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# Alteration of Gene Expression in Normal-Appearing Colon Mucosa of *APC*$^{min}$ Mice and Human Cancer Patients

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## ABSTRACT

**The expression of many genes is altered in colon cancer, but the roles of these genes in carcinogenesis are unclear. Using real-time quantitative PCR, we demonstrated that several genes previously implicated in human colon cancer undergo altered expression in the *APC*$^{min}$ mouse adenomatous polyp, a precursor of cancer, as well as in normal-appearing surrounding mucosa. The five genes that were most highly up-regulated in mouse polyp were also significantly up-regulated in polyp-free colon mucosa. Similar changes occurred in morphologically normal mucosa of surgical sections taken from human cancer patients, frequently extending to the margins. Thus, morphologically normal colon mucosa in *APC*$^{min}$ mice and in human cancer patients is not metabolically normal. Altered gene expression in this tissue does not appear to result from a field effect because there was no correlation between extent of altered regulation and distance from polyp or tumor. Our data suggest that alterations of expression levels of these genes may be an early event in carcinogenesis and a marker of risk for the development of colon cancer.**

## INTRODUCTION

Colon cancer is thought to result from a series of mutations and other genetic derangements that result in pathological changes in key metabolic pathways within the cell. Large-scale screening of gene expression profiles of colon cancers, using such methods as cDNA arrays or reverse transcription-PCR, have identified many of these alterations (1–6). However, screening of advanced carcinomas cannot distinguish changes in gene expression that are critical to the process of carcinogenesis from those that result from the progressive derangement of the genome and accompanying disruption of large metabolic networks.

One approach to identifying the essential metabolic events targeted in cancer is to manipulate certain genes in colon cancer cell lines and determine the effects on proliferation, invasiveness, and other properties of these cells (7, 8). However, the success of this approach depends on selecting, from the potentially large number of candidates identified by large-scale screening, a relatively small number of promising genes. Moreover, altered expression of a particular gene may have effects in a tumor cell line without being involved in carcinogenesis *in vivo*. An alternative approach is to identify metabolic events that are altered early in carcinogenesis by screening precancerous tissue (1, 9, 10). Most colon cancers begin as an adenomatous polyp, which may progress to a carcinoma as a result of the accumulation of genetic alterations. Therefore, screening at this stage may provide greater insight into cancer pathogenesis.

The *APC*$^{min}$ mouse carries a mutation in the adenomatous polyposis coli (*APC*) gene. As in humans, this mutation leads to the early development of intestinal adenomas that can progress to locally invasive carcinomas (11). This makes it possible to screen morphologically normal tissue from these animals for the expression of genes that may be involved in carcinogenesis, with colon tissue from wild-type animals providing a control.

We hypothesized that some of the metabolic alterations observed in colon carcinomas occur early in carcinogenesis, *i.e.,* before morphological alterations are apparent. To test this hypothesis, we screened a panel of genes to determine whether some have altered expression levels at earlier stages of carcinogenesis. We screened a panel of 15 genes that are altered (up- or down-regulated) in the late stages of human colon cancers. These genes function in several pathways related to cancer development, including the APC/β-catenin pathway, the nuclear factor-κB pathway, cell cycle, and inflammation, and therefore may represent the much larger set of genes that are altered in colon cancer.

We report here that we identified several candidate genes that have altered expression levels in adenomatous polyps as well as in morphologically normal colon mucosa from *APC*$^{min}$ mice. We also found altered expression levels of some of these same genes in apparently normal mucosa from human colon cancer patients. Our findings suggest that altered expression of these genes may be a useful indicator of the presence of colon cancer and may aid in identifying patients at risk of developing cancer.

## MATERIALS AND METHODS

**Animals.** C57BL/6J-min/+ mice and their wild-type littermates were obtained from The Jackson Laboratory (Bar Harbor, ME). The *APC* genotype was confirmed by use of primers specific for the mutated *APC* gene.

**Human Subjects.** Samples of colon cancer and adjacent grossly normal-appearing tissue were obtained at the time of surgery from patients undergoing colon surgical resection at California Pacific Medical Center (San Francisco, CA). We also obtained biopsies of grossly normal-appearing colon tissue from patients with no adenomatous polyps and no known family history or previous colon cancer who had submitted to routine colonoscopic examination. Patients ranged in age from 50–83 years and included both males and females. The research protocols for removal of both surgical samples and normal biopsies were approved by the California Pacific Medical Center Institutional Review Board. The appropriate procedure for obtaining informed consent was followed for all individuals participating in these studies.

**Preparation of Mouse Colonic Mucosal Cells.** The entire colon was removed from the *APC*$^{min}$ and wild-type mice, opened longitudinally, and washed extensively in cold PBS. Any visible adenomatous polyps were removed completely and analyzed. The colon was then divided into six equal segments from the proximal to the distal end. Colonic mucosa samples were isolated from each segment by 2 min of vigorous vortexing of the segment in 1 ml of cold PBS and centrifugation at $40 \times g$ for 5 min.

**Preparation of Human Colonic Mucosal Cells.** Colon cancer and adjacent grossly normal-appearing mucosa (4–20 samples/patient) were obtained from patients at the time of segmental resection to remove colon cancer. Control samples consisted of biopsies (six to eight from the rectosigmoid and three to four from the ascending colon per patient) obtained in the course of colonoscopic examination of patients with no personal or family history of colon cancer and with no polyps. For the surgical samples, the easily separable mucosal layer from multiple sites of the adjacent normal tissue was lifted and dissected from the submucosal layer. All samples were snap-frozen on dry ice as soon as possible within 30 min of surgery, or 1 min of biopsy, and then were taken immediately to the laboratory for RNA preparation (see below).

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**Extraction and Preparation of RNA.** Total RNA was extracted from the individual adenomatous polyps and the isolated normal mucosal cells with use of RNAeasy kits from Qiagen (Valencia, CA). RNA samples were treated with RNase-free DNase to remove any genomic DNA contamination and were reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA) with oligo(dT) and random primers (Invitrogen). Fifty ng of cDNA from each sample were used as template for PCR amplification with specific oligonucleotide primers in the Applied Biosystems 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). PCR reactions were performed according to the manufacturer's instructions, using the SYBR Green PCR Core Kit (PE Applied Biosystems). The identities of the PCR products were confirmed by melting temperatures and dissociation curves.

**Analysis of Gene Expression.** We analyzed 15 genes, all of which have previously been shown to be altered in expression in human colon cancer. They fall into four groups, including those involved in the (*a*) APC/β-catenin pathway, including c-myc, cyclin D1, and proliferating peroxisome activating receptor-α (PPARα; Refs. 12, 13); (*b*) nuclear factor-κB/inflammation pathway, including growth-related oncogene-α (Gro-α), osteopontin (OPN), macrophage-colony-stimulating factor (MCSF-1; Ref. 9), cyclooxygenase-1 (COX-1) and -2 (COX-2), Gro-γ [or its mouse homolog, macrophage inflammatory protein-2 (MIP-2)], interleukin-8 [IL-8; or its mouse homolog, stroma-derived factor (SDF-1)], and CXC cytokine receptor 2 (CXCR2); (*c*) cell cycle/transcription factors, including $p21^{cip/waf1}$, cyclin D1, c-myc, and PPARα, -δ, and -γ (1, 14); and (*d*) cell communication signals, including IL-8, PPARα and -γ, CXCR2, CD44, and OPN. Most of these genes have been reported to be up-regulated in human colon cancers, although some, such as $p21^{cip/waf1}$, are down-regulated.

Specific primers against each gene were designed with the Primer Express Software (PE Applied Biosystems). Primer length was 21–27 nucleotides, with a theoretical melting temperature of 58–60°C. Sizes of amplicons ranged from 66 to 150 bp. Primers were designed to amplify only cDNA template but not genomic DNA template whenever possible. The specificities of the primers used were demonstrated by the appearance of a single product on 10% PAGE and a single dissociation curve of the PCR product.

All of the cDNA samples were tested for genomic DNA contamination by use of primers for β-actin genomic DNA. With these primers, PCR products derived from the genomic DNA have a different melting temperature and length from the PCR product derived from cDNA. Only cDNA samples without genomic DNA contamination were used.

For quantitation of gene expression, the fluorescence of the SYBR Green dye bound to the PCR products was measured after each cycle, and the cycle numbers were recorded when the accumulated signals crossed an arbitrary threshold ($C_T$ value). To normalize this value, a $\Delta C_T$ value was determined as the difference between the $C_T$ value for each gene and the $C_T$ value for β-actin or histidyl tRNA synthetase (his-tRNA synthetase; Ref. 3). All PCR reactions were performed in duplicate when cDNA samples were available. The average $\Delta C_T$ values were used in the following analysis. In addition, PCR for β-actin or his-tRNA synthetase were repeated in each experiment as references and were shown not to vary significantly under the different experimental conditions used in this study. For each gene, a $\Delta\Delta C_T$ value was determined as the difference between the $\Delta C_T$ value for each individual sample and the average $\Delta C_T$ value for this gene obtained from the control samples. These $\Delta\Delta C_T$ values were then used to calculate relative gene expression values as described. (Applied Biosystems; User Bulletin 2; December 11, 1997). Because we had limited amounts of RNA from human biopsies, his-tRNA synthetase was assayed only in approximately half of the samples. The results indicated that expression of both β-actin and his-tRNA synthetase did not vary between the cancer patient group and the control group. Therefore, calculations of $\Delta\Delta C_T$ and relative gene expression values yielded similar results when we used either β-actin or his-tRNA synthetase as reference. The results of statistical analyses were obtained with β-actin as the reference.

**COX-2 Activity Assay.** Colonic mucosal cells were prepared as described above. The cells were homogenized in 0.1 M Tris-HCl (pH 7.8) in the presence of 1 mM EDTA. COX activity was assayed with the COX activity assay kit from Cayman Chemical (Ann Arbor, MI), which measures the peroxidase activity component of the COXs by colorimetric monitoring of oxidized *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine at 590 nm. The COX-1-specific inhibitor was included in the assay to obtain COX-2 specific activity. Assays were carried out in duplicate with 200 μg of protein.

**Immunohistochemical Analysis.** Five-μm sections of formalin-fixed, paraffin-embedded mouse colonic tissues were analyzed by immunostaining according to previously published methods (15). After deparaffinization and antigen retrieval with 10 mM citrate buffer (pH 6.0), sections were blocked in 1% normal sheep serum for 1 h and then incubated for 60 min at room temperature with the following antisera: 1:600 dilution of rabbit antimouse COX-2 antiserum from Cayman Chemical; 1:300 dilution of rabbit antimouse CXCR2 (IL-8RB); 1:300 dilution of goat antimouse OPN antiserum; or 1:300 dilution of goat antimouse Gro-α antiserum from Santa Cruz Biotechnology (Santa Cruz, CA). After washing, slides were incubated with biotinylated goat antirabbit or rabbit antigoat secondary antibody at a 1:300 dilution (DAKO, Carpenteria, CA), followed by streptavidin-horseradish peroxidase at a 1:300 dilution (Amersham, Arlington, IL). Color was developed with a 3,3′-diaminobenzidine tetrahydrochloride peroxidase substrate kit (Vector Labs, Burlingame, CA). Sections were counterstained with Mayer's hematoxylin and mounted with Permount (Fisher Scientific, Santa Clara, CA).

**Statistical Analysis.** In this study, expression patterns of several genes were compared between $APC^{min}$ mice/cancer patients *versus* controls. Rather than testing the expression of each gene separately and adjusting for multiple comparisons by methods that reduce statistical power, we tested expression patterns of all genes by multivariate ANOVA, a global test that accounts for correlations among expression levels. If the global test was significant, indicating that there was evidence that the overall expression patterns differed, we used univariate *t* tests to determine which genes were contributing to the global difference. All multivariate ANOVA tests were based on Wilks' λ criterion and were carried out on log(base 2) values for the expression because this transformation was required to achieve normal distribution of values.

## RESULTS

**Altered Gene Expression in Adenomatous Polyps from $APC^{min}$ Mice.** $APC^{min}$ mice (ages 6–23 weeks) had polyps ranging from 1 to 4 mm in both the colon and small intestine. Typically, ≥30 polyps were present in the small intestine and 0–3 in the colon of each mouse. In general, more polyps were found in older mice than younger ones. A total of 14 colonic polyps from eight mice were analyzed. All were adenomatous polyps. These were classified as low-grade dysplastic based on glandular architecture; nuclear hyperchromasia, stratification, and pleomorphism; and cytoplasmic mucus content. Table 1 lists the relative expression levels of all genes that were examined in these polyps relative to expression in colon mucosa from wild-type mice. A wide range of expression levels was observed;

Table 1 *Relative gene expression levels in colon polyps of $APC^{min}$ mice (mean ± SE)*

Gene expression levels were determined as described in the "Materials and Methods." In nos. 1~5, $n = 13$ in the wild-type littermate group, and $n = 14$ in the individual polyp group; in nos. 6~15, $n = 6$ in the wild-type littermate group, and $n = 10$ in the individual polyp group. Significance was determined by *t* test.

| No. | Gene | Wild-type littermate | Individual polyp | *P* |
|---|---|---|---|---|
| 1 | OPN[a] | 1.62 ± 0.60 | 430 ± 125 | <0.01 |
| 2 | MIP-2 | 1.74 ± 1.60 | 203 ± 43 | <0.001 |
| 3 | Gro-α | 1.40 ± 0.32 | 122 ± 19 | <0.001 |
| 4 | CXCR2 | 1.41 ± 0.35 | 105 ± 23 | <0.001 |
| 5 | COX-2 | 1.41 ± 0.25 | 82 ± 16 | <0.001 |
| 6 | Cyclin D1 | 1.34 ± 0.34 | 19 ± 3 | <0.001 |
| 7 | SDF-1 | 1.23 ± 0.34 | 11 ± 2 | <0.01 |
| 8 | c-myc | 1.09 ± 0.18 | 6.49 ± 0.96 | <0.001 |
| 9 | MCSF-1 | 1.05 ± 0.15 | 4.26 ± 1.60 | NS |
| 10 | CD44V6 | 1.17 ± 0.28 | 3.78 ± 0.61 | <0.01 |
| 11 | COX-1 | 1.07 ± 0.15 | 3.24 ± 0.60 | <0.01 |
| 12 | PPAR-γ | 1.13 ± 0.22 | 0.86 ± 0.24 | NS |
| 13 | $p21^{cip/waf1}$ | 1.11 ± 0.17 | 0.51 ± 0.07 | <0.05 |
| 14 | PPAR-δ | 1.16 ± 0.27 | 0.44 ± 0.05 | <0.05 |
| 15 | PPAR-α | 1.04 ± 0.12 | 0.17 ± 0.03 | <0.001 |

[a] OPN, osteopontin; MIP-2, macrophage inflammatory protein-2; Gro-α, growth-related oncogene-α; CXCR2, CXC cytokine receptor 2; COX-1 and -2, cyclooxygenase-1 and -2; SDF-1, stroma-derived factor-1; MCSF-1, macrophage-colony-stimulating factor; NS, not significant; PPAR-α, -δ, and -γ, proliferating peroxisome-activating receptor-α, -δ, and -γ.



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several genes were up-regulated markedly, whereas others were down-regulated. Five genes—COX-2, GRO-α, CXCR2, OPN, and MIP-2, had a particularly high degree of altered expression in the polyps. All of these genes are up-regulated in human colon cancer or other cancers, although not to such a high degree (1, 8, 16, 17).

**Altered Gene Expression in Morphologically Normal Colon of *APC^min^* Mice.** We next analyzed the expression of these five genes in morphologically normal colon tissue from *APC^min^* mice at three different ages—6, 13, and 23 weeks—compared with normal colon tissue from wild-type littermates. After we removed all of the polyps from the *APC^min^* mice, we divided the polyp-free colons of the *APC^min^* mice and the age-matched wild-type littermates into six equal segments ∼1.5 cm in length. Colonic mucosa was isolated as described in "Materials and Methods," and the expression of the five genes was analyzed.

In Fig. 1, each point represents the level of expression of a single gene in the mucosa of a single colon segment of a single mouse. Although the expression levels by segment and age showed little variability among wild-type animals, there was considerable variation among the *APC^min^* mice. To determine the significance of the differences between these latter values and those for wild-type animals, we applied multivariate analysis on the data presented in Fig. 1. In each age group, some genes were significantly up-regulated in at least some segments in *APC^min^* mice ($P < 0.01$). As shown in Table 2, all of these genes except OPN were significantly up-regulated in at least some segments of 23-week-old *APC^min^* mice, particularly in segment 6.

The distribution of these metabolic alterations was not correlated with the presence of polyps. As shown in Fig. 1*C*, in the 23-week-old *APC^min^* mice, a total of four polyps were detected in segments 1 and 2, one in segment 5, and none in the other segments; however, the greatest degree of up-regulation for most of these genes was observed in segment 6. This finding suggests that the observed changes in gene expression were not the result of a field effect caused by escape of altered cells from the polyps, but were intrinsic to the morphologically normal cells of the colon where they were detected.

**Expression of COX-2 Protein Was Elevated in Grossly Normal Colon Mucosa of *APC^min^* Mice.** To determine whether the up-regulation of RNA expression in the morphologically normal colon mucosa of *APC^min^* mice also resulted in protein overexpression, we assayed COX-2 activity in the grossly normal mucosa from six colonic segments in both *APC^min^* mice and the wild-type littermates. As illustrated in Fig. 2, COX-2 activity was elevated 5–8-fold in the grossly normal mucosa of 23-week-old *APC^min^* mice ($P < 0.05$ for segments 1, 2, 5, and 6, Mann–Whitney rank-order test).

To determine the cellular locations of the up-regulation of these genes, we performed immunohistochemical analysis of Gro-α, OPN, CXCR2, and COX-2 in the grossly normal colon mucosa from segments 1, 3, and 5 of two 23-week-old *APC^min^* mice and two age-matched wild-type mice. A total of nine aberrant crypt foci (ACF) were found in the samples from *APC^min^* mice (three in segment 1, one in segment 3, and five in segments 5 of the two *APC^min^* mice). No ACF were found in the samples from wild-type mice. Fig. 3 shows representative immunostaining results for COX-2. We did not detect COX-2 staining in the mucosa of wild-type mice (Fig. 3*A*). However, in adenomatous polyps from *APC^min^* mice, we observed strong staining in the macrophages and moderate staining in the epithelial cells (Fig. 3*D*). Macroscopically normal mucosa of *APC^min^* mice are shown in Fig. 3, *B* and *C*. Notably, COX-2 expression was prominent in macrophages from *APC^min^* mice (Fig. 3*B*). Weak COX-2 staining was observed in the epithelial cells of all of the ACF from *APC^min^* mice (Fig. 3*C*). However, in all samples analyzed, the epithelial cells of the normal crypt foci from *APC^min^* mice exhibited very weak or unde-

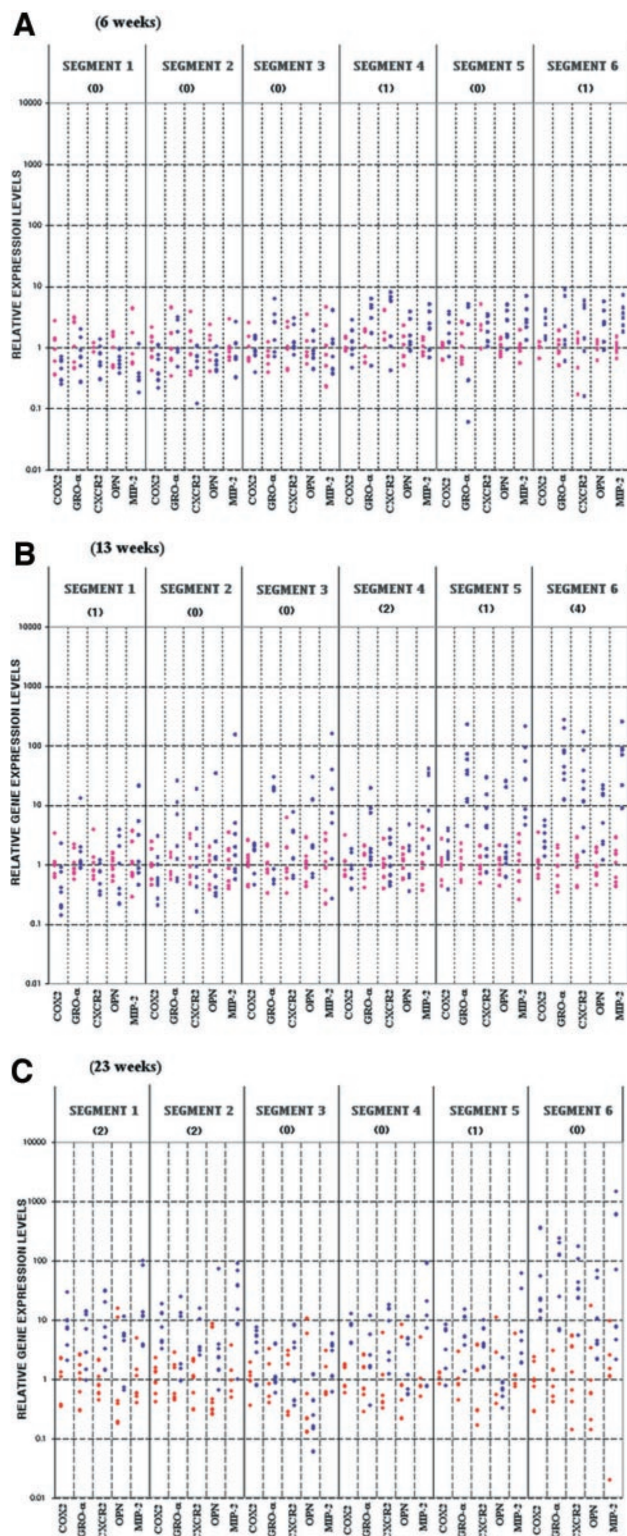


Fig. 1. Altered gene expression levels in segments of *APC^min^* mouse colon. *APC^min^* mice were sacrificed at 6 (*A*), 13 (*B*), and 23 (*C*) weeks of age ($n = 6–8$ at each age), and the colons were removed and divided into six segments from the most proximal (*segment 1*) portion to the rectum (*segment 6*). Mucosal tissue was removed from each segment, and total RNA was extracted from each sample. Expression levels of the indicated genes were determined by reverse transcription-PCR with specific primers for each gene, as described in the "Materials and Methods," and compared with a β-actin control. Relative expression levels were obtained by comparing the changes in threshold cycle values for each gene with the mean value of that gene for all wild-type animals ($n = 6–8$). Each *dot* represents the relative expression level of one gene from one wild-type (*red dots*) or *APC^min^* (*blue dot*) mouse. The total number of polyps found in all of the mice of a particular age for each segment is shown in *parentheses* at the *top* of the figure. *COX2*, cyclooxygenase-2; *GRO-α*, growth-related oncogene-α; *CXCR2*, CXC cytokine receptor 2; *OPN*, osteopontin; *MIP-2*, macrophage inflammatory protein-2.

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Table 2 *Multivariate analysis of gene expression in normal-appearing colon mucosa of 23-week-old APC$^{min}$ mice compared with colon mucosa of wild-type mice*

Colons were removed from animals, and any polyps were removed. The colons were then divided into six segments, the colon mucosa was isolated, and gene expression was determined as described in the "Materials and Methods," with values for $APC^{min}$ mice compared with those for wild-type mice. Multivariate analysis was performed on these values as described in the "Materials and Methods," in which the significance of the difference in expression between $APC^{min}$ and wild-type mice was determined for each gene in the presence of all of the other genes. For this analysis, six mice were used for each group ($APC^{min}$ and wild-type), and one mucosa sample was analyzed per segment per mouse.

| | Colon segment[a] | | | | | |
|---|---|---|---|---|---|---|
| Gene | 1 | 2 | 3 | 4 | 5 | 6 |
| Cox-2[b] | ++ | ++++ | + | ++++ | + | +++ |
| CXCR2 | + | ++ | − | ++ | + | ++++ |
| MIP-2 | ++ | ++ | − | ++ | + | +++ |
| Gro-α | − | + | − | − | + | +++ |
| OPN | − | − | − | − | − | − |

[a] −, $P > 0.05$; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$; ++++, $P < 0.0001$.

[b] Cox-2, cyclooxygenase-2; CXCR2, CXC cytokine receptor-2; MIP-2, macrophage inflammatory protein-2; Gro-α, growth-related oncogene-α; OPN, osteopontin.

tectable Cox-2 staining (Fig. 3*B*). We were unable to demonstrate unequivocal staining for Gro-α, OPN, and CXCR2 (data not shown).

**Altered Gene Expression in Normal Colon Mucosa Adjacent to Carcinoma in Human Patients.** We next analyzed colon samples from human patients who had undergone surgery to remove colon carcinomas. Although all of the genes we analyzed in mice are differentially regulated (up- or down-regulated) in human colon cancers, previous studies have generally assumed that morphologically normal colon mucosa adjacent to the tumor is metabolically normal and have used such tissue as a baseline for comparison. Because of our findings with $APC^{min}$ mice, we were interested in determining whether this assumption is valid or whether there are altered gene expression profiles in morphologically normal colon mucosa from cancer patients. We therefore compared gene expression levels in morphologically normal-appearing colon mucosa from cancer patients with those in mucosa from patients without cancer. We analyzed two sets of data, one set consisting of samples from patients with cancer in the sigmoidal-rectal region, the other from patients with cancer in the ascending colon. In both studies, we examined expression levels of the same 15 genes that were analyzed in $APC^{min}$ mice, except for Gro-γ, the human analog of MIP-2 in mice, and IL-8, a close relative of SDF-1 in mice.

In both studies, the values obtained from the cancer patients were highly variable, much more so than the corresponding values from the controls (Table 3). This finding parallels the observations we made in $APC^{min}$ and wild-type mice, although the variation in humans was even higher. Despite the great variability, expression levels for several genes were much higher in some samples from cancer patients than for any samples from controls. For example, four of the genes that were significantly up-regulated in normal-appearing mucosa of $APC^{min}$ mice—CXCR2, GRO-α, COX-2, and OPN—were up-regulated in normal-appearing mucosa from some cancer patients to levels 50–200 times greater than those in controls. In addition, in some cancer patients, PPARα, -δ, and -γ were down-regulated 50–100-fold.

To evaluate the significance of these differences, we conducted a series of multivariate tests. In this analysis, seven genes were significantly up-regulated in morphologically normal mucosa from patients with sigmoidal-rectal cancer relative to controls: MCSF-1, OPN, IL-8, COX-2, CXCR2, p21, and CD44. Two genes—PPARδ and -γ—were significantly down-regulated (Table 3). Similar results were obtained for the ascending colon. Six of the seven genes significantly up-regulated in sigmoidal-rectal mucosa were also up-regulated in the ascending colon—MCSF-1, OPN, IL-8, COX-2, CXCR2, and CD44—along with COX-1. Likewise, PPARδ and -γ were significantly down-regulated in ascending colon (Table 3).

The difference between cancer patients and controls was even more striking when the relative expression levels of three of the most up-regulated genes—COX-2, OPN, and MCSF-1—were considered together. In Fig. 4, the log(base 2) of expression level of each of these three genes in each patient sample is plotted in a three-dimensional set of axes. In virtually every sample from a cancer patient, at least one of these three genes was significantly up-regulated relative to its expression level in any sample from a control. This analysis thus suggests that expression levels of these three genes considered together may be sufficient to distinguish normal colon mucosa in colon cancer patients from colon mucosa in controls.

**Altered Gene Expression in Normal Mucosa Adjacent to Carcinomas Is Not a Result of Field Effect.** The samples of normal-appearing mucosa from cancer patients were taken randomly from all areas of the surgical section. The distribution of samples taken from a single cancer patient is shown in Fig. 5, which indicates the approximate expression level in each sample of COX-2 and OPN. There was no correlation of expression level with distance from the cancer. Similar results were obtained with other, differently regulated genes (data not shown). These observations suggest that the differently regulated areas of gene expression in normal-appearing colon mucosa of cancer patients did not result from a field effect of cells spreading from the original cancer.

## DISCUSSION

The major goal of this study was to identify metabolic alterations that precede overt colon cancer and that might occur early in carci-

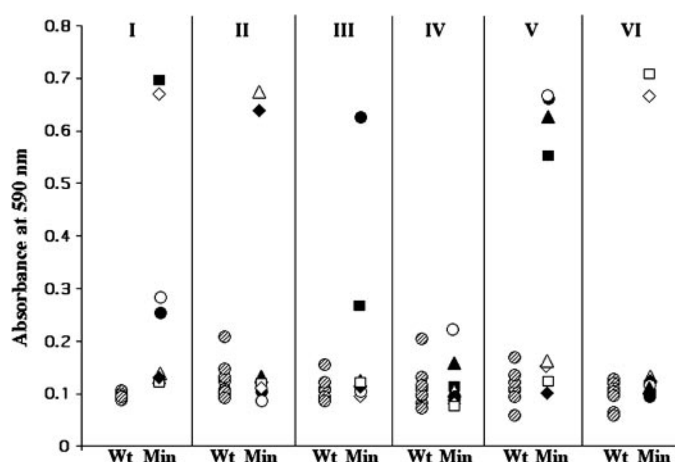


Fig. 2. Cyclooxygenase-2 enzymatic activity in the normal-appearing colonic mucosa of $APC^{min}$ mice. $APC^{min}$ mice (*Min*) and the wild-type littermates (*Wt*) were sacrificed at 23 weeks of age ($n = 8$ for each group). The *hatched circles* represent wild-type mice; the *other symbols* represent individual $APC^{min}$ mice. In $APC^{min}$ mice, the same symbol in different segments represents mucosa cells from the same $APC^{min}$ mouse. Colonic mucosa cells were obtained as described in "Materials and Methods." Cells were homogenized, and 200 μg of protein were used for each cyclooxygenase-2 enzymatic activity assay. The *Roman numerals* indicate the segment numbers.



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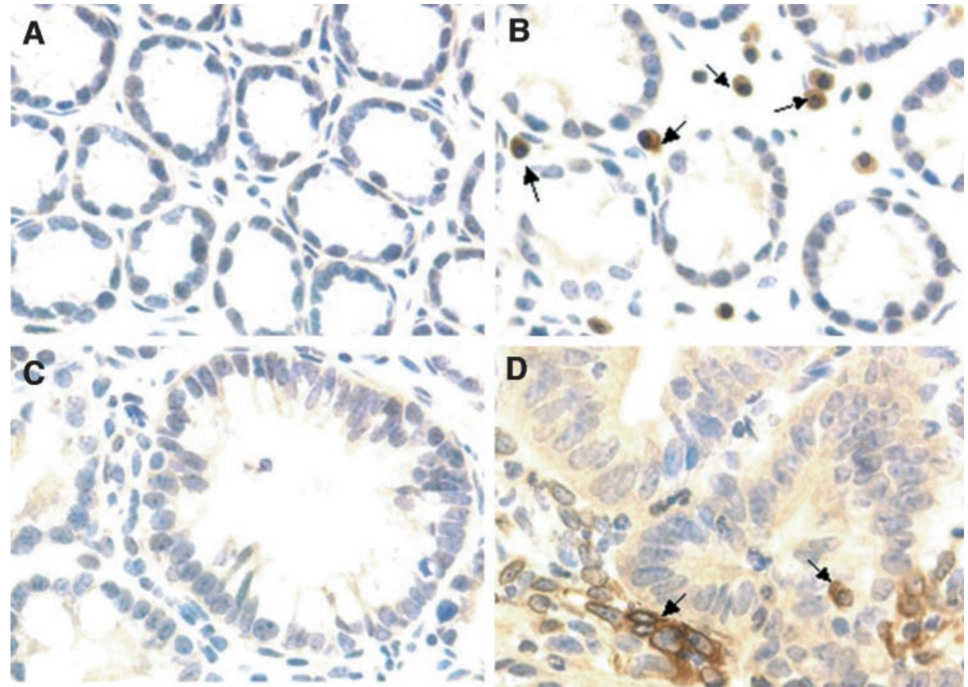


Fig. 3. Immunohistochemical analysis of cyclooxygenase-2 protein expression in grossly normal colonic mucosa of *APC^min^* mice. Representative sections of formalin-fixed, paraffin-embedded tissues from macroscopically normal colonic mucosa from a wild-type mouse (*A*) and a *APC^min^* mouse (*B* and *C*), and colonic adenomatous polyp tissue from a *APC^min^* mouse (*D*) were stained with anti-cyclooxygenase-2 antibody by the immunoperoxidase method. Nuclei were counterstained with Mayer's hematoxylin. *Arrows* in *B* and *D* indicate strongly stained macrophages. *Panel C* illustrates the presence of aberrant crypt foci in the macroscopically normal colonic mucosa of an *APC^min^* mouse. All photographs were taken at a ×40 magnification.

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nogenesis. Our aim was not to identify all possible alterations but to analyze a representative sample. Accordingly, we selected 15 genes from four groups of genes that are altered in human colon cancer (1, 2, 4–6, 8).

Most studies of gene expression in colon cancer have used as controls morphologically normal tissue from the same patient or group of patient (1, 3, 9). The assumption underlying this practice is that such tissue is also metabolically normal, with a gene expression profile identical to that of healthy colon mucosa. There are at least two reasons for questioning this assumption. First, in the *APC^min^* mouse (and in humans with familial adenomatous polyposis), multiple areas of the colon may develop polyps. Thus, before the appearance of polyps, any area in the colon may be in a precancerous state. Even in humans with no known hereditary risk for cancer, the emergence of multiple polyps or of a single cancer is associated with a greater risk for developing another cancer (18). Second, the formation of an adenomatous polyp or cancer may depend not only on genetic alterations within certain cells, but also on certain environmental events in the colon mucosa that may have a widespread distribution. Thus, one might expect that cancer would be associated with widespread metabolic changes in the colon.

**Differential Gene Expression in Adenomatous Polyps of *APC^min^* Mice.** Most of the genes we examined were regulated differently in polyps of *APC^min^* mice (Table 1). This is not surprising; human studies have reported differential regulation of hundreds of genes in adenomas as well as carcinomas of the colon (1, 9). However, the patterns of expression in the mice were somewhat different from those observed in human cancer. Most notably, several genes were up-regulated dramatically, at levels 80–400 times greater than in wild-type controls. Up-regulation to this degree has not been observed in human colon cancer. At least part of the difference may be attributable to use as a baseline colon mucosa from wild-type mice rather than from *APC^min^* mice, because expression of many genes was frequently higher in portions of the latter (Fig. 1).

These highly up-regulated genes have several functions. OPN, a ligand involved in cell signaling and communication, also affects inflammation and the response to cellular insults (19). Because of its role as a cell adhesion molecule, it is thought to facilitate metastasis. COX-2 is an inducible, rate-limiting enzyme that catalyzes the formation of prostaglandins from arachidonic acid. In addition to its involvement in the inflammation response, an increase in COX-2 expression is associated with many cancers, and COX-2 inhibitors are used as a cancer preventative treatment. Tumor growth has been reported to be decreased in double-knockout *APC^min^* mice in which COX-2 was also inactivated (20).

Of the other three gene products that demonstrated early up-regulation in *APC^min^* mice, two (MIP-2 and Gro-$\alpha$) are ligands for a third, the CXCR2 receptor. Gro-$\alpha$ is a known oncogene that has an autocrine action on CXCR2 to stimulate growth (21). The observation that three functionally related genes were all up-regulated raises the possibility that the initial event was up-regulation of one of these genes, followed by up-regulation of the others through compensatory mechanisms.

**Differential Gene Expression in Normal-Appearing Colon Mucosa of *APC^min^* Mice.** The five genes that were up-regulated to the highest degree in polyps—Gro-$\alpha$, OPN, MIP-2, CXCR2, and COX-2—were also up-regulated in polyp-free areas of the colons from these animals. In agreement with our reverse transcription-PCR results, we found that COX-2 activity was up-regulated in the grossly normal mucosa from *APC^min^* mice.

In mice that did have polyps, the location of the up-regulated areas was not correlated with the location of the polyps (Fig. 1); *i.e.,* the areas of greatest alteration of gene expression were just as likely to be distant from a polyp as close to it. This observation suggests that these areas of differential gene expression did not result from a field effect from a polyp but were independent loci. Because we did not examine this tissue under a microscope, we cannot rule out the possibility that it contained microscopic lesions such as ACF, which may have altered expression of certain genes (22). However, previous studies have reported that there are relatively few ACF in the colon of *APC^min^* mice (23, 24).

There are many cell types in the colonic mucosa, including epithelial cells, fibroblasts, and blood-borne cells. It is unlikely that the altered levels of gene expression that we observed can be attributed to

Table 3 *Gene expression levels in morphologically normal colon mucosa from human colon cancer patients as compared with noncancer subjects*

For Table 3A, colon mucosa samples were isolated from the sigmoidal-rectal region of noncancer subjects (78 samples from 12 individuals) and from the adjacent normal-appearing mucosa of patients with sigmoidal-rectal cancer (62 samples from 5 patients). For Table 3B, the colon mucosa samples were isolated from the ascending region of noncancer subjects (39 samples from 11 individuals) and from the adjacent normal-appearing mucosa of patients with ascending colon cancer (65 samples from 4 patients). Samples were analyzed for gene expression as described in the "Materials and Methods." Means ± SD are given for noncancer subjects, and ranges are given for cancer patients. Multivariate analysis was then performed on each gene taken in relation to all of the other genes to determine the significance of the difference between individuals with or without cancer.

| No. | Gene | Normal subjects (mean ± SD) | Cancer patients (range) | $P$ |
|---|---|---|---|---|
| A. Sigmoidal-rectal colon | | | | |
| 1 | CXCR2[a] | 1.30 ± 1.11 | 0.81–210 | <0.01 |
| 2 | Gro-α | 2.93 ± 6.93 | 0.78–105 | NS[b] |
| 3 | IL-8 | 2.25 ± 2.63 | 1.22–82 | 0.0001 |
| 4 | COX-2 | 1.80 ± 2.63 | 0.91–66 | 0.001 |
| 5 | OPN | 1.55 ± 2.04 | 0.94–58 | 0.0001 |
| 6 | Gro-γ | 1.92 ± 3.34 | 0.80–37 | NS |
| 7 | MCSF-1 | 1.54 ± 1.40 | 1.54–31 | 0.0001 |
| 8 | COX-1 | 1.22 ± 0.87 | 0.12–9.58 | NS |
| 9 | CD44 | 1.12 ± 0.56 | 0.54–6.52 | <0.05 |
| 10 | c-myc | 1.24 ± 0.82 | 0.12–4.76 | NS |
| 11 | Cyclin D | 1.28 ± 0.84 | 0.43–4.44 | NS |
| 12 | PPAR-α | 1.10 ± 0.62 | 0.02–2.87 | NS |
| 13 | PPAR-δ | 1.15 ± 0.55 | 0.02–1.90 | <0.01 |
| 14 | p21 | 1.04 ± 0.29 | 0.40–1.68 | <0.01 |
| 15 | PPAR-γ | 1.07 ± 0.40 | 0.01–1.28 | <0.01 |
| B. Ascending colon | | | | |
| 1 | CXCR2 | 1.32 ± 1.08 | 1.90–90 | <0.05 |
| 2 | Gro-α | 1.60 ± 2.08 | 0.46–30 | NS |
| 3 | IL-8 | 1.66 ± 1.62 | 1.32–183 | <0.05 |
| 4 | COX-2 | 1.84 ± 3.04 | 2.96–153 | 0.0001 |
| 5 | OPN | 1.53 ± 1.31 | 9.24–153 | 0.0001 |
| 6 | Gro-γ | 1.40 ± 1.41 | 0.63–11 | NS |
| 7 | MCSF-1 | 1.68 ± 1.62 | 4.01–40 | 0.0001 |
| 8 | COX-1 | 1.17 ± 0.75 | 0.84–45 | <0.001 |
| 9 | CD44 | 1.11 ± 0.51 | 0.99–14 | 0.0001 |
| 10 | c-myc | 1.16 ± 0.63 | 0.39–10.82 | NS |
| 11 | Cyclin D | 1.38 ± 1.08 | 0.12–13.15 | NS |
| 12 | PPAR-α | 1.16 ± 0.58 | 0.22–4.09 | NS |
| 13 | PPAR-δ | 1.13 ± 0.55 | 0.02–7.08 | <0.05 |
| 14 | p21 | 1.09 ± 0.40 | 0.04–2.66 | NS |
| 15 | PPAR-γ | 1.08 ± 0.42 | 0.01–1.14 | 0.01 |

[a] CXCR2, CXC cytokine receptor 2; Gro-α and -γ, growth-related oncogene-α and -γ; IL-8, interleukin-8; COX-1 and -2, cyclooxygenase-1 and -2; OPN, osteopontin; MCSF-1, macrophage-colony-stimulating factor; PPAR-α, -δ, and -γ, proliferating peroxisome-activating receptor-α, -δ, and -γ.

[b] NS, not significant at $P < 0.05$ level.

alterations localized to ACF alone. Indeed, COX-2 may not be expressed in the colonic epithelial cells; it is expressed in interstitial cells at an early stage (25). There are some reports of expression of COX-2 in stromal macrophages in tumor tissues (26–28). Elevated expression levels of COX-2 were also demonstrated in macrophages in the lamina propria of histologically normal epithelium of $APC^{min}$ mice but not in the mucosa of wild-type mice (26). In agreement with the literature, we also observed prominent expression of COX-2 in macrophages but only weak COX-2 staining in epithelial cells of ACF. Mucin secreted by colon cancer cells can induce the expression of COX-2 in macrophages located in the surrounding area, and it has been suggested that activated macrophages may provide a favorable microenvironment for epithelial cell growth (28).

We were unable to detect immunohistochemical staining of Gro-α and OPN, perhaps because both are secreted, unstable proteins and therefore evanescent in tissue sections. In addition, the $C_T$ values obtained from quantitative reverse transcription-PCR indicates that the absolute expression level of CXCR2 was low, which may explain why we could not demonstrate CXCR2 staining. If the absolute RNA quantities are sufficient, RNA *in situ* hybridization may be a better method to determine the cellular locations of up-regulation of these genes. Regardless of the cell types responsible for the overexpression of these genes, our results demonstrate that the RNA expression level is a more sensitive indicator of abnormal colon mucosa than cytological or architectural changes.

**Differential Gene Expression in Normal-Appearing Colon Mucosa of Human Cancer Patients.** As with $APC^{min}$ mice, several of the same genes were significantly up-regulated, and several other genes were significantly down-regulated, in the morphologically normal mucosa from patients with colon cancer (Table 3). These alterations were not restricted to certain regions of colon mucosa because they occurred in patients with ascending colon carcinomas or sigmoidal-rectal carcinomas and most of the genes involved were similar in the two regions. However, there was considerable variability in expression level from one area of the colon to another, and these differences did not correlate with distance from the tumor. Thus, as with the mice, these metabolic alterations in normal-appearing colon mucosa are apparently not the consequence of a field effect.

**Are Metabolic Disturbances Related to Carcinogenesis?** Our findings in $APC^{min}$ mice and human colon cancer patients raise the

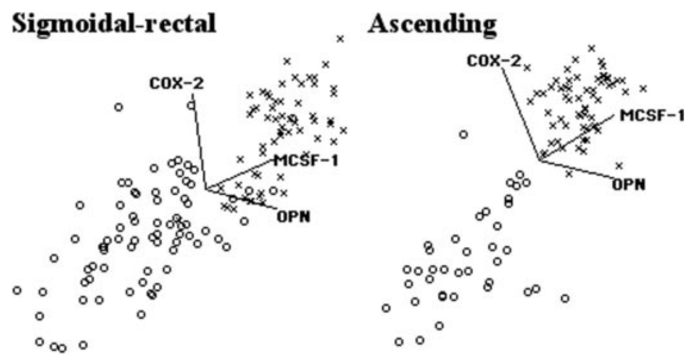


Fig. 4. Three-gene expression analysis of normal-appearing colon mucosa in patients with cancer in sigmoidal-rectal colon and ascending colon. Mucosa samples were taken from each colon cancer patient (see Table 3 for number of patients and number of samples/patient). Biopsies were also taken from noncancer patients (per subject from sigmoidal-rectal area and per subject from ascending colon). Each sample was analyzed for expression levels of cyclooxygenase-2 (*COX-2*), osteopontin (*OPN*), and macrophage-colony-stimulating factor (*MCSF-1*), and the values are plotted on three perpendicular axes. **X**, values for a cancer patient; ○, values for a noncancer patient. *Left*, sigmoidal-rectal colon; *right*, ascending colon.

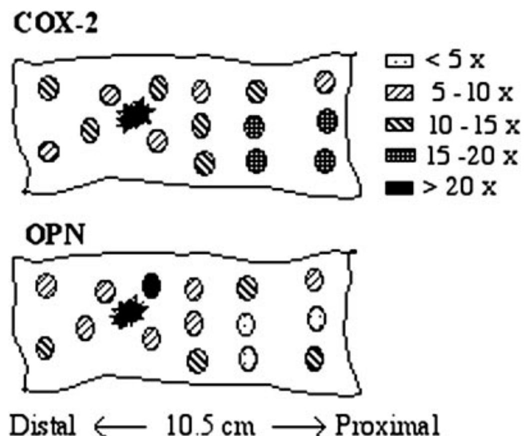


Fig. 5. Distribution of cyclooxygenase-2 (*COX-2*) and osteopontin (*OPN*) expression in sigmoidal-rectal colon of a single cancer patient. The patient had a cancer in the sigmoidal-rectal colon, as indicated by the *spot* with *jagged edges*. Locations of mucosa samples removed for analysis are indicated by the *stippled*, *hatched* and *meshed circles*. Levels of COX-2 and OPN expression in each sample were determined by reverse transcription-PCR and are indicated approximately by various patterns as shown. The mean levels of expression of COX-2 and OPN in colon mucosa of non-cancer patients were 1.80 and 1.55, respectively (as shown in Table 3).

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question of what defines a "normal" colon. Although expression levels of many of the genes we examined were much higher in polyps and cancer than apparently normal mucosa in surrounding tissue, expression levels in the apparently normal mucosa were significantly higher than those in controls, indicating that this surrounding tissue is not metabolically normal. Use of such tissue as a control or baseline in gene expression studies for carcinoma is likely to underestimate the degree of altered regulation of certain genes.

What is the meaning of these metabolic alterations in morphologically normal tissue? Given that these alterations are more marked in polyps and cancer, we speculate that these changes may indicate a predisposition to develop cancer. For example, overexpression of COX-2 in mice increased the likelihood that cells would be transformed after exposure to mutagens (29). Thus, our observation of gene up-regulation in morphologically normal colon mucosa suggests that these cells may be abnormally sensitive to events that do not affect metabolically normal mucosa.

Some of the genes up-regulated in the normal mucosa from cancer patients are involved in inflammation. These include not only COX-2, but also cytokines and chemokines such as Gro-$\alpha$, MCSF-1, IL-8, and MIP-2. Many cancers arise from sites of infection, chronic irritation, or inflammation (30), and COX-2 inhibition may be a useful chemopreventive agent for colon cancer (31). The other genes we have identified that have altered expression before adenomatous polyp formation may also be useful targets for cancer prevention as well as early markers for cancer screening and diagnosis.

Regardless of the underlying mechanisms, our finding of metabolic changes in normal-appearing colon mucosa of cancer patients suggests that analysis of this tissue, through a rectal smear test or lavage, for example, might be used as a simple, noninvasive screening test for colon cancer. Although a significantly higher or lower level of expression of a single gene might not be a very reliable indicator, our multivariate analysis suggests that analysis of a group of genes might provide a much better indicator of increased risk.

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