Ectopic Expression of P-Cadherin Correlates with Promoter Hypomethylation Early in Colorectal Carcinogenesis and Enhanced Intestinal Crypt Fission In vivo

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Abstract

P-cadherin is normally expressed in the basal layer of squamous epithelia and absent from the healthy intestine and colon. We have previously shown it to be expressed in all inflamed, hyperplastic, and dysplastic intestinal and colonic mucosa. This study aimed to better understand the mechanisms controlling the expression of P-cadherin and the biological effects of its ectopic presence in the intestine and colon. We investigated the CpG methylation status of the P-cadherin (CDH3) promoter and P-cadherin mRNA and protein expression in cases of familial and sporadic colorectal cancer (CRC). The CDH3 promoter was hypomethylated in colonic aberrant crypt foci, in CRC, and, occasionally, in the normal epithelium adjacent to cancer, demonstrating a potential “field effect” of cancerization. The hypomethylation was also associated with induction of P-cadherin expression in the neoplastic colon (P < 0.0001). We then created transgenic mice that overexpressed P-cadherin specifically in the intestinal and colonic epithelium under the liver fatty acid binding protein promoter. Forced ectopic expression of P-cadherin accompanied by indomethacin-induced inflammation resulted in a 3-fold higher crypt fission rate within the small and large intestines in the homozygous mice compared with the wild-type animals (P < 0.02). We conclude that epigenetic demethylation of the P-cadherin promoter in the human intestine permits its ectopic expression very early in the colorectal adenoma-carcinoma sequence and persists during invasive cancer. Induced P-cadherin expression, especially in mucosal damage, leads to an increased rate of crypt fission, a common feature of clonal expansion in gastrointestinal dysplasia. [Cancer Res 2008;68(19):7760–8]

Introduction

Colorectal cancer (CRC) is currently the fourth most common malignancy and the second most common cause of death from cancer in the United States and the United Kingdom. It is typified by the adenoma to carcinoma sequence of tumor progression, with mutations in the adenomatous polyposis coli (APC) gene believed to be one of the earliest genetic aberrations and present in up to 80% of sporadic CRCs (1, 2). A heritable form of CRC, characterized by formation of multiple polyp adenomas, familial adenomatous polyposis (FAP), also arises from APC gene mutations. APC binds to β-catenin and modulates its intracellular levels by marking it for degradation. β-Catenin is a potent regulator of oncogene transcription and mediator of the Wnt signaling pathway but has an additional role in cell-cell contact and adhesion through binding to cadherins (3).

Cadherins, as key cell adhesion molecules involved in organ development and morphogenesis, display tissue-specific distribution, identifying epithelial (E), neuronal (N), and placental (P) cadherin isoforms (reviewed in ref. 4). Through the interaction with β-catenin, cadherins are able to integrate cellular adhesion and tissue morphology with cell signaling and differentiation. P-cadherin is most strongly expressed in the stratified squamous epithelia, such as the esophagus in humans and placenta in mice (5). “Placentation” is characterized by physiologic invasion and cellular division during gestation. In contrast to E-cadherin, which is present throughout the squamous epithelium and responsible for the maintenance of stratification and differentiation, the expression of P-cadherin is restricted to the basal cell layer, governing cell proliferation and glandular architecture (5). Altered expression of cadherins has been linked to changes in cellular behavior and tumorigenesis (6). Down-regulation of E-cadherin, often through epigenetic promoter silencing, has been associated with progression to breast cancer (7), esophageal cancer, (8) and CRC (9), whereas up-regulation of P-cadherin has been documented in esophageal, gastric, pancreatic, bladder, and breast cancer (10–14).

One study addressed the potential role of P-cadherin overexpression in tumorigenesis and observed changes in cell morphology, weakened cell-cell adhesion, and increased cell motility in a pancreatic cancer cell line expressing P-cadherin. P-cadherin overexpression induced cytoplasmic accumulation of catenin p120ctn, a tyrosine kinase substrate involved in SRC signaling. P-cadherin expression in pancreatic cancer cells promoted cell proliferation and migration (20). In colorectal adenomas and carcinomas, increased P-cadherin expression has been associated with increased proliferative activity and loss of differentiation (21, 22). In colorectal cancer cell lines, P-cadherinexpression has been found to be associated with decreased cell-cell adhesion, increased cell motility, and increased metastatic potential (23). P-cadherin expression has also been associated with an increased proliferation rate and resistance to apoptosis in colorectal cancer cell lines (24).}

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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9 www.cancerresearchuk.org
growth factor receptors epidermal growth factor (EGF) and platelet-derived growth factor (PDGF; ref. 15).

We have previously shown universal ectopic P-cadherin expression across different regenerative and hyperplastic lesions in the colon, such as colon injury, inflammation, restitutition, hyperplasia, and neoplasia (16, 17). In CRC, P-cadherin is present within the earliest morphologically recognized neoplastic lesion, the aberrant crypt focus (ACF). This is observed before and independent of disturbances in E-cadherin, catenins, or APC expression and persists into hyperplastic and adenomatous polyps (16). However, the mechanism underlying the ectopic P-cadherin expression in the ACF is unknown, as is the effect of P-cadherin on intestinal crypt biology. Gene-activating mutations and chromosomal amplifications of the P-cadherin chromosomal region 16q22.1 are not common in CRC10 and regulation of P-cadherin expression in CRC by epigenetic alterations, such as CpG island methylation, has not been reported to date. CRC is often coupled with epigenetic changes (18), although reports describing aberrant gene silencing through DNA hypermethylation outnumber examples of gene hypomethylation in CRC (19).

It has been shown that colorectal adenomas and hyperplastic polyps develop as a result of an increased rate of intestinal crypt proliferation, which occurs through the process of crypt fission, originating at the base of the crypt (20, 21). The majority of new crypts form during a short postnatal period, after which their numbers increase only gradually after a cycle of around 3 months in mouse and 9 to 18 years in man (21). Adenomatous polyps bearing APC mutations expand through increased rates of intestinal crypt fission, identifying APC as one of the key precursors of adenoma progression (21). However, other factors which affect catenin/cadherin biology might also deregulate crypt fission and therefore influence the progression, as well as initiation, of colorectal tumors.

Here, we show ectopic expression of P-cadherin in the colon mucosa from early stages of CRC, which is likely to be the result of aberrant APC signaling leading to altered crypt fission. This is supported by observation of a disturbed cell proliferation and is consistent with the previously published data on the loss of APC expression. The mechanism underlying this process remains unclear. However, this study demonstrates that P-cadherin expression is increased in colorectal neoplastic lesions, which may be of relevance to the biology of these lesions.

Materials and Methods

Collection and characterization of tissue samples. Internationally accepted criteria, such as the Vienna classification, were used to characterize normal and CRC tissue in patients. Samples of normal colon were collected from patients with gastrointestinal complaints undergoing colonoscopy, upon which no lesions were found. Samples of colon mucosa containing aberrant crypt foci, phenotypically normal colon (from outside the tumor margins), and CRC were collected at time of colonoscopy or CRC resection, respectively. The samples were selected by applying a 0.05 false discovery rate threshold. Differential gene expression in adenoma samples was measured relative to the median signal of all normal samples.

Immunohistochemistry. Sections (5 μm) of paraffin-embedded tissue were stained, as previously described (16). Mouse anti-human P-cadherin antibody (clone 56, BD Biosciences) was used at 1:100 dilution followed by biotinylated goat anti-mouse/rabbit IgG (Dako) at 1:200 dilution and avidin-biotin complex method (ABC; Vector Laboratories) diluted 1:1,000. Goat anti-mouse P-cadherin primary antibody (AF761, R&D Systems) was used at 1:100 dilution and secondary rabbit anti-goat biotinylated antibody (Dako) at 1:1,000 dilution, followed by ABC/HRP (Dako) diluted 1:1,000. Primary rabbit anti-mouse Rac1/Cdc42 polyclonal antibody and 61 catenin phosphorylated Y228 (p120ctn) monoclonal antibodies (Abcam) were used at 1:50 and 1:75 dilution, respectively, each followed by secondary goat anti-rabbit biotinylated antibody (Dako) at 1:300 dilution and ABC/HRP (Dako) diluted 1:1,000. The sections were analyzed on BH-2 light microscope (Olympus).

DNA extraction for methylation analysis. DNA was extracted from frozen samples of colon mucosa with aberrant crypt foci (n = 15), normal colon mucosa (n = 19), and phenotypically normal mucosa adjacent to cancer (n = 20), and CRC specimens (n = 15) using the GenElute Mammalian Genomic DNA kit (Sigma-Aldrich) by homogenizing 20 mg of tissue in the after-lysis buffer: 50 mmol/L KCl, 10 mmol/L Tris-HCl, 2.5 mmol/L MgCl2, 0.01% gelatin, 0.5% NP40, and 0.5% Tween 20 containing 200 μg/mL proteinase K (Sigma-Aldrich).

Methylation-specific PCR. The methylation status of a 149-bp fragment upstream of the P-cadherin exon 1 was analyzed using bisulfite modification, as previously published (23). Briefly, NaOH-denatured DNA was treated with 5.5 mol/L sodium bisulfite in the presence of hydroquinone for 4 h at 55 °C. Modified DNA was desalted using the Wizard DNA Purification Resin (Promega) and amplified with oligonucleotides specific for unmethylated and methylated DNA. The primers used for detection of unmethylated DNA (product size, 149 bp) 5′-TTGTAGGGGGTGTTGAGTTTGTGC3′ (forward), 5′-ATAAAGAACACTCACCGACACAAACACCCAAAC3′ (reverse), and methylated DNA (141 bp) 5′-CGAGGGGGCGGGATTTCGTGGC3′ (forward), 5′-AACAATCGCAGCGACGACAGCGA3′ (reverse). PCR conditions used for unmethylated DNA: 5 min at 95 °C, followed by 35 cycles of 95 °C (1 min), 61 °C (1 min), 72 °C (1 min), and final extension at 72 °C for 7 min. Conditions used for methylated DNA were the same apart from 64 °C annealing temperature.

Sequencing of the methylation-specific PCR products. The products of methylation-specific PCR were sequenced with reference to a previously published method (24). The PCR products were purified using Wizard PCR Prep DNA Purification System (Promega) and inserted into vector pGEM-T using the LigAfast DNA Ligation System (Promega); 50 ng vector DNA were used in each ligation. The quantity of insert DNA used in ligation reactions was calculated by using the equation: mass insert (ng) = mass vector (mg) × size of insert (kb) / size of vector (kb) × molar ratio of insert/vector. Vector and insert DNA were mixed with DNA ligation buffer and ligase and incubated at room temperature for 15 min before being placed on ice. Cloned inserts were sequenced with T7 oligonucleotide as a primer (5′-TAATACGACTCACTATAGG-3′) and reverse primer (5′-ACGGCTCCACACCCAGGG-3′) using the total RNA kit (Sigma-Aldrich). Adenoma DNA was screened for protein-truncating somatic APC mutations using fluorescence-SSCP and AB13100 sequencers (22). Adenomas from five patients with known APC mutations were compared with normal samples for gene expression using Affymetrix HG-U133A array. Data were imported into GeneSpring (Agilent Technologies), processed using MAS5.2 and subsequently median centered. Reporter genes called as absent in all cases were removed from the analysis, and each gene was scaled to its median value within the normal control group. Differentially expressed genes between the tumor and normal control groups were selected by applying a 0.05 false discovery rate threshold. Differential gene expression in adenoma samples was measured relative to the median signal of all normal samples.

Reverse transcription–PCR of human P-cadherin. The cDNA was synthesized from RNA using the Reverse Transcription System (Promega). RNA at 1 mg/mL was added to the reverse transcription mix containing random hexamer oligonucleotides and heated to 19°C (10 min), 42°C (50 min), 99°C (5 min), then placed on ice. Primers used for amplifying the P-cadherin cDNA were forward 5′-ACGGCTCCACACCCAGGG-3′ and reverse 5′-ACGGCTCCACACCCAGGG-3′.

10 Our unpublished observations.
reverse 5'-TGGCTGTGGAGGTTGGGAG-3' using the following PCR conditions: 3 min at 95°C; followed by 35 cycles of 95°C (1 min), 56°C (1 min), 72°C (1 min); and final extension at 72°C for 7 min.

**Generation of P-cadherin transgenic mice.** Genetic modification of animals and all animal procedures were approved by the appropriate local and national authorities. P-cadherin mouse transgene construct was created by inserting mouse P-cadherin cDNA flanked by EcoRV sites (gift from Dr. S. Hirohashi; ref. 25) into pL596hGHΔB plasmid (gift from Dr. J. Gordon; ref. 26). This construct contains nucleotides −596 to −21 of the promoter for the rat fatty acid binding protein gene (L-FABP), expressed in the liver and the intestine to induce intestine-specific expression of the transgene. The L-FABP/mP-cad construct was introduced into F1 × F1 embryos (F1 from CBA × B6 cross) by pronuclear injection and mice maintained on this background by breeding to F1 mice. Two lines of experimental wild-type, heterozygous, and homozygous animals were generated after four generations of breeding from two founder females. All animals were fed a standard chow diet *ad libitum*.

**PCR of the mP-cadherin transgene.** Transgenic animals were screened for the presence of the P-cadherin transgene using whole tail lysis-PCR and the primers 5'-CCCATTCTGATTTTGATTTTTATCGTT-3' (forward) and 5'-CTCGGAGACCACGCTGCGTAG-3' (reverse), resulting in a 250-bp ampli-
con. PCR conditions used were 5 min at 95°C, followed by 35 cycles of 95°C (1 min), 60°C (1 min), 72°C (1 min), and final extension at 72°C for 7 min.

**Microdissection of intestinal crypts from P-cadherin transgenic mice and assessment of cell proliferation and crypt fission.** Wild-type, homozygous, and heterozygous mice were injected ip with vincristine-
sulfate (David Bull Laboratories) at 1 mg/kg body weight, which causes cell arrest in metaphase. Mice were sacrificed 2 h later in a CO2 chamber followed by cervical dislocation. The indomethacin-treated mice were injected with indomethacin (85 mg/kg body weight in fresh 5% bicarbonate)

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**Figure 1.** A, immunohistochemical analysis of P-cadherin expression in normal and colorectal colon cancer mucosa. Images were taken at 100× and 250× magnification (inserts). A, top row, normal colon mucosa (no evidence of colorectal neoplasia) showing absence of P-cadherin; phenotypically normal colon mucosa adjacent to CRC from a patient with sporadic CRC (P-cadherin expression was found in 1 sample of 20); colon mucosa from sporadic CRC showing P-cadherin expression. Bottom row, colon mucosa from a patient with FAP showing P-cadherin expression; negative control (esophageal epithelium, no primary antibody); positive control (esophageal epithelium, constitutive P-cadherin expression within the basal layer). B, expression heat-map for the top 10 up-regulated and down-regulated genes in FAP adenomas. Colorectal polyp adenomas and matched normal control tissue from five FAP patients with known germline APC mutations (total of 16 adenoma and 8 normal samples) were analyzed for differential gene expression on a microarray of 14,500 genes. The red/green signals (columns 1–16) represent the adenoma samples relative to the median of all normal samples. Negative controls (columns 1–8) are the normal samples relative to the median of all normal samples, showing low variability among the normal samples. The genes are presented in decreasing order of expression. Top (DEFA6), most up-regulated; bottom (CCL19), most down-regulated. P-cadherin (CDH3, blue rectangle) was the second highest up-regulated gene present in polyp adenomas in an excess of 29-fold compared with normal colon mucosa. Log2 ratio scale is shown below the map.
or vehicle (sham controls) 24 h before sacrifice. The entire gastrointestinal tract was isolated, rinsed in cold PBS, fixed in Carnoy’s fixative for 3 h, transferred to 75% ethanol, and stored until microdissection. For immunohistochemistry, gut preparations from ethanol were rolled up into a “Swiss-roll” and embedded in paraffin.

Assessment of proliferation and fission throughout the gut was performed using the “crypt microdissection” method, as previously described (21, 27). Briefly, representative samples of tissue from the proximal, mid, and distal small intestine and colon were hydrated, hydrolyzed, and stained with the Feulgen reaction, as described before (28). For studying metaphase arrest, the mucosal crypts were gently teased apart with needles and gently compressed with a coverslip. The number of metaphases per crypt was counted in 20 crypts per animal using a light microscope at 160× magnification. The number of crypt fission events per 200 crypts per animal was determined under a dissecting microscope at 45× magnification (29). All samples were counted in a blinded fashion.

Statistical analysis. All statistical analyses were performed using GraphPad Prism software and P values constrained with a 95% confidence interval. For the statistical analyses of metaphase and crypt fission, individual two-sided, nonparametric tests were performed, followed by Bonferroni correction for multiple comparisons.

Results

P-cadherin is ectopically expressed in both sporadic and familial CRC. We have previously shown a lack of P-cadherin (CDH3) expression in the normal colon mucosa and its overexpression in sporadic colorectal adenomas (16), as well as in the injured and regenerating colon (17). The overexpression of P-cadherin is first noted in the aberrant crypt foci, the earliest phenotypic precursors of CRC (16). Here, we investigated the presence of P-cadherin protein and mRNA expression in samples of normal colon mucosa from patients with no evidence of colorectal inflammation or dysplasia and cancer samples from patients with sporadic CRC and from phenotypically normal mucosa adjacent to the cancer, as well as samples from patients with FAP.

As expected, all samples of normal colon mucosa from non-CRC subjects were negative for P-cadherin (n = 19; Fig. 1A). However, 1 of 20 samples of phenotypically normal but adjacent to cancer mucosa from sporadic CRC patients showed aberrant P-cadherin expression, as did all of the cancer samples (n = 15). The staining was confined to the epithelial cells (columnar and goblet). Positive staining for P-cadherin was also noted in all FAP samples (n = 5). No staining was observed in samples from patients with a mild inflammation of the colon—diverticular colitis (data not shown).

Normal esophageal squamous tissue, which constitutively expresses P-cadherin in the basal layer, was used as positive tissue control and, without the primary antibody, as the negative experimental control (Fig. 1A).

To investigate the timing and significance of P-cadherin expression in relation to APC aberration, a known early change in CRC, we used samples from patients with FAP. We studied the expression of P-cadherin in colorectal polyp adenomas from FAP patients using microarray gene expression analysis (Fig. 1B). Biopsies of colorectal adenomas (n = 16) and normal tissue (n = 8) were collected from five FAP patients with known germline mutations in the APC gene (S1190X, Q163X, 798FS, 1157FS, and R213X). All biopsies were bisected for subsequent DNA and RNA extraction and snap-frozen. From each adenoma, the DNA extracted from half the biopsy was analyzed for APC mutations. Biopsies with germline APC mutations along with the normal mucosa were subsequently analyzed for gene expression using the Affymetrix HG-U133A gene array with 14,500 human genes.

We identified 205 significantly up-regulated and 616 significantly down-regulated genes in the analysed adenomas (Table 1). These comprised 15 previously identified Wnt targets (IL-8, MYB, CCND1, MMP7, CD44, DKK3, EPHB3, MSX1, MYC, SOX9, EN1, EPHB2, ETS2, MET, MSX2). Remarkably, P-cadherin was the second highest up-regulated gene (after Defensin), present in an excess of 29-fold over normal colon mucosa. A member of the Frizzled family of proteins (FZD3), which transmit Wnt signals through interaction with β-catenin, was found to be up-regulated 12-fold. Among the most significantly down-regulated genes were several chemokine receptors and members of the complement pathway. A heat map with a scale representing the log_2-fold ratio for the 10 most up-regulated and down-regulated genes in the adenoma samples is shown in Fig. 1B; the transcripts and their fold-changes are summarized in Table 1.

Table 1. Summary of the 10 most up-regulated and down-regulated transcripts and their fold-change relative to the median value of the normal samples

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Log2 ratio (fold change up-regulated)</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Log2 ratio (fold change down-regulated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEFA6</td>
<td>Defensin</td>
<td>5.13 (35×)</td>
<td>DARC</td>
<td>Blood group-duffy system</td>
<td>−4.03 (16×)</td>
</tr>
<tr>
<td>CDH3</td>
<td>P-cadherin</td>
<td>4.84 (29×)</td>
<td>C3</td>
<td>Complement component 3</td>
<td>−4.11 (17×)</td>
</tr>
<tr>
<td>KIAA1199</td>
<td>Inner ear protein</td>
<td>4.57 (24×)</td>
<td>CR2</td>
<td>Complement component receptor 2</td>
<td>−4.12 (17×)</td>
</tr>
<tr>
<td>MSX2</td>
<td>Muscle segment homeobox 2</td>
<td>4.45 (22×)</td>
<td>MLY9</td>
<td>Myosin light chain 9</td>
<td>−4.30 (20×)</td>
</tr>
<tr>
<td>SLC01B3</td>
<td>Propionate transporter 1</td>
<td>4.21 (19×)</td>
<td>IGLV3-10</td>
<td>Immunoglobulin variable region</td>
<td>−4.32 (20×)</td>
</tr>
<tr>
<td>PCSK1</td>
<td>Proprotein convertase 1</td>
<td>3.96 (16×)</td>
<td>CXCL13</td>
<td>Chemokine ligand 13</td>
<td>−4.34 (20×)</td>
</tr>
<tr>
<td>TACSTD2</td>
<td>Tumor-associated Ca signal transducer 2</td>
<td>3.84 (14×)</td>
<td>CCL21</td>
<td>Chemokine ligand 21</td>
<td>−4.43 (22×)</td>
</tr>
<tr>
<td>LCN2</td>
<td>Lipocalin 2</td>
<td>3.39 (12×)</td>
<td>PYY</td>
<td>Peptide YY</td>
<td>−4.62 (24×)</td>
</tr>
<tr>
<td>XIST</td>
<td>X inactivation-specific transcript</td>
<td>3.38 (12×)</td>
<td>FBLN1</td>
<td>Fibulin 1</td>
<td>−4.63 (25×)</td>
</tr>
<tr>
<td>FZD3</td>
<td>Frizzled homologue 3</td>
<td>3.55 (12×)</td>
<td>CCL19</td>
<td>Chemokine ligand 19</td>
<td>−5.33 (40×)</td>
</tr>
</tbody>
</table>

11 J. Jankowski, unpublished observations.
the P-cadherin promoter. The upstream region of the P-cadherin gene has a GC content of 75% and contains 14 CpG dinucleotides grouped into three CpG islands. The third CpG island spans a 300-bp region proximal to exon 1 and harbors the CDH3 transcription initiation site.

We hence investigated the methylation pattern of the P-cadherin promoter in samples of sporadic CRC. This is the common form of CRC and can provide a more complete spectrum of samples in different stages of cancer development. In contrast to sporadic CRC, patients with FAP generally undergo prophylactic colonic resections before cancer, reducing the availability and diversity of samples across the adenoma-carcinoma sequence. To analyze the P-cadherin promoter methylation status, we used bisulfate DNA treatment followed by methylation-specific PCR for the CDH3 promoter (National Center for Biotechnology Information sequence accession no. X95824).

Methylation analysis was carried out on DNA isolated from sporadic CRC biopsies (n = 15), samples of cancer-adjacent, phenotypically normal colon mucosa (n = 20) and normal colon (n = 19), and samples of aberrant crypt foci (n = 15). Normal colon samples showed presence of both methylated and unmethylated P-cadherin promoter DNA (Fig. 2A), as did 16 of 20 samples of "normal" mucosa adjacent to CRC, suggesting an inactive (silenced) promoter. However, in the remaining four samples, only unmethylated PCR products were detected (Fig. 2B). Sequencing of the methylated PCR products revealed either partial or complete methylation of all 14 CpG dinucleotides. In contrast to the normal mucosa, all cancer samples showed the presence of only unmethylated DNA (Fig. 2C), indicating an active P-cadherin promoter. Likewise, the P-cadherin promoter was unmethylated in all of the ACF samples, indicating that this methylation change occurs early in the development of CRC (Fig. 2D).

To assess whether P-cadherin promoter methylation changes correlate with its transcription, the expression of P-cadherin mRNA was analyzed in the cancer and cancer-adjacent normal colon mucosa samples using reverse transcription–PCR (RT-PCR; Fig. 3). Integrity of mRNA was assessed by amplification of β-actin (Fig. 3A and B, bottom). Consistent with the CDH3 promoter methylation patterns, cancer-adjacent normal colon mucosa (with the exception of one sample) showed the absence of P-cadherin mRNA. The single sample expressing P-cadherin mRNA was one of the four samples that showed lack of promoter methylation and
P-cadherin expression (Figs. 1A and 2B). There was no detectable P-cadherin RNA in the remaining three unmethylated normal mucosa samples, giving an overall correlation coefficient of 0.84 (Pearson r; P < 0.0001) between promoter methylation status and mRNA expression in the tissue adjacent to cancer. All of the cancer samples fully correlated with the unmethylated status of the promoter and were found to express P-cadherin mRNA (Fig. 3B).

**Phenotypic effects of induced mP-cadherin expression in the mouse intestine in vivo.** To investigate the in vivo effects of ectopic P-cadherin expression alone, in the context of intact APC, we generated transgenic mice expressing P-cadherin in the intestine under the promoter for L-FABP, which is expressed only in the mouse intestine, colon, and liver. This promoter has been extensively used to induce foreign gene expression in the mouse intestine and colon (26). The plasmid construct, containing the mouse P-cadherin transgene (mP-cad) and L-FABP promoter, L-FABP/mP-cad, was introduced into wild-type mice by pronuclear injection (see Materials and Methods). The presence of the mP-cad

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Figure 4. Analysis of the L-FABP/mP-cad transgenic mice for the presence of the mP-cad transgene. A, transgene-specific PCR of the tail tissue lysate. The wild-type mice were negative for the mP-cad transgene (250 bp) and all of the homozygous transgenic mice show the presence of mP-cad. B, immunohistochemical labeling of intestinal tissue from wild-type (n = 4) and transgenic mice (n = 4). Magnification, 100×, 200×, and 400×. No transgene is detected in the small intestine of the wild-type mice (top left), whereas the intestines of homozygous mice specifically express mP-cad (top middle). Transgene mP-cad expression in the homozygous mice varied between animals, from medium intensity cytoplasmic to strong membranous. An intestinal crypt in fission is shown marked by a red asterisk (top right), as well as an example of membranous staining within a villus (bottom left). Primary antibody was omitted when staining mouse placenta as the negative control (bottom middle), whereas the positive control (bottom right) shows membranous and cytoplasmic expression of native P-cadherin in mouse placenta. C, demonstration of a functional P-cadherin transgene through Rac1/Cdc42 up-regulation and p120ctn translocation. Top row, wild-type mice (n = 4); bottom row, homozygous mice (n = 4) stained for Rac1/Cdc42 (left-hand panels) and p120ctn (right-hand panels) shown at 200× magnification. In the wild-type mice, Rac1/Cdc42 staining is low and correlates with membrane-bound p120ctn. In the presence of the mP-cad transgene (homozygous mice), up-regulated Rac1/Cdc42 expression is associated with translocation of p120ctn into the nucleus, as well as the cytoplasm.
transgene in the transgenic mice was confirmed by PCR of whole tail tissue lysate (Fig. 4A) in all of the homozygous mice (total of 251 of 505 genotyped mice). Tissue-specific expression of the P-cadherin transgene transcript in the homozygous mice was confirmed by RT-PCR and protein expression in the intestine and colon shown using immunohistochemistry (Fig. 4B). To confirm the functionality of the P-cadherin transgene, we investigated the events induced by P-cadherin overexpression, such as EGF/PDGF signaling evident through up-regulation of Rac1 and Cdc42 and p120ctn translocation from the membrane into the cytoplasm and nucleus (15) in four mice from each group. Wild-type mice showed very weak Rac1/Cdc42 expression and strictly membrane-associated p120ctn. In the intestines of homozygous mice, there was a notable up-regulation of Rac1/Cdc42 and translocation of p120ctn from the membrane into the cytoplasm and, in some regions, the nuclei. Particularly strong up-regulation of Rac1/Cdc42 coincided with the translocation of p120ctn into the nucleus (Fig. 4C). The heterozygous mice had regions of increased Rac1/Cdc42 staining and some cytoplasmic but no nuclear localization of p120ctn (data not shown).

The phenotypic effects of ectopic P-cadherin expression, specifically observing mucosal cell proliferation and intestinal crypt fission (as illustrated in Fig. 4B), were assessed microscopically in the wild-type and homozygous animals (n = 12 in each group). The degree of cell proliferation was evaluated as the number of cells in metaphase per crypt, and cell division and crypt fission were assessed by bromodeoxyuridine incorporation or proliferating cell nuclear antigen staining (29). A nonsignificant increase in the crypt fission rate was calculated as the number of dividing crypts per 200 crypts per animal, which has been shown as a method superior to the more traditional approaches using bromodeoxyuridine incorporation or proliferating cell nuclear antigen staining (29). A nonsignificant increase in the crypt fission was found in homozygous mice (uncorrected P = 0.053; Fig. 5B). Among the heterozygous mice, the rate of crypt fission occurred, again being intermediate at three of the analyzed sites along the small and large intestine (see Supplementary Fig. S2).

Mice homozygous for L-FABP/mP-cad showed a marginally significant trend of fewer dividing cells per crypt (P = 0.048), which decreased to P = 0.053 after correction for multiple comparisons. In the heterozygous mice, the number of cells in metaphase was intermediate of those observed in wild-type and homozygous mice, but there was no statistical significance. The intestinal crypt fission rate was calculated as the number of dividing crypts per 200 crypts per animal, which has been shown as a method superior to the more traditional approaches using bromodeoxyuridine incorporation or proliferating cell nuclear antigen staining (29). A nonsignificant increase in the crypt fission was found in homozygous mice (uncorrected P = 0.053; Fig. 5B). Among the heterozygous mice, the rate of crypt fission varied, again being intermediate at three of the analyzed sites along the small and large intestine (see Supplementary Fig. S2).

We have previously shown in the jejunum of transgenic mice that other genes regulating mucosal physiology may be biologically neutral unless epithelial integrity is compromised (30). We therefore investigated the phenotypic effects of the transgene in the context of proximal to middle gut intestinal inflammation. Animals analyzed: wild-type (WT, n = 8), transgenic homozygous (TG_hz, n = 6), WT + indomethacin (n = 9) and TG_hz + indomethacin (n = 7). C, number of dividing cells per crypt shows no significant difference between the wild-type and the homozygous animals with or without indomethacin injection. D, intestinal crypt division rate was higher in the proximal colon of the homozygous mice compared with the wild-type. Statistically significant difference was found between the wild-type and the homozygous animals treated with indomethacin in both terminal small intestine (*, P = 0.04) and proximal colon (**, P = 0.02).
L-FABP/mP-cad animals was significantly higher after indomethacin injection compared with wild-type mice (Fig. 5D; \( P = 0.04 \) for terminal small intestine and \( P = 0.02 \) for proximal colon after Bonferroni correction). Indomethacin injury alone had no effect on either cell division or crypt fission, as treated wild-type animals did not differ significantly from the untreated ones. There was a trend to decreased fission in wild-type animals following indomethacin. These results strongly suggest that ectopic expression of P-cadherin in the intestine, exacerbated by mucosal damage, can lead to increased crypt fission.

**Discussion**

The expression of P-cadherin within tissues is strictly programmed, due to its role in the development of normal stratified squamous epithelium (5). This study shows that in CRC the control of P-cadherin expression is disrupted through abnormal promoter methylation, induced gene transcription, and ectopic presence of P-cadherin from the earliest stages of carcinoma development. Increased P-cadherin expression was observed in both sporadic cancer and heritable colorectal adenomas, and gene expression analysis showed high up-regulation of P-cadherin in familial adenomatous polyposis. Forced expression of P-cadherin, accompanied by inflammation in the mouse intestine in vivo, resulted in an increased rate of intestinal crypt fission.

Overexpression through promoter hypomethylation of P-cadherin, a gene not classically associated with imprinting or a defined proto-oncogene, has been reported previously in a proportion of breast cancer cases (31). Furthermore, in inflamed premalignant gastrointestinal mucosa, such as Barrett’s metaplasia and ulcerative colitis, similar de novo expression of P-cadherin occurs early in carcinogenesis (32), implying that these changes could have similar epigenetic control. Our study shows that a CpG island in the P-cadherin (CDH3) promoter is methylated in the normal colon mucosa and demethylated in CRC. Importantly, we show an early disruption of normal P-cadherin promoter methylation within aberrant crypt foci, potential precursors of CRC, indicating a putative role of P-cadherin in the initiation of CRC. Hypomethylation of the P-cadherin promoter in CRC seems to be gene-specific and unlikely due to genome-wide demethylation, because P-cadherin maps adjacent to E-cadherin, whose promoter is hypermethylated in a proportion of CRCs (9).

We have previously reported early ectopic P-cadherin expression, both transient and sustained, in the reparative colon mucosa and hyperplastic crypts and benign adenomatous tumors (16, 17). This study shows that the aberrant expression of P-cadherin persists in sporadic CRC. We show that the ectopic expression of P-cadherin in the colon is not limited to the neoplastic region but can extend into the adjacent, phenotypically normal, colon mucosa. This finding resembles the previously described “field effect” phenomenon, which suggests that precancerous cells neighboring cancer have some, but not all, of the genetic alterations of the fully developed tumor (33, 34). This concept has been illustrated with hypermethylation in CRC (35) but has not been documented in the context of methylation loss until now. The observed field effect indicates that demethylation of P-cadherin might be an early event in the colorectal mucosa before any morphologic changes. It is possible that this early epigenetic change triggers downstream events, which, in an inflamed gut niche, can lead to bifurcation, clonal expansion, and ultimately neoplastic changes. One of the candidate downstream pathways could be the up-regulation of Rac1 and nuclear translocation of p120ctn, as observed in the mice homozygous for P-cadherin transgene. Up-regulation of Rac1 has been shown to induce nuclear localization of p120ctn (36), which has been associated with early stages of lobular breast cancer (37).

Crypt fission is the dominant mechanism of early adenoma development in the intestine, whereas top-down growth into adjacent crypts is prevalent in advanced dysplasia later in carcinogenesis (21, 38). Our present findings further support a potential role of P-cadherin in early adenoma formation and neoplastic development. The enhanced (albeit statistically non-significant) rate of intestinal crypt fission induced by the transgenic P-cadherin in our murine model was further increased after the injection of indomethacin. Indomethacin, given as a single injection, induces acute epithelial inflammation and mucosal injury, which reach maximum 1 day postinjection and resolve completely after 3 to 7 days (39). Our previous work shows up-regulation of P-cadherin in the colon mucosa after severe injury (radiation proctitis; ref. 17). In the current study, both injury and P-cadherin transgene maximally resulted in crypt fission. This might indicate that prior endogenous P-cadherin expression is required to maximally exploit the exogenous inflammatory stimuli through activation of downstream factors, the “fertile soil” theory (40) or that a threshold of P-cadherin level necessary for a significant increase in rate of crypt proliferation was reached by a subtle up-regulation of the native P-cadherin due to indomethacin-induced injury combined with the transgene expression.

In sporadic colorectal adenomas, ectopic P-cadherin is functional with respect to β-catenin binding and, importantly, expressed in the absence of disturbances in the APC gene (16), which are viewed as a key initiating event of malignant transformation in the colon (2, 41, 42). In addition, other members of the Wnt signaling pathway are known to play critical roles in colorectal tumorigenesis in the presence of wild-type APC and β-catenin (43, 44). Mutations in APC and β-catenin have been shown to give rise to CRC independently (45), and an APC-independent mode of β-catenin degradation has also been reported (46). Another report showed that increased β-catenin signaling can lead to up-regulation of P-cadherin expression (47), whereas a recent study further highlighted the complexities of the Wnt pathway by uncovering that one of its targets, C-myc oncogene, can rescue the perturbed cell differentiation and proliferation phenotype induced by APC loss in the mouse intestine (48). We show here that in vivo expression of P-cadherin, in the context of an intestinal inflammation and intact APC and β-catenin, is sufficient to trigger an increased rate of crypt proliferation. In this regard, P-cadherin might be implicated in governing the niche of stem cells, as has been shown for other cadherins at other epithelial sites (49, 50).

In conclusion, we show here that ectopic expression of P-cadherin in the mouse intestine can lead to increased crypt fission under conditions of intestinal damage and provide another example of gene promoter hypomethylation associated with ectopic protein expression very early in CRC development.

**Disclosure of Potential Conflicts of Interest**

N.A. Wright: employment, Takeda; commercial research grant, AstraZeneca. J.A.Z. Junkowska: employment and commercial research grant, AstraZeneca; commercial research grant, Cancer Research UK; commercial research support, Pfizer and AstraZeneca; honoraria from speakers bureau, Ferring and AstraZeneca; consultant/
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**References**


Ectopic Expression of P-Cadherin Correlates with Promoter Hypomethylation Early in Colorectal Carcinogenesis and Enhanced Intestinal Crypt Fission In vivo

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