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ENHANCED INHIBITION OF PROLIFERATION IN HUMAN COLON CANCER DLD1 CELLS OVEREXPRESSING PPARγ

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Abstract

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear hormone receptor that regulates many important functions, including adipogenesis, glucose homeostasis and cancer. Loss of function of this protein has been associated with increased incidence of human colorectal cancer. The function of this nuclear receptor was examined using human DLD1 colorectal adenocarcinoma cells stably overexpressing PPARγ. Overexpression of PPARγ was confirmed at both the mRNA and protein levels by real-time quantitative PCR and western blot analysis. Enhanced activation of a PPARγ target gene was also observed in response to ligand activation of PPARγ as compared to control cells. The xCELLigence System from Roche® was used to monitor cell proliferation in real time, providing quantitative assessment of cell number over a period of 120 hours. A clonogenic assay was used to monitor the clone forming potential of the cells. In both assays, ligand activation of PPARγ in DLD1 cells overexpressing this nuclear receptor caused enhanced inhibition of proliferation as compared to controls. Western blot analysis was used to measure levels of PARP cleavage, a marker for apoptosis. There was increased PARP cleavage in DLD1 cells overexpressing PPARγ compared to controls. Results from this study show that activating PPARγ in DLD1 cells that overexpress this receptor can effectively enhance inhibition of proliferation as compared to controls, and this may occur through activation of apoptosis in these cells. This data suggests that approaches that increased expression of PPARγ in colon cancer cells could be developed as a new strategy for colon cancer chemoprevention.

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Acknowledgements

I am incredibly grateful to Dr. Jeffrey Peters for taking me into his laboratory nearly three years ago and providing me the environment to learn and practice good scientific technique. He has supported me throughout my time at Penn State and provided opportunities that played a huge role in shaping my education and future direction towards a career in the biomedical sciences. It was his hard work securing grants that made this work possible. I would like to thank my first mentor, Dr. Prajakta Palkar, who taught me the foundations for all of my lab skills. I would also like to thank Bokai Zhu for sharing bench space and always patiently answering my questions. I am grateful to everyone in the Peters lab for their help along the way.

Introduction

According to the American Cancer Society, colorectal cancer is the third leading cause of cancer-related deaths in both men and women in the United States (1). A combination of screening and preventative methods has yielded decreased occurrence and death due to colon cancer in the past decade (1). Early detection and removal of potentially threatening polyps in the colon can often avoid progression to metastatic stages. Early stage colon cancer is usually treated with surgical removal in combination with chemotherapy and radiation, while advanced stages often require biologically targeted therapies (2). Research into the cellular and molecular mechanisms of colorectal tumorigenesis is being conducted in order to further understand the genetic causes that lead to tumor development as well as to identify natural mechanisms of the body that block tumor growth and may be targeted for drug intervention.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily of ligand activated transcription factors. PPARγ has been studied extensively and is known to regulate a variety of processes including adipogenesis, glucose homeostasis, and cancer (3). PPARγ regulates gene expression by heterodimerizing with RXR (retinoid X receptor) and binding DNA sequences called peroxisome proliferator response elements (PPREs) in the promoters of target genes (4) (figure 1.A.).

PPARγ is activated by ligand binding. Endogenous ligands for this receptor include unsaturated fatty acids and prostaglandins (5). One class of synthetic PPARγ ligands are the thiazolidinediones (TZDs) which were developed for their insulin sensitizing properties in the treatment of diabetes mellitus type 2 (6). This family of small molecules includes rosiglitazone (Avandia, figure 1.B.), pioglitazone (Actos), and troglitazone (Rezulin). Ligand binding to PPARγ elicits a conformational change in the receptor that leads to dissociation of co-repressors like histone deacetylases (HDACs), heterodimerization with RXR, recruitment of co-activators like histone acetyl transferases (HATs) and subsequent activation of target genes (7).

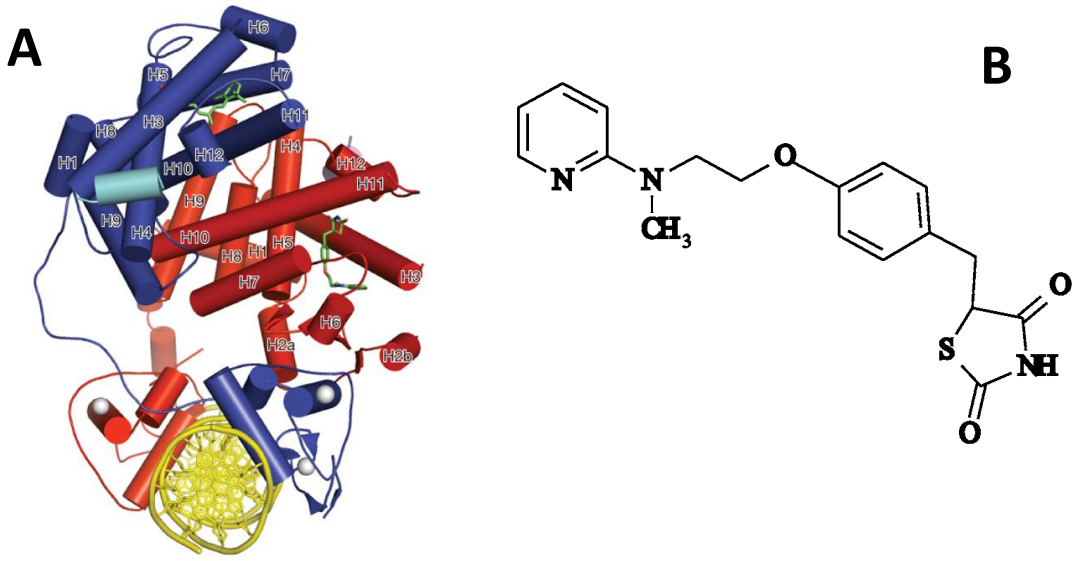

Figure 1. (A) Crystal structure of the PPARγ-RXRα heterodimer bound to DNA (8) **and (B) Chemical structure of the PPARγ agonist rosiglitazone.** RXR-α is shown in blue and PPARγ is shown in red. Rosiglitazone and 9-cis-retinoic acid ligands are bound to the complex, shown in green. Zn2+ ions are shown in white.

PPARγ mRNA and protein are expressed in many human tissues, including adipose, skeletal muscle, liver, kidney, small and large intestine, and colon (9). PPARγ is highly abundant in adipose tissue, where it functions in fatty acid homeostasis by regulating genes that control fatty acid oxidation and storage (10). Activation of PPARγ induces genes that regulate adipocyte differentiation from pre-adipocyte precursors (5; 11–13). PPARγ is expressed at high levels in the colon epithelium, comparable to amounts found in adipose tissue (14; 15). In the colon, PPARγ controls expression of genes involved in growth and maturation, making it an important target of study in colorectal carcinogenesis (16). PPARγ is expressed in most human colorectal cancer cell lines (15; 17).

Epidemiological evidence suggests an interesting potential role for PPARγ in colon cancer. PPARγ is activated by natural ligands that are produced as the result of fatty acid metabolism. Populations with diets that are typically high in fat, such as Americans, coincide with populations in which colon cancer is one of the most common cancers, whereas populations with diets consisting mainly of fruits, vegetables and unsaturated fats, such as the Japanese, have lower incidence of this type of cancer (18). It is possible that these associations may be related to the biology of PPARγ regulated pathways.

Additionally, mutations causing loss of function of PPARγ have been associated with a higher occurrence of colon cancer in humans (19; 20).

Several TZDs have been used as selective PPARγ agonists for the treatment of diabetes. However, there is some controversy over the effect of these drugs on colon polyp formation. Mice with nonsense mutations in the adenomatous polyposis coli (APC) tumor suppressor gene (*APC*$^{min/+}$), used as a model for human familial adenomatous polyposis (FAP), have been used to study the possible role of PPARγ in TZD-induced colon carcinogenesis. In this model, C57BL/6JAPC$^{min/+}$ mice are naturally susceptible to intestinal neoplasia due to a nonsense mutation in the APC tumor suppressor gene. Tumor formation occurs spontaneously in the small intestines of these mice, with low numbers in the colon.

Some studies showed that TZDs promote colon cancer in the APC$^{min/+}$ model. Mice treated with 1500-2000 ppm troglitazone, a selective PPARγ agonist, in the diet had increased size and number of adenocarcinomas in the colon (21; 22; 23). Troglitazone caused increased tumor formation mainly in the colon epithelium, where PPARγ expression is high. Similar proliferative results were observed in the APC-mutated HT-29 human colon cancer cell line, which exhibited increased cellular proliferation when treated with PPARγ ligand (24). One suggested mechanism by which PPARγ agonists may promote colon tumors in APC$^{+/min}$ mice is by increasing levels of β-CATENIN, a potential oncogene, in the colon (11; 25–27). β-CATENIN is responsible for activating several target genes associated with colon tumorigenesis and may also regulate cell-cell contact through association with E-CADHERIN, which is involved in tumorigenesis and metastasis (28).

However, other studies showed that TZDs inhibit colon cancer in APC$^{min/+}$ mice. Niho et. al. reported that pioglitazone, another TZD derivative and selective PPARγ agonist, had a suppressive effect on colon polyp formation in APC$^{min/+}$ mice at doses of 100-1600 ppm (29). The authors suggested that the previously reported enhancement of polyp formation by troglitazone in APC$^{min/+}$ mice may have been due to PPARγ-independent mechanisms, but the exact reason for the discrepancy remains unclear (29). Another study by McAlpine et. al. showed that intestine-specific knockouts of PPARγ, either

heterozygous or homozygous, resulted in enhanced numbers of tumors in the small intestine and colon of $APC^{mic/+}$ mice (30). These results suggest that PPARγ protects against tumor formation in the colon.

The vast majority of remaining evidence suggests that PPARγ ligands suppress colon tumorigenesis. Colon tumor cell lines that express functional PPARγ and were treated with PPARγ ligand showed growth inhibition and induction of cell cycle arrest that correlated with the level of functional PPARγ present (31). Another study showed that, rosiglitazone, a synthetic PPARγ ligand, inhibited the growth of HT-29 colon cancer cells expressing wild-type PPARγ, but not HCT-15 cells that express mutant PPARγ (32). Other studies have reported similar results showing a PPARγ dependent mechanism of inhibited cellular proliferation with treatment of several different PPARγ ligands (33–36).

There is evidence, however, suggesting that the antitumor effects of PPARγ ligands may occur independent of ligand activation of the receptor. Some TZDs induce apoptosis in cancer cells more potently than others, suggesting that the antitumor effects may be structure specific to individual TZDs (37). Concentrations that are required to cause antitumor effects are often much higher (three orders of magnitude larger) than concentrations that cause PPARγ activation (37). Structurally similar, but PPARγ-inactive analogues of TZDs showed different antitumor activity in cancer cells than parent compounds (38; 39). Additionally, in some tumor types the level of PPARγ expression does not correlate with sensitivity to the antitumor effects of TZDs (37; 40; 41).

There are a variety of mechanisms by which PPARγ ligands may elicit antitumor effects. The majority of available data suggests that PPARγ activation promotes terminal differentiation, inhibits cell growth, and increases apoptosis in human cancer cell lines (42). Many studies have shown that TZDs and endogenous PPARγ ligands cause cell cycle arrest in human cancer cell lines (3; 31–33; 35; 36). This may occur through modulation of cell cycle regulatory molecules such as CYCLIN D1, p21, p27, β-CATENIN, and may occur through the PPARγ receptor, or in a receptor independent fashion (42).

Another possible mechanism by which PPARγ ligands may alter cellular proliferation is by inducing apoptosis in human cancer cell lines by activating PPARγ (33; 34; 43). PPARγ activation can cause upregulation of the pro-apoptotic factors BAX and BAD, or inhibit the anti-apoptotic BCL-X and

BCL-2 function (42). Activation of PPARγ was shown to induce apoptosis in HT-29 colon cancer cells, with corresponding decreased levels of NF-κB and BCL-2 expression (44). Also, PPARγ activation was shown to induce PTEN, a tumor suppressor protein, in colon cancer cells (16).

Previous reports have shown that PPARγ mediates differentiation in several colon cancers, and this may be another mechanism by which PPARγ ligands induce inhibition of proliferation in these cells (14; 32; 45). PPARγ's role in controlling differentiation in cancer cells correlates with the established role of the receptor in mediating differentiation of pre-adipocytes (10). Some key molecules that PPARγ may regulate include KERATIN 18, 19, and 20, carcinoembryonic antigen (CEA), and E-CADHERIN (3; 42). All of these molecules induce differentiating effects, such as exit from cell cycle and inhibited proliferation.

While there remains some uncertainty regarding the role of PPARγ in cancer, in particular bladder cancer (42), the evidence suggesting that activating PPARγ will prevent colon cancer is considerably stronger than that suggesting that activating PPARγ will promote colon cancer. The focus of my study was to test the hypothesis that inhibition of cellular proliferation occurs due to ligand activation of PPARγ in a receptor-dependent mechanism. To test this, I created a stable PPARγ overexpression model in DLD1 colon cancer cells to show that increased levels of the receptor coordinated with increased sensitivity to the antitumor effects of the TZD, rosiglitazone, as compared to controls.

Materials and Methods

Materials

5-{p-[2-(methyl-2-pyridylamino)ethoxy]benzyl}-2,4-thiazolidinedione maleate (rosiglitazone) was dissolved in dimethylsulfoxide (DMSO). The pMigR1 vector (MigR1) and pψ-Ampho have been previously described (46). The MigR1-PPARγ construct was created by cloning pcDNA3.1-hPPARγ, kindly provided by Dr. Curtis Omiecinski (The Pennsylvania State University, University Park, PA), into the cloning site of the MigR1 vector. The murine stem cell virus promoter upstream of the cloning site drives expression of the inserted sequence of interest encoded, followed by an internal ribosomal entry site (IRES), and the sequence encoding enhanced green fluorescence protein (eGFP), allowing for identification of cells that have successfully integrated the viral DNA via fluorescence imaging.

Cell culture

Human colon cancer DLD1 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin. HEK293T cells were kindly provided by Dr. Yanming Wang (The Pennsylvania State University, University Park, PA) and cultured in Dulbecco's Minimal Essential Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin. Both cell lines were incubated at 37°C and 5% carbon dioxide.

Establishment of MigR1 stable cell lines

The human PPARγ cDNA from pcDNA3.1-hPPARγ was subcloned into the cloning site of the MigR1 vector to generate the MigR1-PPARγ construct. The construct was confirmed by sequencing at the Penn State University Nucleic Acid Facility. HEK293T cells were transfected with both the MigR1-PPARγ overexpression construct or MigR1 empty vector construct and pψ-Ampho packaging plasmids using Lipofectamine® transfection reagent and the manufacturer's protocol. Retrovirus particles were collected from the supernatant of the HEK293T cells and used to infect DLD1 cells. Successful stably infected cells expressing the eGFP fluorescent marker protein were isolated via fluorescence-activated

cell sorting with an InFlux V-GS Cytometry Workbench and the Spigot software (BD Biosciences, San Jose, CA).

Characterization of MigR1 over-expression cell lines

Cells sorted for eGFP expression via fluorescence-activated cell sorting were visualized with a SPOT SP100 cooled CCD camera fitted to a Nikon Eclipse TE 300 upright microscope with normal phase contrast lens and EFD-3 episcopic fluorescence attachment at excitation wavelength 488 nm and emission wavelength 525 nm to ensure eGFP production throughout experimentation. PPARγ mRNA over-expression was verified in the DLD1-MigR1-PPARγ cells via quantitative real-time polymerase chain reaction (RT qPCR). PPARγ protein over-expression was verified in the DLD1-MigR1-PPARγ cells via western blot. In order to analyze the functionality of the over-expressed protein, ligand activation of PPARγ and subsequent target gene upregulation was analyzed via RT qPCR. DLD1 parent, DLD1-MigR1 empty-vector, and DLD1-MigR1-PPARγ cells were treated with medium containing either vehicle (0.02% DMSO) or the PPARγ ligand, rosiglitazone for 24 hours, and target gene expression was measured with RT qPCR.

Cell proliferation analysis

Cell proliferation was monitored in real time with the xCELLigence System (Roche Applied Science, Indianapolis, IN). Electrical impedance was used to measure adherent cell growth. Sensor impedance was expressed in arbitrary units as Cell Index, which was defined as (Rn-Rb)/15, where Rn is the background impedance of the well containing cells and media and Rb is the background impedance of the well containing media only. Measurements were taken every 15 minutes for 112 hours using the RTCES System (ACEA Biosciences, San Diego, CA) for data collection and analysis. DLD1-parent, DLD1-MigR1 empty vector or DLD1-MigR1-PPARγ cells were plated at 400 cells in each E-plate 16 well for each treatment (DMSO control, 0.1, 1, and 10 μM of rosiglitazone), N=3.

Western blot analysis

Protein was isolated from DLD1 cell lines as previously described (47). Briefly, whole-cell protein was isolated with MENG buffer (25mM MOPS, 2mM EDTA, 0.02%NaN_3, and 10% glycerol, pH 7.5) containing 500mM NaCl, 1% Nonidet P-40, and protease inhibitors. Thirty-five to fifty micrograms of protein per sample was resolved using 10% SDS-PAGE and transferred onto a nitrocellulose membrane using an electroblotting method. The membranes were incubated in a 5% dried milk in Tris buffered saline/Tween-20 blocking solution, incubated overnight in primary antibodies, washed, incubated in a biotinylated secondary antibody, washed, and incubated in ^{125}I-streptavaidin for detection and quantification via filmless autoradiographic analysis. The following primary antibodies were used: anti-PPARγ (sc6284; Santa Cruz Biotechnologies), anti-CYCLIN D1 (Cell Signaling Technology, Danvers, MA), anti-poly (ADP-ribose) polymerase (PARP; Cell Signaling Technology), anti-β-actin (ACTIN; Rockland, Gilbertsville, PA), anti-p27 (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-Bcl2-associated X protein (BAX; Santa Cruz Biotechnologies, Santa Cruz, CA).

Quantitative real-time polymerase chain reaction (qPCR) analysis

Total mRNA was isolated from human colon cancer DLD1 cell lines using Ribozol RNA Extraction Reagent (Amresco, Solon, OH) according to the manufacturer's protocol. cDNA was reverse transcribed from 2.5 μg of total RNA using the MultiScribe Reverse Transcriptase kit (Applied Biosciences, Gaithersburg, MD). Primers for *ANGPTL4*, *GAPDH*, *PPARγ*, *PTEN*, *SURVIVIN*, and *CAVEOLIN-1* were purchased from Integrated DNA Technologies (IDT, Coralville, IA).

Data analysis

Data were analyzed for statistical significance using one-way analysis of variance (ANOVA) and the Bonferroni's multiple comparison tests or Student's t-test as described in the figure legends. All data are presented as the mean ± standard error of the mean (SEM) using Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

Results

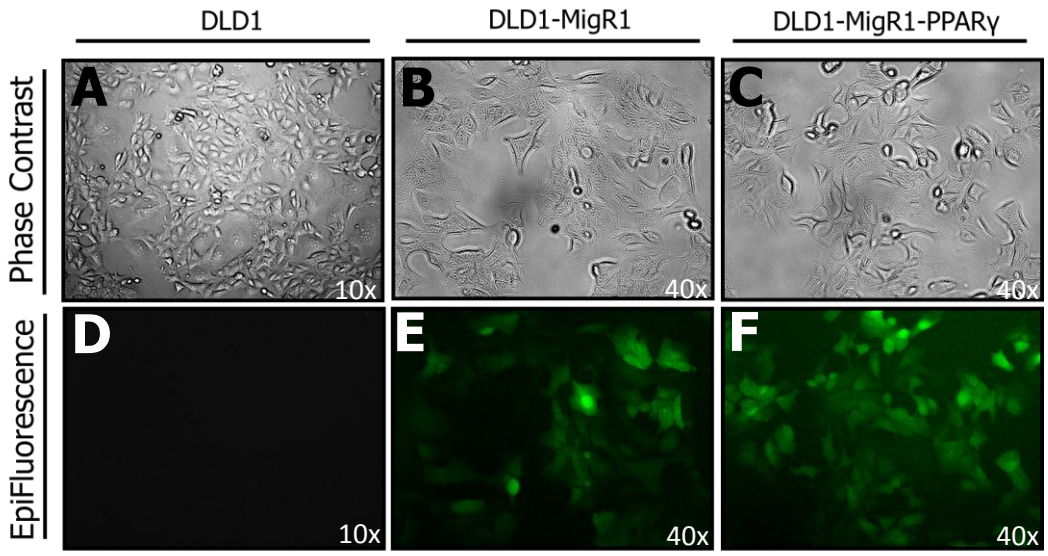

Figure 1. eGFP fluorescence in DLD1, DLD1-MigR1, and DLD1-MigR1-PPARγ cells. DLD1 human colon cancer cells were infected with the viral MigR1 empty vector or MigR1-PPARγ construct. In addition to the sequence for PPARγ inserted at the cloning site, the vector contained a constitutively expressed sequence for enhanced green fluorescent protein (eGFP) that allowed for identification and sorting of successful stably infected cells. Infected cells were isolated using fluorescence-activated cell sorting set to excite at 488 nm and detect at 525 nm. The cells were gated to select for those expressing a minimum of 100-fold increased emission at the wavelength for eGFP, compared to non-infected parent DLD1 cells. Sorted cells were cultured to form a robust clonal population. Cells were visualized with a SPOT SP100 cooled CCD camera fitted to a Nikon Eclipse TE 300 upright microscope with a normal phase contrast lens and EFD-3 episcopic fluorescence attachment set at an excitation wavelength of 488 nm and an emission wavelength of 525 nm to ensure eGFP production throughout experimentation.

Parent DLD1 cells were not infected with the MigR1 construct containing the eGFP coding sequence, and did not show any fluorescence at excitation and emission wavelengths of 488 and 525 nm, respectively (figure 1, panel D). The cells appeared healthy under normal light microscopy (figure 1, panel A). DLD1-MigR1 cells stably expressed the MigR1 empty-vector construct containing the eGFP

coding sequence, and fluoresced at excitation and emission wavelengths of 488 and 525 nm, respectively (figure 1, panel E). The cells appeared healthy under normal light microscopy (figure 1, panel B). DLD1-MigR1-PPARγ cells stably expressed the MigR1 construct containing PPARγ and eGFP coding sequences and fluoresced at excitation and emission wavelengths of 488 and 525 nm, respectively (figure 1, panel F). The cells appeared healthy under normal light microscopy (figure 1, panel C).

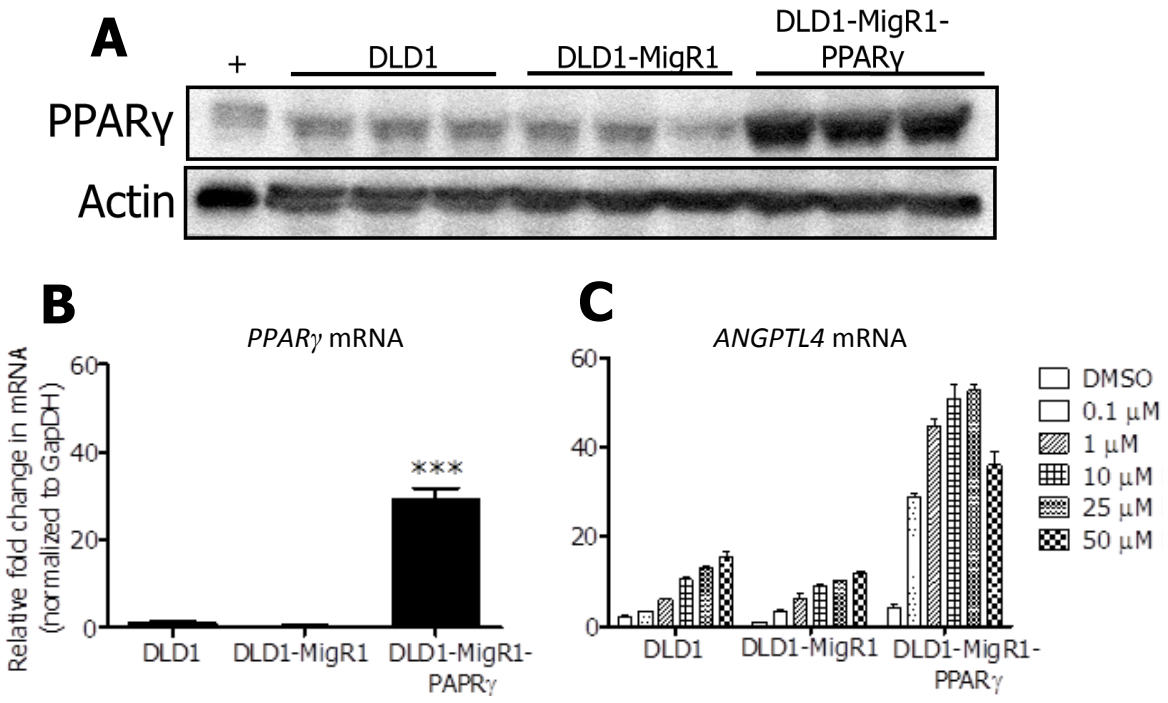

Figure 2. Characterization of DLD1 cells over-expressing PPARγ. Overexpression of PPARγ was confirmed at protein and mRNA levels in DLD1-MigR1-PPARγ cells, compared to DLD1 parent and DLD1-MigR1 empty vector control cells. (A) PPARγ protein expression was measured in DLD1 parent, DLD1-MigR1 empty vector, and DLD1-MigR1-PPARγ cells in triplicate by western blotting with 35 µg of whole cell lysate protein extract and anti-PPARγ and anti-ACTIN antibodies. Protein extract from bacterial COS-1 cells engineered to express PPARγ protein was run in the first lane as a positive control. Protein was quantified with Opti-Quant software and normalized to ACTIN. (B) Levels of *PPARγ* mRNA were quantified via RT qPCR from 2.5 µg of total mRNA using primers for human *PPARγ* mRNA normalized to *GAPDH* internal control mRNA levels. Values represent the mean ± S.E.M., N = 3. (C).

Expression levels for *ANGPTL4* mRNA, a known target gene for PPARγ, were measured in response to treatment of each cell type with DMSO (vehicle) or rosiglitazone at 0.1, 1, 10, 25, or 50 μM. Values represent the mean ± S.E.M., N = 3. Data were analyzed for statistical significance using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison tests using GraphPad Prism 5.0 software.

DLD1 parent and DLD1-MigR1 empty vector cells had moderate levels of PPARγ protein expression that were approximately equal (figure 2.A). DLD1-MigR1-PPARγ cells had approximately 30 fold increased levels of PPARγ protein compared to DLD1 parent and DLD1-MigR1 empty vector cells (figure 2.A). DLD1 parent and DLD1-MigR1 empty vector cells had approximately equal levels of *PPARγ* mRNA (figure 2.B). DLD1-MigR1-PPARγ cells had approximately 30 fold increased levels of *PPARγ* mRNA compared to DLD1 parent and DLD1-MigR1 empty vector cells (Figure 2.B). *ANGPTL4*, a known target gene for PPARγ, was expressed in each of the cell types in a dose-dependent manner in response to DMSO (vehicle) or 0.1, 1, 10, 25, or 50 μM of rosiglitazone. DLD1-MigR1-PPARγ cells had enhanced ligand activation, with 5-10 fold increased target gene expression. There was a modest decrease in target gene expression in DLD1-MigR1-PPARγ cells treated with 50 μM rosiglitazone compared to the level seen at 25 μM rosiglitazone.

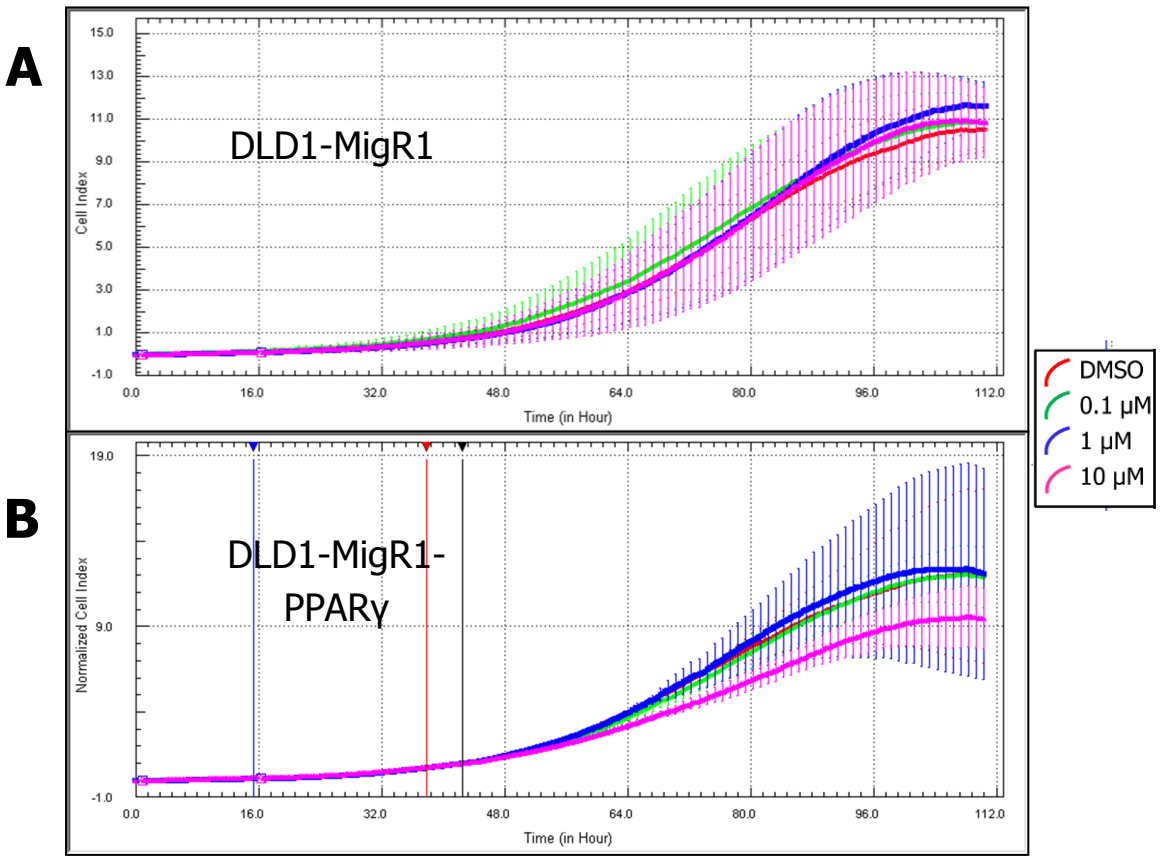

Figure 3. Inhibited proliferation of DLD1-MigR1-PPARγ cells treated with rosiglitazone. Cells were plated at 400 cells per well on an electro-sensitive E plate 16 that was normalized for background media readings and then treated with DMSO (vehicle) or 0.1, 1 or 10 µM of rosiglitazone approximately 24 hours later. Resistance measurements were taken every 5 minutes for 112 hours using the real time monitoring xCELLigence system from Roche. The cell index was normalized to values obtained 3 hours following the addition of treatment. Values represent the mean ± S.E.M., N = 3.

DLD1-MigR1 empty vector control cells showed no inhibited proliferation at any concentration of rosiglitazone treatment, as evidenced by nearly identical growth curves for all treatments (figure 3.A). DLD1-MigR1-PPARγ cells exhibited inhibited proliferation at the highest concentration of rosiglitazone treatment administered, 10 µM, when compared to the DMSO control. All other treatments elicited nearly identical growth curves to the DMSO control (figure 3.B).

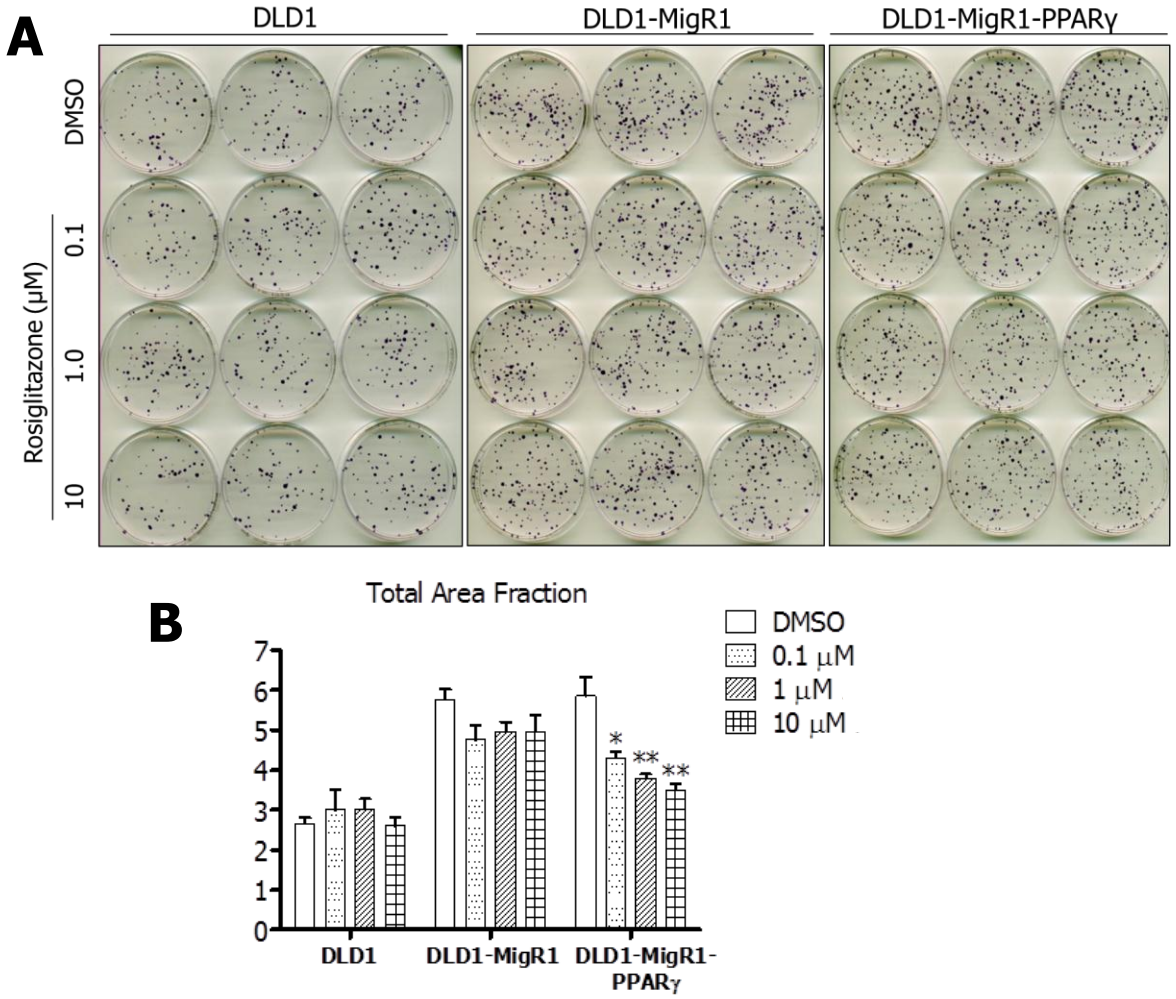

Figure 4. Ligand activation of PPARγ results in inhibited clonogenicity of DLD1 cells over-expressing PPARγ. Cells were plated at low density and cultured in DMSO (vehicle), 0.1, 1, or 10 μM of rosiglitazone for 15 days. At day 15, clones were fixed with 6% gluteraldehyde and stained with 0.5% crystal violet. Clones were quantified with Image J software (National Institutes of Health, Bethesda, MD). Values represent the mean ± S.E.M., N = 3.Data were analyzed for statistical significance using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison tests using GraphPad Prism 5.0 software.

A clonogenic assay was used to measure the effect of PPARγ activation on the ability of DLD1-MigR1-PPARγ cells to form colonies. DLD1 parent cells showed no statistically significant changes in clonogenicity at any dose of rosiglitazone administered. DLD1-MigR1 empty vector cells had higher

overall total area fraction values compared to DLD1 parent cells, but did not differ significantly among treatment groups. DLD1-MigR1-PPARγ cells showed statistically significant inhibition of clonogenicity at all doses of rosiglitazone administered (0.1, 1, 10 μM) when compared to the DMSO control.

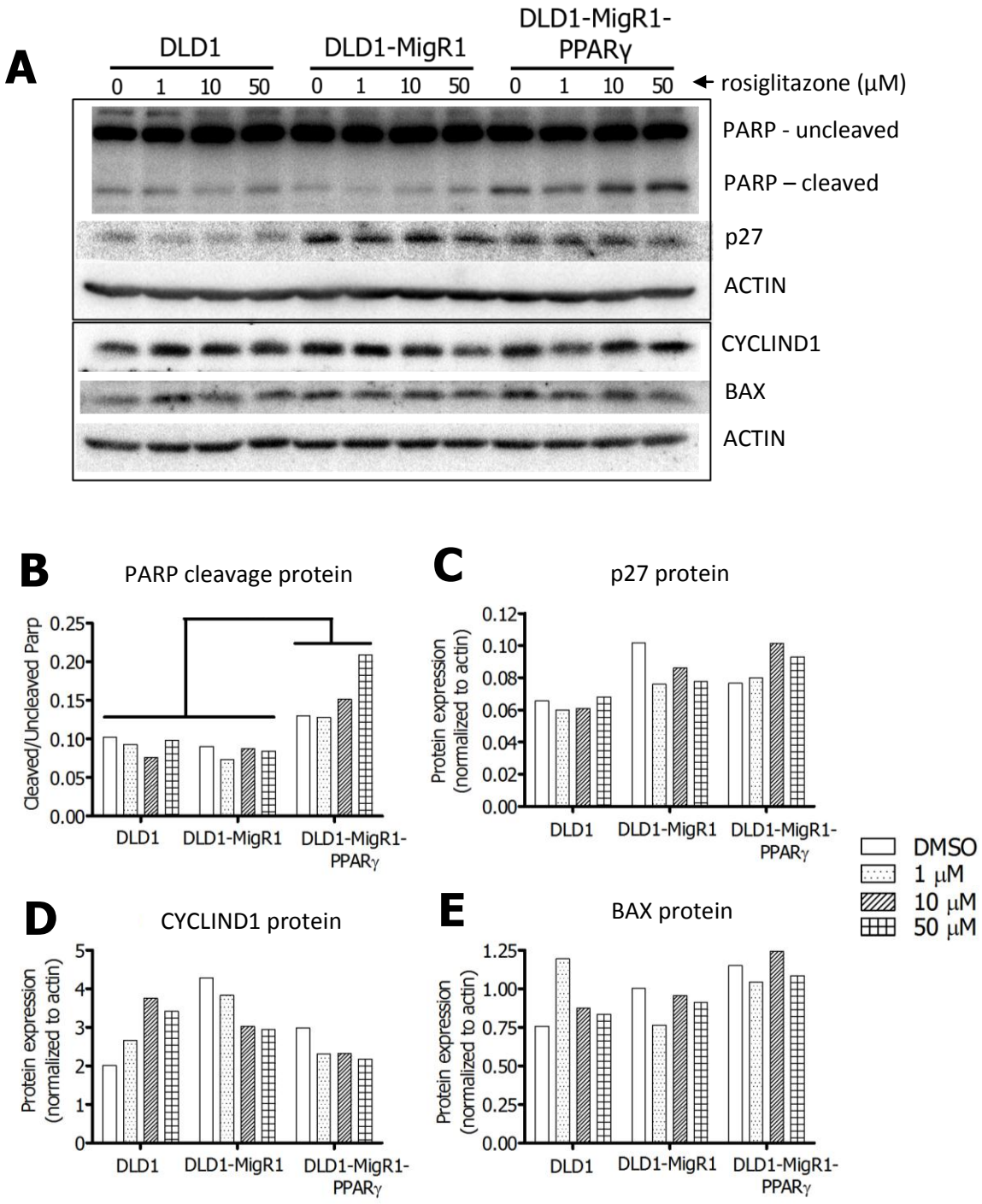

Figure 5. Levels of apoptosis and cell cycle associated proteins in DLD1-MigR1-PPARγ. DLD1, DLD1-MigR1 and DLD1-MigR1 PPARγ cells were treated with DMSO (vehicle), 1, 10 or 50 μM of rosiglitazone for 24 hours and then harvested for whole cell protein extraction. 35 μg of protein was separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes via electroblotting. The membranes were blocked in 5% milk, incubated overnight in primary antibodies, and incubated in biotinylated secondary antibodies. Proteins were detected after incubation with ^{125}I-streptavadin by autoradiographic analysis. Protein levels were quantified using Image J software and normalized to ACTIN control. Values were graphed using GraphPad Prism 5.0 software.

Western blot was used to analyze levels of signaling proteins that may be involved in inhibition of cellular proliferation. The ratio of cleaved to uncleaved PARP protein, a marker for apoptosis, was significantly higher in DLD1-MigR1-PPARγ cells treated with ligand, compared to DLD1 parent and DLD1-MigR1 empty vector cells treated with the same concentrations of ligand. Levels of the cell cycle protein CYCLIND1 protein tended to be lower in DLD1-MigR1-PPARγ, but the values were not statistically significant. Levels of BAX and p27 proteins did not show significant changes following ligand treatment, but tended to increase in DLD1-MigR1-PPARγ cells.

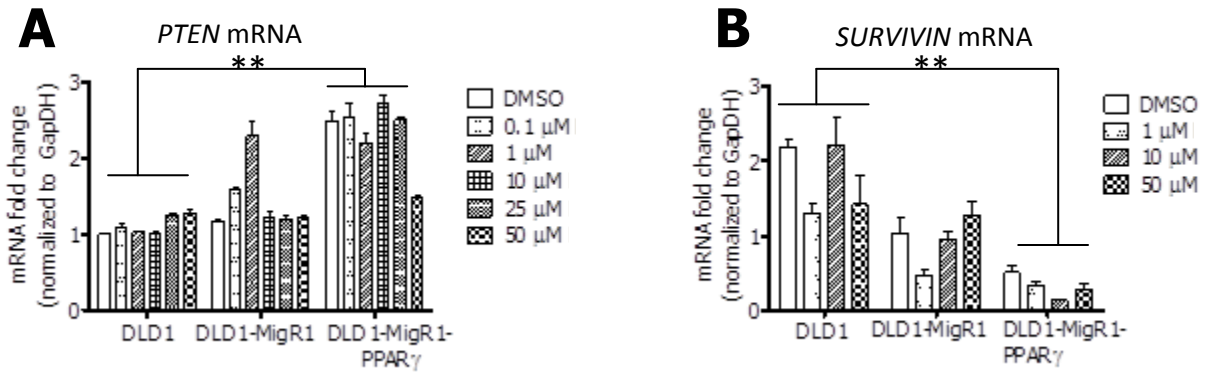

Figure 6. Effect of ligand activation on expression levels of mRNAs involved in apoptosis. Cells were treated with DMSO (vehicle), 0.1, 1, 10, 25, or 50 μM of rosiglitazone for 24 hours and then harvested for mRNA isolation. 2.5 μg of mRNA was reverse transcribed to make cDNA and then analyzed via RT

qPCR. Ct values were normalized to GAPDH internal control. Values represent the mean ± S.E.M., N = 3. Data were analyzed for statistical significance using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison tests using GraphPad Prism 5.0 software.

RT qPCR was used to analyze levels of mRNA molecules that may be affected by or influence the inhibited cellular proliferation observed. *PTEN* mRNA was significantly increased in DLD1-MigR1-PPARγ cells when compared to DLD1 controls for all treatments except 50 μM rosiglitazone. *SURVIVIN* mRNA expression was lower in DLD1-MigR1-PPARγ cells when compared to DLD1 controls, independent of treatment.

Discussion

A stable PPARγ overexpression model was created in DLD1 colon cancer cells to show that increased levels of the receptor coordinated with increased sensitivity to the antitumor effects of rosiglitazone, as compared to empty vector and parent controls. Increased sensitivity with higher levels of the receptor would indicate that ligand activation of the receptor is causing these effects. PPARγ was expressed at higher levels in DLD1-MigR1-PPARγ cells compared to DLD1-MigR1 empty vector and DLD1 parent cells and this overexpressed protein was functional, indicating that the overexpressed protein is capable of affecting transcription of target genes involved in regulating growth.

PPARγ target gene expression in DLD1-MigR1-PPARγ cells treated with rosiglitazone was significantly enhanced compared to controls, and occurred in a dose-dependent manner except at the highest dose of rosiglitazone administered. This suggests that PPARγ activation was enhanced when it was overexpressed, since the target gene expression correlated to the level of PPARγ present in the cells and was dependent on the dose of ligand administered. There was a modest decrease in target gene expression at the 50 μM rosiglitazone. This decrease in target gene activation at high concentrations of ligand may be due to saturation of the receptor. The remaining agonist may have altered gene expression in a receptor-independent manner above this concentration.

Ligand activation of PPARγ in DLD1 cells over-expressing PPARγ caused enhanced inhibition of proliferation compared to controls. Both the xCELLigence proliferation assay and clonogenic assay showed inhibited proliferation at high doses of rosiglitazone treatment in DLD1-MigR1-PPARγ cells compared to controls. In the xCELLigence assay, DLD1-MigR1-PPARγ cells were sensitive to rosiglitazone induced growth inhibition at 10 μM, but not at 0.1 or 1 μM. In the clonogenic assay, cells were plated more sparsely, and had increased sensitivity to rosiglitazone. The cells showed inhibited proliferation in this assay at all levels of treatment (0.1, 1, and 10 μM). The increased sensitivity to lower concentrations of rosiglitazone in the clonogenic assay may have been due to decreased cell-to-cell contact and proliferative growth factors from the surrounding culture.

It is possible that the increased sensitivity of DLD1-MigR1-PPARγ cells to ligand induced inhibition of proliferation at lower concentrations of rosiglitazone may involve signaling through the WNT/β-CATENIN pathway. Previous studies have implicated the WNT/β-CATENIN pathway in the signaling of activated PPARγ in colon cancer (26). β-CATENIN regulates cell-cell adhesion and cellular proliferation through the WINGLESS/WNT pathway (11). Decreased levels of β-CATENIN can lead to inhibited proliferation (11; 26; 27). Further studies are needed to determine if β-CATENIN degradation is involved in the inhibited proliferation that is observed in DLD1-MigR1-PPARγ cells treated with rosiglitazone.

Ligand treatment of DLD1 cells over-expressing PPARγ caused increased markers for apoptosis compared to DLD1-MigR1 empty vector and DLD1 parent cells, suggesting that apoptosis is activated by PPARγ signaling. PARP is a DNA repair protein that is cleaved by caspase-3, and is a marker for apoptosis. Cells over-expressing PPARγ had higher levels of PARP cleavage in response to ligand treatment, and this tended to increase with higher concentrations of the rosiglitazone in DLD1-MigR1-PPARγ cells. Further studies are needed to confirm a dose-dependent relationship with the observed increase in apoptosis. Further studies should be conducted to look for more markers of apoptosis mediated cell death such as increased expression of pro-apoptotic factors such as BAX and BAD, or decreased expression of anti-apoptotic factors such as BCL-X and BCL-2 in order to validate the current findings. In order to further support the idea that apoptosis caused the inhibited proliferation seen in DLD1-MigR1-PPARγ cells, caspase inhibitors could be employed to attempt to inhibit the apoptotic downstream effector molecules. It would be expected that proliferation would no longer be inhibited if caspases were inactive and unable to mediate the cellular destruction.

It is possible that the overexpressed receptor may influence gene expression independently of ligand treatment in some circumstances, perhaps due to activation of the receptor by endogenous ligands always present in the cell. This idea is supported by the mRNA expression data that showed increased expression of *PTEN* and decreased expression of *SURVIVIN* independently of the dose of rosiglitazone administered in DLD1-MigR1-PPARγ cells.

It is likely that activation of PPARγ is involved in rosiglitazone-induced inhibition of cell growth. Clonogenic and xCELLIgence proliferation assays showed increased inhibition of cell growth only in cells over-expressing PPARγ and treated with ligand, whereas ligand did not induce inhibited proliferation in control cell groups. This mechanism may involve increased apoptosis in cells overexpressing the receptor and activated by ligand, as demonstrated by higher levels of PARP cleavage. These results confirm the hypothesis that inhibition of cellular proliferation occurs due to ligand activation of PPARγ in a receptor-dependent mechanism.

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Education

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B.S. Biochemistry and Molecular Biology
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Work Experience

April 2010-Present **Independent Researcher**
Peters Research Group, Center for Molecular Toxicology and Carcinogenesis, Penn State University, University Park, PA. Supervised by Dr. Jeffrey M. Peters, Distinguished Professor of Molecular Toxicology and Carcinogenesis, Primary Investigator.

- Develop, plan, and execute independent thesis project: "Enhanced inhibition of cell proliferation in human colon cancer DLD1 cells over-expressing PPARγ."
- Formulate data into thesis project for the Schreyer Honors College, poster for Society of Toxicology 2012 conference, and manuscript for publication.

May 2011-Aug. 2011 **NIH Summer Internship Program**
Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD. Supervised by Dr. Frank J. Gonzalez, Head, Nucleic Acids Section, Laboratory Chief.

- Characterize gene expression and signaling pathways contributing to hepatocyte proliferation in HNF4α-null mice resulting in publication in *J. Biol. Chem.* Presented research to Laboratory of Metabolism, NIH: "HNF4α is a master regulator of liver proliferation."
- Metabolomic analysis of AZT induced hepatotoxitcity in mice. Poster presented at NIH Summer Intern Poster Session, August 2011.
- Present data at weekly lab meetings.

Mar. 2009-Present **Research Assistant**
Peters Research Group, Center for Molecular Toxicology and Carcinogenesis, Penn State University, University Park, PA. Supervised by Dr. Jeffery M. Peters, Distinguished Professor of Molecular Toxicology and Carcinogenesis, Primary Investigator.

- Work collaboratively with post-doctoral researchers and graduate students, contributing to ongoing research project.
- Present data at weekly lab meetings.

Selected Laboratory Skills and Techniques

Cell culture (primary and immortalized lines), extraction and purification of DNA, protein and RNA from mouse tissue and cultured cells, western blotting, Real Time qPCR, mouse dissection and tissue/organ isolation, dosing, mating and maintenance of mouse lines, FACS, expression cloning, SDS-PAGE.

Computer Skills

Microsoft Office (Word, Excel, PowerPoint), GraphPad Prism, Image J, OptiQuant, UNIX based bioinformatics tools (FastQC, fastX, BWA aligner, Samtools, Integrative Genomics Viewer, BEDTools, Tabix, inGAP, ggplot2, bowtie2), metabolomics software (Masslynx, SIMCA-P+), Genomics tools (BLAST, Galaxy, USCS Genome Browser), statistical software (R, Minitab)

Publications

Zhy, B., Khozoie, C., Bility, M.T., **Ferry, C.H.**, Blasanin, N., Glick, A.B., Gonzalez, F.J., Peters, J.M. "PPARβ/δ crosstalks with E2F and attenuates mitosis in HRAS-expressing cells." Mol. Cell. Biol., [Accepted 2012].

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Abstracts

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Achievements and Awards

- Undergraduate Research Conference Travel Support Award, Penn State University, 2012
- Recipient of the Cancer Research Training Award, National Cancer Institute, 2011
- Schreyer Honors College Scholar, Penn State University, 2010-present
- Dean's list standing all semesters, Penn State University, 2008-present
- The President's Freshman award, Penn State University, 2009

Activities and Affiliations

- Penn State Outing Club, trip leader, 2010-2012
- Undergraduate Affiliate, Society of Toxicology. 2010-present
- Undergraduate Education Program at the Society of Toxicology National Conference, 2010
- Shaver's Creek Environmental Center volunteer, 2010-present
- Penn State Vedic Society, yoga and meditation instructor, 2010
- Penn State Environmental Society, member, 2009-2010