

# Inhibition of Proliferation and Estrogen Receptor Signaling by Peroxisome Proliferator-activated Receptor $\gamma$ Ligands in Uterine Leiomyoma<sup>1</sup>

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## ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)  $\gamma$  is an important signaling molecule in cells of mesenchymal origin, inducing differentiation and regulating cell proliferation in several cell types such as vascular smooth muscle cells. Leiomyomas arise from smooth muscle cells of the uterine myometrium with an incidence rate as high as 70% in women of reproductive age. PPAR signaling has not been characterized in these tumors, although prostaglandins, natural PPAR ligands, are known effectors of key biological functions in the normal myometrium. Leiomyomas and tumor-derived cells isolated from a rat model for this disease were characterized by Western analysis and found to express all three PPAR isoforms, suggesting that signaling pathways mediated by these receptors were intact in this tumor type. *In vitro* experiments with a leiomyoma-derived cell line demonstrated that the pan-PPAR ligand *cis*-4,7,10,13,16,19-docosahexaenoic acid and PPAR $\gamma$ -specific ligands 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>, troglitazone, and ciglitazone inhibited 17 $\beta$ -estradiol-stimulated cell proliferation. This inhibitory effect was not observed with PPAR $\alpha$ - or PPAR $\beta$ -specific ligands. Although both PPAR and estrogen receptor (ER) signaling pathways were intact in leiomyoma cells, in addition to growth inhibition, stimulation of PPAR $\gamma$  signaling also inhibited ER-mediated gene expression. Human leiomyomas were also found to express all three PPAR isoforms, and primary cultures of these cells were sensitive to the inhibitory effects of PPAR $\gamma$  ligands. These results suggest that in uterine leiomyomas PPAR $\gamma$  activation is growth inhibitory and that this inhibition is mediated at least in part by negative cross-talk between ER and PPAR signaling pathways.

## INTRODUCTION

The ligand-activated PPARs<sup>3</sup> are members of the nuclear hormone receptor superfamily of transcription factors. Expression of three known PPAR isoforms,  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$ , has been described in multiple species. Whereas PPAR $\beta/\delta$  is ubiquitously expressed, PPARs  $\alpha$  and  $\gamma$  are expressed in a tissue-specific manner (1). After ligand binding, PPARs heterodimerize with the ligand-bound RXR (2) and modulate gene expression via binding to a specific DNA-regulatory element called the PPRE. The tissue-specific expression of PPARs suggests that these receptors are involved in a variety of specialized biological functions.

PPAR $\alpha$  was the first PPAR identified (3) and is highly expressed in the liver, kidney, and heart of the adult rat (1). This isoform modulates the transcription of genes encoding enzymes involved in fatty acid

oxidation (4, 5), as well as apolipoproteins that function to control cholesterol levels in serum (6). The ubiquitous PPAR $\beta/\delta$  is expressed at higher levels than PPAR $\alpha$  or PPAR $\gamma$  in most tissues, although much less is known about the biological function of this isoform. PPAR $\gamma$  plays an important role in adipocyte differentiation and has been described in both humans and rodents. Furthermore, PPAR $\gamma$  ligands have been shown to inhibit proliferation and/or induce apoptosis in many cell types (7–9). The two variants of this isoform, PPAR $\gamma_1$  and PPAR $\gamma_2$ , are derived from distinct transcription start sites and result from alternative splicing during transcription. Although there is no evidence supporting a functional difference between the PPAR $\gamma$  variants, the relative expression of these variants is not equal in all tissues. PPAR $\gamma_1$  seems to be ubiquitously expressed, whereas higher levels of PPAR $\gamma_2$  are expressed in adipose tissue (10, 11).

Uterine leiomyomas, or “fibroids,” are benign smooth muscle tumors originating from the myometrium. These tumors have a reported incidence of as high as 77% in women of reproductive age and are the leading indication for hysterectomy in the United States (12, 13). Common symptoms associated with these tumors are dysmenorrhea, menorrhagia, infertility, and morbidity (12). In the majority of cases, alterations in hormonal milieu appear to underlie the impact of risk factors associated with fibroid development (14–18). The growth of uterine leiomyoma is thought to be modulated by the ovarian hormones, estrogen (E<sub>2</sub>) and progesterone. Hormone-dependent leiomyoma growth is evidenced by the fact that most of these tumors are diagnosed during the reproductive years, change in size during pregnancy, and regress after the onset of menopause (12), events coinciding with changes in hormonal milieu. Furthermore, treatment with gonadotropin-releasing hormone agonists, which interfere with signaling pathways of the hypothalamic-pituitary axis, halts or reverses uterine leiomyoma growth through induction of a hypoestrogenic state (19, 20).

The Eker rat is a well-characterized animal model for spontaneous uterine leiomyoma (21, 22). Heterozygous (*Tsc2*<sup>EK/+</sup>) female Eker rats carrying a germ-line mutation of the tuberous sclerosis (*Tsc2*) tumor suppressor gene develop grossly observable tumors with an incidence of approximately 65% by 12–16 months of age (23). Eker rat leiomyomas are histologically similar to human leiomyomas and express the smooth muscle markers desmin and smooth muscle  $\alpha$ -actin (21). An Eker leiomyoma tumor-derived cell line (ELT-3) was characterized with respect to ER and PR expression (24) and has been successfully used in many studies to investigate the hormonal modulation of leiomyomas (25–27).

The role of PPAR signaling in leiomyoma cells has not been elucidated to date. In the present study, PPAR expression was characterized in normal and neoplastic myometrial tissues, and the ability of PPAR ligands to inhibit the proliferation of leiomyoma cells was determined. These preclinical data demonstrated that treatment of leiomyoma cells with PPAR $\gamma$  ligands specifically inhibited E<sub>2</sub>-dependent proliferation and gene expression, demonstrating cross-talk between these two signaling pathways in these tumors and suggesting that PPAR $\gamma$  ligands may have clinical relevance for the treatment of uterine leiomyoma.

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<sup>3</sup> The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; ER, estrogen receptor; PR, progesterone receptor; E<sub>2</sub>, 17 $\beta$ -estradiol; DHA, *cis*-4,7,10,13,16,19-docosahexaenoic acid; Tro, troglitazone; Cig, ciglitazone; Bez, Bezafibrate; 15 $\Delta$ PGJ<sub>2</sub>, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>; Ros, rosiglitazone; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; FBS, fetal bovine serum; ERE, estrogen response element; TBS, tris buffered saline; TBST, TBS containing 0.5% Tween 20; PBST, phosphate buffered saline containing 0.5% Tween 20.

## MATERIALS AND METHODS

**Cell Lines and Media.** As described previously (25), the Eker rat tumor-derived ELT-3 uterine leiomyoma cell line was maintained in 5% CO<sub>2</sub> at 37°C in DF8 medium containing 10% FCS (Hyclone Laboratories Inc., Logan UT). Serum-free, phenol red-free DF8 basal medium containing 1% BSA (Sigma Chemical Co., St. Louis, MO) was used to treat ELT-3 cells with test compounds. LM2 cells were originated and characterized in the Copland laboratory. These cells express vimentin and desmin but do not express cytokeratin. Cells were used at early passages (passages 4–10) and maintained in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 5% FBS (Atlanta Biologics, Inc., Atlanta, GA) and 2% penicillin-streptomycin-antimycotic (Life Technologies, Inc.). Cells were grown at 37°C in a humidified air atmosphere that was 5% CO<sub>2</sub>.

**Chemical Compounds.** Tro was a gift from M. M. Gottardis (Bristol Myers-Squibb, Princeton, NJ) or a gift from Sankyo Company Ltd. (Tokyo, Japan). BMS-263990-01-001 was a gift from M. M. Gottardis (Bristol Myers-Squibb), and 15 $\Delta$ PGJ<sub>2</sub> was purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA) or Cayman Chemical Co. (Ann Arbor, MI). Cig and Wy-14643 were purchased from BIOMOL Research Labs Inc. Ros was a gift from Sankyo Company Ltd. Bez, E<sub>2</sub>, DMSO, and ethanol were purchased from Sigma Chemical Co.

**Cell Growth Kinetics.** ELT-3 cells were plated at 5000 cells/well in 24-well plates and grown for 48 h in DF8 media containing 10% FBS. Cells were counted and then treated on day 0 in E<sub>2</sub>-free media with vehicle, E<sub>2</sub>, PPAR ligand and vehicle, or PPAR ligand and E<sub>2</sub>. In the instance where dual treatments were administered, two vehicle controls were used. Cells were washed once with 1 $\times$  PBS and then collected using 3 $\times$  trypsin and counted on days 3, 5, 6, and 7 (if possible) using a Coulter Z1 counter (Coulter Electronics, Hialeah, FL).

**Rat Tissues.** Eker rats were maintained on a 14-h light/10-h dark cycle, with food and water provided *ad libitum*. Eker rats between the ages of 12 and 16 months were sacrificed by CO<sub>2</sub> asphyxiation. Normal myometrial tissue pooled from 12–16-month-old animals was obtained after removing the endometrial lining of the uterus by scraping using a sterile scalpel, followed by PBS rinse; snap-frozen in liquid N<sub>2</sub>; and then stored at –80°C. Leiomyomas collected from tumor-bearing animals were snap-frozen and stored at –80°C. Rats were maintained and handled according to NIH guidelines and in Association for the Accreditation of Laboratory Animal Care-accredited facilities. The protocols involving use of these animals were approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

**Human Tissues.** Leiomyoma and normal myometrium were collected from surgical hysterectomy specimens submitted to the Department of Pathology, University of Texas M. D. Anderson Cancer Center. Both leiomyoma and normal myometrium were snap-frozen in liquid nitrogen and stored at –80°C.

**Western Blots.** All tumor and normal myometrium samples were pulverized using mortar and pestle in liquid N<sub>2</sub> and then immediately transferred to radioimmunoprecipitation assay buffer containing protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride, and aprotinin) and incubated for 1 h at 4°C. After a 10-min spin at 10,000  $\times$  g, the supernatant containing the total cell lysate was quantitated using BCA Protein Assay Reagent (Pierce, Rockford, IL). Thirty  $\mu$ g (or 15  $\mu$ g for LM2 cells) of total cell lysate were resolved by SDS-PAGE using a 4–20% gradient or 7.5% gel (Bio-Rad Laboratories, Hercules, CA) or 10% gel. Proteins were transferred overnight to polyvinylidene difluoride membrane and blocked for 1–2 h in 5% milk TBST or 2% milk PBST. A 1:1000 dilution of primary antibodies recognizing PPAR $\alpha$ , PPAR $\beta$  (Affinity Bioreagents, Denver, CO), PPAR $\gamma$ , ER,  $\alpha$ -tubulin, and  $\beta$ -actin [1:6000 (Santa Cruz Biotechnology, Santa Cruz, CA)] or PR (Calbiochem, La Jolla, CA) was hybridized in 1% milk TBST for 2 h or in 4% milk PBST for 2 h. The membranes were then washed once with Tris-buffered saline, followed by three washes with TBST and one final wash with Tris-buffered saline each for 5'. PPAR $\gamma$  antibody recognized M<sub>r</sub> 56,000 and M<sub>r</sub> 52,000 variants that correspond to PPAR $\gamma$ <sub>1</sub> and PPAR $\gamma$ <sub>2</sub>. Antirabbit or antimouse IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was hybridized for 1 h in 1% milk TBST. The wash sequence was the same as that stated previously. Whole cell lysates from rat liver were used as positive controls for Western analysis of PPAR isoforms. All hybridizations and washes were performed at room temperature. LumiGLO (KPL, Gaithersburg, MD) was used for visualization. Consistent protein amounts

were determined by staining the membrane after hybridization with Ponceau S (Sigma Chemical Co.), and  $\alpha$ -tubulin or  $\beta$ -actin expression was used to assess consistent loading between samples. PR-A expression was quantitated densitometrically and normalized using  $\alpha$ -tubulin expression.

**Reporter Gene Assays.** ELT-3 cells were plated at 15,000 cells/well in 12-well plates and grown for 24 h in DF8 media containing 10% FBS. Effectene Transfectant Reagent kit (Qiagen, Valencia, CA) was used to transfect cells with pCMV- $\beta$ -galactosidase (a gift from Dr. A. Butler; University of Texas M. D. Anderson Cancer Center) and vit-ERE-Luc plasmids (28). Cells were washed twice with 1 $\times$  PBS and then treated with increasing doses of PPAR ligand for 24 h, followed by treatment with vehicle or E<sub>2</sub> for 24 h. At that time, luminescence and  $\beta$ -galactosidase values were determined using the Promega Luciferase Assay System (Madison, WI) and Tropix Galactolight (Bedford, MA) according to the manufacturer's instructions. Luminescence was detected using a Dynex-MLX Luminometer (Chantilly, VA). Luciferase activity was normalized with  $\beta$ -galactosidase values to correct for transfection efficiency. Cells were plated at 100,000 cells/60-mm plate in DMEM containing 5% FBS and transfected the following day with 2  $\mu$ g of PPRE-luc plasmid (a gift from Dr. R. Evans) and 0.1 ng of *Renilla* luciferase plasmid (Promega) using FuGene transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) at a ratio of 1  $\mu$ g DNA:3  $\mu$ l FuGene. On the following day, cells were treated with either a 1:1,000 dilution of DMSO or an appropriate concentration of PPAR ligand. Twenty-four h later, cells were lysed, and firefly luciferase and *Renilla* luciferase activities were measured in a Lumat luminometer.

**Cell Proliferation Assay.** A fluorometric assay, implementing Hoechst 33258 (bisbenzimidazole), was used for DNA quantitation. LM2 cells (25,000 cells/well) were plated on a 12-well plate in 1 ml of DMEM media supplemented with FBS and penicillin-streptomycin-antimycotic (described above). The cells were allowed to attach overnight at 37°C, and then the media were replaced with media containing the appropriate treatment of 15 $\Delta$ PGJ<sub>2</sub>, Tro, or Ros. Control cells were treated with a 1:1,000 dilution of DMSO. The cells were incubated for 3 days, followed by cell lysis and DNA content determination using Hoechst dye solution (10  $\mu$ l/100 ml distilled H<sub>2</sub>O). Fluorescence was measured (DyNA Quant 200; Hoefer Pharmacia Biotech) after excitation at 365 nm and fluorescence at 458 nm. Calf thymus DNA (Sigma) was used as a standard to determine DNA concentration. Antisense and sense phosphorothioate-modified oligodeoxynucleotides were designed using the PPAR $\gamma$  nucleotide cDNA sequence. The PPAR $\gamma$  antisense (5'-CTC-TGT-GTC-AAC-CAT-GGT-CAT-3') and sense (5'-ATG-ACC-ATG-GTT-GAC-ACA-GAG-3') oligonucleotides were provided by Sigma-Genosys (Woodlands, TX). Cells were plated as described above and treated daily with either sense or antisense oligonucleotides at a final concentration of 10  $\mu$ M. On the second day of treatment with oligonucleotide, cells were treated with PPAR $\gamma$  agonists and then allowed to proliferate for 3 days. DNA content was then determined.

**Statistical Analysis.** Statview 5.01 (SAS Institute, Cary, NC) was used for statistical calculations (ANOVA, means and SE).

## RESULTS

**Leiomyomas Express All Three PPAR Isoforms.** Western analysis was performed using Eker rat myometrium, leiomyoma, and ELT-3 leiomyoma cells to characterize PPAR expression patterns. All

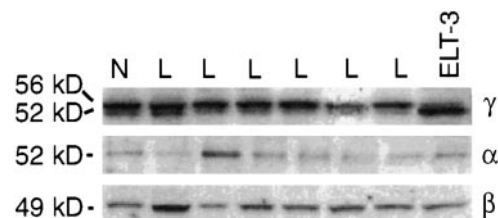


Fig. 1. PPAR expression in Eker rat tissues and tumor-derived cells. Western analysis of six Eker rat leiomyomas (L), pooled myometrium from 12–16-month-old Eker rats (N) and ELT-3 cells.

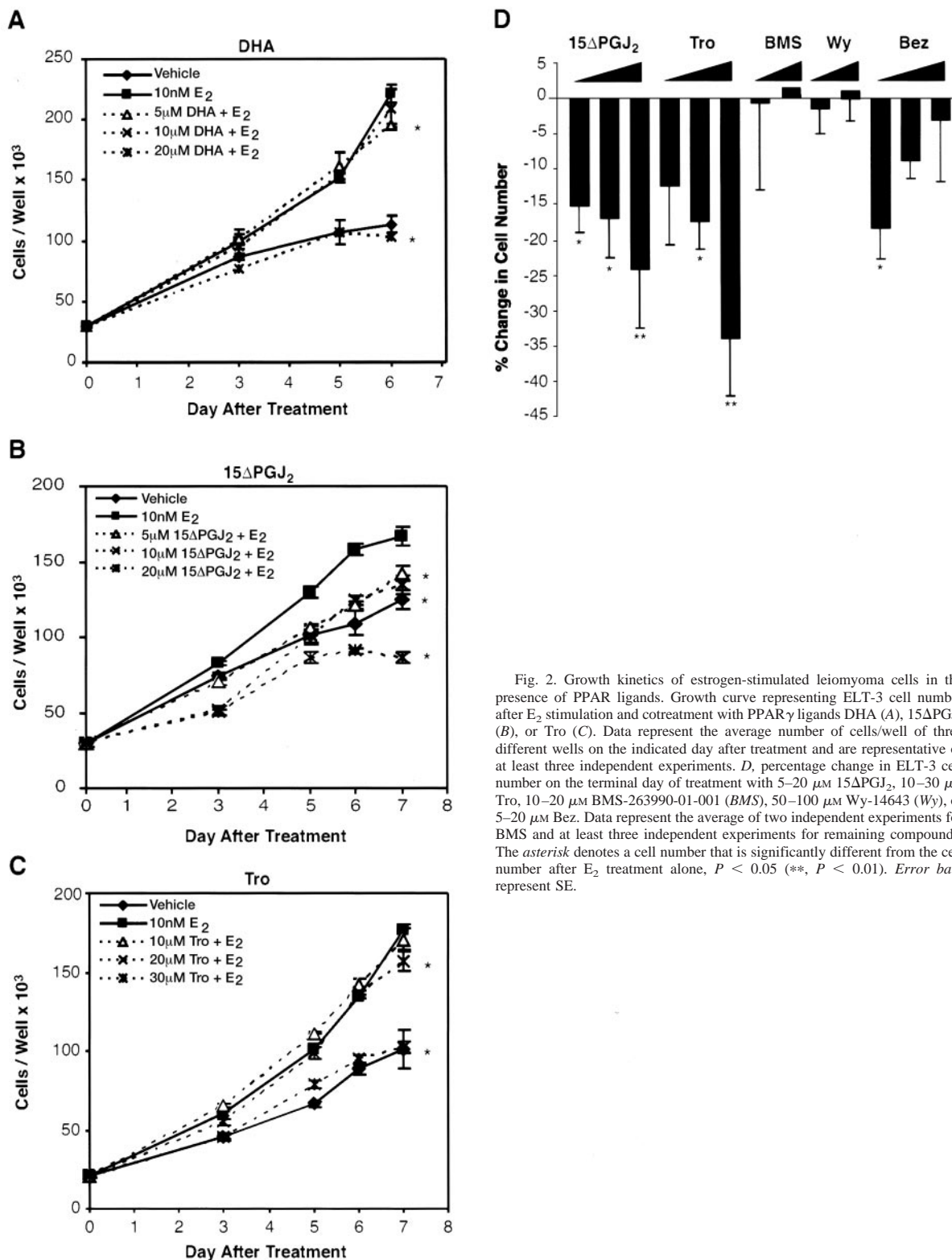


Fig. 2. Growth kinetics of estrogen-stimulated leiomyoma cells in the presence of PPAR ligands. Growth curve representing ELT-3 cell number after E<sub>2</sub> stimulation and cotreatment with PPAR $\gamma$  ligands DHA (A), 15 $\Delta$ PGJ<sub>2</sub> (B), or Tro (C). Data represent the average number of cells/well of three different wells on the indicated day after treatment and are representative of at least three independent experiments. D, percentage change in ELT-3 cell number on the terminal day of treatment with 5–20  $\mu$ M 15 $\Delta$ PGJ<sub>2</sub>, 10–30  $\mu$ M Tro, 10–20  $\mu$ M BMS-263990-01-001 (BMS), 50–100  $\mu$ M Wy-14643 (Wy), or 5–20  $\mu$ M Bez. Data represent the average of two independent experiments for BMS and at least three independent experiments for remaining compounds. The asterisk denotes a cell number that is significantly different from the cell number after E<sub>2</sub> treatment alone,  $P < 0.05$  (\*\*,  $P < 0.01$ ). Error bars represent SE.

three PPAR isoforms were expressed in normal myometrium and leiomyomas from Eker rats as well as in tumor-derived ELT-3 cells. Furthermore, there was no consistent difference in the levels of expression between normal myometrium and leiomyomas (Fig. 1).

**PPAR $\gamma$  Ligands Inhibit E<sub>2</sub>-induced Leiomyoma Cell Proliferation.** The growth of uterine leiomyomas *in vivo* is dependent on steroid hormones, and Eker rat leiomyoma-derived cells proliferate *in*

*vitro* in response to E<sub>2</sub> (24). Initially, the pan-PPAR ligand DHA was used to determine whether a PPAR-activating compound could modulate the growth of ELT-3 leiomyoma cells. E<sub>2</sub> treatment significantly increased ELT-3 cell number over vehicle controls, and this effect was significantly inhibited by DHA in a dose-dependent manner. DHA (20  $\mu$ M) specifically inhibited the E<sub>2</sub>-induced increase in cell number, and a 10  $\mu$ M dose of DHA also caused a slight decrease in proliferation,

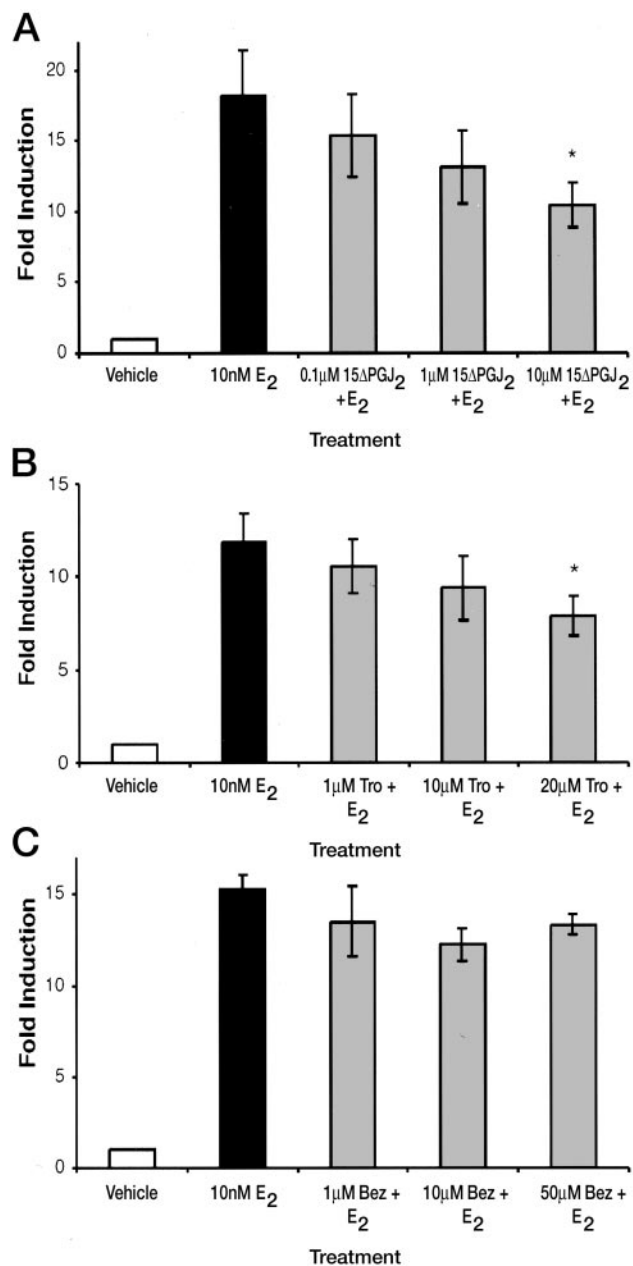


Fig. 3. ER modulated transcription after PPAR $\gamma$  ligand treatment. Transactivation of vit-ERE promoter-driven luciferase gene in the presence of 10 nM E<sub>2</sub> after 24-h pretreatment with vehicle or increasing doses of 15ΔPGJ<sub>2</sub> (A), Tro (B), or Bez (C). The asterisk denotes fold induction that is significantly different from fold induction with E<sub>2</sub> treatment alone,  $P < 0.05$ . Data shown are the average data for three (Bez) or five (15ΔPGJ<sub>2</sub> and Tro) independent experiments. Error bars represent SE.

which, although less dramatic than that induced by the 20  $\mu$ M dose, was significant and reproducible (Fig. 2A).

To determine whether a particular PPAR isoform was responsible for this inhibition, cell growth kinetics in response to E<sub>2</sub> in the presence of isoform-specific PPAR ligands were determined. The PPAR $\gamma$  ligand 15ΔPGJ<sub>2</sub> significantly inhibited E<sub>2</sub>-induced proliferation of ELT-3 cells when treated with 5–20- $\mu$ M doses (Fig. 2B). To confirm the effect of PPAR $\gamma$  ligands on ELT-3 cell growth, the synthetic PPAR $\gamma$ -activating thiazolidinediones, Tro and Cig, were also evaluated for the ability to inhibit E<sub>2</sub>-stimulated proliferation. Treatment of ELT-3 cells with Tro (Fig. 2C) and Cig (data not shown) in the presence of E<sub>2</sub> resulted in a decrease in ELT-3 cell number when compared with growth with E<sub>2</sub> treatment alone. To determine

whether growth inhibition mediated by PPAR was specific for PPAR $\gamma$ -activating compounds, E<sub>2</sub>-stimulated cells were treated with 50–100  $\mu$ M Wy-14643 (PPAR $\alpha$  ligand) or 5–20  $\mu$ M BMS-263990-01–001 (PPAR $\beta$  ligand). These compounds had no effect on ELT-3 cell number in the presence of E<sub>2</sub> treatment at the tested doses (Fig. 2D). A 5- $\mu$ M dose of the PPAR $\alpha/\beta$  ligand Bez (29) significantly inhibited ELT-3 cell growth, whereas 10- and 20- $\mu$ M doses did not inhibit the E<sub>2</sub>-stimulated growth of this cell type (Fig. 2D). These results indicate that PPAR $\gamma$ -activating compounds, unlike PPAR $\alpha$ - or PPAR $\beta$ -activating compounds, can inhibit the estrogen-dependent proliferation of leiomyoma cells.

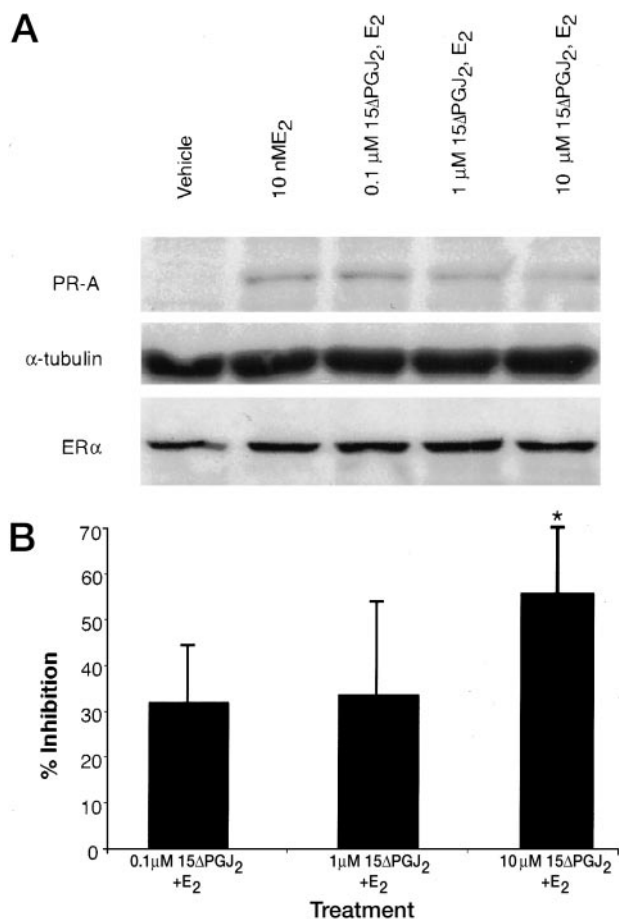


Fig. 4. Expression of endogenous targets of ER after PPAR ligand treatment. A, representative Western blots for PR-A and ER $\alpha$  after 24-h pretreatment with 15ΔPGJ<sub>2</sub> followed by treatment with 10 nM E<sub>2</sub> for 24 h. The expression of  $\alpha$ -tubulin was used to determine consistent protein loading between samples and for normalization. B, average percentage of inhibition of E<sub>2</sub>-stimulated PR-A expression after 15ΔPGJ<sub>2</sub> pretreatment. Bands were quantitated densitometrically, and PR-A band intensity was normalized with  $\alpha$ -tubulin expression. Data presented are the average of three independent experiments, and error bars represent SE.



Fig. 5. PPAR expression in human normal and leiomyoma samples. Western analysis of PPAR expression in human leiomyoma and myometrium is shown. Data are representative of 24 matched samples. Consistent protein loading was determined by staining the membrane with Ponceau S solution.

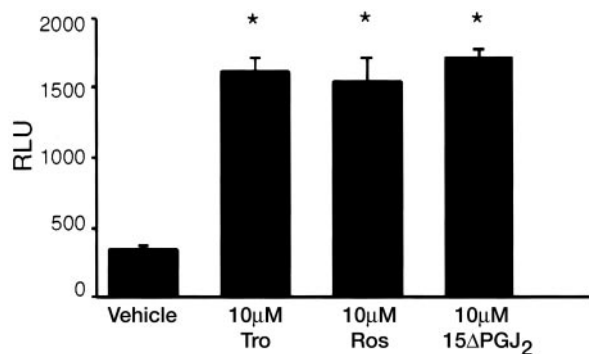


Fig. 6. Transactivation of a PPRE in primary human leiomyoma cells. Activation of a transiently transfected PPRE-driven luciferase gene in LM2 cells after treatment with the indicated PPAR $\gamma$  ligand for 24 h. Data are representative of three independent experiments. The asterisk denotes significant difference from vehicle treatment,  $P < 0.05$ . Error bars represent SD for three different wells.

Except for the highest dose of 15 $\Delta$ PGJ<sub>2</sub>, none of the above treatments with PPAR ligands significantly altered ELT-3 cell proliferation in E<sub>2</sub>-free media, as assessed by a decrease in ELT-3 cell number (data not shown). These data indicate that treatment with PPAR $\gamma$  ligands specifically inhibits the E<sub>2</sub>-induced proliferation of ELT-3 cells and, with the exception of the highest dose of 15 $\Delta$ PGJ<sub>2</sub>, does not appear to have any overt toxicity.

**PPAR $\gamma$  Ligands Modulate ER Activity in ELT-3 Cells.** The ability of PPAR $\gamma$  ligands to specifically inhibit E<sub>2</sub>-induced cell growth suggested that these compounds could modulate ER-mediated signaling. To confirm that this was the case, leiomyoma cells were transfected with a vit-ERE-Luc reporter plasmid and treated with E<sub>2</sub> and vehicle or E<sub>2</sub> and various PPAR ligands. Twenty-four-h pretreatment of ELT-3 cells with 15 $\Delta$ PGJ<sub>2</sub> or Tro, compounds known to activate PPAR $\gamma$ , inhibited the transactivation of a vit-ERE-Luc reporter gene in response to E<sub>2</sub> in a dose-dependent manner (Fig. 3, A and B, respectively). The PPAR $\alpha$ / $\beta$ -activating compound Bez failed to inhibit E<sub>2</sub>-induced ERE transactivation in leiomyoma cells (Fig. 3C). Likewise, the PPAR $\alpha$ -activating compound Wy-14643 did not inhibit E<sub>2</sub>-mediated transactivation of this reporter in leiomyoma cells (data not shown). Although Wy-14643 did not significantly inhibit transactivation of the reporter in the presence of E<sub>2</sub> at 1–50- $\mu$ M doses, there was a slight decrease in transactivation with a 50- $\mu$ M dose (data not shown). Although it was not significant, this inhibition was reproducible.

In addition to inhibiting the transactivation of a reporter gene in leiomyoma cells, 15 $\Delta$ PGJ<sub>2</sub> pretreatment also inhibited the E<sub>2</sub>-dependent expression of PR-A, an endogenous target of ER transactivation (Fig. 4A, top panel). Average data from three independent experiments revealed that 0.1-, 1-, and 10- $\mu$ M doses of 15 $\Delta$ PGJ<sub>2</sub> inhibited the expression of PR-A by 32%, 33%, and 57% respectively. Only the highest dose of 15 $\Delta$ PGJ<sub>2</sub> significantly inhibited PR-A expression (Fig. 4B). Tro pretreatment also resulted in diminished PR-A expression in response to E<sub>2</sub>, but to a lesser extent than was observed after 15 $\Delta$ PGJ<sub>2</sub> pretreatment (data not shown). One trivial explanation for diminished downstream ER activity is a decrease in ER expression after treatment with PPAR $\gamma$  ligands. As shown by Western analysis, the levels of ER remained constant in ELT-3 cells regardless of treatment, indicating that the decrease in ER transactivation is not due to a decrease in ER in the cells after treatment with PPAR $\gamma$  ligands (Fig. 4A, bottom panel). These data demonstrate the ability of PPAR $\gamma$ -specific ligands to interfere with the action of ER in response to E<sub>2</sub> and suggest that negative cross-talk between ER and PPAR signaling pathways is a

potential mechanism by which leiomyoma cell growth can be inhibited by PPAR $\gamma$  ligands.

**PPAR Expression in Human Myometrium and Leiomyoma.** To extrapolate data obtained in the Eker rat model to the human disease, the pattern of PPAR expression in matched human leiomyoma and normal myometrium was examined by Western analysis, using leiomyomas and matched myometrium from 24 individuals. Similar to data collected from the Eker rat, all three PPAR isoforms were expressed in the normal and neoplastic human myometrium (Fig. 5). PPAR expression varied in the leiomyoma samples compared with the matched myometrium; however, no consistent pattern could be determined.

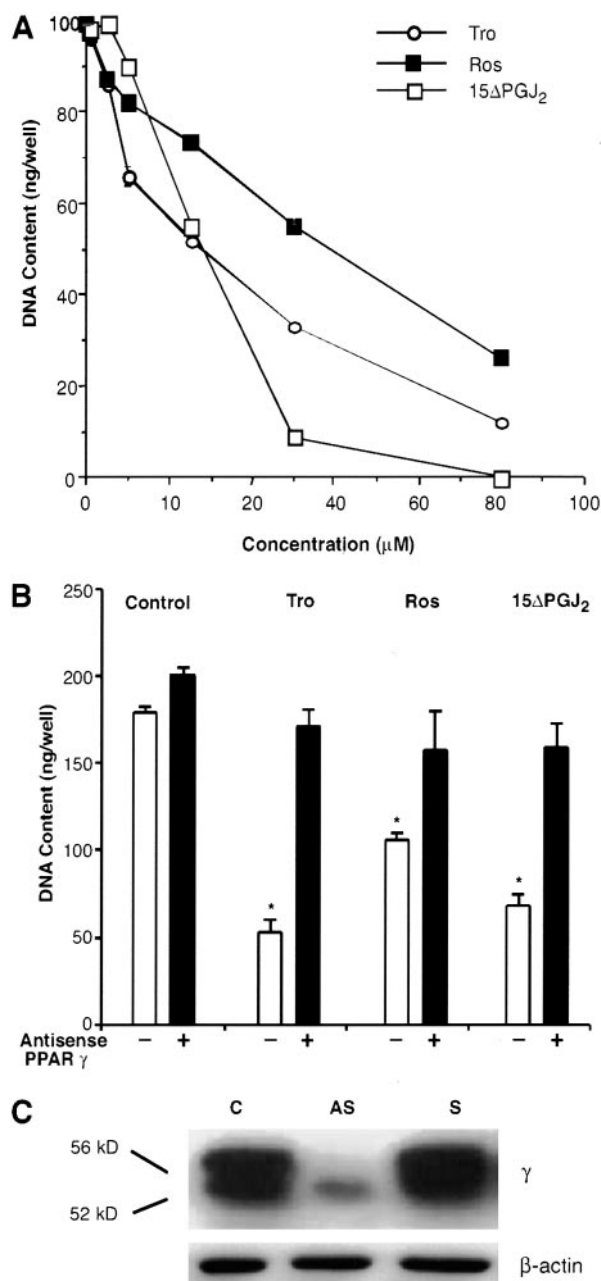


Fig. 7. PPAR $\gamma$ -treated human primary leiomyoma cells. A, DNA content of LM2 cells after treatment with PPAR $\gamma$  ligands. Data are representative of three experiments. B, DNA content of LM2 cells after treatment with PPAR $\gamma$  ligands in the presence of antisense PPAR $\gamma$  oligonucleotides. Data are representative of three experiments, and error bars represent SD of mean for triplicate wells. The asterisk denotes significant difference from vehicle treatment,  $P < 0.05$ . C, expression of PPAR $\gamma$  after transfection of control (C) antisense PPAR $\gamma$  (AS), and sense PPAR $\gamma$  (S) oligonucleotides.

**PPAR $\gamma$  Treatment Inhibits the Growth of Human Leiomyoma Cells.** Primary cultures of human leiomyoma cells (LM2) were treated with multiple PPAR $\gamma$  ligands to assess the ability of these compounds to initiate transcription in this cell type and determine the viability of these cells in response to treatment. As shown by relative luciferase values, compounds known to activate PPAR $\gamma$  (15 $\Delta$ PGJ<sub>2</sub>, Tro, and Ros) enhanced the transcription of a luciferase reporter driven by a PPRE-containing promoter (Fig. 6). Treatment of LM2 cells with PPAR $\gamma$  ligands 15 $\Delta$ PGJ<sub>2</sub>, Tro, and Ros resulted in diminished proliferation of this cell type compared with vehicle-treated cells (Fig. 7A). To determine whether this effect was PPAR $\gamma$  dependent, LM2 cells were transfected with PPAR $\gamma$  antisense oligonucleotides before treatment. The introduction of these oligonucleotides reversed the decrease in cell number observed in response to 15 $\Delta$ PGJ<sub>2</sub>, Tro, and Ros, suggesting that inhibition was PPAR $\gamma$  dependent (Fig. 7B). The efficacy of the antisense oligonucleotide depletion of endogenous PPAR $\gamma$  was confirmed by measuring PPAR $\gamma$  protein levels by Western blot (Fig. 7C). These data suggest that, similar to results obtained in the Eker rat model, PPAR $\gamma$  ligands inhibit proliferation of primary human leiomyoma cells in a PPAR $\gamma$ -specific manner.

## DISCUSSION

In the present study, we show that both myometrium and leiomyomas from humans and Eker rats express the three known PPAR isoforms consistent with a biologically relevant role for these receptors in this tissue. In human leiomyomas, PPAR expression levels showed no consistent pattern of variation when compared with patient-matched myometrium, although there appears to be a great deal of variation in isoform expression between some tumor and normal tissues. Cotreatment of leiomyoma cells with E<sub>2</sub> and PPAR $\gamma$  ligands in E<sub>2</sub>-responsive leiomyoma cells (rat) resulted in a significant decrease in cell number when compared with E<sub>2</sub> treatment alone. PPAR $\alpha$  and PPAR $\beta$  ligands had no inhibitory effect on proliferation in these cells. The ability of PPAR $\gamma$ -activating compounds to inhibit leiomyoma cell proliferation was also confirmed in human primary leiomyoma cultures, although growth inhibition in these cells was not determined in estrogen-free conditions. Furthermore, an inhibition of E<sub>2</sub>-dependent ERE activation and a reduction in E<sub>2</sub>-induced PR levels were demonstrated in ELT-3 cells in response to PPAR $\gamma$  ligands.

Previous studies have demonstrated that PPAR $\alpha$  can modulate ERE transactivation. In an artificial promoter context, PPAR $\alpha$ -RXR heterodimers can bind and transactivate an ERE-containing promoter (30). This transactivation was not achieved in the more natural, ERE-containing promoter of the PS2 gene. Negative PPAR-ER cross-talk demonstrated in this system was attributed to activated PPAR $\alpha$ -RXR heterodimers physically blocking ER binding to the ERE of this promoter, thus inhibiting ER-mediated transcription of the associated reporter gene. Our data demonstrated PPAR $\gamma$  ligand-mediated inhibition of ER-responsive gene transactivation and ER-induced protein expression. The mechanism of nuclear receptor cross-talk between PPAR $\gamma$  and ER in leiomyoma responsible for this observation has not yet been determined. Interestingly, Tro has been shown to inhibit the growth of breast cancer cells, and ER-positive breast cancer cells are more sensitive to this inhibition (7). It will be important to further investigate the ability of PPARs to modulate the activity of steroid hormone receptors in these and other hormonally responsive cell types.

A possible tumor suppressor role for PPAR $\gamma$  has been previously postulated for colon carcinoma; approximately 7% of human colon carcinomas tested (4 of 55) had loss of function mutations in

PPAR $\gamma$  (31). Furthermore, the growth of transplanted colon cancer cells in nude mice was significantly inhibited when treated with Tro (8). In contrast to a previous study of PPARs in leiomyoma that indicated that PPAR $\gamma$  levels were increased in human leiomyomas (32), no increased PPAR $\gamma$  expression was observed in rat or human leiomyoma samples relative to matched myometrium. The size of the immunoreactive band ascribed to PPAR $\gamma$ 2 ( $M_r$  >60,000) in that study differed significantly from the  $M_r$  ~52,000 (PPAR $\gamma$ 1) and  $M_r$  ~56,000 (PPAR $\gamma$ 2) bands recognized in our study and reported by other investigators (33, 34). Consistent with our observation that PPAR $\gamma$  expression is unchanged in leiomyoma compared with matched myometrium, PPAR $\gamma$  message levels were previously reported to be the same in human leiomyomas compared with adjacent myometrium (35). Furthermore, Tro treatment prevented the formation of abdominal leiomyoma induced by E<sub>2</sub> in hormone-treated guinea pigs (32). These data suggest that Tro had a protective effect on the development of uterine leiomyoma in the guinea pig, findings consistent with a growth-inhibitory role for PPAR $\gamma$  in leiomyoma. Clearly, much more work needs to be focused on understanding the involvement of PPARs in this tissue and the possible role of these receptors as ER modulators.

The regulation of leiomyoma growth by ovarian steroid hormones has been well described (12, 19, 20). Similar to human leiomyomas, the ELT-3 leiomyoma cells retain expression of ER and PR as well as the ability to respond to steroid hormones (24–27). This differs from human leiomyoma cell cultures, which have been reported to undergo a 75% decrease in ER and PR expression within 8 h of culture (36), and these cultures consistently lose hormone responsiveness (37). Therefore, ELT-3 cells are a unique, *in vitro* tool to study the effects of steroid hormones on leiomyoma growth and evaluate steroid receptor signaling mechanisms. In this study, we used the ELT-3 cells to demonstrate that PPAR $\gamma$  ligands exhibited transdominant suppression of ER action in leiomyoma cells, inhibiting proliferation and ER signaling response to steroid hormones. To date, this is the first report suggesting that nuclear receptor cross-talk is a mechanism by which PPAR $\gamma$  ligands may exert antiproliferative effects. Although the ability of activated PPAR $\gamma$  to interfere with ER action will continue to be explored, these data provide evidence for PPAR $\gamma$ -ER cross-talk and suggest that PPAR $\gamma$  ligands should be examined as candidate therapeutic agents for uterine leiomyoma.

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