Potentiation of Colon Cancer Susceptibility in Mice by Colonic Epithelial PPAR-δ/β Overexpression

Xiangsheng Zuo, Min Xu, Jiang Yu, Yuanqing Wu, Micheline J. Moussalli, Ganiraju C. Manyam, Sun II Lee, Shoudan Liang, Mihai Gagea, Jeffrey S. Morris, Russell R. Broaddus, Imad Shureiqi

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Correspondence to: Imad Shureiqi, MD, Department of Gastrointestinal Medical Oncology, Unit 426, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030-4009 (e-mail: ishureiqi@mdanderson.org).

Background The nuclear receptor peroxisome proliferator-activated receptor-δ/β (PPAR-d) is upregulated in human colorectal cancers, but its role in colonic tumorigenesis remains controversial.

Methods We generated a novel mouse model of intestinally targeted PPAR-d overexpression to simulate PPAR-d upregulation in human colon carcinogenesis. Colon-specific PPAR-d overexpression was confirmed by real-time reverse transcription polymerase chain reaction, immunoblotting, and activity assays. Mice with and without targeted PPAR-d overexpression were tested for azoxymethane (AOM)-induced colonic tumorigenesis. Mouse whole-genome transcriptome microarray analyses were performed to identify PPAR-d target genes to promote tumorigenesis. We used linear models to test for PPAR-d overexpression trend effects on tumor multiplicity. All statistical tests were two-sided.

Results Targeted PPAR-d overexpression markedly increased colonic tumor incidence (from 0 of 10 wild-type [WT] littermate mice to 9 of 10 mice [P < .001] in 2 FVB/N background mouse lines [villin-PPAR-d-1 and villin-PPAR-d-2] at a 5-mg/kg AOM dose) and multiplicity (number of tumors per mouse per mg/kg dose of AOM increased from 0.47 [95% confidence interval [CI] = 0.22 to 0.72] for the WT littermates to 2.15 [95% CI = 1.90 to 2.40] [P < .001] for the villin-PPAR-d-1 mice and from 0.44 [95% CI = 0.09 to 0.79] for the WT littermates to 1.91 [95% CI = 1.57 to 2.25] [P < .001] for the villin-PPAR-d-2 mice). PPAR-d overexpression reversed resistance to AOM-induced colonic tumorigenesis in C57BL/6 mice. PPAR-d overexpression modulated expression of several novel PPAR-d target genes in normal-appearing colonic epithelial cells of mice with PPAR-d overexpression in a pattern that matched the changes in colonic tumors.

Conclusions Our finding that PPAR-d upregulation profoundly enhances susceptibility to colonic tumorigenesis should impact the development of strategies of molecularly targeting PPAR-d in cancer and noncancerous diseases.


The nuclear receptor proliferator-activated receptor-δ/β (PPAR-d), the most widely expressed member of the PPAR ligand-activated transcription factor family in human cells, modulates many cellular functions critical for both health and disease, including fatty acid metabolism, obesity, wound healing, apoptosis, and inflammation (1). PPAR-d agonists have been developed and tested clinically to treat metabolic disorders, including dyslipidemia (2,3). The major challenge facing development of PPAR-d therapeutic targeting is that the role of PPAR-d in tumorigenesis remains unclear and highly controversial (1,4). Testing PPAR-d agonists in diseases such as dyslipidemia and obesity usually requires only short-term studies, during which any pro-tumorigenic effects of PPAR-d might be missed. Availability of PPAR-d agonists for general use in treatment of diseases such as dyslipidemia or obesity, which have incidences reaching epidemic proportions, could endanger the health of millions of individuals before any cancer risk in humans becomes evident. Furthermore, if PPAR-d upregulation is confirmed to promote cancer, this could open new opportunities to develop PPAR-d inhibitors to treat cancer. Therefore, data to clearly establish the role of PPAR-d in tumorigenesis are much needed.

Several studies have shown that PPAR-d is upregulated in human colorectal adenomas and cancers (5–11). However, mouse studies designed to test the role of PPAR-d in colonic tumorigenesis have been limited to genetic deletion studies, and these studies have produced contradictory results (12). For example, nontargeted PPAR-d knockout in APCmin mouse models non-statistically significantly reduced the incidence of intestinal tumorigenesis in one study (13), increased the incidence in another study (14), and strongly inhibited intestinal tumorigenesis in a third study (15). Even after publication of a report that targeted intestinal PPAR-d knockout strongly inhibited colonic tumorigenesis in mice (12), the
controversy regarding the role of PPAR-d in colonic tumorigenesis continues (4).

Genetic deletion of PPAR-d might be inadequate to study the impact of PPAR-d overexpression on tumorigenesis because the deletion could artificially alter cell biology by reducing PPAR-d expression to levels below constitutive levels in normal cells. We therefore developed a novel transgenic mouse model in which PPAR-d overexpression is targeted to the intestinal epithelial cells to simulate PPAR-d upregulation in human colon carcinogenesis.

**Methods**

**Generation of Villin-PPAR-d Mice**

We subcloned mouse PPAR-d cDNA into a villin promoter-driven expression construct that has been successfully used to produce targeted gene expression in mouse intestinal epithelial cells (16,17). The resulting targeting construct was injected using a pronuclear injection approach into fertilized mouse FVB/N (FVB) and C57BL/6 (B6) oocytes to generate villin-PPAR-d founder mice. Mice were housed and bred in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care at the University of Texas MD Anderson Cancer Center. Mice were maintained on a 12-hour light/12-hour dark cycle. Experiments were conducted according to protocols approved by the MD Anderson Institutional Animal Care and Use Committee. The mice were treated in accordance with the US Public Health Service Guide for the Care and Use of Laboratory Animals. Buprenorphine was administered at a dose of 0.5 to 2.5 mg/kg subcutaneously every 6 to 12 hours for analgesia.

**Colonic Tumorigenesis Induction by Azoxymethane**

Six- to eight-week-old PPAR-d-overexpressing mice and wild-type (WT) littermates (n = 6–10 per group) were injected intraperitoneally with azoxymethane (AOM; Sigma-Aldrich, St. Louis, MO) once a week for 6 weeks. FVB mice were injected with one of three doses of AOM: 5.0 mg/kg, 7.5 mg/kg, or 10 mg/kg body weight. B6 mice were injected with AOM at a dose of 10 mg/kg body weight. The mice were monitored until they were killed at 16 weeks (FVB) or 32 weeks (B6) after the last AOM injection, similar to what was previously described (12).

**Whole-Mouse Gene Transcriptome Studies**

Global transcriptome studies were performed using Agilent’s Whole Mouse Genome Oligo-4 × 44K microarray (Agilent Technologies, Palo Alto, CA, catalog No. G4122F) according to the manufacturer’s protocol. This array, developed using information from leading public databases to represent all known murine genes and resulting transcripts, is composed of 41,534 oligonucleotide probes (60-mer) representing more than 41,000 mouse genes and transcripts. RNA was isolated from normal and tumor samples of villin-PPAR-d-3 mice (villin-PPAR-d mouse line with B6 background) and their WT littermates treated with AOM 10 mg/kg and followed as described for the B6 mice. Excised tumor samples were pooled from each individual mouse for RNA extraction. Isolated normal colonic crypt epithelial cells were used for RNA isolation, as described in the Supplementary Methods (available online). Normal colonic crypt epithelial cells from villin-PPAR-d-3 tumor-bearing mice were used for comparison with their paired tumor samples. In the second comparison, normal colonic epithelial RNA samples from tumor-bearing villin-PPAR-d-3 mice were used for comparison with normal samples from their WT littermate mice, none of which developed tumors.

Briefly, 200 mg of total RNA/sample were reverse-transcribed into cDNA using an Agilent Quick Amp labeling kit (Agilent Technologies) by T7 oligo-dT promoter primer incorporation before production of fluorescent cRNA. The labeled cRNA was purified using a QiaGen RNeasy Mini Kit (Qiagen, Germantown, MD) and quantified using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Waltham, MA). A Gene Expression Hybridization Kit (Agilent Technologies) was used to hybridize Cy3-labeled (reference) and Cy5-labeled (sample) cRNAs to the array. The hybridized arrays were incubated in Agilent SureHyb chambers for 17 hours at 65°C, washed according to the manufacturer’s instructions, and then scanned on an Agilent’s dual-laser-based scanner.

Agilent Feature Extraction software GE2-v5.95 was used to link a feature to a design file and determine the relative fluorescence intensity between the two samples. Gene lists were created using P value information from the internal replicates within the microarray.

**Bioinformatics Analyses of Transcriptome Data and Canonical Pathway Analysis**

MA plots were used to verify the quality of the two-color arrays. Cluster analyses were performed using hierarchical clustering and principal component analysis. Quality-control analysis with MA plots of the arrays suggested no further normalization on the data. Differential expression analysis was performed using t tests by comparing various sample groups with samples of normal colonic epithelial cells from mice that developed tumors (villin-PPAR-d-3 mice). The false discovery rate was obtained from the P values by the beta-uniform mixture method (18). The P values from the t tests and fold-change (log 2 ratio) between tumors and paired normal tissue in villin-PPAR-d-3 mice and between normal cells in villin-PPAR-d-3 mice and their WT littermates were used to derive the transcripts with differential expression in both comparisons.

Differentially expressed genes were subjected to canonical pathway analysis using Ingenuity Pathway Analysis software (Ingenuity Systems, http://www.ingenuity.com) to characterize pathways that were differentially modulated by PPAR-d overexpression in colonic epithelial cells. The probability of association between these differentially modulated genes and canonical pathways was calculated using Fisher’s exact test.

**Statistical Analyses**

Quantitative outcome measures for one factor in experimental conditions were compared using one-way analysis of variance, and Bonferroni adjustments were used for all multiple comparisons. We used two-way analysis of variance to analyze data involving the simultaneous consideration of two factors. Tumor incidence was compared using Fisher’s exact tests. Poisson regression was used for tumor count analyses, as previously described (12). We used linear models to test for PPAR-d overexpression trend effects on tumor multiplicity (tumor count per mouse) per milligram per kilogram
dose of AOM. The model included a linear term for AOM dose with separate slopes for villin-PPAR-d-1, villin-PPAR-d-2, and their negative littermate groups and no intercept, and a contrast statement was used to test for difference in slopes between the two groups. The data were log-transformed as necessary to accommodate the normality and homoscedasticity assumptions implicit to the statistical procedures used. All tests were two-sided and conducted at a significance level of \( P \) less than .05. Data were analyzed using SAS software version 9.2 (SAS Institute, Cary, NC). Further methods details are available in the Supplementary Methods, available online.

**Results**

**Generation of Mice With Targeted Intestinal PPAR-d Overexpression**

We generated mice with targeted intestinal PPAR-d overexpression by subcloning mouse PPAR-d cDNA into a villin promoter-driven expression construct (p12.4Kvill-PPAR-d) that was used for pronuclear injection into B6 and FVB fertilized oocytes. Two mouse lines on FVB background (villin-PPAR-d-1 and villin-PPAR-d-2) and one mouse line on B6 background (derived from founder 1; villin-PPAR-d-3) were selected for testing (Figure 1A; Supplementary Figure 1, A and B, available online). In all tested

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**Figure 1.** Generation of villin-PPAR-d-1 and villin-PPAR-d-2 mouse lines with targeted intestinal peroxisome proliferator-activated receptor-\( \delta/\beta \) (PPAR-d) overexpression. A) Identification of villin-PPAR-d founders of FVB genetic background. Quantitative polymerase chain reaction was used to measure genomic PPAR-d cDNA level of littermates after pronuclear injections of the villin-PPAR-d cDNA construct into fertilized oocytes. The genomic levels of PPAR-d cDNA were calculated relative to the level of a calibrator sample (founder 2). Neg indicates no detectable genomic PPAR-d cDNA. Values are means with 95% confidence intervals of duplicate measurements. Three repeated independent experiments showed similar results. *\( P < .001 \) for founders vs negs (one-way analysis of variance [ANOVA]). B and C) Targeted intestinal PPAR-d overexpression in villin-PPAR-d-1 (B) and villin-PPAR-d-2 (C) mice. PPAR-d mRNA expression was quantified by quantitative real-time polymerase chain reaction in isolated intestinal crypts from small and large intestine and samples from liver and spleen. Values are means with 95% confidence intervals of triplicate experiments. WT = wild-type littermates. PPAR-d indicates either villin-PPAR-d-1 or villin-PPAR-d-2 mice. *\( P < .001 \) (two-way ANOVA) for intestine (large or small) vs other organs. D) PPAR-d protein expression in villin-PPAR-d-1 and villin-PPAR-d-2 mice. Representative photographs of normal colonic mucosa stained for PPAR-d by immunohistochemistry. Scale bar = 100 \( \mu \)M. E) PPAR-d protein expression from intestinal crypts of small intestine (S) and colon (C) of villin-PPAR-d-1 and villin-PPAR-d-2 mice. + represents PPAR-d positive control. F and G) Effects of PPAR-d overexpression on PPAR-d activity in villin-PPAR-d-1 and villin-PPAR-d-2 mice. Primary colonic epithelial cells were isolated from mice, and the cells were treated with either 1 \( \mu \)M GW0742 (PPAR-d agonist) or vehicle solution (control) for 4 hours. Expression of the PPAR-d target gene PLIN2 was measured by quantitative real-time polymerase chain reaction. Values are means with 95% confidence intervals of triplicate measurements. *\( P < .001 \) for PLIN2 expression in villin-PPAR-d-1 and villin-PPAR-2 vs WT by two-way ANOVA are as shown. All statistical tests were two-sided.
mouse lines, PPAR-d mRNA expression was statistically significantly upregulated in the small and large intestines but not in other organs (Figure 1, B–E; Supplementary Figure 1, C and D, available online). The upregulation of PPAR-d mRNA expression levels in the liver of villin-PPAR-d-1 and villin-PPAR-d-2 mice was less than 5% of that of the intestinal PPAR-d mRNA expression levels: ratios of mean liver/small intestinal relative expression levels were 0.051 (95% confidence interval [CI] = 0.054 to 0.048) for villin-PPAR-d-1 and 0.045 (95% CI = 0.0453 to 0.0438) for villin-PPAR-d-2. The hepatic PPAR-d upregulation in these mice was biologically inconsequential because it resulted in a non-statistically significant increase in PLIN2 (formerly ADRP; adipose differentiation-related protein), a known PPAR-d target (19). PLIN2 mRNA relative expression levels compared with the levels in negative littermates were 1.0 (95% CI = 0.98 to 1.03) for villin-PPAR-d-1 and 1.16 (95% CI = 1.12 to 1.21) for villin-PPAR-d-2. The overexpressed PPAR-d protein was transcriptionally active in the intestine and increased PLIN2 mRNA in colonic crypt cells (Figure 1, F and G) to levels similar to those achieved with PPAR-d agonist treatment of WT littermates.

Villin-PPAR-d mice appeared to develop normally and were fertile. Randomly selected villin-PPAR-d-1 and villin-PPAR-d-2 mice and their WT littermates were killed at 10 and 50 weeks of age, necropsied, and examined grossly and histologically to characterize the effects of intestinal PPAR-d overexpression. Villin-PPAR-d-1 and villin-PPAR-d-2 mice had intestinal tract changes (described below) and marked depletion of the abdominal adipose tissue from the mesentery and around the abdominal organs. Otherwise, villin-PPAR-d-1 and villin-PPAR-d-2 mice did not differ from WT mice (Supplementary Tables 1 and 2, available online).

At 10 weeks of age, the small intestines of villin-PPAR-d mice were diffusely pale with mild to moderate increased thickness of the intestinal wall and dilation of the intestinal lumen (Supplementary Figure 2A, available online). The small intestines of villin-PPAR-d mice were statistically significantly longer than the small intestines of control WT littermate mice (P = .03) (Supplementary Table 1 and Supplementary Figure 2, B and C, available online). Microscopic examination of hematoxylin and eosin–stained sections revealed mild to moderate epithelial hyperplasia of small intestinal mucosa of 10-week-old villin-PPAR-d mice (Supplementary Figure 2, D and E, available online). The crypt proliferative zone, measured by Ki-67 immunohistochemistry, was markedly longer in villin-PPAR-d mice than in WT mice (Supplementary Figure 2, F and G, available online).

At 50 weeks of age, all villin-PPAR-d-1 and villin-PPAR-d-2 mice had marked thickening of the entire gastrointestinal tract, but the stomach and small intestine were more severely affected (Figure 2, A–D). The small intestines were diffusely white and markedly thickened with a dilated lumen. The large intestine (cecum and colon) had mildly to moderately increased thickness. The small intestine was statistically significantly longer in villin-PPAR-d mice (P < .003) (Figure 2E) than in WT mice. Epithelial hyperplasia of the mucosa of glandular stomach and small intestines of villin-PPAR-d mice became more evident at 50 weeks of age; the large intestine also showed hyperplasia, and the small intestine showed consistently diffuse villous blunting that seemed more extensive than at earlier time points (Figure 2, F–K). In addition, two of seven villin-PPAR-d mice examined had colonic adenomas (Figure 2K). The crypt proliferative zone, measure by Ki-67 immunohistochemistry, was statistically significantly longer in villin-PPAR-d mice than in WT mice (P < .001) (Figure 2, L and M).

Effects of PPAR-d Overexpression in Intestinal Epithelial Cells on AOM-Induced Colonic Tumorigenesis

We tested the susceptibility of these mouse lines with targeted intestinal PPAR-d overexpression to AOM-induced colonic tumorigenesis. AOM exposure is currently the best available method to simulate human colorectal tumorigenesis in mice (20). FVB mice are susceptible to AOM-induced colonic tumorigenesis at an AOM dose of 10 mg/kg (21). We therefore tested whether PPAR-d upregulation increased susceptibility to colon tumorigenesis by lowering the dose of AOM needed to induce colonic tumorigenesis. We used the two FVB mouse lines (villin-PPAR-d-1 and villin-PPAR-d-2) and three AOM dose levels: 10, 7.5, and 5 mg/kg. Colonic PPAR-d overexpression dramatically increased susceptibility to AOM-induced colonic tumorigenesis in both tested FVB mouse lines. For the villin-PPAR-d-1 mice, at the AOM dose of 5 mg/kg, tumors formed in zero of 10 WT littermates and nine of 10 mice (90%) with targeted PPAR-d overexpression (P < .001) (Figure 3A). The mean numbers of tumors per mouse were as follows: at the AOM dose of 5 mg/kg, 0 for the WT littermates and 3.6 (95% CI = 1.92 to 5.28) for the villin-PPAR-d-1 mice; at the AOM dose of 7.5 mg/kg, 2 (95% CI = 0.83 to 3.16) for the WT littermates and 13.8 (95% CI = 12.62 to 18.98) for the villin-PPAR-d-1 mice; and at the AOM dose of 10 mg/kg, 7.33 (95% CI = 5.19 to 9.48) for the WT littermates and 25.78 (95% CI = 22.19 to 29.51) for the villin-PPAR-d-1 mice (P < .001 for all comparisons) (Figure 3A). Similarly, for the villin-PPAR-d-2 mice, at the AOM dose of 5 mg/kg, tumors formed in zero of 10 WT littermates and nine of 10 mice (90%) with targeted PPAR-d overexpression (P < .001) (Figure 3B). The mean numbers of tumors per mouse were as follows: at the AOM dose of 5 mg/kg, 0 for the WT littermates and 2.3 (95% CI = 1.13 to 3.47) for the villin-PPAR-d-2 mice; at the AOM dose of 7.5 mg/kg, 1.1 (95% CI = 0.25 to 1.95) for the WT littermates and 10.7 (95% CI = 6.8 to 14.6) for the villin-PPAR-d-2 mice; and at the AOM dose of 10 mg/kg, 7.33 (95% CI = 4.33 to 10.34) for the WT littermates and 27.22 (95% CI = 21.22 to 33.22) for the villin-PPAR-d-2 mice (P < .001 for all comparisons) (Figure 3B). We used statistical modeling to test for PPAR-d overexpression trend effects on number of tumors per mouse per milligram per kilogram dose of AOM. The number of tumors per mouse per milligram per kilogram dose of AOM increased from 0.47 (95% CI = 0.22 to 0.72) for the WT littermates to 2.15 (95% CI = 1.9 to 2.4; P < .001) for the villin-PPAR-d-1 mice, a 4.57-fold increase (Supplementary Figure 3A, available online). Similarly, the number of tumors per mouse per mg/kg dose of AOM increased from 0.44 (95% CI = 0.09 to 0.79) for the WT littermates to 1.91 (95% CI = 1.57 to 2.25; P < .001) for the villin-PPAR-d-2 mice, a 4.34-fold increase (Supplementary Figure 3B, available online).

The increase in colonic tumor incidence in the villin-PPAR-d mice was observed for all tumor size categories (<1.5 mm, 1.5–3 mm, and >3 mm) for both mouse lines (Figure 3, C and D). Additionally, villin-PPAR-d mice had more microadenoma formation in grossly appearing colonic mucosa than WT littermates after AOM treatment (Supplementary Figure 3C, available online). Microscopically,
Figure 2. Effects of peroxisome proliferator-activated receptor-δ (PPAR-δ) overexpression on gastrointestinal tract. Randomly selected villin-PPAR-δ (villin-PPAR-δ-1 and villin-PPAR-δ-2) mice (villin-PPAR-δ) (n = 7 mice) and their wild-type (WT) littermates (n = 6 mice) were killed at 50 weeks of age, necropsied, and examined grossly and histologically. A and B) Gross images of gastrointestinal tract (1: stomach; 2: small intestine; 3: cecum; 4: colon). C and D) Hematoxylin and eosin-stained sections of “Swiss roll” preparations of small intestine show increased thickness of intestinal wall in villin-PPAR-d mice (D) in comparison with WT mice (C). Magnification x5; scale bar = 2 mm. E) Small intestinal length for the villin-PPAR-d and WT mice shown in panels A and B. Values are means ± 95% confidence interval. *P* for two-sided t test. F and G) There is marked epithelial hyperplasia of duodenal mucosa of villin-PPAR-d mice (G) in comparison with normal duodenum of WT mice (F). Magnification x100; scale bar = 200 μM. H and I) The jejunal mucosa of villin-PPAR-d mice has marked epithelial hyperplasia (I) in comparison with normal jejunum of WT mice (H). Magnification x200; scale bar = 100 μM. J and K) Adenoma of colonic mucosa (black arrows) of villin-PPAR-d mouse in comparison with normal colon of WT mouse. Magnification x200; scale bar = 1 mM. L and M) Proliferative zone lengths in small intestine of mice described in panel A and B were measured by Ki-67 immunohistochemistry staining of villin-PPAR-d and WT mouse (L). Values (M) are means ± 95% confidence intervals. *P* for two-sided t test.
Figure 3. Effects of targeted intestinal peroxisome proliferator-activated receptor-δ (PPAR-δ) overexpression on azoxymethane (AOM)-induced colonic tumorigenesis in FVB mice. A and B) Mice from 2 PPAR-δ-overexpressing lines (villin-PPAR-δ-1 and villin-PPAR-δ-2) and wild-type (WT) littermate FVB mice were treated with AOM weekly for 6 weeks at the indicated doses, killed, and examined for tumor formation as described in the Methods. Photographs were taken of the dissected colons of all the mice from each group. C and D) Distribution of tumor multiplicity for panels A and B according to tumor size categories by AOM dose for villin-PPAR-δ-1 (C) and villin-PPAR-δ-2 mice (D) and WT littermates. Values are means with 95% confidence intervals of numbers for 9 to 10 mice/group. *P < .001 and **P < .05 for villin-PPAR-δ-1 and villin-PPAR-δ-2 vs WT by two-way analysis of variance are as shown. E) Microscopic appearance of colon tumors in mice with targeted intestinal PPAR-δ overexpression. Representative photomicrographs of colorectal adenomas induced by AOM in villin-PPAR-δ-1 mice as described for panel A. Adenomas in villin-PPAR-δ-1 mice had broader bases and less demarcation (arrow) from surrounding normal colonic epithelium than adenomas in WT littermates, which had a fairly thin stalk and clear demarcation from adjacent nonneoplastic epithelium. F and G) Length of colonic crypt proliferative zones was determined by Ki-67 immunohistochemistry. F) Representative pictures of immunohistochemistry staining for AOM-treated villin-PPAR-δ-2 and WT mice. G) Mean and 95% confidence interval of crypt length measurements. Each dot represents the average measurement for proliferative zones for one mouse. P by two-sided t test.
adenomas in villin-PPAR-d mice had broader bases and less demarcation from surrounding normal colonic epithelium than adenomas in WT littermates, which had a fairly thin stalk and clear demarcation from adjacent nonneoplastic epithelium (Figure 3E). PPAR-d protein expression in intestinal epithelial cells, measured by immunohistochemistry, was higher in villin-PPAR-d mice than in WT mice; PPAR-d expression further increased in tumors compared with normal mucosa (Supplementary Figure 3D, available online). The crypt proliferative zone in normal mucosa, measured by Ki-67 immunohistochemistry, was markedly longer in AOM-treated villin-PPAR-d mice than in WT littermates (Figure 3, F and G).

B6 mice are resistant to AOM-induced tumor formation (21). Targeted intestinal PPAR-d overexpression overcame this resistance, as evidenced by the formation of colonic tumors with AOM (10 mg/kg) in villin-PPAR-d-3 mice with B6 genetic background but not in their WT littermates (Figure 4).

**PPAR-d Modulation of Intestinal Epithelial Cell Transcriptome to Promote Colonic Tumorigenesis**

Given PPAR-d’s known function as a transcription factor (1), we conducted mouse whole-genome transcriptome microarray analyses to identify target genes that were modulated by PPAR-d to promote tumorigenesis. We studied gene expression profiles in

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**Figure 4.** Effects of targeted intestinal peroxisome proliferator-activated receptor-δ/β (PPAR-d) overexpression on azoxymethane (AOM)-induced colonic tumorigenesis in B6 mice. A) Villin-PPAR-d-3 mice and wild-type (WT) littermate B6 mice were treated with AOM 10 mg/kg weekly for 6 weeks, killed, and examined for tumor formation as described in the Methods. Photographs were taken of the dissected colons of all the mice from each group. B) Scatter plots of tumor multiplicity for villin-PPAR-d-3 heterozygote (+/-) and homozygote (+/+) mice and WT littermates. The mean number of tumors per mouse was statistically significantly higher for the villin-PPAR-d-3 homozygote mice than for WT littermates (two-sided Poisson test). Horizontal lines represent means. C) Representative photomicrograph of a colorectal adenoma with high-grade dysplasia from a villin-PPAR-d-3 mouse (hematoxylin and eosin staining, ×200).
AOM-treated normal colonic epithelial cells and tumors of villin-PPAR-d-3 mice in which AOM induced colorectal tumors despite the B6 background. We also compared gene expression profiles in AOM-treated normal colonic epithelial cells between these mice and their WT littermates (n = 3 mice/group) that were resistant to AOM-induced colonic tumorigenesis. A large number of transcripts were differentially expressed between the tumor and normal colonic epithelial cells of villin-PPAR-d-3 mice (Figure 5A), which is in agreement with what we expected for colonic tumorigenesis (22). More important, however, a large number of transcripts were differentially expressed between the normal colonic epithelial cells of the villin-PPAR-d-3 mice and their WT littermates (Figure 5B). This differential expression in normal-appearing colonic epithelial cells demonstrated the ability of PPAR-d overexpression to differentially modulate gene expression in early stages preceding the development of morphological changes associated with tumors.

We next identified transcripts that were statistically significantly different not only between tumor and normal colonic epithelial cells of villin-PPAR-d-3 mice but also between normal colonic epithelial cells from villin-PPAR-d-3 mice and WT littermates. A transcript-level change was considered statistically significant if the fold change was equal to or greater than twofold in magnitude with P less than .05 on a t test. On the basis of these criteria, 29 transcripts were identified as differentially expressed for both comparisons (Figure 5C; Supplementary Table 3, available online). The 29 transcripts identified 14 upregulated and five downregulated genes with known function. These differentially expressed genes were evaluated using the Ingenuity Pathway Knowledge Base to determine the functionality of the pathways that were perturbed by PPAR-d overexpression in colonic epithelial cells. The calculated probability (P value) for each identified canonical pathway is a measurement of the likelihood that the association between this pathway and PPAR-d overexpression can be attributed to random chance. PPAR-d overexpression in colonic epithelial cells was statistically significantly associated with alteration of various canonical pathways (Figure 5D), including ones that contribute substantially to tumorigenesis promotion (eg, cell cycle, cellular movement, cell death and survival, and cellular growth and proliferation pathways).

To confirm that the differential gene expression profile associated with PPAR-d overexpression was not due to multiple-comparison false discoveries, we validated the differential expression of selected differentially upregulated and downregulated genes identified by the transcriptome screening studies (Figure 5C) using normal and tumor colonic epithelial cells from an independent mouse line (villin-PPAR-d-2) and using a different methodology to measure mRNA expression levels (quantitative real-time polymerase chain reaction). Differentially upregulated genes were selected for validation if their levels were at least five times as high in tumor as in normal colonic epithelial cells and at least 2.5 times as high in normal colonic epithelial cells of villin-PPAR-d-3 mice as in WT littermates. Differentially downregulated genes were selected for validation if their expression levels were less than or equal to 50% as high in normal colonic epithelial cells as in tumor and less than or equal to 60% as high in normal colonic epithelial cells of villin-PPAR-d-3 mice as in WT littermates. In this confirmation, which was limited to the selected genes by the later criteria and not all 29 genes, we robustly validated that expression of NRG1 and IFITM3 was statistically higher in tumor than normal colonic epithelial cells and statistically higher in normal colonic epithelial cells of villin-PPAR-d-2 mice than WT littermates (Figure 6A and B). We also robustly validated that ADAMTS18 expression was statistically lower in tumor than normal colonic epithelial cells and in normal colonic epithelial cells of villin-PPAR-d-2 mice than WT littermates (Figure 6C).

To examine whether PPAR-d as a transcriptional factor directly altered the expression of the identified target genes, we used chromatin immunoprecipitation assay to test the binding of PPAR-d to the promoter of the IFITM3 gene, expression of which was robustly altered by PPAR-d overexpression (Figure 6B). PPAR-d binding to the IFITM3 promoter and IFITM3 mRNA expression levels in the intestinal crypt epithelial cells were increased in villin-PPAR-d-2 mice compared with WT littermates (Figure 6, D and F). To examine the applicability of these findings to human colon cancer cells, we transfected PPAR-d by a plasmid vector into LoVo human colon cancer cells and measured PPAR-d binding to the IFITM3 promoter and IFITM3 mRNA expression. PPAR-d overexpression enhanced binding to the IFITM3 promoter and increased IFITM3 mRNA levels (Figure 6, E and G), which demonstrated that PPAR-d overexpression can act through its transcriptional function to promote the transcription of protumorigenic genes such as IFITM3.

**Discussion**

Here we have shown that PPAR-d overexpression in colonic epithelial cells strongly increased susceptibility to colonic tumorigenesis. Mice with PPAR-d overexpression developed intestinal hyperplasia that increased with age and resulted in formation of spontaneous colonic adenomas in some of these mice at the age of 50 weeks even without carcinogen treatment. The intestinal hyperplasia was driven by marked increase of crypt proliferation zones. Treatment of villin-PPAR-d mice with AOM resulted in marked promotion of colorectal tumorigenesis. The increase in AOM-induced tumorigenesis was potentiated at early and late tumorigenesis stages with increase of microadenoma and small and large polypl formation. This potentiation of tumorigenesis was associated with PPAR-d markedly expanding the epithelial cell proliferative zone in the colonic crypts, which is known to be a critical event for the development of colonic tumorigenesis in humans (23,24). PPAR-d overexpression promotion of colorectal tumorigenesis was not mouse line or strain background specific because it was observed in three independent mouse lines and with two different genetic backgrounds (B6 and FVB). In the FVB background strains, which are known to be susceptible to AOM-induced colonic tumorigenesis, PPAR-d–targeted intestinal expression lowered the AOM dose required to induce colonic tumorigenesis by approximately 50% (from 10 mg/kg to 5 mg/kg). Furthermore, the number of tumors induced by AOM was increased by more than fourfold. In the B6 strain, which is known to be resistant to AOM-induced colonic tumorigenesis, PPAR-d–targeted intestinal overexpression reversed this resistance. Although prior studies with genetic deletion of PPAR-d have produced mixed results (12–15), our novel results using a mouse model with targeted intestinal PPAR-d expression to simulate PPAR-d overexpression in human colorectal
Figure 5. Differential expression profile for colonic epithelial cells with and without peroxisome proliferator-activated receptor-δ/β (PPAR-d) overexpression. A) Heat map for 413 transcript probes that were differentially expressed between normal and cancerous colonic epithelial cells of villin-PPAR-d-3 mice using cutoff of false discovery rate of 0.1 ($P = .004$). B) Heat map for 67 transcript probes that were differentially expressed between normal colonic epithelial cells of villin-PPAR-d-3 mice and their wild-type (WT) littermates using cutoff of false discovery rate of 0.15 ($P < .001$). C) Heat maps for transcripts with statistically significantly different expression (> log2 ratio (1/-1)) and $P < .05$ in each of two comparisons: 1) paired normal and tumor colonic epithelial cells of villin-PPAR-d-3 mice and 2) normal colonic epithelial cells of villin-PPAR-d-3 mice and their WT littermates. D) Canonical pathway analysis of genes differentially modulated by PPAR-d overexpression. Differentially expressed genes shown in panel C were subjected to Ingenuity Pathway Analysis. $P$ values represent the probability that random chance could produce the association between identified canonical pathways and PPAR-d overexpression in colonic epithelial cells.
cancer clearly demonstrate the strong potential of PPAR-d overexpression to enhance tumorigenesis. In contrast with the prior models that used the strategy of genetically deleting PPAR-d, which is constitutively expressed in normal cells, our new model directly simulated PPAR-d overexpression in colorectal epithelial cells as occurs in human colorectal tumorigenesis. Although deletion of the constitutive function of PPAR-d in normal cells could have artificially altered cell function and could arguably be considered one of the reasons for the divergent results of the genetic deletion models, data from our new model, which directly simulates overexpression of PPAR-d as occurs in human colorectal tumorigenesis, clarify the role of PPAR-d overexpression in colorectal tumorigenesis.

We observed that PPAR-d overexpression modulated the gene expression profile of colonic epithelial cells in a pattern that was strongly compatible with tumorigenesis promotion. Canonical pathway analysis of genes differentially expressed by PPAR-d overexpression in colonic epithelial cells showed that PPAR-d overexpression was statistically significantly associated with alteration of various canonical pathways that are critical to tumorigenesis, including cell cycle, antiapoptotic, prosurvival, and cellular movement pathways. These findings support a protumorigenic role of PPAR-d. The PPAR-d protumorigenic alterations in the gene expression profile occurred in normal-appearing colonic epithelial cells, resembled the differential expression pattern in tumor cells, and included several genes that were already known to strongly impact colorectal tumorigenesis: neuregulin 1 (NRG1) and interferon induced transmembrane protein 3 (IFITM3). Our results identify these genes for the first time as PPAR-d targets. NRG1,

Figure 6. Effects of targeted intestinal peroxisome proliferator-activated receptor-δ (PPAR-d) overexpression on colonic-tumorigenesis-related genes. A–C) Villin-PPAR-d-2 mice and wild-type (WT) littermates were treated with azoxymethane (AOM) 10 mg/kg and killed as described in the Supplementary Methods (available online). Normal and tumor colonic epithelial cells were evaluated for expression of NRG1 (A), IFITM3 (B), and ADAMTS18 (C) by quantitative real-time polymerase chain reaction. Values are means with 95% confidence intervals of triplicate measurements for pooled samples from seven mice per group. * P < .001 for comparison between tumor and normal colonic epithelial cells in villin-PPAR-d-2 mice and between tumor and normal colonic epithelial cells in WT littermates. ** P < .001 for comparison of normal colonic epithelial cells between villin-PPAR-d-2 mice and WT littermates. P values for all comparisons are from two-sided, two-way analysis of variance tests. D and E) PPAR-d binding to IFITM3 promoter to induce IFITM3 expression. D) Isolated colonic epithelial cells from villin-PPAR-d-2 mice and WT littermates were formaldehyde cross-linked and subjected to chromatin immunoprecipitation (ChIP) assays using PPAR-d antibody. A 138-bp fragment of the human IFITM3 promoter was amplified by polymerase chain reaction as described in the Supplementary Methods (available online). NS IgG = non-specific (control) immunoglobulin G. Input is the total DNA before immunoprecipitation. E) LoVo human colon cancer cells were transfected with PPAR-d overexpression or control vector. Cells were formaldehyde cross-linked 48 hours after transfection and subjected to ChIP assays as described for panel D but using antihuman PPAR-d antibodies or NS IgG. A 108-bp fragment of the human IFITM3 promoter was amplified by polymerase chain reaction. F and G) IFITM3 mRNA expression was measured by quantitative real-time polymerase chain reaction in RNA extracted from colonic epithelial cells (F) and LoVo cancer cells (G) that were obtained as described in panels D and E. P is from two-sided t tests for triplicate measurements from one of three repeated independent experiments showing similar results.
a member of the neuregulin family, is a glycoprotein that, by interacting with NEU/ERBB2 receptor tyrosine kinase, increases its phosphorylation, thus acting as a signaling protein to activate various pathways (eg, ERK and PI3K/AKT pathways) for cell–cell interaction and growth and development; NRG1 dysregulation has been linked to various human diseases, including cancer (25,26). Activation of NRG1 is common in various types of cancer cells and contributes to malignant transformation (27). NRG1 promotes apoptosis resistance and survival of colon cancer cells (28). IFITM3 is an interferon-induced transmembrane protein that helps inhibit virus entry into host cells (26,29,30). IFITM3 promotes colonic tumorigenesis and is upregulated early in colonic tumorigenesis (31,32); this upregulation adversely impacts patient prognosis (33).

PPAR-δ is a transcriptional factor, and its overexpression likely upregulated the expression of these genes directly to increase their transcription. In support of this notion is our finding that PPAR-δ bound to the IFITM3 promoter and increased its expression in colonic epithelial cells. IFITM3 increases colonic epithelial cell proliferation (33), and thus its upregulation by PPAR-δ could contribute to PPAR-δ’s effect of increasing the length of colonic crypt proliferative zone, which is well known to enhance colorectal tumorigenesis, including its induction by carcinogen in mouse models (34). Our finding that PPAR-δ overexpression downregulated genes such as ADAM metallopeptidase with thrombospondin type 1 motif 18 (ADAMTS18) is in agreement with a prior observation that PPAR-δ on occasion acts as a transcriptional repressor for some genes (35). ADAMTS18, a tumor suppressor gene, is a member of the ADAMTS protein family, which is characterized by sharing several protein modules (a propeptide region and disintegrin, metalloproteinase, and thrombospondin domains) (26,36). Thus, PPAR-δ overexpression alters the expression of various genes (eg, IFITM3, NRG1, ADAMTS18) that have been linked to tumorigenesis in protumorigenic fashion.

PPAR-δ has been identified as a target of aberrant β-catenin activation in colon cancer (5). However, studies of PPAR-δ knock-out in Apc mice have generated conflicting data, as discussed. In these miarray studies, several of the genes differentially expressed in colonic epithelial cells with PPAR-δ overexpression (eg, PROX1, MMP9, SPP1) (Supplementary Table 3, available online) are targets of β-catenin, including IFITM3, which we confirmed as being directly transcriptionally targeted by PPAR-δ (Figure 6) (32,37–39). These findings suggest that PPAR-δ overexpression could positively enhance Wnt/β-catenin signaling for promotion of colonic tumorigenesis. Future in-depth mechanistic studies are needed to elucidate the mechanisms of the interaction between PPAR-δ and β-catenin.

Potential limitations of this study are that mouse modeling is limited by nature in simulating human colorectal tumorigenesis because of species differences and targeting PPAR-δ overexpression to epithelial intestinal cells provides no information on the possible contribution of altering PPAR-δ expression in stromal cells to colorectal tumorigenesis.

In conclusion, our findings, generated from studying the effects of targeted intestinal PPAR-δ overexpression in colonic epithelial cells on colonic tumorigenesis in a novel in vivo model, provide strong evidence that PPAR-δ upregulation strongly enhances susceptibility to colonic tumorigenesis. These new findings indicate the need for great caution in development of therapeutic modalities to increase PPAR-δ activation for treatment of noncancerous illnesses. These data also indicate that efforts should be directed to developing PPAR-δ inhibitors for the treatment and prevention of cancer.

References


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**Affiliations of authors:** Department of Gastrointestinal Medical Oncology (XZ, MX, JY, IS), Department of Clinical Cancer Prevention (XZ, YW, IS), Department of Pathology (MJM, RRB), Department of Bioinformatics & Computational Biology (GCM, SL), Department of Veterinary Medicine and Surgery (MG), and Department of Biostatistics (JSM), University of Texas MD Anderson Cancer Center, Houston, TX.