Functional Characterization of Peroxisome Proliferator-Activated Receptor-β/δ Expression in Colon Cancer

Jennifer E. Foreman,[1] Wen-Chi L. Chang,[2] Prajakta S. Palkar,[1] Bokai Zhu,[1] Michael G. Borland,[1] Jennie L. Williams,[3] Lance R. Kramer,[1] Margie L. Clapper,[2] Frank J. Gonzalez,[4] and Jeffrey M. Peters[1]*

[1]*Department of Veterinary and Biomedical Sciences and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, Pennsylvania*
[2]*Cancer Prevention and Control Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania*
[3]*Cancer Prevention Laboratory, State University of New York at Stony Brook, Stony Brook, New York*
[4]*Laboratory of Metabolism, National Cancer Institute, Bethesda, Maryland*

This study critically examined the role of PPARβ/δ in colon cancer models. Expression of PPARβ/δ mRNA and protein was lower and expression of CYCLIN D1 protein higher in human colon adenocarcinomas compared to matched non-transformed tissue. Similar results were observed in colon tumors from $Apc^{+/\text{Min-FCCC}}$ mice compared to control tissue. Dietary administration of sulindac to $Apc^{+/\text{Min-FCCC}}$ mice had no influence on expression of PPARβ/δ in normal colon tissue or colon tumors. Cleaved poly (ADP-ribose) polymerase (PARP) was either increased or unchanged, while expression of 14-3-3ε was not influenced in human colon cancer cell lines cultured with the PPARβ/δ ligand GW0742 under conditions known to increase apoptosis. While DLD1 cells exhibited fewer early apoptotic cells after ligand activation of PPARβ/δ following treatment with hydrogen peroxide, this change was associated with an increase in late apoptotic/necrotic cells, but not an increase in viable cells. Stable over-expression of PPARβ/δ in human colon cancer cell lines enhanced ligand activation of PPARβ/δ and inhibition of clonogenicity in HT29 cells. These studies are the most quantitative to date to demonstrate that expression of PPARβ/δ is lower in human and $Apc^{+/\text{Min-FCCC}}$ mouse colon tumors than in corresponding normal tissue, consistent with the finding that increasing expression and activation of PPARβ/δ in human colon cancer cell lines inhibits clonogenicity. Because ligand-induced attenuation of early apoptosis can be associated with more late, apoptotic/necrotic cells, but not more viable cells, these studies illustrate why more comprehensive analysis of PPARβ/δ-dependent modulation of apoptosis is required in the future.

Key words: apoptosis; clonogenicity; tumorigenicity

INTRODUCTION

Peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) is a ligand-activated transcription factor that has critical cellular functions mediated by receptor-dependent modulation of target gene expression in addition to epigenetic activities (reviewed in Refs. [1–3]). PPARβ/δ is expressed in most tissues, but is expressed at the highest levels in epithelium, in particular skin and intestine [4–8]. Interestingly, in cells where PPARβ/δ is expressed, the receptor is found in the nucleus and can be co-immunoprecipitated with its heterodimerization partner, retinoid X receptor (RXR), suggesting that PPARβ/δ has an important constitutive biological function(s) that is/are possibly mediated by endogenous ligands [6]. The best-characterized role for PPARβ/δ to date is its involvement in the promotion of terminal differentiation in many cell types including epithelium (reviewed in Refs. [1–3]). In addition, preclinical and clinical trials have demonstrated that ligand activation of PPARβ/δ stimulates potent anti-inflammatory activities [9], increases serum HDL-cholesterol concentrations [10–13], improves exercise endurance [14] and is central in the regulation of lipid and glucose homeostasis [15–17].

In contrast to the established effects of activating PPARβ/δ that are of interest for the treatment and prevention of metabolic diseases including obesity, diabetes, and dyslipidemias, the role of PPARβ/δ in cancer remains uncertain. Some studies

Abbreviations: PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; APC, adenomatous polyposis coli; TCF4, transcription factor 4; NSAID, non-steroidal anti-inflammatory drugs; Bcl-2, B-cell CLL/lymphoma 2l; PARP, poly (ADP-ribose) polymerase; ADRP, adipocyte differentiation-related protein; ANGPTL4, angiopoietin-like protein 4; PCR, polymerase chain reaction; PI, propidium iodide; eGFP, enhanced green fluorescent protein

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*Correspondence to: Department of Veterinary and Biomedical Sciences and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, PA 16802.

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indicate that PPARβ/δ promotes tumorigenesis while others suggest that PPARβ/δ attenuates tumorigenesis (reviewed in Refs. [1–3]). It was originally hypothesized that PPARβ/δ was upregulated by the adenomatous polyposis coli (APC)/β-CATENIN/transcription factor 4 (TCF4) pathway during colon carcinogenesis and facilitated tumor growth by modulating a group of yet-to-be identified target genes [18]. This idea was based in part on the observed correlation of decreased PPARβ/δ expression and increased APC expression in a human colon cancer cell line and the reported increase in PPARβ/δ expression in four human colon tumors as compared to normal tissue [18]. Since this preliminary report, results from a number of studies that examined expression of PPARβ/δ in human and experimental models of colon cancer have failed to provide support for the view that PPARβ/δ is increased in colon cancer cells or that PPARβ/δ is upregulated by the APC/β-CATENIN/TCF4 pathway ([19], reviewed in Ref. [3]). Some reports have also suggested that the chemopreventive effects of non-steroidal anti-inflammatory drugs (NSAIDs) are mediated in part by reduced expression of PPARβ/δ in colon cancer models, but this result is not observed consistently (reviewed in Refs. [2,3]). In fact, in some cases, upregulation of PPARβ/δ is found in human colon cancer cells following treatment with NSAIDs [19]. Several technical limitations are commonly associated with the latter observation including the use of immunohistochemistry to assess expression, a sole focus on mRNA expression, evaluation of a small number of independent samples, and failure to quantify PPARβ/δ expression by Western blotting. Thus, whether expression of PPARβ/δ is increased, unchanged, or decreased during colon carcinogenesis remains unclear.

The effect of ligand activation of PPARβ/δ during colon carcinogenesis also remains unclear. There is evidence to suggest that ligand activation of PPARβ/δ promotes cell proliferation during colon tumor formation by regulating unidentified target genes that modulate cell cycle progression (reviewed in Refs. [2,3]). It has also been hypothesized that ligand activation of PPARβ/δ prevents apoptosis induced by NSAIDs or hydrogen peroxide in colon cancer cell lines. This hypothesis is based on the idea that PPARβ/δ prevents chemically induced apoptosis by increasing expression of 14-3-3ε whose elevated levels enhance sequestration of Bad, a pro-apoptotic member of the B-cell CLL/lymphoma 2 (Bcl-2) family [20–23]. In contrast, a large body of literature suggests that ligand activation of PPARβ/δ has either no effect on, or inhibits, colon tumorigenesis by enhancing terminal differentiation, and promoting apoptosis (reviewed in Refs. [2,3]). These inconsistent results dictate a need to further examine the functional role of PPARβ/δ in colon cancer development.

The present studies were designed to characterize the functional role of PPARβ/δ in colon cancer. Expression of PPARβ/δ was measured at both the protein and mRNA level in colon tissue (tumors and matched control) from cancer patients, and in intestinal tissue from a unique strain of Multiple Intestinal Neoplasia ($Apc^{+/\text{Min-FCCC}}$) mice that develop multiple colorectal adenomas [24]. The effect of ligand activation of PPARβ/δ on apoptosis was also examined by flow cytometry of human colon cancer cells following chemically induced apoptosis. Lastly, the clonogenicity of stable human colon cancer cell lines over-expressing PPARβ/δ was examined.

MATERIALS AND METHODS

Cell Culture

RKO, DLD1, and HT29 were obtained from American Type Culture Collection (Manassas, VA). RKO cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA). DLD1 cells were cultured in RPMI 1640 medium (Invitrogen). HT29 cells were cultured in McCoy's 5A medium (Invitrogen). All cell lines were cultured in medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% carbon dioxide.

Mouse Colon Cancer Model

Male C57BL/6J wild-type or $Apc^{+/\text{Min-FCCC}}$ [24], aged 6–8 wk were used for this study. Mice of both genotypes (9 wild-type and 10–12 $Apc^{+/\text{Min-FCCC}}$ mice) were fed either control diet (Purina Rodent Chow 5013, PMI-Nutrition International, Inc., Brentwood, MO) or diet containing sulindac (300 ppm) for 45 d. At the time of euthanasia, the colons were excised, flushed with phosphate buffered saline, and examined grossly for the presence of tumors. Identifiable tumors were collected while the remaining colonic mucosa was isolated from the tissue by scraping it with a glass slide. All samples were snap frozen for protein and mRNA isolation as described below.

Human Colon Tissue Samples

Matched pairs of frozen normal colon tissue and colon or rectal adenocarcinomas were obtained from The Penn State Hershey Cancer Institute Tissue Bank. A summary of the sample demographics is provided in Table 1. Protein or mRNA was prepared from these samples as described below.

Induction of Apoptosis in Human Colon Cancer Cell Lines

For human colon cancer cell lines, RKO (wild-type APC and β-catenin), DLD1, and HT29 (mutant APC and wild-type β-catenin) cells were

Table 1. Demographics of Colon and Rectal Adenocarcinomas

		Age		Sex		
Tumor type	*N*	Mean ± SD	Range	Male	Female	Unknown
Colon	19	64.8 ± 16.6	33–86	7	10	2
Rectal	14	58.8 ± 13.8	33–85	4	10	0

cultured on 60 mm dishes and maintained in culture medium as described above, until they were approximately 80% confluent on the day of treatment. Cells were then pretreated for 1 h with either 0.1% DMSO or the selective PPARβ/δ agonist GW0742 (0.1, 1.0, and 10 μM) and then treated for 24 h with 800 μM indomethacin, 150 μM sulindac, and 160 μM sulindac sulfide, or 4 h with 0.5 mM hydrogen peroxide in the presence of ligand. The selected concentrations of GW0742 are known to activate specifically PPARβ/δ in these cell lines [19,25]. The concentrations of indomethacin, sulindac, and sulindac sulfide were chosen based on previous studies showing inhibition of cell proliferation [19], and PPARβ/δ-dependent attenuation of NSAID-induced apoptosis in the same human colon cancer cell lines [20]. Preliminary analyses from this group indicate that 0.5 mM hydrogen peroxide is the optimal concentration to increase apoptosis sufficiently without causing excessive cell death (data not shown). The same exposure paradigm was used to suggest PPARβ/δ-mediated attenuation of hydrogen peroxide-induced apoptosis in endothelial cells due to suppression of 14-3-3ε [21]. Untreated cells were used as a negative control, while cells treated with only 2 μM staurosporine for 5 h served as a positive control.

Western Blot Analysis

Protein extracts were prepared from the mouse colonic mucosa, tumor samples, or human colon cancer cell lines as described previously [6]. Snap frozen human tissue samples were ground to a powder using a mortar and pestle in liquid nitrogen. This ground tissue was used for isolation of protein or mRNA. Whole-cell protein extracts were prepared using MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, and 10% glycerol, pH 7.5) containing 500 mM NaCl, 1% Nonidet P-40, and protease inhibitors. Twenty-five to fifty micrograms of protein per sample was resolved using 10% SDS–PAGE and transferred onto a polyvinylidene fluoride membrane using an electroblotting method. Membranes were incubated with primary antibodies, washed, incubated with a biotinylated secondary antibody, washed, and then incubated with ^{125}I-streptavidin to allow for detection. Membranes were exposed to plates and the level of radioactivity quantified with filmless autoradiographic analysis. The following primary antibodies were used: anti-14-3-3ε (sc1020; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-PPARγ (sc6284; Santa Cruz Biotechnologies), anti-PPARβ/δ (human) (ab21209; Abcam Inc., Cambridge, MA), anti-PPARβ/δ (mouse) [6], anti-CYCLIN D1 (Cell Signaling Technology, Danvers, MA), anti-poly (ADP-ribose) polymerase (PARP; Cell Signaling Technology), anti-β-actin (ACTIN; Rockland, Gilbertsville, PA), and anti-lactate dehydrogenase (LDH; Rockland). Hybridization signals for the proteins of interest were normalized to the hybridization signal of either ACTIN or LDH. The ratio of normalized cleaved PARP to normalized uncleaved PARP was used as a measure of relative apoptosis.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from colon tissue, tumor samples or human colon cancer cell lines using Ribozol (Amresco, Solon, OH) according to the manufacturer's protocol. The mRNAs encoding PPARβ/δ, and its target genes adipocyte differentiation-related protein (*ADRP*) and angiopoietin-like protein 4 (*ANGPTL4*) were quantified using quantitative real-time polymerase chain reaction (qPCR). cDNA was generated using 2.5 μg total RNA with MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Primers were designed for real-time PCR using the Primer Express software (Applied Biosystems). The sequence and GenBank accession number for the forward and reverse primers used to quantify mRNAs were: human *PPARβ/δ* (AY919140) forward, 5′-GACAGTGACCTGGCCCTATTCA-3′ and reverse, 5′-AGGATGGTGTCCTGGATAGCCT-3′, mouse *PPARβ/δ* (NM_011145) forward, 5′-TTGAGCCCAA GTTCGAGTTTGCTG-3′ and reverse, 5′-ATTCTAGA GCCCGCAGAATGGTGT-3′, mouse *PPARγ* (NM_011146) forward, 5′-GACAGTGACCTGGCCCTAT TCA-3′ and reverse, 5′-AGGATGGTGTCCT GGA-TAGCCT-3′, *ADRP* (NM_000122) forward, 5′-CTGCTCTTCGCCTTTCGCT-3′, and reverse, 5′-ACCACCCGAGTCACCACACT-3′, and *ANGPTL4* (NM_020581) forward, 5′-TTCTCGCCTACCAGA-GAAGTTGGG-3′ and reverse, 5′-CATCCACAG-CACCTACAACAGCAC-3′. Expression of mRNA

was normalized to *GAPDH* mRNA (BC083149) that was quantified using the following primers: forward, 5′-GGTGGAGCCAAAAGGGTCAT-3′ and reverse, 5′-GGTTCACACCCATCACAAACAT-3′. Real-time PCR reactions were carried out using SYBR green PCR master mix (Finnzymes, Espoo, Finland) in the iCycler and detected using the MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The following conditions were used for PCR: 95°C for 15 s, 94°C for 10 s, 60°C for 30 s, and 72°C for 30 s and repeated for 45–55 cycles. The PCR included a no template reaction to control for contamination and/or genomic amplification. All reactions had >85% efficiency.

To control for interindividual variability in PPARβ/δ expression, the ratio of normalized PPARβ/δ mRNA for each tumor relative to normalized PPARβ/δ mRNA of each matched control was calculated. This type of analysis creates a positively skewed data distribution, giving a greater range of values for those samples that exhibit higher expression of PPARβ/δ mRNA in the tumor as compared to the matched control (1–∞) in comparison to samples that exhibit lower expression of PPARβ/δ mRNA in the tumor as compared to the matched control (0–1). To control for the skew associated with this type of analysis, the data were log 2-transformed to make a symmetrical data distribution centered around 0. This gives a normal distribution and allows for statistical analyses [26–28].

Examination of Apoptosis and Cell Viability by Flow Cytometry

RKO, DLD1, or HT29 cells were plated on 24-well dishes and cultured as described above until they were approximately 80% confluent on the day of treatment. Cells were pretreated for 1 h with either 0.02% DMSO or GW0742 (0.1, 1.0, and 10 μM) and then treated for 4 h in 0.0, 0.5, or 5.0 mM hydrogen peroxide in the presence or absence of GW0742 (0.1, 1.0, and 10 μM). After these treatments, culture medium was removed and the cells were trypsinized, pelleted, and resuspended in annexin V-binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Prior to analysis, the cells were incubated with a FITC-labeled anti-annexin V antibody for 15 min after which propidium iodide (PI; 1 μg/μl) was added to each sample. Approximately 10,000 cells/sample were analyzed using an EPICS-XL-MCL flow cytometer (Beckman Coulter, Miami Lakes, FL) fitted with a single 15 mW argon ion laser (excitation at 488 nm). Cells stained with FITC were monitored through a 525 nm bandpass filter. Viable cells were defined as the percentage of cells that were annexin V-negative and PI-negative. Early apoptosis was defined as the percentage of cells that were annexin V-positive and PI-negative, and late apoptosis/necrosis was defined as the percentage of cells that were annexin V-negative and PI-positive or annexin V-positive and PI-positive. Values were calculated from a minimum of three independent samples per treatment.

Generation of Stable Cell Lines Over-Expressing PPARβ/δ

The pMigr1 vector (Migr1) and pCL-Ampho have been previously described [29]. The Migr1 retroviral vector contains the mouse stem cell virus promoter that drives expression of cDNA cloned into a cloning site, followed by an internal ribosome entry site (IRES) and a sequence encoding enhanced green fluorescent protein (eGFP) [29]. This bi-cistronic vector allows for expression of a protein of interest and eGFP, which facilitates identification and sorting of cells that have stably integrated the Migr1 retroviral vector. The pcDNA3.1-hPPARβ/δ construct was kindly provided by Dr. Curt Omiecinski (The Pennsylvania State University, University Park, PA). The Migr1-hPPARβ/δ vector was made by subcloning the human PPARβ/δ cDNA sequence from pcDNA3.1-hPPARβ/δ into the Migr1 vector. The coding sequence was confirmed by sequencing at the Penn State University Nucleic Acid Facility. Stable Migr1 (vector control) and Migr1-hPPARβ/δ cell lines were established by retrovirus spinoculation as described previously [29]. Briefly, each construct and pCL-Ampho plasmids were co-transfected into HEK293T cells to produce retrovirus using the Lipofectamine® transfection reagent and the manufacturer's recommended protocol. Forty-eight hours after transfection, the supernatant containing the retrovirus was passed through a 0.22 μm filter and used to spinoculate RKO, DLD1, or HT29 cells. eGFP-positive cells were isolated by fluorescence-activated cell sorting using an InFlux V-GS Cytometry Workbench and Spigot software (BD Biosciences, San Jose, CA). Forward-scatter and side-scatter dot plots gave the cellular physical properties of size and granularity and allowed gating for live cells. Fluorescence was excited at 488 nm (eGFP), and emission was collected using a 525 nm band-pass filter. Collected eGFP cells possessed a minimum of 100-fold higher eGFP expression than non-GFP cells. Fluorescence photomicrographs were obtained with a SPOT SP100 cooled CCD camera fitted to a Nikon Eclipse TE300 upright microscope with EFD-3 episcopic fluorescence attachment. The presence of eGFP fluorescence was routinely checked using the Nikon fluorescence microscope.

Colony Formation Assay

Control (parent human colon cancer cell line, RKO, DLD1, or HT29), cells stably expressing the Migr1-empty vector, or cells stably expressing

PPARβ/δ were plated in 60 mm culture dishes. RKO cells were plated at a concentration of 300 cells/well and allowed to adhere for 24 h before treatment. DLD1 and HT29 cells were plated at a concentration of 400 or 600 cells/well, respectively, and allowed to adhere for 8 h before treatment. Adhered cells were treated with medium containing either: 0 (DMSO control), 0.1, 1.0, or 10 μM GW0742. RKO, DLD1, and HT29 cells were cultured for 14, 13, or 15 d, respectively, after which the cell colonies were fixed with 6% (v/v) glutaraldehyde and stained with 0.5% (w/v) crystal violet. Colony number was quantified using Image J software (version 1.37, National Institutes of Health, Bethesda, MD). The plating efficiency and surviving fraction were calculated as described previously [30,31].

Statistical Analysis

Statistical significance was determined using either a *t*-test, or, where applicable, analysis of variance and the Bonferroni post-test (Prism 5.0, GraphPad Software Inc., La Jolla, CA).

RESULTS

PPARβ/δ Expression Is Lower in Human Colon Adenocarcinomas as Compared to Control Tissue

Expression of PPARβ/δ protein was markedly lower in 19 human colon adenocarcinomas as compared to control colon tissue (Figure 1A, Supplemental Figure 1A). In contrast, expression of CYCLIN D1 was higher in human colon adenocarcinomas as compared to control colon tissue (Figure 1A, Supplemental Figure 1A). Expression of PPARγ1 protein was not different in human colon adenocarcinomas as compared to control colon tissue (Figure 1A). No significant difference was found in expression of PPARβ/δ or PPARγ1 protein in human rectal adenocarcinomas as compared to control tissue, and while expression of CYCLIN D1 was higher in some human rectal adenocarcinomas, this was not statistically significant (Figure 1A, Supplemental Figure 1A). No difference in relative expression of PPARβ/δ or PPARγ1 was observed between male and female samples in either colon adenocarcinomas or rectal

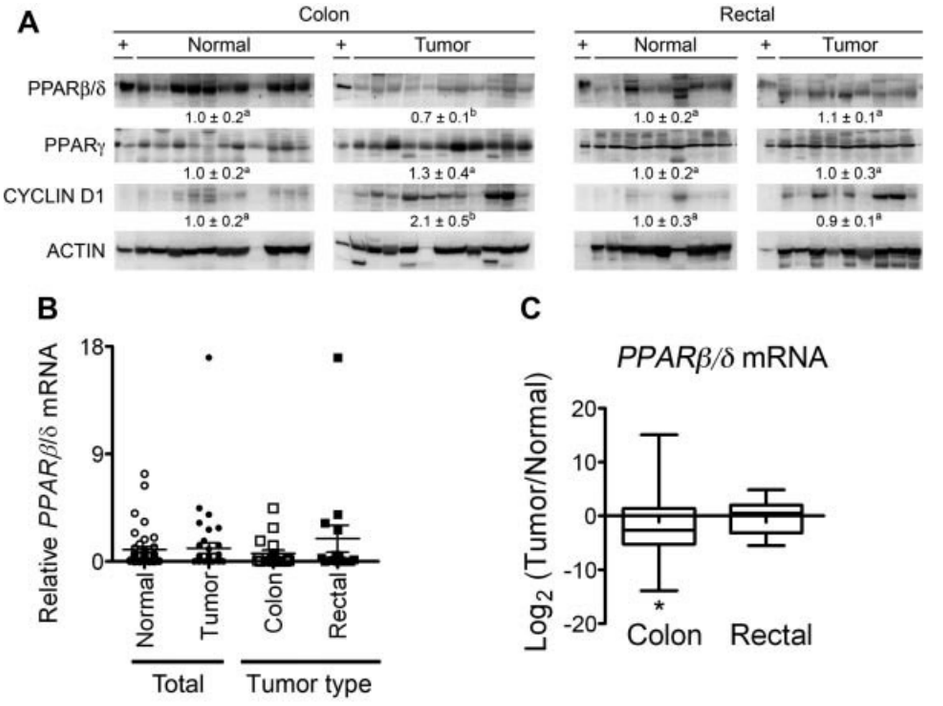

Figure 1. Expression of PPARβ/δ protein and mRNA is lower in human colon tumors compared to matched control tissue. (A) Representative quantitative Western blot of PPARβ/δ, PPARγ, and CYLCIN D1 expression in normal colon or rectal tissue and colon or rectal tumors. A total of 19 colon tumors or matched control colon tissue from the same patient, or 14 rectal tumors or matched rectal tissue from the same patient were examined. + = positive control: lysate from COS1 cells transfected with human *PPARβ/δ* expression vector or, in vitro translated human PPARγ1. Values were normalized to ACTIN. Values represent the mean ± SEM from all independent samples. Values with different superscripts are significantly different at $P \leq 0.05$. (B) Quantitative real-time PCR was performed to measure expression of mRNA encoding *PPARβ/δ*. Normalized values were calculated from the independent samples described in (A) and represent the mean ± SEM from all independent samples. Comparisons were made between normal tissue and both colon and rectal tumors combined (left panel), or by tumor type (right panel). (C) The ratio of relative *PPARβ/δ* mRNA between tumor and matched control tissue was calculated and log-transformed. *Statistically lower in tumors as compared to matched tissue control, $P \leq 0.05$.

adenocarcinomas as compared to control colon (data not shown). Expression of *PPARβ/δ* mRNA between control tissue and both colon and rectal tumors was not different when normalized expression was compared between control tissue *PPARβ/δ* mRNA and both colon and rectal *PPARβ/δ* mRNA, respectively (Figure 1B). Similarly, average expression of *PPARβ/δ* mRNA in only colon or rectal tumor types was not different as compared to expression of *PPARβ/δ* mRNA in control tissue, respectively (Figure 1B). However, the log 2-transformed ratio of normalized *PPARβ/δ* mRNA for each tumor relative to normalized *PPARβ/δ* mRNA of each matched control indicates that average expression of *PPARβ/δ* mRNA is lower in colon adenocarcinomas as compared to matched control tissue from each sample (Figure 1C).

PPARβ/δ Expression Is Lower in $Apc^{+/\text{Min-FCCC}}$ Mouse Colon Tumors as Compared to Control Tissue

Expression of PPARβ/δ in colon tumors was also examined in the $Apc^{+/\text{Min-FCCC}}$ mouse model. Additionally, the effect of feeding a diet containing sulindac was also determined because previous studies suggest that treatment with NSAIDs inhibits expression of PPARβ/δ in human colon cancer cell lines [20,32,33]. Average colon tumor multiplicity was 6.3 ± 0.8 in control $Apc^{+/\text{Min-FCCC}}$ mice and 4.3 ± 0.8 in $Apc^{+/\text{Min-FCCC}}$ mice fed sulindac ($P = 0.057$). PPARβ/δ protein was markedly lower (~60%), and expression of CYCLIN D1 was markedly higher (~400%) in control $Apc^{+/\text{Min-FCCC}}$ mouse colon tumors as compared to control colon mucosa (Figure 2A, Supplemental Figure 1B). Similarly, expression of PPARγ1 was markedly lower (~70%) in colon tumors as compared to colon mucosa in $Apc^{+/\text{Min-FCCC}}$ mice (Figure 2A). Similar changes in PPARβ/δ, PPARγ1, and CYCLIN D1 were observed in $Apc^{+/\text{Min-FCCC}}$ mouse colon tumors following treatment with dietary sulindac as compared to sulindac-treated colon mucosa (Figure 2A, Supplemental Figure 1B). The expression pattern of *Pparβ/δ* and *Pparγ* mRNA (Figure 2B) closely paralleled the observed decrease in PPARβ/δ and PPARγ1 protein expression found in colon tumors from $Apc^{+/\text{Min-FCCC}}$ mice (Figure 2A). Expression of *Pparβ/δ* and *Pparγ* mRNA was lower in colon tumors from $Apc^{+/\text{Min-FCCC}}$ mice fed either the control or sulindac diet (Figure 2B). Interestingly, while protein expression of PPARβ/δ was not different in colon mucosa from $Apc^{+/\text{Min-FCCC}}$ mice fed sulindac as compared to colon mucosa from $Apc^{+/\text{Min-FCCC}}$ mice fed the control diet, expression of *Pparβ/δ* mRNA was higher in colon mucosa from $Apc^{+/\text{Min-FCCC}}$ mice fed sulindac as compared to colon mucosa from $Apc^{+/\text{Min-FCCC}}$ mice fed the control diet (Figure 2B).

Effect of Ligand Activation of PPARβ/δ on NSAID or Hydrogen Peroxide-Induced Apoptosis

Previous studies suggest that one mechanism by which NSAIDs inhibit colon cancer cell proliferation is through decreasing expression of PPARβ/δ resulting in increased apoptosis, possibly mediated by PPARβ/δ-dependent downregulation of 14-3-3ε [20,32,33]. Similarly, it was suggested that ligand activation of PPARβ/δ results in anti-apoptotic activity via direct upregulation of 14-3-3ε [21]. These hypothetical mechanisms were critically evaluated in the present study using the same human colon cancer cell lines and concentrations of NSAIDs by examining quantitative expression of PPARβ/δ and 14-3-3ε, cleavage of PARP, and quantification of annexin V-positive cells using flow cytometry. In contrast to previous studies [20,32,33], expression of PPARβ/δ was either unchanged or increased in RKO, DLD1, or HT29 cells treated with indomethacin, sulindac, sulindac sulfide, or hydrogen peroxide as compared to controls not treated with the indomethacin, sulindac, sulindac sulfide, or hydrogen peroxide (Supplemental Figure 2). Expression of PPARβ/δ was unchanged in DLD1 cells following treatment with indomethacin or sulindac sulfide, with and without co-treatment with GW0742 as compared to controls not treated with the NSAIDs (Supplemental Figure 2). Expression of PPARβ/δ was also unchanged in RKO, DLD1, and HT29 cells following treatment with hydrogen peroxide, with and without co-treatment with GW0742 as compared to controls not treated with the hydrogen peroxide (Supplemental Figure 2). In contrast, expression of PPARβ/δ was increased in RKO and HT29 cells treated with indomethacin or sulindac sulfide, and in RKO, DLD1, and HT29 cells treated with sulindac; with and without co-treatment with GW0742 as compared to controls not treated with the NSAIDs (Supplemental Figure 2). Increased cleavage of PARP was observed in RKO and DLD1 cells, but not in HT29 cells, cultured with indomethacin and sulindac sulfide, as compared to controls (Figure 3, Supplemental Figure 3). A significant increase in PARP cleavage was observed in DLD1 cells co-treated with indomethacin and GW0742 (0.1–10 μM) as compared to control DLD1 cells cultured with only indomethacin (Figure 3, Supplemental Figure 3). A similar effect was also observed in DLD1 cells co-cultured with indomethacin and another PPARβ/δ ligand GW501516 (Supplemental Figure 4). While ligand activation of PPARβ/δ with GW0742 did not influence PARP cleavage in HT29 cells co-treated with sulindac sulfide, an increase in PARP cleavage was observed in RKO cells co-treated with sulindac sulfide and GW0742 (1.0 and 10 μM) and in DLD1 cells co-treated with sulindac sulfide and

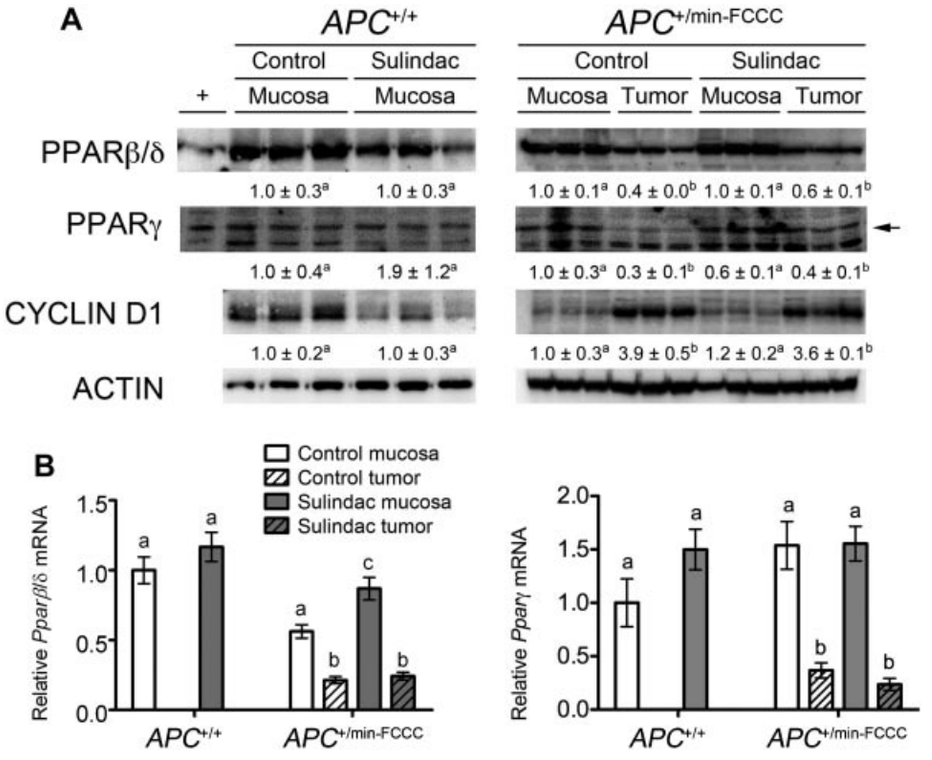

Figure 2. Expression of PPARβ/δ and PPARγ protein and mRNA in colon and colon tumors from wild-type and $APC^{+/Min\text{-}FCCC}$ mice. (A) Tissue extracts were prepared from colon (normal and tumor) from wild-type or $APC^{+/Min\text{-}FCCC}$ mice fed either a control diet or one containing sulindac as described in the Materials and Methods Section. Representative Western blots were performed to measure expression of PPARβ/δ, PPARγ, or CYCLIN D1 and normalized to ACTIN. + = positive control: lysate from COS1 cells transfected with mouse PPARβ/δ expression vector or in vitro translated mouse PPARγ1. A total of nine independent samples per group were examined. Arrow indicates immunoreactive band for PPARγ1. (B) Quantitative real-time PCR was performed to measure expression of mRNA encoding *Pparβ/δ* or *Pparγ1* and normalized to *Gapdh* mRNA. Normalized values were calculated from the nine independent samples described in (A) and represent the mean ± SEM from all independent samples. Values within a row with different superscripts are significantly different at $P \leq 0.05$.

GW0742 (0.1 and 10 μM; Figure 3, Supplemental Figure 3). Hydrogen peroxide (0.5 mM) did not cause a large increase in cleavage of PARP in RKO, DLD1, or HT29 cells as compared to controls, and co-treatment with GW0742 and hydrogen peroxide had no influence on PARP cleavage in any of the human colon cancer cell lines evaluated (Figure 3, Supplemental Figure 3). Expression of 14-3-3ε was not influenced by treatment with any NSAID, hydrogen peroxide, GW0742, or co-treatments, except for RKO cells co-treated with hydrogen peroxide and 10 μM GW0742, where a significant increase was observed (Figure 3).

Since PARP cleavage reflects later stages of apoptosis, earlier stages of apoptosis were examined by quantifying the presence of annexin V by flow cytometry. For this analysis, hydrogen peroxide was used because it can effectively increase apoptotic signaling after acute exposure. Based on exploratory experiments, it was determined that a reasonable range of apoptosis could be achieved by treating the three different cell lines with hydrogen peroxide at a concentration of either 0.5 or 5.0 mM for 4 h (data not shown). RKO cells were relatively resistant to hydrogen peroxide-induced apoptosis. While the percentage of cells undergoing late apoptosis/necrosis was marginally higher and the percentage of viable cells was marginally lower in RKO cells treated with 5.0 mM hydrogen peroxide for 4 h as compared to controls, these trends were not statistically significant (Figure 4A). Ligand activation of PPARβ/δ in RKO cells treated with hydrogen peroxide did not influence the percentage of cells undergoing apoptosis in response to either 0.5 or 5.0 mM hydrogen peroxide (Figure 4A). In DLD1 cells, 0.5 and 5.0 mM hydrogen peroxide caused an increase in the percentage of cells undergoing late apoptosis/necrosis that was associated with a decrease in viable cells compared to untreated control DLD1 cells (Figure 4B). Ligand activation of PPARβ/δ in DLD1

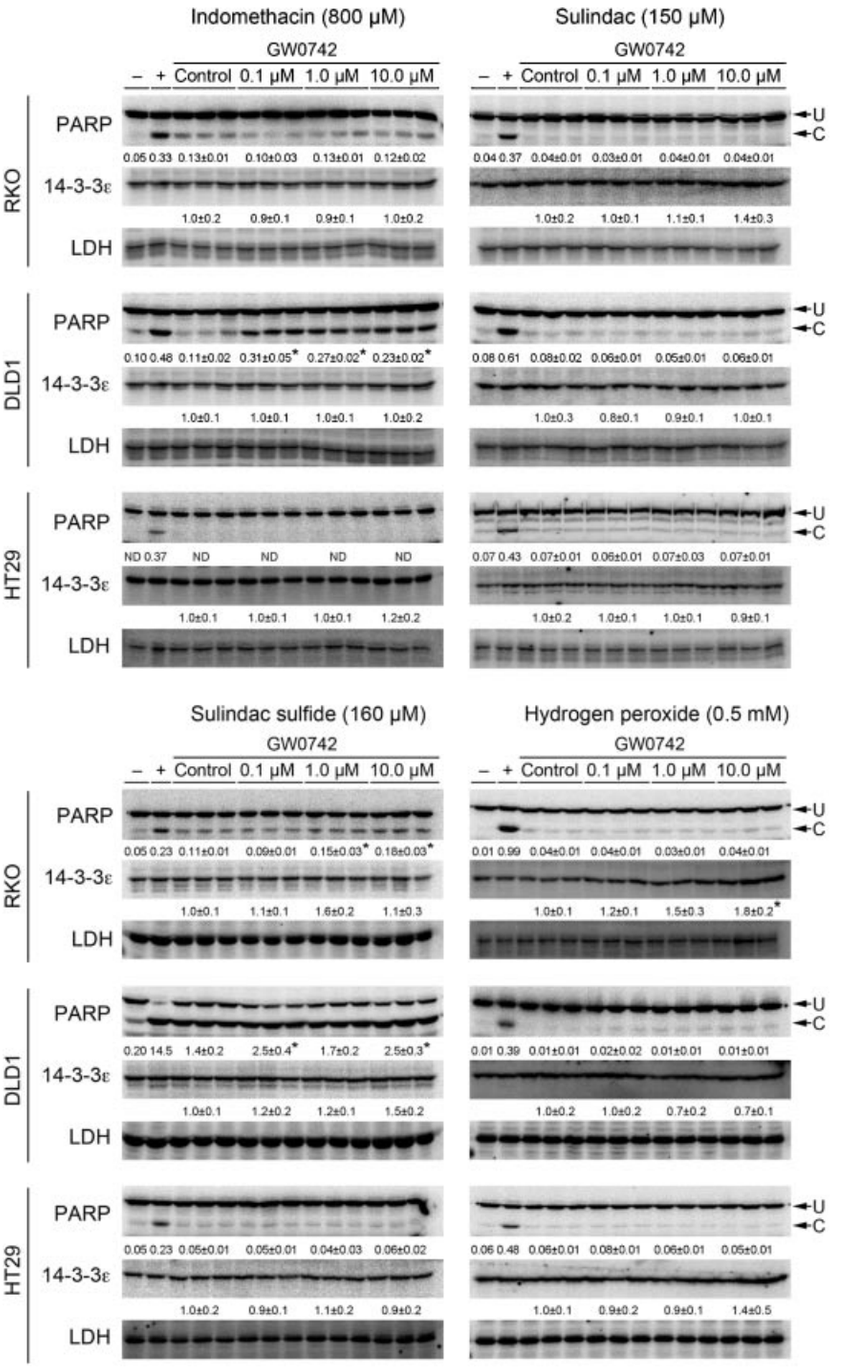

Figure 3. Effect of ligand activation of PPARβ/δ on markers of apoptosis in human colon cancer cell lines following induction of apoptosis by NSAIDs or hydrogen peroxide. Human colon cancer cell lines (RKO-wild-type APC/β-CATENIN, DLD1, and HT29-constitutively active APC/β-CATENIN) were cultured with the indicated concentrations of GW0742 and 800 μM indomethacin (upper left panel), 150 μM sulindac (upper right panel), 160 μM sulindac sulfide (lower left panel), or 0.5 mM hydrogen peroxide (lower right panel). For indomethacin, sulindac, and sulindac sulfide treatment, cells were cultured for 24 h and whole-cell lysates prepared at the end of this treatment period. For hydrogen peroxide treatment, cells were cultured for 4 h and whole-cell lysates prepared at the end of this treatment period. Cells cultured without the NSAID, hydrogen peroxide, or GW0742 served as a negative control (–). Cells cultured without the NSAID, hydrogen peroxide, or GW0742 but in the presence of 2 μM staurosporine for 5 h served as a positive control (+). Expression of uncleaved (U) and cleaved (C) PARP and 14-3-3ε were measured by quantitative Western blots. The immunoreactive signals for uncleaved PARP, cleaved PARP, or 14-3-3ε were normalized to the immunoreactive signals for LDH. PARP expression is shown as the ratio of the normalized values of cleaved/uncleaved PARP and is presented as the mean ± SEM. Normalized 14-3-3ε expression is shown as the fold change as compared to control cells for each respective group and is presented as the mean ± SEM. A total of at least three independent samples were examined for each treatment except for the negative and positive controls. *Significantly different from control (NSAID-treated without GW0742) at $P \leq 0.05$. ND = cleaved PARP not detected.

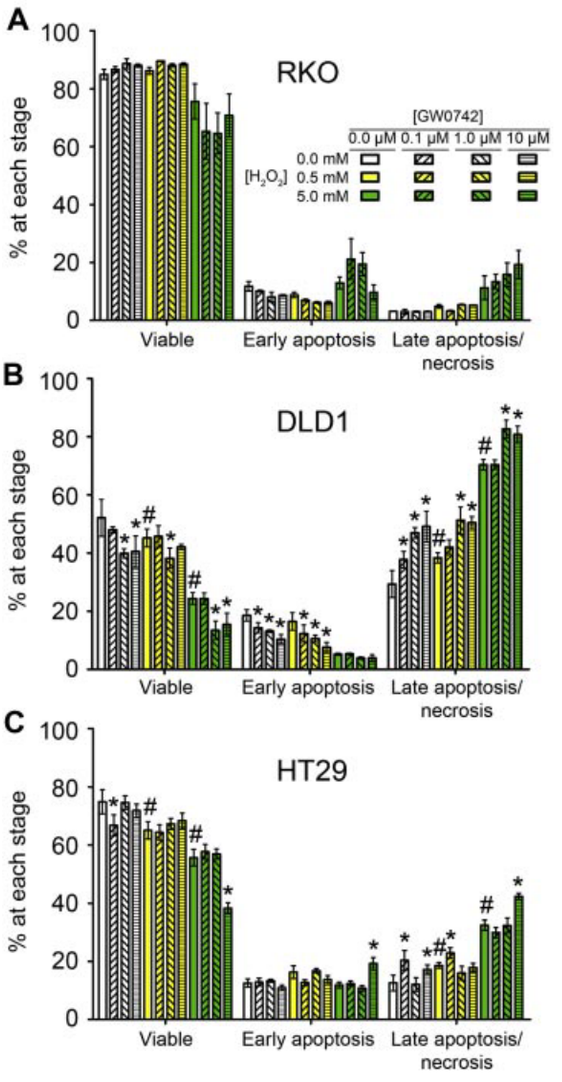

Figure 4. Modulation of hydrogen peroxide-induced apoptosis and cell viability following ligand activation of PPARβ/δ. (A) RKO, (B) DLD1, or (C) HT29 human colon cancer cell lines were cultured to approximately 80% confluency and then pretreated for 1 h with either 0.02% DMSO (control) or GW0742 (0.1, 1.0, and 10 μM). Cells were then treated for 4 h in medium containing either 0.0, 0.5, or 5.0 mM hydrogen peroxide in the presence of the indicated concentration of GW0742. Apoptosis and viability was determined by flow cytometry after labeling with PI and a FITC-labeled anti-annexin V antibody. Viable cells were defined as the percentage of cells that were annexin V-negative and PI-negative. Early apoptosis was defined as the percentage of cells that were annexin V-positive and PI-negative, and late apoptosis/necrosis was defined as the percentage of cells that were annexin V-negative and PI-positive or annexin V-positive and PI-positive. The percentage of cells within each category was calculated as described in the Materials and Methods Section. Values represent the mean ± SEM from at least three independent samples. #Significantly different from untreated control at $P \leq 0.05$. *Significantly different from respective control at $P \leq 0.05$.

cells caused a decrease in the percentage of cells undergoing early apoptosis in control cells and this effect was also observed in cells co-treated with 0.5 mM hydrogen peroxide and GW0742 (Figure 4B). However, these changes were reflected by an increase in cells undergoing late apoptosis/necrosis and a decrease in the percentage of viable cells (Figure 4B). Hydrogen peroxide exposure caused a dose dependent increase in the percentage of HT29 cells undergoing late apoptosis/necrosis and this was associated with a lower percentage of viable cells (Figure 4C). Co-treatment with hydrogen peroxide and GW0742 only caused an increase in the percentage of cells undergoing late apoptosis/necrosis (Figure 4C). Most notably, co-treatment of 5.0 mM hydrogen peroxide and 10 μM GW0742 in HT29 cells caused an increase in the percentage of early and late apoptotic/necrotic cells and a decrease in viable cells (Figure 4C).

Stable Over-Expression of PPARβ/δ Enhances Sensitivity to Ligand Activation and Inhibition of Clonogenicity

Since expression of PPARβ/δ protein is quantitatively lower in human colon adenocarcinomas and mouse colon tumors, the effect of over-expression of PPARβ/δ in human colon cancer cell lines was examined. The Migr1 expression vector was used for this purpose. This system allows for isolation of cells stably expressing PPARβ/δ because the bicistronic vector allows for expression of not only the protein of interest (e.g., PPARβ/δ) but also eGFP; the latter of which allows for efficient sorting. Indeed, high expression of eGFP is observed in RKO, DLD1, or HT29 cells that have stably integrated the Migr1 vector alone, or Migr1-hPPARβ/δ, while eGFP expression is lacking in the parent cell lines (Figure 5A). Expression of PPARβ/δ is also increased in RKO, DLD1, and HT29 cells that have stably integrated Migr1-hPPARβ/δ, as compared to control cells with the Migr1 vector alone or the parent cell line (Figure 5B). To determine whether the increase in PPARβ/δ expression was functional, the effect of ligand activation on target gene expression was examined. For this purpose, expression of *ADRP* was measured in RKO and *ANGPTL4* in DLD1 cells because previous work demonstrated that these genes were more responsive in these cell lines, respectively [19]. The increase in PPARβ/δ expression observed in human colon cancer cell lines that had stably integrated Migr1-hPPARβ/δ correlates well with increased efficacy of target gene induction in response to ligand activation of PPARβ/δ in RKO and DLD1 cells (Figure 5C). The efficacy of target gene induction in response to ligand activation of PPARβ/δ in HT29 cells that had stably integrated Migr1-hPPARβ/δ was only comparable to the efficacy observed in the parent HT29 cells (Figure 5C); however, it is worth noting that relative expression of Migr1-hPPARβ/δ was somewhat lower in HT29 cells as compared to RKO and DLD1 cells (Figure 5B).

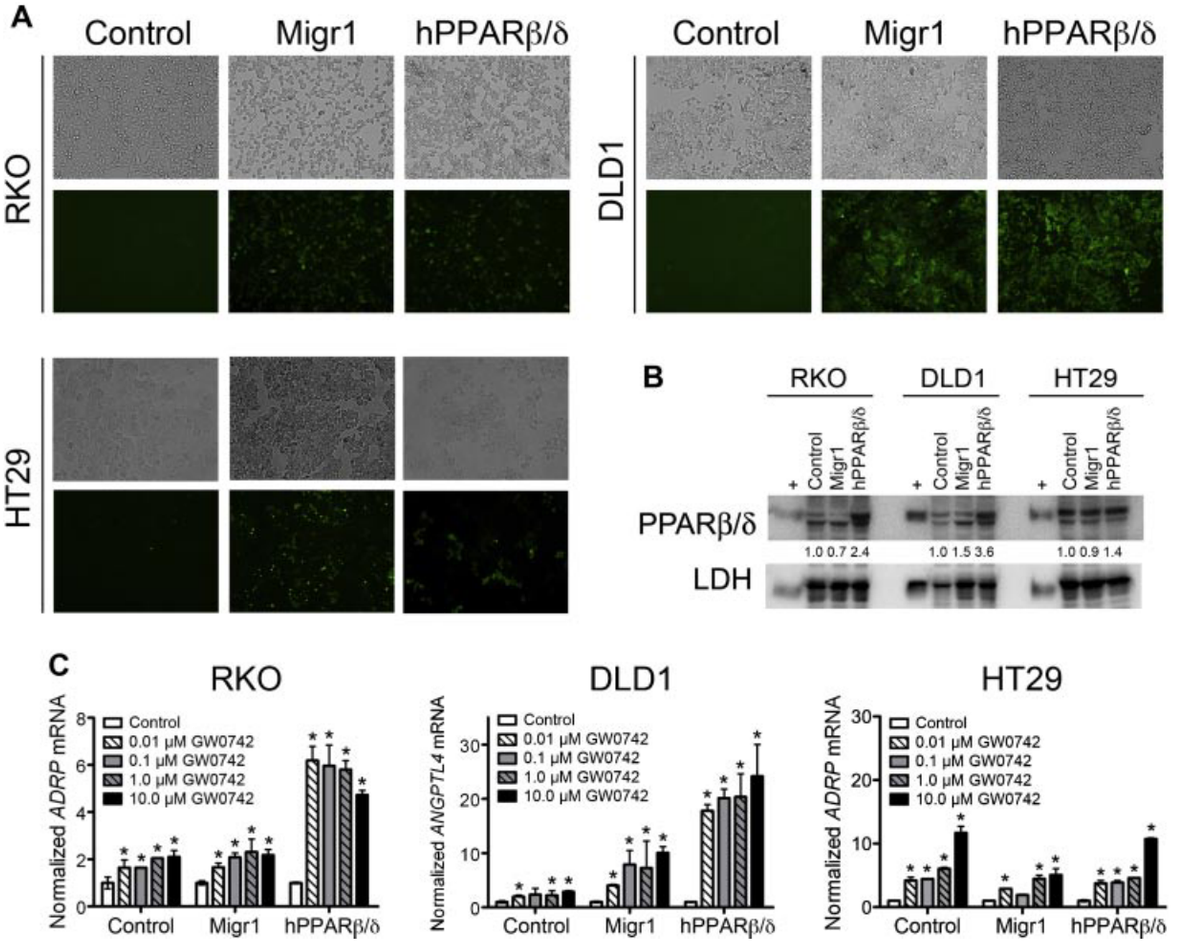

Figure 5. Characterization of stable human colon cancer cell lines over-expressing PPARβ/δ. (A) Human colon cancer cell lines (RKO, DLD1, and HT29) were used to generate stable cell lines over-expressing PPARβ/δ and eGFP as described in Materials and Methods Section. Representative photomicrographs using phase contrast microscopy (upper panels) or fluorescence (lower panels) of control (parent cell line), cells stably expressing the Migr1-empty vector (Migr1), or cells stably expressing PPARβ/δ (hPPARβ/δ). (B) Quantitative Western blots were performed to measure expression of PPARβ/δ in control (parent cell line), cells stably expressing the Migr1-empty vector (Migr1), or cells stably expressing PPARβ/δ (hPPARβ/δ). Values represent PPARβ/δ expression normalized to LDH control as compared to control cells. (C) Quantitative real-time PCR was performed to determine the effect of ligand activation of PPARβ/δ by GW0742 in control (parent cell line), cells stably expressing the Migr1-empty vector (Migr1), or cells stably expressing PPARβ/δ (hPPARβ/δ). Normalized *ADRP* or *ANGPTL4* mRNA were measured as markers of PPARβ/δ transcriptional activity. *Significantly different from respective control at $P \leq 0.05$.

Based on the known correlation between clonogenicity and in vivo tumorigenesis, colony formation assays were performed. Relative clonogenicity was inhibited in parent RKO cells in response to ligand activation of PPARβ/δ by treatment with either 0.01 or 1.0 μM GW0742 (Figure 6). Comparable inhibition of clonogenicity was also observed in RKO cells with stable integration of Migr1 or Migr1-hPPARβ/δ, cultured in medium containing between 0.01 and 10 μM GW0742 (Figure 6). Relative clonogenicity was inhibited in parent DLD1 cells in response to ligand activation of PPARβ/δ by treatment with 10 μM GW0742 (Figure 7), but no inhibition of clonogenicity was observed in DLD1 cells with stable integration of Migr1 or Migr1-hPPARβ/δ cultured in medium containing between 0.01 and 10 μM GW0742 (Figure 7). Relative clonogenicity was not changed in parent HT29 cells in response to ligand activation of PPARβ/δ by treatment with 0.01–10 μM GW0742 (Figure 8). Inhibition of clonogenicity was observed in HT29 cells with stable integration of Migr1 following treatment with only 10 μM GW0742 (Figure 8). Moreover, clonogenicity was inhibited in HT29 cells with stable integration of Migr1-hPPARβ/δ, cultured in medium containing between 0.01 and 10 μM GW0742 (Figure 8). Similar inhibition of clonogenicity was

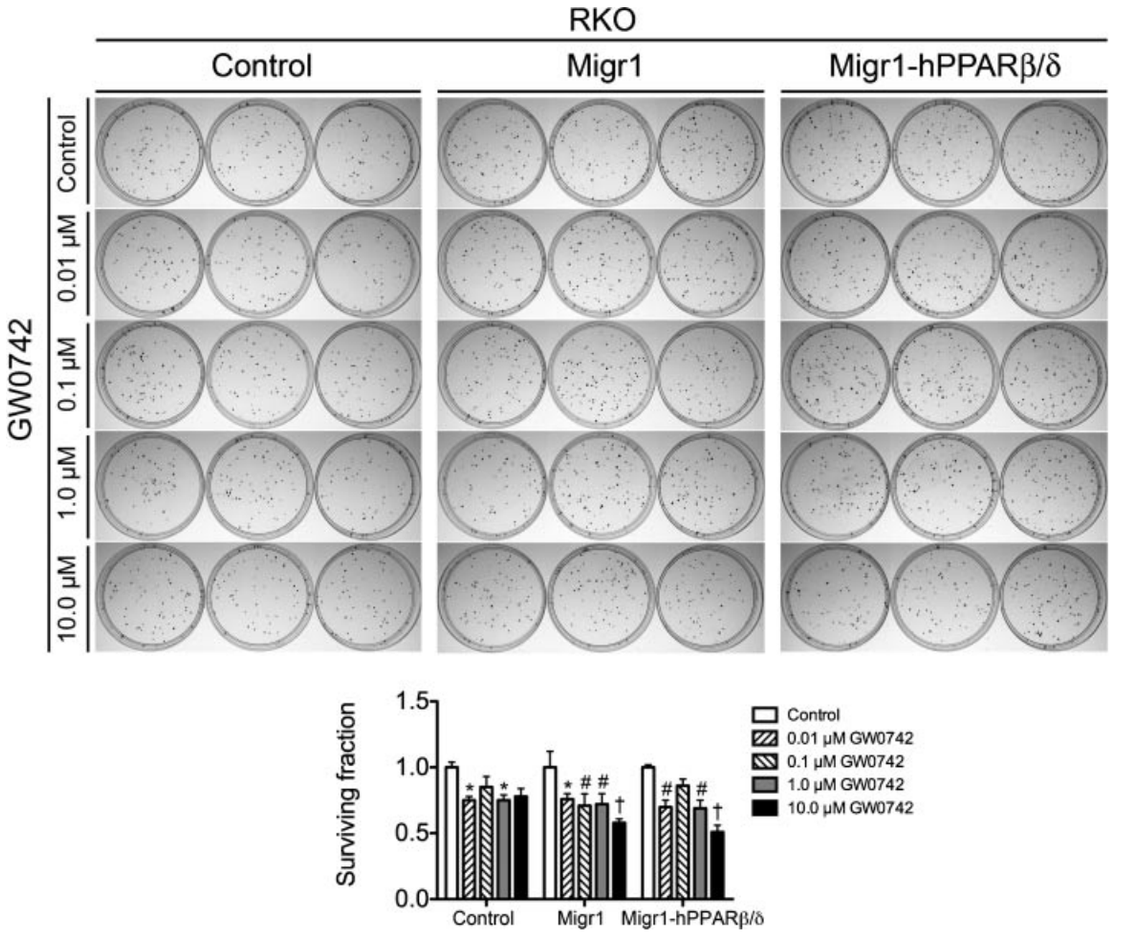

Figure 6. Effect of ligand activation of PPARβ/δ on colony formation in human colon cancer cell lines over-expressing PPARβ/δ. Control (parent RKO cell line), RKO cells stably expressing the Migr1-empty vector (Migr1), or RKO cells stably expressing PPARβ/δ (Migr1-hPPARβ/δ) were plated at 300 cells/well. After allowing the cells to adhere for 24 h, cells were treated with medium containing either: 0 (DMSO control), 0.1, 1.0, or 10 μM GW0742. Plating efficiency (%) was 32 ± 1, 48 ± 6, and 55 ± 1 for parent RKO, Migr1, and Migr1-hPPARβ/δ cells, respectively. The surviving fraction was calculated after colony number was quantified using Image J software (version 1.37, National Institutes of Health, Bethesda, MD). The surviving fraction is presented as the mean ± SEM. *Significantly different from control, $P < 0.05$. #Significantly different from control, $P < 0.01$. †Significantly different from control, $P < 0.001$.

observed in HT29 cells with stable integration of Migr1-hPPARβ/δ, cultured in medium containing 0.1 μM GW501516 (Supplemental Figure 5).

DISCUSSION

The present studies were undertaken to focus on several fundamental questions that remain concerning the functional role of PPARβ/δ in colon carcinogenesis. The first question addressed was whether expression of PPARβ/δ is altered in human and/or rodent colon tumors. It was originally hypothesized that expression of PPARβ/δ is directly upregulated by the APC/β-CATENIN/TCF4 pathway, similar to that observed for CYCLIN D1 and c-MYC [18]. While increased expression of PPARβ/δ in colon tumors has also been reported by other laboratories, the weight of evidence indicating that PPARβ/δ expression is not upregulated by the APC/β-CATENIN/TCF4 pathway is increasing (reviewed in Refs. [2,3]). For example, expression of PPARβ/δ is not increased in human colon cancer cell lines with gain-of-function mutations in the APC/β-CATENIN/TCF4 pathway, despite clear upregulation of expression of CYCLIN D1 and/or c-MYC [19]. To date, no studies have quantitatively examined expression of PPARβ/δ protein from cohorts of tumors and corresponding control tissue from human colon cancer patients. Thus, results from the present study are the first to demonstrate that expression of PPARβ/δ protein is lower in 19 human colon adenocarcinomas as

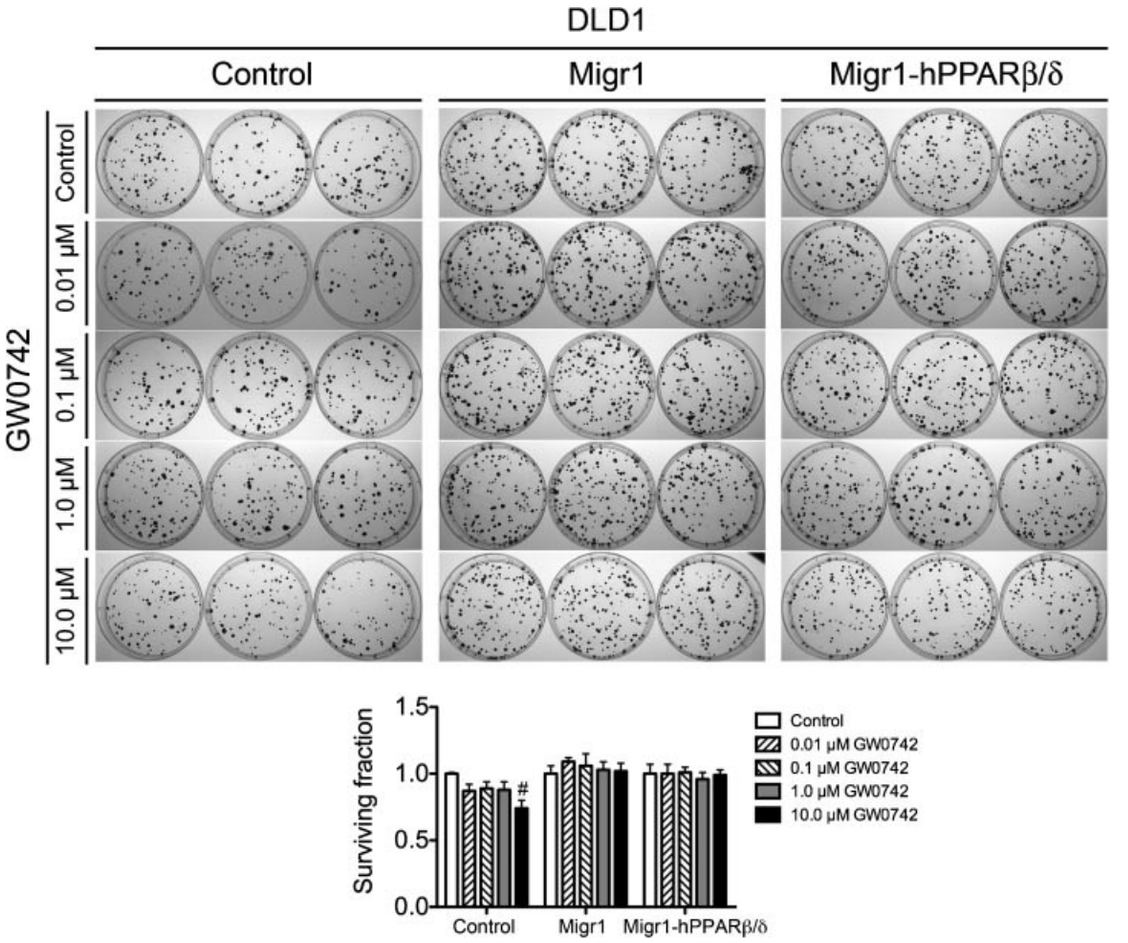

Figure 7. Effect of ligand activation of PPARβ/δ on colony formation in human colon cancer cell lines over-expressing PPARβ/δ. Control (parent DLD1 cell line), DLD1 cells stably expressing the Migr1-empty vector (Migr1), or DLD1 cells stably expressing PPARβ/δ (Migr1-hPPARβ/δ) were plated at 400 cells/well. After allowing the cells to adhere for 8 h, cells were treated with medium containing either: 0 (DMSO control), 0.1, 1.0, or 10 μM GW0742. Plating efficiency (%) was 20 ± 1, 27 ± 2, and 24 ± 2 for parent DLD1, Migr1, and Migr1-hPPARβ/δ cells, respectively. The surviving fraction was calculated after colony number was quantified using Image J software (version 1.37, National Institutes of Health, Bethesda, MD). The surviving fraction is presented as the mean ± SEM. #Significantly different from control, $P < 0.01$.

compared to matched control colon tissue. It is also important to note that this decrease in colon adenocarcinoma PPARβ/δ was associated with increased expression of CYCLIN D1. The decrease in expression of PPARβ/δ was specific for colon adenocarcinomas as similar changes were not found in a cohort of human rectal tumors. The observed decrease in PPARβ/δ expression in nine colon tumors from $Apc^{+/\text{Min-FCCC}}$ mice as compared to matched control colon mucosa is also highly consistent with the changes observed in human colon adenocarcinomas, as decreased expression of PPARβ/δ was also associated with markedly higher expression of CYCLIN D1. While previous work showed no change in expression of PPARβ/δ expression in small intestine tumors from $Apc^{+/\text{Min}}$ mice [19], it is important to note that the present study examined expression from colon tumors from $Apc^{+/\text{Min}}$ mice and that whole-cell lysates were used rather than nuclear fractions. Collectively, this is the most robust data set published to date that definitively demonstrates that expression of PPARβ/δ protein is decreased, not increased, during colon tumorigenesis.

Most studies to date examining expression of PPARβ/δ during colon carcinogenesis have focused primarily on *PPARβ/δ* mRNA expression. Previous studies suggesting that expression of PPARβ/δ is increased during colon tumorigenesis are often limited because expression of protein is not compared with that of mRNA (reviewed in Refs. [2,3]). Additionally, some studies suggesting increased expression of PPARβ/δ during colon tumorigenesis are limited to immunohistochemical analysis

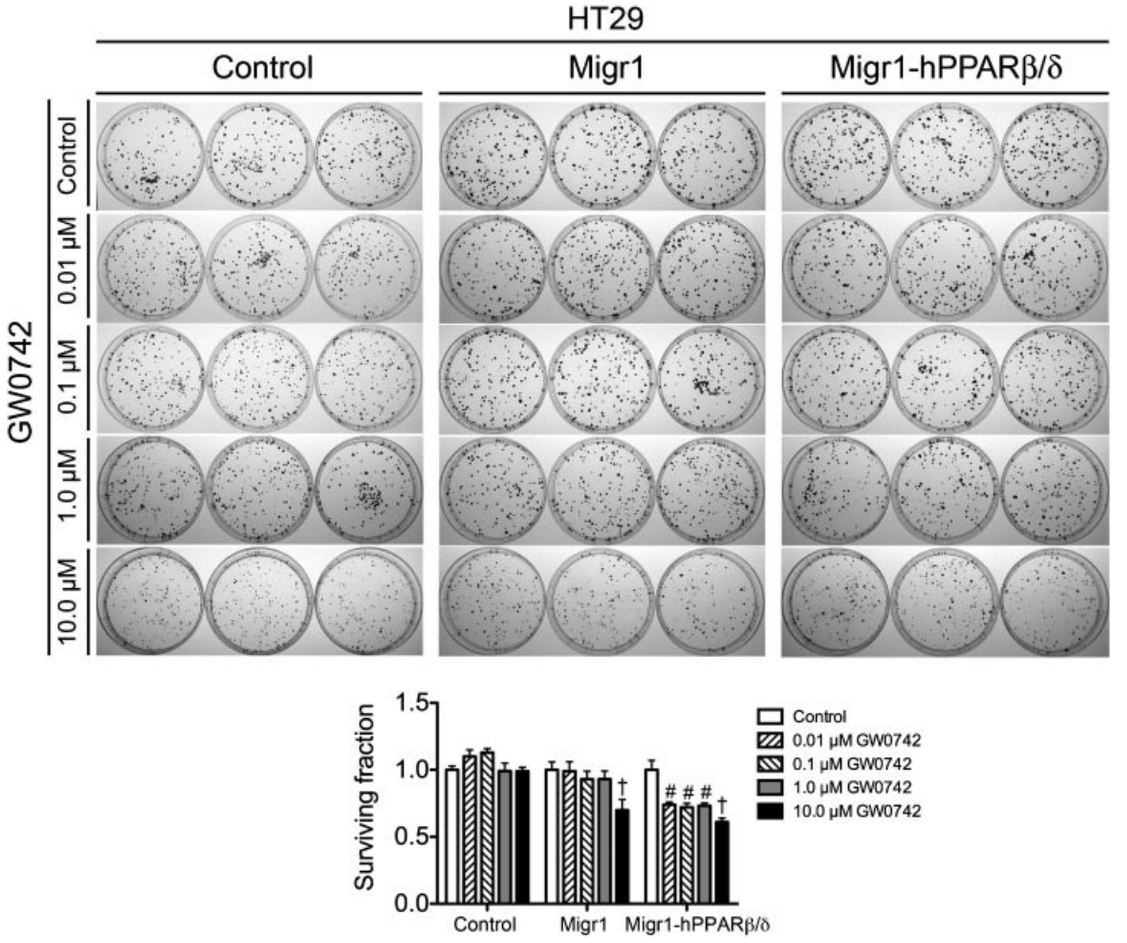

Figure 8. Effect of ligand activation of PPARβ/δ on colony formation in human colon cancer cell lines over-expressing PPARβ/δ. Control (parent HT29 cell line), HT29 cells stably expressing the Migr1-empty vector (Migr1), or HT29 cells stably expressing PPARβ/δ (Migr1-hPPARβ/δ) were plated at 600 cells/well. After allowing the cells to adhere for 8 h, cells were treated with medium containing either: 0 (DMSO control), 0.1, 1.0, or 10 μM GW0742. Plating efficiency (%) was 20 ± 1, 25 ± 2, and 27 ± 2 for parent HT29, Migr1, and Migr1-hPPARβ/δ cells, respectively. The surviving fraction was calculated after colony number was quantified using Image J software (version 1.37, National Institutes of Health, Bethesda, MD). The surviving fraction is presented as the mean ± SEM. #Significantly different from control, $P < 0.01$. †Significantly different from control, $P < 0.001$.

[34,35]. This is problematic because immunohistochemical analysis of PPARβ/δ expression is unreliable due to considerable non-specific immunoreactivity of PPARβ/δ antibodies. For example, while one study suggested that expression of nuclear PPARβ/δ is higher in mouse colon tumors based on immunohistochemical analysis [34], subsequent Western blot analysis using samples from the same study revealed no changes in nuclear expression of PPARβ/δ [19]. The hypothesis that expression of PPARβ/δ is increased during colon tumorigenesis as suggested by some (reviewed in Refs. [2,3]) is also at odds with the findings that colon and small intestine exhibit the highest expression of PPARβ/δ in mice [6]. Further, recent evidence from antibody proteomic analysis indicates that while expression of PPARβ/δ is strong in human colon cells, expression of PPARβ/δ is weak to negligible in human colorectal cancer [4,8]. Since there is considerable debate whether expression of PPARβ/δ is either increased or decreased during colon tumorigenesis, it is surprising that many reports fail to point out findings reporting that PPARβ/δ is not increased during colon carcinogenesis. Based on the definitive findings from the present work, it is clear that future studies should rigorously examine expression of PPARβ/δ protein to confirm changes in mRNA expression.

It is also of interest to note that expression of PPARγ1, was markedly lower in colon tumors from $Apc^{\text{Min-FCCC}}$ mice, but not in human colon or

rectal adenocarcinomas. Similar results have also been observed in small intestine polyps from $Apc^{+/Min}$ mice [19]. Additionally, decreased PPARγ mRNA was also reported to occur in colon polyps from azoxymethane-treated mice that correlated with reduced protein expression based on immunohistochemical analysis [36]. In contrast, other studies reported no change in expression of PPARγ in $Apc^{+/Min}$ mice [37,38] or even increased expression of PPARγ in colon polyps from azoxymethane-treated rats or $Apc^{+/Min}$ mice [39–41]. The reason for these differences cannot be determined from the present work. The reason why decreased expression of PPARγ1 was not found in human colon or rectal tumors in the present study is also uncertain. While one study showed no difference in expression of PPARγ mRNA between colonic epithelial cells and tubular adenomas [42], decreased PPARγ mRNA has been found in colon tumors from acromegalic patients [43,44]. Further studies are necessary to determine why colon tumors from $Apc^{+/Min\text{-}FCCC}$ mice exhibit decreased expression of PPARγ1, while human colon and rectal tumors do not.

The second issue addressed by the present study is whether NSAIDs downregulate expression of PPARβ/δ during colon carcinogenesis, which in turn promotes apoptotic signaling. This notion is based on the hypothesis that PPARβ/δ is anti-apoptotic and prevents NSAID-induced apoptosis by increasing expression of the 14-3-3ε that enhances sequestration of Bad, a pro-apoptotic member of the Bcl-2 family [20,21,23]. This hypothesis is based on studies using human colon cancer cell lines (DLD1 and HT29) and endothelial cells treated with NSAIDs (sulindac sulfide and indomethacin) or hydrogen peroxide to induce apoptosis. Thus, the present study used the same human colon cancer cell lines and the same concentrations of NSAIDs to critically examine the hypothesis that NSAID- or hydrogen peroxide-induced apoptosis is mediated by downregulation of PPARβ/δ due to decreased expression of 14-3-3ε that leads to increased apoptosis. In contrast to several reports [20,21,23], results from the present study demonstrate that NSAIDs (sulindac, sulindac sulfide, and indomethacin) do not decrease expression of PPARβ/δ, but rather, expression of PPARβ/δ is either unchanged or increased by these drugs. This observation is consistent with a number of other studies (reviewed in Refs. [2,3]) including the recent observation that indomethacin increases expression and function of PPARβ/δ in RKO human colon cancer cell lines [19]. Additionally, in vivo analyses reveal that nimesulide does not alter expression of PPARβ/δ in the mouse colon [45], and that sulindac does not alter expression of PPARβ/δ in the colon or colon tumors from $Apc^{+/Min\text{-}FCCC}$ mice as shown from the present studies. While expression of 14-3-3ε was increased in RKO cells co-treated with hydrogen peroxide and 10 μM GW0742, this change in expression was not associated with anti-apoptotic activity, and no changes in 14-3-3ε were observed in all other treatment paradigms. Collectively, these observations suggest that NSAIDs do not downregulate expression of PPARβ/δ in colon cancer models and emphasizes the need to critically examine the hypothesis that PPARβ/δ is anti-apoptotic and prevents NSAID-induced apoptosis by increasing expression of the 14-3-3ε that enhances sequestration of Bad as suggested by others [20,21,23].

Since NSAIDs do not downregulate PPARβ/δ expression in either human colon cancer cell lines or colon tumors from $Apc^{+/Min\text{-}FCCC}$ mice, it is not surprising that ligand activation of PPARβ/δ did not attenuate PARP cleavage following treatment with either sulindac, sulindac sulfide, indomethacin, or hydrogen peroxide. In fact, the only change observed in PARP cleavage was that co-treatment of NSAIDs with GW0742 enhanced PARP cleavage in DLD1 and RKO cells. These findings demonstrate that ligand activation can promote apoptosis in human colon cancer cells when combined with indomethacin or sulindac sulfide, rather than attenuate apoptosis as suggested by others (reviewed in Refs. [2,3]). It is thus noteworthy that a dose-dependent decrease in the percentage of DLD1 cells undergoing early apoptosis was observed in response to ligand activation of PPARβ/δ following induction of apoptosis with hydrogen peroxide. This is important to note, because this change was associated with a concomitant increase in the percentage of cells undergoing late apoptosis/necrosis and a decrease in the percentage of viable cells. These observations might explain why others suggest that PPARβ/δ promotes anti-apoptotic activities, when in fact, this change is associated with more cells that have already undergone apoptosis/necrosis, but not with more viable cells. This also illustrates the need for future studies to comprehensively examine the effect of PPARβ/δ on apoptosis, including examination of different stages of apoptosis and cell viability.

The effect of increasing expression of PPARβ/δ in human colon cancer cells was the last important issue examined by the present study. Whether PPARβ/δ promotes or attenuates colon tumorigenesis remains uncertain. Results from the present study showing markedly lower expression of PPARβ/δ in both human colon adenocarcinomas and colon tumors from $Apc^{+/Min\text{-}FCCC}$ mice suggest that increasing expression of PPARβ/δ will attenuate colon tumorigenesis. Indeed, PPARβ/δ attenuation of colon tumorigenesis has been observed in some null mouse models [45–47], but not all [48,49]. Similarly, knockdown of PPARβ/δ in

HCT116 human colon cancer cells is reported to increase cell proliferation in one model [50], but prevent xenograft tumorigenesis in another [51]. Thus, examination of PPARβ/δ over-expression is an alternative approach to those that have been used previously to examine the role of PPARβ/δ expression in colon carcinogenesis. Over-expression of PPARβ/δ in RKO and DLD1 cells increased the efficacy of ligand activation as target gene expression is enhanced in cells over-expressing PPARβ/δ as compared to control cells. Why enhanced target gene expression in HT29 cells over-expressing PPARβ/δ compared to control was not observed cannot be determined from this work, but could be due to differences in the presence of co-activators, co-repressors, or other accessory proteins (e.g., RXR), epigenetic differences in the promoter regions of PPARβ/δ target genes, to the fact that HT29 cells have two mutant copies of the APC allele and/or to differences related to site(s) of integration of the retroviral vector. Ligand activation of PPARβ/δ inhibited clonogenicity in RKO cells, but over-expression of PPARβ/δ did not markedly enhance this effect. Ligand activation of PPARβ/δ with GW0742 inhibited clonogenicity in DLD1 cells, but only at a concentration of 10 μM. No change in clonogenicity was found in either Migr1-control DLD1 cells or in DLD1 cells over-expressing PPARβ/δ in response to ligand activation of PPARβ/δ. The lack of an enhanced effect in cells with stable integration of either Migr1 or Migr1-hPPARβ/δ suggests that the Migr1 vector contributes to this phenotype. Interestingly, despite the lack of enhanced efficacy on target gene expression, over-expression of PPARβ/δ caused enhanced inhibition of clonogenicity in HT29 cells as compared to control HT29 cells. The reason why enhanced inhibition of clonogenicity was only observed in HT29 cells that modestly over-expressed PPARβ/δ and co-treated with the highly specific PPARβ/δ GW0742, but not in control HT29 cells treated with GW0742, is unclear. This could be due to differences in the ability of PPARβ/δ to alter gene expression and function in the different cell lines through undefined mechanisms. The inhibition of clonogenicity suggests that the observed decrease in expression of PPARβ/δ found in human colon adenocarcinomas could be causally related to colon tumor progression, and that restoring or activating PPARβ/δ may be a suitable target for preventing colon tumorigenesis. This is consistent with previous work showing PPARβ/δ-dependent inhibition of colon tumorigenicity following ligand activation of PPARβ/δ in mice [45–47]. Importantly, in the presence of increased expression of PPARβ/δ, human colon cancer cell line clonogenicity is either unaffected or is inhibited further in response to ligand activation of PPARβ/δ. No increase in clonogenicity was observed in any of the three different models.

Combined, the results from these studies significantly advance the field because they are the first to provide quantitative evidence from both human and mouse models of colon cancer demonstrating that expression of PPARβ/δ is lower during colon tumorigenesis. These findings increase the body of evidence supporting the hypothesis that activating PPARβ/δ prevents colon tumorigenesis. The reason(s) why some studies suggest that PPARβ/δ promotes tumorigenesis through a variety of unconfirmed mechanisms remain unclear. However, given the findings from the present studies, future work should include definitive examination of PPARβ/δ expression, and not rely on past reports to suggest increased expression of PPARβ/δ during colon tumorigenesis. Results from the present study also emphasize that comprehensive analysis of apoptosis including cytometric analysis of viable and apoptotic cells are recommended. Finally, there remains a need for more fundamental research on the role of PPARβ/δ in colon cancer to help resolve conflicting reports in the literature.

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