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Peroxisome proliferator-activated receptor gamma overexpression suppresses proliferation of human colon cancer cells

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ABSTRACT

Peroxisome proliferator-activated receptor gamma (PPARγ) plays an important role in the differentiation of intestinal cells and tissues. Our previous reports indicate that PPARγ is expressed at considerable levels in human colon cancer cells. This suggests that PPARγ expression may be an important factor for cell growth regulation in colon cancer. In this study, we investigated PPARγ expression in 4 human colon cancer cell lines, HT-29, LOVO, DLD-1, and Caco-2. Real-time polymerase chain reaction (PCR) and Western blot analysis revealed that the relative levels of PPARγ mRNA and protein in these cells were in the order HT-29 > LOVO > Caco-2 > DLD-1. We also found that PPARγ overexpression promoted cell growth inhibition in PPARγ lower-expressing cell lines (Caco-2 and DLD-1), but not in higher-expressing cells (HT-29 and LOVO). We observed a correlation between the level of PPARγ expression and the cells' sensitivity for proliferation.

1. Introduction

Colon cancer is a malignancy that develops in colon and rectal tissues. Colon cancer cells can also spread to other parts of the body, e.g., the liver and the lung, and form a new tumor. If detected early, colon cancer is treatable; however, metastatic colon cancer is associated with high mortality [1,2]. Furthermore, the prognosis for metastatic colon cancer remains poor [3,4]. Therefore, novel therapeutic options are needed to reduce colon cancer mortality.

Peroxisome proliferator-activated receptor gamma (PPARγ) is member of the nuclear receptor superfamily that is involved in the regulation of lipid metabolism, glucose homeostasis, and cell differentiation [5]. Alternative splicing and differential promoter utilization generate 2 protein isoforms, PPARγ1 and PPARγ2 [6–8]. To date, no functional difference between PPARγ1 and PPARγ2 has been identified, and the significance of the different mRNAs also remains to be determined; however, cell- or tissue-specific expression patterns have been observed. Human PPARγ2 contains 30 extra amino acids (28 in mice) in its N-terminus compared with PPARγ1. Genetic deletion of PPARγ1 is embryonically lethal [9]; however, deletion of PPARγ2 causes minimal alterations to lipid metabolism [10]. PPARγ is overexpressed in many types of cancer, including colon, lung, breast, and stomach cancers, suggesting that regulation of PPARγ might affect cancer pathogenesis. The ability of PPARγ activation to inhibit growth and induce apoptosis in colon cancer cells has been discussed in the literature [11–13].

Some reports indicate that PPARγ is expressed at considerable levels in human colon cancer cells and tissues, and that treatment with PPARγ agonists and antagonists reduces the cell growth rate [14,15]. Moreover, PPARγ ligands prevent adhesion to the extracellular matrix and reduced FAK activation [16,17]. Although PPARγ expression has been examined in human colon cancer cells and tissues [18,19], its significance in human colon cancer remains uncertain. Because PPARγ ligands have been shown to have a variety of PPARγ-dependent and -independent effects, the role of PPARγ remains unknown [20–22]. To determine the correlation between the PPARγ protein level in colon cancer cell lines and cell proliferation rate, we studied the functional significance of PPARγ overexpression in human colon cancer cells.

2. Materials and methods

2.1. Materials

Anti-PPARγ rabbit polyclonal antibody (sc-7196) and anti-β-actin mouse monoclonal antibody (sc-47778) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-FLAG M2 antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Abbreviations: PPARγ, peroxisome-proliferator activated receptor-gamma; PPRE, peroxisome-proliferator response element; ROSI, rosiglitazone.

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2.2. Plasmids

The pSV40-β-galactosidase and pcDNA3.1 plasmids were purchased from Promega (Madison, WI, USA) and Invitrogen Corp. (Carlsbad, CA, USA), respectively. The pcDNA3.1-PPARγ and pGL3b-PPRE (ACO)-Fluc plasmids were constructed as described previously [23,24]. pcDNA3.1-FLAG-PPARγ was purchased from Addgene (Cambridge, MA, USA).

2.3. Cell culture

The human colon cancer cell lines HT-29, LOVO, and Caco-2 were obtained from American Type Culture Collection (Manassas, VA, USA). DLD-1 human adenocarcinoma cells were obtained from Health Science Research Resources Bank (Osaka, Japan). Cells were grown in Dullbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) or RPMI 1640 medium (Nacalai Tesque) containing 10% (v/v) fetal bovine serum, at 37 °C in a humidified incubator with 5% CO_2.

2.4. Western blot analysis

Cells were seeded onto 6-well plates (Iwaki, Tokyo, Japan) at a density of 1×10^5 cells/well. After the indicated treatment, cells were lysed on ice for 30 min in cell lysis buffer (20 mM Tris–HCl [pH 7.4], 10% [v/v] glycerol, 100 mM NaCl, 1% [v/v] Triton X-100, 1/100 protease inhibitor cocktail [Sigma], 1 mM dithiothreitol) and centrifuged at 16,000*g* for 20 min at 4 °C. The supernatants were saved as cell lysates and assayed for protein content by the Bradford method, using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates were then separated on 5–20% sodium dodecyl sulfate (SDS)-polyacrylamide gels (e-PAGEL; ATTO, Tokyo, Japan) and electrotransferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were blocked by Block Ace (DS Parma Biomedical Co. Ltd., Osaka, Japan) for 1 h and incubated with a primary antibody in TBS-T with 5% Block Ace for 12 h at 4 °C. Bands were visualized with EzWestLumi plus (ATTO).

2.5. Quantitative real-time PCR analysis

Total RNA was prepared using NucleoSpin RNA II (Takara, Shiga, Japan) from 4 cell lines. Total RNA (0.5 μg) was used for the subsequent synthesis of cDNA with the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) as recommended by the manufacturer. mRNA levels were measured with an ECO Real-Time PCR system (Illumina Inc., San Diego, CA, USA) and SYBR Green Realtime PCR Master Mix-Plus- (Toyobo) with the following primer pairs: PPARγ1, 5′-TCT CTC CGT AAT GGA AGA CC-3′ (F) and 5′-GCA TTA TGA GAC ATC CCC AC-3′ (R); PPARγ2, 5′-GCT GTT ATG GGT GAA ACT CTG-3′ (F) and 5′-ATA AGG TGG AGA TGC AGG TTC-3′ (R); 18S rRNA, 5′-CAG CCA CCC GAG ATT GAG CA-3′ (F) and 5′-TAG TAG CGA CGG GCG GTG TG-3′ (R). All PCRs were performed in a 10-μL volume, using 48-well PCR plates (Illumina). The cycling conditions were 95 °C for 10 min (polymerase activation) followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s. To determine which housekeeping genes were most suitable for subsequent normalization of data, we initially selected 3 candidates:

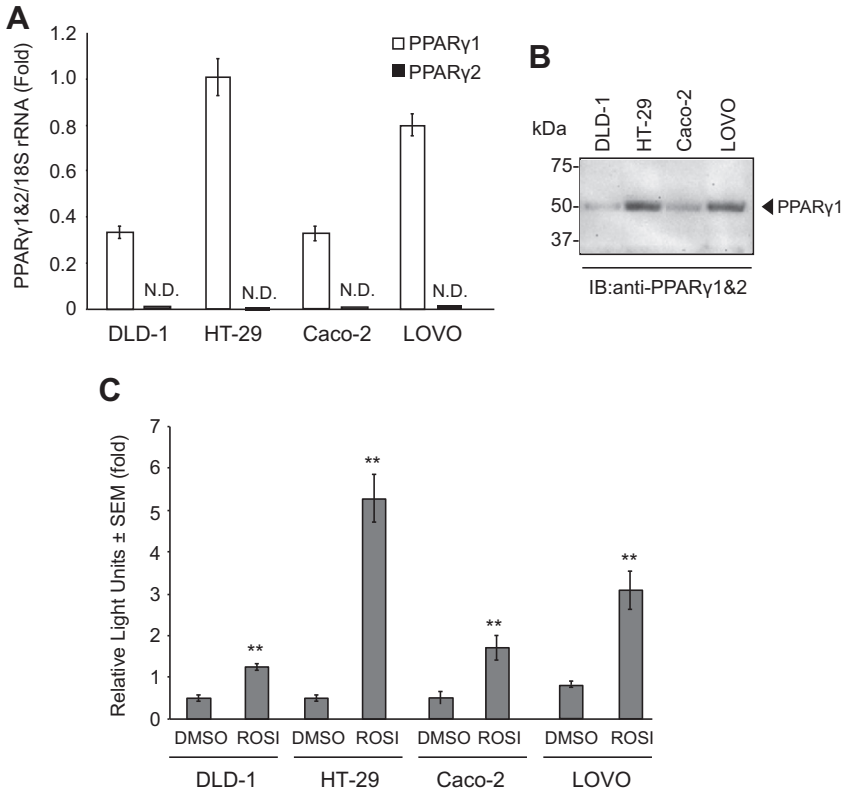

Fig. 1. Comparison of endogenous PPARγ expression. (A) Real-time PCR measurement of PPARγ1 and PPARγ2 mRNA expression in the tested cell lines. PCR was performed using specific primers for PPARγ1 and PPARγ2. The relative PPARγ1 and PPARγ2 levels normalized to 18S rRNA are expressed as mean ± SEM ($n = 3$), $^{**}p < 0.01$. (B) Representative Western blot of PPARγ1 and PPARγ2 expression. Cell lines were separated into the nuclear and cytoplasmic fractions, and 50 μg protein from the cytoplasmic fraction was analyzed by SDS–PAGE and visualized with enhanced chemiluminescence as described in Section 2. (C) Cells express functional PPARγ. The indicated cell lines were transfected with the PPRE-Luc and CMV-β-galactosidase plasmids, and treated with vehicle (DMSO) or rosiglitazone (10 μM) for 20 h. The level of luciferase activity was measured in lysates of treated cells and normalized to β-galactosidase activity. Data are presented as mean ± SEM ($n = 3$), $^{**}p < 0.01$.

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *β-actin*, and *18S-rRNA*, which are commonly used as internal controls in mammalian cells. After amplification, the samples were slowly heated from 55 to 95 °C with continuous reading of fluorescence to obtain a melting curve. The relative mRNA level was calculated using the arithmetic formula $2^{-\Delta\Delta Cq}$, where ΔCq is the difference between the threshold cycle of a given target cDNA and that of an endogenous reference cDNA. Derivation of the formulas and validation tests have been described in Applied Biosystems User Bulletin No. 2.

2.6. Measurement of cell proliferation

PPARγ plasmid was performed in serum-free OPTI-MEM I by cationic liposome transfection using Lipofectamine 2000 as per the manufacture's protocol (Invitrogen). PPARγ was overexpressed in 4 cell lines were seeded in 96-well culture plates (5×10^3 cells/well) and incubated in 5% CO_2 incubator. Cell proliferation was determined using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Ten microliter of Cell Counting Kit-8 was added to the medium and incubated for 2 h in 5% CO_2 incubator. The orange formazan dye generated can be measured by determining absorbance at 450 nm in a microplate reader (Awareness Technology Inc., Palm City, FL, USA).

2.7. Reporter gene assays

PPARγ activation was determined in cells transfected with 125 ng of pGL3-PPRE-acyl-CoA oxidase luciferase, 62.5 ng of pcDNA3.1-PPARγ, and 12.5 ng of pSV-β-galactosidase (Promega), constructed as previously reported [23,24]. Briefly, cells were seeded on a 96-well plate at a concentration of 2×10^4 cells/well. Twenty-four hours after transfection, cells were treated with Opti-MEM (Invitrogen) containing the test compound dissolved in DMSO (up to 0.1%) and cultured for an additional 20 h. Luciferase activity was measured with ONE-Glo Luciferase Assay System (Promega), using a LuMate microplate luminometer (Awareness Technology).

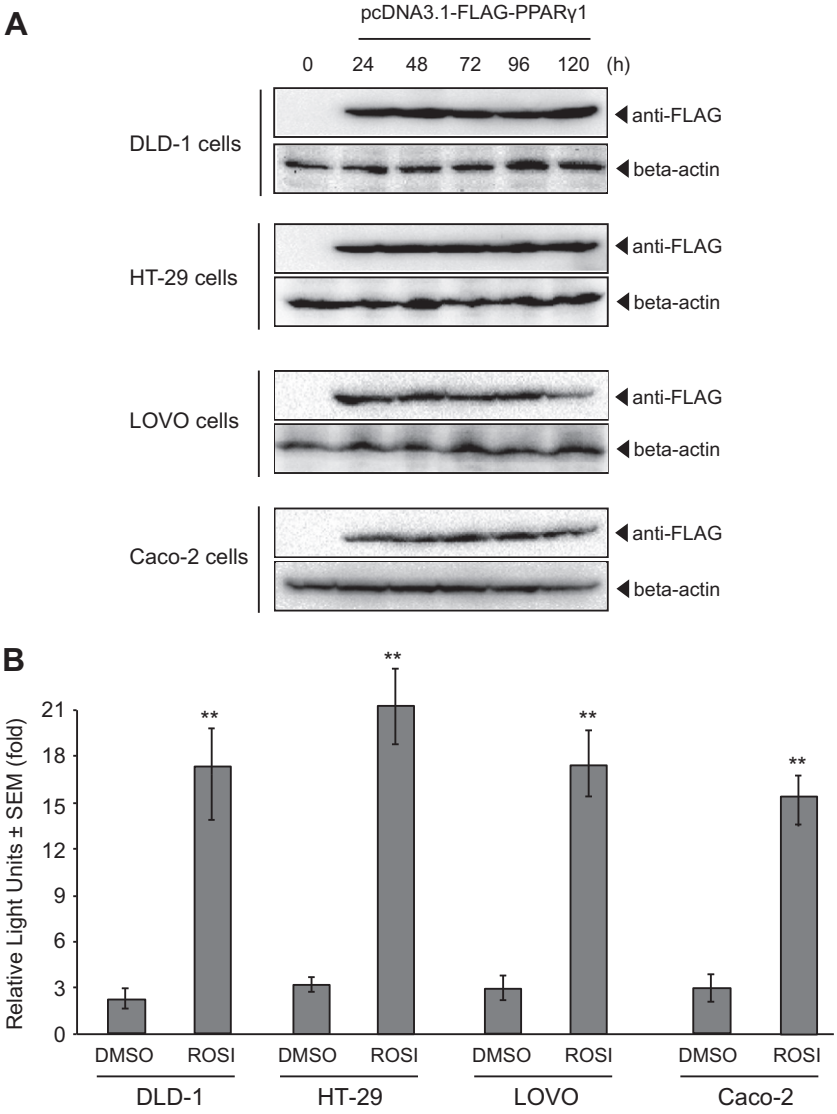

Fig. 2. PPARγ is functionally active in the tested cell lines. (A) Cells were transfected with expression plasmids encoding FLAG-PPARγ for 24 h. Cells were harvested 24, 48, 72, 96, and 120 h after transfection, and cell lysates were used for Western blotting with anti-FLAG antibody. β-Actin was used as the internal protein standard for loading control and was detected by mouse anti-β-actin. (B) Cells were transfected with PPRE-Luc and CMV-β-galactosidase plasmids with control vector or FLAG-PPARγ expression vector, and treated with 10 μM rosiglitazone (ROSI) or control buffer (DMSO) for 20 h. Luciferase activity was measured in lysates of treated cells and normalized to β-galactosidase activity. Data are presented as mean ± SEM ($n = 3$), $^{**}p < 0.01$.

2.8. Statistical analysis

Student's *t*-test was used for statistical comparisons. Differences were considered significant at the $p < 0.05$ level.

3. Results and discussion

3.1. Comparison of endogenous PPARγ expression in colon cancer cell lines

The existence of 2 PPARγ isoforms, PPARγ1 and PPARγ2, which originate from alternative splicing, has been shown in humans [6–8]. To evaluate the function of PPARγ in human colon cancer cells, we used 4 well-characterized transformed colonic epithelial cell lines, DLD-1, HT-29, LOVO, and Caco-2. We first examined the expression of PPARγ1 and PPARγ2 mRNA and protein in cells. In our previous work, we reported that the only PPARγ isoform detected was PPARγ1 in HT-29 cells [14]. As shown in Fig. 1A, PPARγ1 mRNA was expressed at high levels in HT-29 and LOVO, and at an intermediate level in DLD-1 and Caco-2. Fig. 1B shows the expression of PPARγ protein in these cell lines. We observed that the 4 human colon cancer cell lines exhibit different expression levels of PPARγ. The protein had a molecular mass of approximately 50 kDa, which is consistent with the reported value for PPARγ1 protein [25]. Furthermore, the expression levels of PPARγ protein were in good agreement with those of the mRNA. Next, to determine if the PPARγ expressed in the cell lines was functional, we transfected the 4 cell lines with a pGL3-PPRE-acyl-CoA oxidase luciferase reporter plasmid. Over 20 h, luciferase activity in cells treated with 10 μM rosiglitazone (ROSI), a full agonist for PPARγ, for 20 h was approximately 1.5-fold (DLD-1), 5-fold (HT-29), 3-fold (LOVO), and 2-fold (Caco-2) higher than that in vehicle (DMSO)-treated cells (Fig. 1C). These results together suggest that ROSI can activate the PPRE-ACox-Luc reporter gene dependently of the expression of PPARγ in the 4 cell lines.

3.2. Exogenous PPARγ expression and PPRE transactivation in colon cancer cell lines

We have previously shown that HT-29 colon cancer cells highly express PPARγ and that exposure to certain PPARγ ligands induces cell death, suggesting that PPARγ may be a therapeutic target for colon cancer [14]. To determine the functional consequences of PPARγ overexpression in colon cancer cells, we overexpressed the plasmid-encoded cDNA of human FLAG-PPARγ in the 4 cell lines. Exogenous PPARγ expression was tested using an anti-FLAG antibody. At the protein level, FLAG-PPARγ increased in transfected cells, reaching maximum expression at 48 h (Fig. 2A). These results suggest that PPARγ was synthesized at high levels and is stable from 24 h after transfection in all tested cell lines. Next, to test whether this overexpressed PPARγ protein is functional and could bind to its promoter sequence, we examined its transcriptional activity by using the PPRE-ACox-Luc reporter gene system. All cell lines were transfected with PPARγ, the reporter gene construct, and SV40-β-galactosidase for normalization. As shown in Fig. 2), 24 h post-transfection, cells were treated with 10 μM ROSI, after which the cell lysates were collected. Cells transfected with the FLAG-PPARγ vector exhibited a significant increase in promoter activity, as assessed by an increase in luciferase, compared with that of empty vector control, confirming the functionality of the overexpressed PPARγ.

3.3. PPARγ overexpression reduces cell viability

The role of PPARγ overexpression and cell proliferation in colon cancer cells has not been elucidated to date. Thus, we determined the effect of PPARγ overexpression on cell viability by using a colorimetric assay, as described in Section 2. As shown in Fig. 3A, PPARγ overexpression severely inhibited cell proliferation (~50%) in DLD-1 and Caco-2 cells, which have a lower endogenous level of PPARγ compared with the other cell lines. However, HT-29

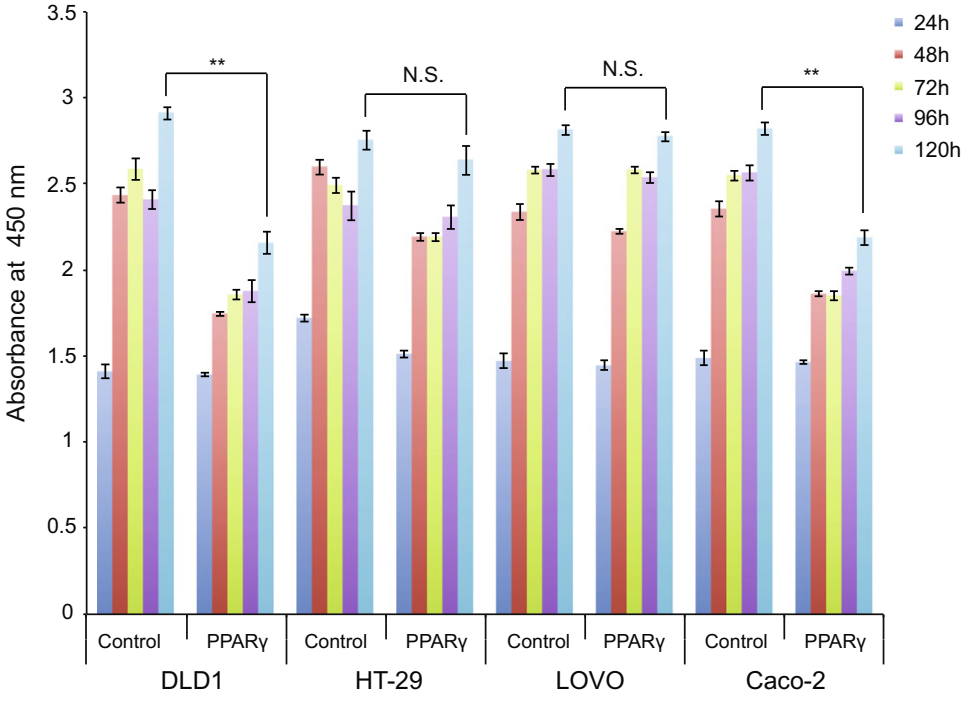

Fig. 3. PPARγ overexpression in 4 different colon cancer cell lines reduced cell proliferation. (A) At 24 h post-transfection, cells were replated in 96-well plates (5×10^3 cells/well) and incubated for 24, 48, 72, 96, and 120 h. Cell proliferation was determined using Cell Counting Kit-8 (Dojindo). Ten microliter of Cell Counting Kit-8 was added to the medium (100 μL) and incubated for 2 h in a 5% CO_2 incubator. Data are presented as mean ± SEM ($n = 3$), $^{**}p < 0.01$, N.S., nonsignificant at the $p = 0.05$ level.

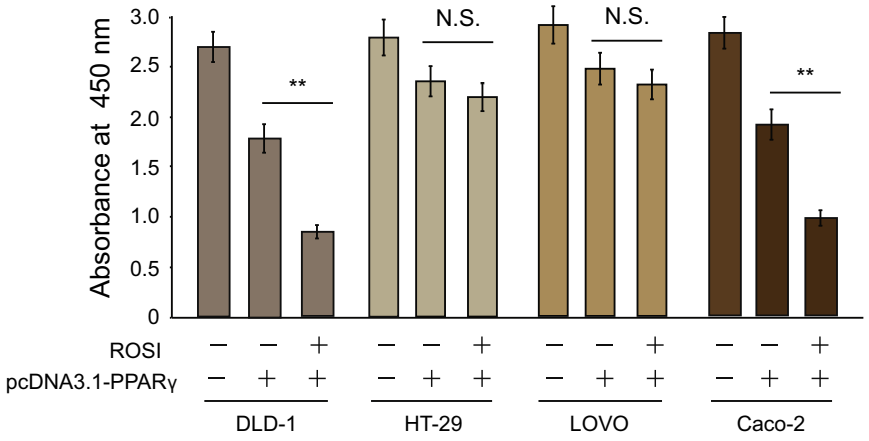

Fig. 4. Effects of combination treatment with a PPARγ agonist. Cells were transfected with pcDNA3.1-FLAG-PPARγ plasmid for 24 h. The medium was replaced with fresh medium, and 10 μM ROSI was added to cells. After 48 h, cell proliferation was determined using Cell Counting Kit-8. Ten microliters of Cell Counting Kit-8 was added to the medium and incubated for 2 h in 5% CO_2 incubator. Data are presented as mean ± SEM ($n = 3$), $^{**}p < 0.01$.

and LOVO cells appear more resistant to growth inhibition (Fig. 3A). These results show that PPARγ levels correlate with the cell proliferation rates, and this effect might be dependent on cell type.

3.4. Synergistic effect of cell growth inhibition by a PPARγ agonist

Interestingly, as shown in Fig. 3, PPARγ lower-expressing cell lines such as DLD-1 and Caco-2 showed reduced cell proliferation even in the absence of an exogenous PPARγ ligand. We determined that up-regulation of PPARγ expression in colon cancer cell lines, in combination with administration of ROSI, a selective PPARγ agonist, may have therapeutic potential. As shown in Fig. 4, in the presence of ROSI, the cell proliferation rate was further reduced for PPARγ-overexpressing DLD-1 and Caco-2 cells but not HT-29 and LOVO cells. A significant difference was found between the PPARγ level and the cell proliferation rate. The modulation of the transcriptional activity of PPARγ might be an effective therapy for colon cancer [26,27]. The level of PPARγ in colon tissue is equal to or greater than that in adipose tissue [28]. This observation suggests the special role of PPARγ in the colon, as reflected in part by the cell- or tissue-specific expression of the receptor. In the present study, we examined the hypothesis that PPARγ expression is essential to the underlying mechanism of colon cancer proliferation. Our analysis provides insight into the functional significance of PPARγ overexpression and transactivation in colon cancer in an in vitro model. The results suggest an inverse relation between the sensitivity of cells toward the growth-inhibitory action of PPARγ and the level of PPARγ expression. Ikezoe et al. reported that mutational analysis of colon tumor samples and colon tumor cell lines did not detect mutant PPARγ in any of the samples, which suggests that PPARγ mutation in human colon tumors is rare [29]. These results together suggest that PPARγ expression levels and/or activation status may be an important prognostic marker for colon cancer. However, the physiological context of PPARγ signaling in colon cancer is still unclear. Further clarification might allow the synthesis of novel medicines that can modulate PPARγ.

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