Expression of PPARδ in multistage carcinogenesis of the colorectum: implications of malignant cancer morphology

O Takayama1, H Yamamoto*,1, B Damdinsuren1, Y Sugita1, CY Ngan1, X Xu1, T Tsujino1, I Takemasa1, M Ikeda1, M Sekimoto1, N Matsuura2 and M Monden1

1Department of Surgery, Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita-City, Osaka 565-0871, Japan; 2Department of Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University, Osaka 565-0871, Japan

Whether peroxisome proliferator-activated receptor (PPAR) δ is a good target for the chemoprevention and/or treatment of colorectal cancer (CRC) remains controversial. Our goal was to examine PPARδ expression in multistage carcinogenesis of the colorectum and to assess the relevance of PPARδ in CRC. Immunohistochemical analysis indicated that PPARδ expression increased from normal mucosa to adenomatous polyps to CRC. In cancer tissues, the PPARδ protein was accumulated only in those cancer cells with highly malignant morphology, as represented by a large-sized nucleus, round-shaped nucleus, and presence of clear nucleol. Interestingly, the cancer tissue often contained both PPARδ-positive and -negative areas, each retaining their respective specific morphological features. Moreover, this pattern persisted even when PPARδ-positive and -negative cells were aligned next to each other within a single cancer nest or gland and was present in the majority of CRC cases. Immunohistochemistry for Ki-67 proliferation marker showed no significant correlation between Ki-67 and PPARδ in CRC samples. Based on Western blot analysis and quantitative RT–PCR, high PPARδ protein expression correlated with high PPARδ mRNA levels. Peroxisome proliferator-activated receptor δ may have a supporting role in tumorigenesis, and the close association between PPARδ expression and malignant morphology of CRC cells suggests a pivotal role in cancer tissue.

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. Peroxisome proliferator-activated receptors play a role in normal physiological processes such as lipid metabolism and embryo implantation, and they have been implicated in the disease-related processes of inflammation, diabetes mellitus, and cancer (Kersten et al., 1999; Barak et al., 2003). These two isotypes also have clinical significance in the differentiation (Tontonoz et al., 1999). Various functions of the PPARα and γ isotypes have been described, such as involvement in lipid homeostasis, immunity, and cellular differentiation (Tontonoz et al., 1994; Spiegelman 1998; Kliwer et al., 1999). These two isotypes also have clinical significance in the treatment of dyslipidaemia and type II diabetes mellitus (Rangwala and Lazar, 2004). In contrast, less is known about the physiological role of the PPARβ isoform, although there is some evidence supporting its involvement in embryo implantation and development (Lim et al., 1999; Barak et al., 2002), epidermal maturation and wound healing (Di-Poi et al., 2003), and regulation of fatty acid metabolism (Wang et al., 2003).

Recent studies suggest that PPARδ may play a role in colorectal cancer (CRC). The adenomatous polyposis coli (APC) and K-ras genes are known to play a role in colorectal carcinogenesis (Vogelstein et al., 1988): PPAR expression and/or activity increase after loss of the APC gene or activation of K-ras gene expression (He et al., 1999; Shao et al., 2002). Cyclooxygenase-2 also modulates intestinal tumorigenesis (Oshima et al., 1996), and its metabolite, prostacyclin, increases PPARδ activity in CRC cells (Gupta et al., 2000). In addition, PPARδ has also been shown to be a downstream target of APC/β-catenin/T-cell factor (TCF)-4-mediated transcriptional activation, which is a key mediator in the development of CRC (He et al., 1999). However, it is currently unclear whether PPARδ, like other downstream targets such as c-myc and cyclin D1 (He et al., 1998; Tetsu and McCormick, 1999), contributes to oncogenesis and the development of colon tumours. Several studies using Apcmin mice, designed to evaluate the role of PPARδ in colon tumour development, have produced conflicting findings. Peroxisome proliferator-activated receptorδ was found to be unnecessary for small intestinal polyp formation, but might be required for the development of large-sized intestinal polyps (Barak et al., 2002). In addition, PPARδ attenuates polyp formation in chemical and genetic models (Harman et al., 2004; Reed et al., 2004). In contrast, activation of PPARδ using a synthetic ligand increases the number and size of intestinal polyps (Gupta et al., 2004); indeed, PPARδ-deficient CRC cells can establish tumours when grown as xenografts in nude mice (Park et al., 2001). In this study, we examined expression of PPARδ in multistage carcinogenesis of the colorectum in an effort to elucidate the role of PPARδ in human CRC.

*Correspondence: Dr H Yamamoto;
E-mail: kobunyam@surg2.med.osaka-u.ac.jp
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MATERIALS AND METHODS

Cell lines
The IEC18 intestinal cell line was a generous gift from Dr I Bernard Weinstein (Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, New York, NY, USA). They were grown in Dulbecco’s modified Eagle’s medium plus 10% foetal bovine serum, 100 U ml\(^{-1}\) penicillin, and 100 \(\mu\)g ml\(^{-1}\) streptomycin, in 5% CO\(_2\) at 37°C.

Patients and tissue samples
The expression of PPAR\(\gamma\) was examined by immunohistochemistry in the following set of colorectal samples: normal mucosa (\(n = 32\)), adenomatous polyps (\(n = 23\)), and various stages of carcinomas (\(n = 32\)). Tissue samples were consecutively collected in the years 2002–2003 during surgery or during endoscopic polypectomy at the Department of Surgery, Osaka University. None of the patients had a history of family syndromes for CRC. The samples of normal mucosa were cut in the longitudinal direction, and the polyps and carcinomas were cut across the maximum diameter. These samples were fixed in buffered formalin at 4°C overnight, processed through graded ethanol solutions, and embedded in paraffin. The resected samples were used with the approval of the ethical committee of Osaka University. Adenomatous polyps were 16 tubular and seven tubulovillous adenoma.

There were 23 male and nine female CRC patients, with a mean age of 59.3 years (range, 42–80 years) at surgery. Primary tumours were distributed in the colon (\(n = 14\)) and rectum (\(n = 18\)). The tumours were well-differentiated adenocarcinomas (\(n = 11\)), moderately differentiated adenocarcinomas (\(n = 20\)), and poorly differentiated adenocarcinoma (\(n = 1\)). Eleven patients had lymph node metastasis and 21 patients were node-negative. Dukes’ staging classified nine patients as stage A, 10 patients as stage B, nine patients as stage C, and four patients as stage D.

Antibodies
Rabbit anti-human PPAR\(\gamma\) polyclonal antibody (sc-7197, H-74) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). This antibody recognises amino acids 2–75 mapping at the amino-terminus of PPAR\(\gamma\) of human origin and crossreacts with mouse and rat PPAR\(\gamma\). Mouse anti-human \(\beta\)-catenin monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY, USA). The rabbit anti-human Ki-67 polyclonal antibody was purchased from DAKO (Carpinteria, CA, USA). The rabbit anti-human actin antibody was purchased from Sigma-Aldrich (St Louis, MO, USA).

Haematoxylin and eosin staining and immunohistochemistry
Tissue sections (4 \(\mu\)m thick) were deparaffinised in xylene, rehydrated, and stained with haematoxylin and eosin (H&E) solution. The specimens were histologically diagnosed by two pathologists from the Department of Pathology, Osaka University. For immunostaining, sections were mounted on charged glass slides, boiled for antigen retrieval, and then processed for immunohistochemistry, as described previously (Takemasa et al., 2000; Yamamoto et al., 2003), using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA, USA). In the primary antibody reaction, the slides were incubated with appropriate antibodies for 1 h at room temperature. The dilution of each antibody was 1:40 for PPAR\(\gamma\) antibody, 1:1000 for \(\beta\)-catenin antibody, and 1:50 for Ki-67 antibody. For negative control, nonimmunised rabbit or mouse IgG (Vector Laboratories) or PBS alone was used as a substitute for the primary antibody to exclude possible false-positive responses from secondary antibody binding or from nonspecific binding of IgG. The entire series of samples was stained twice using separately prepared sections, and no discrepant staining results were noted.

Immunohistochemical assessment
All immunostained tissue sections were evaluated by two investigators (TO and HY). Samples were coded without indicating the clinical and pathological background of the patients. In each section, 10 high-power fields were selected, and a total of at least 1000 cells were evaluated. The cell populations exhibiting an association between PPAR\(\gamma\) expression and malignant cancer morphology were assessed in the same manner. The results of cytoplasmic staining were expressed as a percentage of positive cells, and the intensity of staining was estimated on a scale from 0 to 3 (negative, weak, moderate, and strong). The total score was determined by multiplication of the percentage of positive cells and staining intensity, ranging from 0 to 300, as reported previously (Krajewska et al., 1996; Shamma et al., 2000). For the assessment of nuclear expression, the percentage of positive cells was examined because staining intensity was routine.

Transduction of PPAR\(\gamma\) complementary DNA (cDNA)
The mammalian expression vector pCMX-mPPAR\(\gamma\), encoding mouse PPAR\(\gamma\) cDNA (length 1.3 kb) was a generous gift from Professor Ronald M Evans (Salk Institute, San Diego, CA, USA). A pcDNA3 vector encoding a neomycin-resistant sequence was purchased from Invitrogen (Carlsbad, CA, USA). Co-transfection was carried out with pcDNA3 and PPAR\(\gamma\) plasmid or pCMX vector at 0.5 and 2 \(\mu\)g, respectively, into intestinal IEC18 cells using Lipofectin Reagent (Life Technologies Inc. Gaithersburg, MD, USA). Eight hours after transfection, cells were transferred from a 60-mm dish into a 150-mm dish and selected for 10 days in the presence of 0.9 mg ml\(^{-1}\) of G418 (Life Technologies).

Western blot analysis
Western blot analysis was performed as described previously (Yamamoto et al., 1999). Briefly, the protein samples (50 \(\mu\)g) were separated using 10% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, followed by electroblotting onto a polyvinylidene difluoride membrane. The membrane was incubated with the primary antibodies at the appropriate concentrations (1 \(\mu\)g ml\(^{-1}\) for PPAR\(\gamma\) antibody, 1:1000 for actin) for 1 h. Protein bands were detected using the Amersham ECL detection system (Amersham Biosciences Corp., Piscataway, NJ, USA).

Quantitative real-time PCR for PPAR\(\gamma\) mRNA
Total cellular RNA was extracted using TRIZOL reagent (Life Technologies Inc., Gaithersburg, MD, USA). Complementary DNA was generated from 1 \(\mu\)g RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time PCR was performed using LightCycler™ (Idaho Technology Inc., Salt Lake City, Utah, USA), as described previously (Yamamoto et al., 2003). Quantification data from each sample were analysed using LightCycler™ analysis software. The transcription value of PPAR\(\gamma\) was determined by plotting on a standard curve constructed using HCT116 colon cancer cells. The amount of each transcript was normalised according to that of \(\beta\)-actin housekeeping gene quantified with the same sample. The primer sequences were as follows: \(\beta\)-actin sense, 5’-GAAA TCTGGGACACCACCTTGTGTTTTCGGATATG-3’; \(\beta\)-actin antisense, 5’-GGTGAAGGTTTCCGTTGGAATTG-3’; PPAR\(\gamma\) sense: 5’-GGGAGCCTTGGTACTGTC-3’ and PPAR\(\gamma\) antisense: 5’-CTTGGTCCTTGAGAAGATCAGC-3’.
Statistical analysis

Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts Inc., Berkeley, CA, USA). Associations between the discrete variables were assessed using Fisher’s exact tests. Data were reported as mean ± s.d., and mean values were compared using the Mann–Whitney test. P-values < 0.05 were accepted as statistically significant.

RESULTS

Validation of specificity of the anti-PPARδ antibody

Immunocytochemistry showed that PPARδ-transfected cultures displayed intense PPARδ staining in comparison to the weak PPARδ staining noted in the control cultures (Figure 1A). Western blotting using anti-PPARδ antibody showed that PPARδ-introduced cultures displayed prominent bands for the PPARδ protein compared with parental and vector control cells (Figure 1B). These results indicate that PPARδ antibody specifically reacts with the PPARδ protein.

PPARδ expression in CRC tissues

In normal colonic mucosa, PPARδ protein was detected in the epithelial cells on the luminal surface of the mucosal glands (Figure 2A). In adenomatous polyps, PPARδ was weakly expressed in eight of 23 samples (34.8%) in the cytoplasm (Figure 2B). On the other hand, all the carcinoma tissues expressed the PPARδ protein to various extents, in the cytoplasm and/or nucleus (Figures 2C and D). More than half of the cancer tissues exhibited nuclear expression at less than 10%, whereas a considerable number of the cancer tissues showed cytoplasmic PPARδ expression (Table 1). Peroxisome proliferator-activated receptorδ expression in adenomatous polyps and cancer tissues with regard to their localisation and expression extent is summarised in Table 1. The differences in cytoplasmic PPARδ expression between adenomatous polyps and cancers were significant (P < 0.0001).

The cancer specimens were divided into two groups (high expression: n = 15 (46.9%) and low expression: n = 17 (53.1%)) based on the mean value of a cytoplasmic PPARδ score of 160 (see Materials and Methods regarding score determination). Comparison of these two groups showed no differences in the various clinical and pathological parameters listed in Table 2. In addition, nuclear expression level (mean value 8.3% at the cutoff point) did not correlate with clinical and pathological parameters in these two groups (data not shown).

Western blot analysis

Among the above series, three CRC cases with low cytoplasmic PPARδ and four CRC cases with high cytoplasmic PPARδ were subjected to Western blot analysis to determine PPARδ protein levels (Figure 3A). Colorectal cancer samples expressed various levels of the PPARδ protein that correlated well with those detected by immunohistochemistry.

Level of PPARδ mRNA

The same tissue samples used in Western blot analysis were subjected to quantitative RT–PCR for PPARδ mRNA quantification. Samples exhibiting high expression of PPARδ protein generally also exhibited high levels of PPARδ mRNA, whereas those with low PPARδ protein levels exhibited low levels of PPARδ mRNA (Figure 3B).

Relationship between PPARδ expression and Ki-67 expression

To investigate the possible involvement of PPARδ in cell growth, we compared the expression of PPARδ and Ki-67, a cell 891

Figure 2 Immunohistochemistry for PPARδ (A) In normal colonic mucosa, the PPARδ protein was weakly detected in the epithelial cells on the luminal surface of the mucosal glands. (B) In adenomatous polyps, PPARδ was weakly expressed in the cytoplasm. (C) Cytoplasmic expression and (D) nuclear expression in carcinoma tissues. Magnifications: A: × 50; B: × 20; C: × 100; D: × 150.

Table 1 PPARδ expression in colorectal tumour

| Nuclear expression* | % Positive |
|---------------------|-----------|
| Cancer              | 0–10     |
| 10 < X ≤ 30         | 10 (31.3%) |
| 30–100              | 2 (6.2%) |

| Cytoplasmic expression | PPAR score | Adenoma | Cancer |
|------------------------|------------|---------|--------|
| 0–50                   | 0 (0%)     | 18 (78.3%) |
| 50 < X ≤ 150           | 5 (21.7%)  |         |
| 150–300                | 3 (9.4%)   | 3 (40.6%) |
|                        | 16 (50.0%) |

PPARδ = peroxisome proliferator-activated receptor. *Adenomatous polyp showed no nuclear expression. **Determined by multiplication of staining intensity (0–3) and positivity (0–100).
interest that a cancer tissue often contained both PPAR
nucleolus, and preserved cellular polarity (Figure 5B). It was of
type, such as an oval and small nucleus without a distinct
morphological features associated with a low malignant pheno-
(Figure 5D and E). Table 3 summarises the levels of PPAR
dexpression, nuclear size and shape index, and presence or absence
distinct nucleolus in these samples.

During the course of this study, we found that the cancer cells with
cytoplasmic accumulation of PPARδ often exhibited morphologi-
cal features associated with a highly malignant phenotype. These
features included a large nucleus, globular nuclear shape, appear-
ance of distinct nucleolus, and loss of cellular polarity
(Figure 5A). In contrast, PPARδ-negative cancer cells had
morphological features associated with a low malignant pheno-
type, such as an oval and small nucleus without a distinct
nucleolus, and preserved cellular polarity (Figure 5B). It was of
interest that a cancer tissue often contained both PPARδ-positive
and -negative areas, with maintenance of these respective, specific
morphological features (Figure 5C). Moreover, this rule was
maintained even when PPARδ-positive and -negative cells were
aligned next to each other within a single cancer nest or gland
(Figure 5D and E). Table 3 summarises the levels of PPARδ
expression, nuclear size and shape index, and presence or absence
of a distinct nucleolus in these samples.

### Table 2 PPARδ and clinicopathological characteristics

| Clinicopathological characteristic | PPARδ | P-value |
|-----------------------------------|-------|---------|
| Tumour size (cm)*                 | 32    | 0.467   |
| Gender                            | 23    | 0.337   |
| Tumour site                       | 14    | 0.755   |
| Degree of differentiation         | 11    | 0.519   |
| Depth of invasion                 | 6     | 0.316   |
| Lymph node metastasis             | 21    | 0.907   |
| Stage                             | 19    | 0.430   |
| Total                             | 32    |         |

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| Total                             | 32    |         |

Mod = moderately differentiated adenocarcinoma; mp = muscularis propria; PPARA = peroxisome proliferator-activated receptor A; por = poorly differentiated carcinoma; ss = subserosa; well = well-differentiated adenocarcinoma. Data are mean ± s.d. Por tumour was only one that had a low PPARδ expression.

### PPARδ expression and malignant morphology of CRC cells

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of a distinct nucleolus in these samples.

Further microscopy analysis, as indicated in the Materials and
Methods, revealed that this association between PPARδ expression
and malignant morphological features was a common rule in the
majority of cancer samples tested. Thus, 100% of cancer cells
followed the rule in the 10 CRCs, 50 – 99% of cancer cells followed
the rule in 18 CRCs, and 1 – 49% of cells in three CRCs adhered to
the pattern. One CRC sample did not exhibit this association.

### DISCUSSION

With regard to human tissue, increased expression of PPARδ was
first reported in a small set of CRC tumours (He et al, 1999; Gupta
et al, 2000). To elucidate further the expression and role of PPARδ
in human colorectal tumour, we examined expression of PPARδ in multistage carcinogenesis of the colorectum and found that PPARδ expression increased from normal mucosa to adenomatous polyps to cancer tissues. Furthermore, we found that PPARδ expression was tightly associated with highly malignant morphology of colon cancer cells. Thus, the present data suggest a pivotal role of PPARδ in human CRC tissue. These findings are consistent with the recent reports that PPARδ mRNA is overexpressed in more than half of CRCs (Yang et al., 2006) and that PPARδ protein expression is elevated in adenomas in Apcmin mice and in colon tumours familial adenomatous polyposis patients (Knutsen et al., 2005). Although clinicopathological correlations were not obtained with PPARδ expression in CRC, we should emphasise that the relatively small number of CRC specimens examined may have given a low statistical power.

In spite of these indications, however, whether PPARδ is a good target for chemoprevention and/or treatment of CRC remains controversial. It has been reported that a polymorphism in PPARδ modifies the protective effects of nonsteroidal anti-inflammatory drugs on colorectal adenomas (Siezen et al., 2006), but other investigators have not reached the same conclusions in the context of CRC (McGreavey et al., 2005). As mentioned in the Introduction, PPARδ was found to be unnecessary for small intestinal polyp formation (Barak et al., 2002), but PPARδ attenuated polyp formation in chemical and genetic models (Harman et al., 2004; Reed et al., 2004). By contrast, it has been reported that inactivation of the PPARδ gene results in reduced tumorigenicity and in vivo growth of HCT116 colon cancer cells (Park et al., 2001) and that a specific PPARδ agonist enhanced in vivo growth of intestinal adenoma of Apcmin mice (Gupta et al., 2004). Moreover, a decrease in PPARδ expression by nitric-oxide-donating aspirin isomers was found to be proportional to their tumour inhibitory effects in Apcmin mice (Ouyang et al., 2006).

A recent report by Wang et al might be a clue to the puzzle. They reported that prostaglandin E2-mediated enhancement of intestinal adenoma of Apcmin mice was negated in Apcmin/PPARδ−/− mice (Wang et al., 2004a,b). Peroxisome proliferator-activated receptorδ may mediate the antiapoptotic effect through activation by prostacyclin (PGI(2)), a major prostaglandin with antiapoptotic activity (Gupta et al., 2000; Cutler et al., 2003). There is also cumulative evidence regarding the antiapoptotic effects of PPARδ in keratinocyte and colon cancer cells (Michalik et al., 2001; Di-Poi et al., 2002; Shureiqi et al., 2003; Gupta et al., 2004). These findings suggest that PPARδ may play a certain tumour-promoting role in intestinal tumours or CRC cells by modulating cell survival and apoptosis, which is in line with our observation that PPARδ was exclusively expressed in those CRC cells that also exhibited highly malignant morphology.

Cellular atypia is the pathological hallmark for estimating the malignant potential of lesions. Studies with large numbers of CRC patients (N = 343, 100, 90, and 64, respectively) (Ambros et al., 1990; Mitmaker et al., 1991; Fernandez-Lopez et al., 1999; Ikeguchi et al., 1999) have shown that the nuclear area, the large maximum nucleus diameter, or nuclear shape index, when determined with the aid of nuclear morphometry, is associated with cancer metastasis or poor prognosis. During the current study, we found that PPARδ-expressing cancer cells often presented such nuclear features, whereas PPARδ-negative cells did not. Surprisingly, this association was found in the majority of CRC cases and was maintained even when PPARδ-positive cells and PPARδ-negative cells were positioned next to each other. These findings indicate that cytoplasmic accumulation of PPARδ could be a sensitive marker for the malignant potential of lesions.
Table 3  PPARδ expression and cancer cell morphology

| Case | Cell type | Intensity of PPARδ staining | Nuclear size index | Nuclear shape index | Presence of distinct nucleoli |
|------|-----------|-----------------------------|-------------------|-------------------|-----------------------------|
| A    | PPAR (+)  | 77.0                        | 170.4             | 0.68              | Yes                         |
| B    | PPAR (+)  | 3.8                         | 77.0              | 0.55              | No                          |
| C    | PPAR (+)  | 41.3                        | 239.7             | 0.64              | Yes                         |
| D    | PPAR (+)  | 4.0                         | 80.6              | 0.50              | No                          |
| E    | PPAR (+)  | 29.6                        | 275.1             | 0.72              | Yes                         |
| F    | PPAR (+)  | 2.0                         | 50.0              | 0.54              | No                          |

PPARδ = peroxisome proliferator-activated receptor. *Major axis multiplied by minor axis. **Defined as the degree of circularity of the nucleus; minor axis divided by major axis.

A perfect circle was recorded as 1.0.

marker of CRC cells with the potential for high malignancy. Recently, Hinoi et al showed that loss of CDX2 was a marker for large-cell minimally differentiated carcinomas of the colon (Hinoi et al, 2001), and we are not aware of other molecular markers tightly associated with CRC cell morphology.

PPARδ is known as a nuclear receptor, and we indeed found that introduction of PPARδ cDNA resulted in nuclear expression in IEC18 cells, whereas immunohistochemistry showed cytoplasmic accumulation of PPARδ in CRC tissues. We believe that the latter findings do not represent nonspecific binding of the PPARδ antibody in tissue samples; β-catenin staining in the same tumour series indicated that cytoplasmic PPARδ was selectively induced in CRC cells, possibly as a result of aberrant accumulation of oncogenic β-catenin (data not shown, our unpublished data). Therefore, it is postulated that cytoplasmic accumulation of PPARδ may be necessary for the proteins to be available for their nuclear role whenever required. It is also possible that nuclear PPARδ might be present at a low level but was not detectable because of the limited sensitivity of the immunohistochemical analysis.

Our immunohistochemical study of Ki-67 and PPARδ did not identify positive effects of PPARδ on in vitro cell growth. We found by in vitro assay that the growth of intestinal cells was stimulated by the introduction of PPARδ cDNA (data not shown, our unpublished data), findings that are consistent with other reports that PPARδ enhances the in vitro growth of breast and prostate cancer cells (Stephen et al, 2004). These results suggest that other positive and negative regulators could be simultaneously exerting their effects on cell growth.

In conclusion, the present study using CRC tissue samples showed that PPARδ expression increased during multistage carcinogenesis. Our data suggest that the association of PPARδ with CRC malignant cellular morphology suggests a pivotal role for PPARδ in these cells.

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