintained given the perpetual epithelial cell renewal that occurs in crypt–villus units. In fact, the absence of a notable increase in the frequency of villus branching as B6–ROSA26Δ–129/Sv(ΔN89β-catenin) mice age suggests that branched villi have a limited life span. This is consistent with the view that normal intestine continually produces new crypts and villi as a byproduct of its dynamic cell renewal (Totafurno et al., 1987). Branching in our chimeric-transgenic mice may be a default response, reflect-
ing the inability of some of their villi to adequately compensate for a threatened or real increase in their steady-state epithelial cell census beyond some critical threshold value. Such a threat may arise from the increased cell production observed in their crypts, from the inability of crypt apoptosis to compensate for this proliferative response, and/or from the effects of slowed epithelial cell migration. Branching could also represent a direct response to aberrant signals, originating from a genetically manipulated epithelium and operating through interactions with the underlying mesenchyme. The significance of branching is that it provides a potential inroad to deciphering how villus structure is preserved in the face of perpetual, rapid renewal of its principal cellular component.

**ΔN89β-Catenin Produces an Increase in Steady-State Levels of E-Cadherin**

Forced expression of ΔN89β-catenin results in a marked increase in E-cadherin levels at the adherens junction and basolateral surfaces of intestinal epithelial cells. Several recent studies suggest that this is due to activation of E-cadherin gene expression. The mouse E-cadherin gene contains a 7-bp binding sequence for Lef-1/Tcf (Huber et al., 1996). Yanagawa and co-workers (1997) used a *Drosophila* wing imaginal disc cell line to show that stimulation of the Wingless pathway induces *Drosophila* E-cadherin gene transcription and accumulation of DE-cadherin at cellular junctions. They also found that forced expression of Dishevelled, an inhibitor of Zeste-white 3 (the *Drosophila* GSK-3 homologue), led to accumulation of Armadillo (the β-catenin homologue) in the cytosol of these cells, a marked increase in *Drosophila* E-cadherin mRNA, and an elevation in junctional DE-cadherin. In addition, they noted that forced expression of an NH2-terminal truncation mutant of Armadillo produces elevated DE-cadherin mRNA and protein levels.

The ΔN89β-catenin–mediated increase in E-cadherin levels is likely to counteract the signaling activity of ΔN89β-catenin within the intestinal epithelium. Studies in *Xenopus* have shown that the binding of β-catenin to cadherins opposes signaling by sequestering β-catenin (Heasman et al., 1994; Fagotto et al., 1996). The location of myc-tagged ΔN89β-catenin within intestinal epithelial cells provides evidence that a similar process occurs in a mammalian system.

The augmentation in cellular E-cadherin pools is also likely to contribute to the slow migratory phenotype of 129/Sv(ΔN89β-catenin) epithelium. When forced expression of E-cadherin was limited to villus enterocytes in transgenic mice, enterocytic migration from the villus base to villus tip was slowed (Hermiston et al., 1996). In this previous study, there was no augmentation of E-cadherin in the crypt epithelium, no perturbations in crypt proliferation or death, and therefore little likelihood that the observed effect on migration was due to a diminution in net cellular output from the crypt.

**APC and the Effects of ΔN89β-Catenin on Intestinal Epithelial Biology**

ΔN89β-Catenin production was not associated with any detectable abnormalities in the intracellular distribution or levels of APC within crypt-villus units. This may have contributed to two phenotypic features of our chimeric-transgenic mice: (a) slowed migration and (b) lack of neoplastic transformation.

Studies in MDCK cells have indicated that β-catenin affects APC’s ability to organize microtubules (Näthke et al., 1996; Pollack et al., 1997). ΔN89β-catenin–APC complexes are more stable than wild-type β-catenin–APC complexes, leading to accumulation of ΔN89β-catenin at clusters of APC positioned at the tips of MDCK cell membrane extensions (Näthke et al., 1996; Barth et al., 1997; Pollack et al., 1997). Based on these findings, Pollack and co-workers (1997) proposed that this stabilization of ΔN89β-catenin–APC complexes inhibits (MDCK) migration by somehow perturbing APC function.

Regulation of β-catenin degradation appears to play an important role in colorectal tumorigenesis and in the formation of melanomas (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). Levels of soluble β-catenin are elevated in colon tumor cells (Munemitsu et al., 1995). In addition, mutations in the NH2 terminus of β-catenin are oncogenic in cultured cells and are associated with intestinal neoplasms (Morin et al., 1997). The stabilized ΔN89β-catenin mutant was produced in intestinal epithelial cells that were able to maintain normal levels of APC. This may account for the failure of these cells to undergo neoplastic transformation during the 10-mo period that animals were studied. In addition, the β-catenin mutations that have been associated with human colorectal cancer involve alterations in NH2-terminal serine residues. Although it may seem, superficially, that NH2-terminal truncations would have similar biochemical effects as serine substitution (i.e., protein stabilization), NH2-terminal phosphorylation may have other roles, including affects on protein–protein interactions that modulate signaling.

**Prospectus**

The self-renewing intestinal epithelium is able to invoke a variety of compensatory responses when its census is threatened. This capacity to compensate makes sense given the enormous energetic investments required to sustain normal self-renewal. Compensation provides an experimental challenge when trying to decipher the function of molecules such as β-catenin, since dramatic or even noticeable phenotypes may be difficult to elicit. Further analysis of the effects of active β-catenin–mediated signaling on epithelial homeostasis may require forced expression of β-catenin–Lef-1 (or Tcf) fusion proteins or dominant-negative Tcf/Lef-1 mutants (Molenaar et al., 1996). The chimeric-transgenic system described above provides a way of assaying the effects of these mutants in vivo under well-controlled conditions.

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