Activation of Peroxisome Proliferator-Activated Receptor δ Stimulates the Proliferation of Human Breast and Prostate Cancer Cell Lines

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ABSTRACT

The nuclear receptor peroxisome proliferator-activated receptor δ [PPARδ/β (NR1C2)] has been implicated in colorectal carcinogenesis by various molecular genetic observations. These observations have recently been supported by studies of activation of PPARδ by pharmacological agents. Here we present the first report of the stimulation of breast and prostate cancer cell growth using PPARδ selective agonists. Activation of PPARδ with compound F stimulated proliferation in breast (T47D, MCF7) and prostate (LNCaP, PNT1A) cell lines, which are responsive to sex hormones. Conversely, we have found that several steroid-independent cell lines, including colon lines, were unresponsive to compound F. These findings were confirmed with an additional high-affinity PPARδ agonist, GW501516. Conditional expression of PPARδ in MCF7 Tet-On cells resulted in a doxycycline-enhanced response to GW501516, thus providing direct genetic evidence for the role of PPARδ in the proliferative response to this drug. Activation of PPARδ in T47D cells resulted in increased expression of the proliferation marker Cdk2 and also vascular endothelial growth factor α (VEGFα) and its receptor, FLT-1, thus, suggesting that PPARδ may initiate an autocrine loop for cellular proliferation and possibly angiogenesis. Consistent with this hypothesis, we demonstrated a pro-proliferative effect of GW501516 on human umbilical vein endothelial cell cultures and found that GW501516 also regulated the expression of VEGFα and FLT-1 in these cells. Our observations provide the first evidence that activation of PPARδ can result in increased growth in breast and prostate cancer cell lines and primary endothelial cells and supports the possibility that PPARδ antagonists may be of therapeutic value in the treatment of breast and prostate cancer.

INTRODUCTION

The peroxisome proliferator-activated receptor δ/β (PPARδ/β) is an ubiquitously expressed nuclear receptor that has been implicated in adipose tissue formation, brain development, placental function, wound healing, and atherosclerosis (1–5). Three groups have reported targeted disruption of the PPARδ gene in mice. One group reported relatively viable PPARδ null mice (5). In contrast, another group reported over 90% mid-gestation lethality with placental defects (2), and the third group has reported almost complete prenatal lethality (4). The reason for the differences in viability among these three groups remains unknown. In both studies in which viable PPARδ null mice were analyzed, it was found that PPARδ null animals were reduced in size both in utero and neonatally, and had smaller adipose stores. Both PPARδ homozygous and PPARδ heterozygous null mice have been

shown to have altered skin function (5). The skin defect has since been explored in detail using PPARδ null embryonic cells, and this work revealed an important role for PPARδ in the suppression of apoptosis in keratinocytes (6).

PPARδ has been implicated in colorectal carcinogenesis by several studies. PPARδ was first linked with colon cancer by the observation that PPARδ gene expression is greatly repressed on overexpression of the wild-type adenomatous polyposis coli (APC) gene in a colon cancer cell line (7). Analysis of PPARδ expression in matched normal and tumor samples revealed that it is up-regulated in tumors (8) and that PPARδ gene expression is increased by activated Ras signaling (9). It has also been postulated that PPARδ may mediate cellular responses to the chemopreventive agent, sulindac sulfide (5, 7). Furthermore, deletion of the PPARδ gene in a human colon cancer cell line by homologous recombination resulted in a profound loss of tumorigenicity in nude mice (10).

Although all of these results suggest a pro-proliferative role for PPARδ in tumor growth, attempts to demonstrate such an effect by ligand activation of PPARδ have largely been unsuccessful. Studies of the biology of PPARδ have been hampered by the lack of availability of potent and specific ligands for PPARδ. Prostacyclin (PGI₂) is the most selective eicosanoid agonist known for PPARδ, and this has been shown to have no effect on the proliferation of colorectal cancer cell lines (8). In contrast, prostacyclin and its synthetic analogs display antimetastatic potential in vivo, and this appears to be mediated via the membrane-bound prostaglandin receptors (11). Several highly selective and potent ligands for PPARδ have been described that activate PPARδ at low nanomolar concentrations, and recently we have demonstrated that one of these compounds, GW501516, can stimulate the proliferation of the human hepatocarcinoma cell line HepG2 (12). This compound has also recently been shown to stimulate intestinal polyp growth in the adenomatous polyposis coli/min mouse model of colorectal cancer (13).

In this study, we have examined the effect of highly potent and selective PPARδ agonists on the growth of a range of human epithelial cell lines. We have found that, under normal culture conditions, PPARδ activation has no effect on cell growth. However, under conditions of hormonal deprivation, PPARδ activation can stimulate the proliferation of certain sex hormone-sensitive cell lines of the breast and prostate, but not colon cancer cell lines. This study reveals a novel pathway for breast and prostate cancer growth and suggests the potential utility of PPARδ antagonists in the treatment or prevention of breast and prostate cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The epithelial cancer-derived cell lines MCF7, T47D, MDA-MB-231, BT20, HT29, SW480, and HCA-7 were obtained from the cell culture collection of the Cancer Research United Kingdom, Cell Resources Unit. The SV40 large T immortalized prostate cell line, PNT1A, was described previously (14). The DU145, LNCaP, and PC3 cell lines were obtained from American Type Culture Collection. Cells were routinely cultured in high-glucose DMEM (Life Technologies, Inc.), 10% FCS

Table 1 *Primers and probes used in this study*

5′ nuclease assays were designed using ABI primer express software. All of the probes were labeled with 5′ FAM and 3′ TAMRA. Specificity of probes was determined by BLAST analysis against GenBank.

Gene	Forward primer	Reverse primer	Probe
AP2α	CGTGTGCGAAACCGAATTT	AGCAGGTCGGTGAACTCTTTG	ATTCCGATCCCAATGAGCAAGTGACAA
AP2β	CGGCATGAATCTATTGGACCA	TCCCAGGAAGCCGTCTTTATT	AGTTCCTCCAAATCGGTGACTTCTC
AP2γ	AGATGGCAGCTAGGAAGAACATG	GGGCGAGCCTGCTGG	CAACTGTGTAAAGAATTCACAGAACTTCTCAGCCAAG
VEGFα	CTGCTGTCTTGGGTGCATTG	TCGTGATGATTCTGCCCTCC	AGCCTTGCCTTGCTGCTCTACCTCCA
FLT1	TGTACAGTGAAATCCCCGAAATT	TCAGGGATCAAAGTGTCAAGTGG	TGCCGGGTTACGGTCACCTAACATCACTGTTACT
VEGFβ	CCCTGTCTCCCAGCCTGAT	TCCACAGTCAAGGGCACCA	TCATGGATAGATGTGTATACTCGCGCTACCTGCCA
PPARδ	GGGACCACAGCATGCACTTC	TGCAGTTGGTCCAGCAGTGA	CCAGCAGCTACACAGACCTCTCCCGG
CDK2	GCATTCCTCTTCCCCTCATCA	CCCGATGAGAATGGCAGAA	CTATCTGTTCCAGCTGCTCCAGGGCCTA
CYCLIN D1	GCCGAGAAGCTGTGCATCTAC	TTCAATGAAATCGTGCGGG	ATGGAGCTGCTCCTGGTGAACAAGCTC
PS2	CACCCTGGCCGAGGC	AACAGCAGCCCTTATTTGCAC	AGACGTGTACAGTGGCCCCCCGT
ER	GAGTCTGGTCCTGTGAGGGC	GGCAGCTCTTCCTCCTGTTTT	CATAACGACTATATGTGTCCAGCCACCAACCA
ErbB2	GGCCCCAAGCACTCTGACT	TGTTGTAGGTGACCAGGGCTG	CTGCCTCCACTTCAACCACAGTGGC

(Sigma, Life Technologies, Inc.), and 2 mM glutamine. Human umbilical vein endothelial cells (HUVECs) were obtained from TCS CellWorks. These cells were grown in large-vessel endothelial cell growth medium (LVECGM; TCS CellWorks), supplemented with 2% FCS, human epidermal growth factor, fibroblast growth factor with heparin, hydrocortisone, gentamicin (25 μg/ml), and amphotericin B (50 ng/ml).

Generation of Cell Lines for the Conditional Expression of PPARδ. The coding sequence of human PPARδ (hPPARδ) was amplified using primers PRMG15 (5′-CTAG<u>TCTAGA</u>**ATG**GAGCAGCCACAGGAGGAAGC-3′) and PRMG3 (5′-CTAG<u>TCTAGA</u>TTAGTACATGTCCTTGTAGATCTCC-TG-3′; *Xba*I sites underlined and the ATG start codon in bold) and were cloned into plasmid pUHD10-3,[4] thus creating plasmid pMGD7. Plasmid pMGD36 was generated by cloning the *Bam*HI fragment of pMGD7 containing PPARδ into pTREHyg (Clontech). The integrity of the PPARδ coding sequence was confirmed by sequencing.

A doxycycline-responsive derivative of the breast cancer cell line MCF7 producing the Tet-On transactivator protein (MCF7-Tet-On; Clontech) was transfected with plasmid pMGD36 using LipofectAMINE Plus according to the manufacturer's instructions (Invitrogen). Hygromycin B (500 μg/ml)- and G418 (100 μg/ml)-resistant clones were selected and expanded and were maintained under hygromycin B and G418 selection (each at 100 μg/ml).

PPAR Agonists. The structures and pharmacology of compound F and GW501516 have been described previously (1, 12, 13, 15, 16). Compound F was a gift from GlaxoSmithKline (Stevenage, United Kingdom) and GW501516 was synthesized by Synthelec AB, Lund, Sweden; the selectivity of this compound was described by Oliver *et al.* (15). Compound F has an EC$_{50}$ for hPPARδ of 2 nM, activates PPARγ at concentrations >200 nM and PPARα at concentrations >10 μM (1). GW501516 activates hPPARδ with an EC$_{50}$ of 1 nM and does not activate PPARα or γ at concentrations of less than 1 μM (12, 15). In addition, GW501516 has also been shown not to activate any other member of the human nuclear receptor superfamily at the concentrations used in this study (15).

Cell Proliferation Assays. For cell proliferation assays, all of the cell lines apart from endothelial cells were seeded in RPMI 1640 (phenol red-free) containing 5% dextran charcoal-stripped FCS (DCFCS) and 2 mM glutamine for all cells other than MCF7 cells, for which this medium was supplemented with 10^{-12} M estradiol. Cells were seeded at a density of 5000 cells/well in 24-well culture plates. Cells were treated with compound F, with GW501516, or with vehicle (≤0.1% DMSO); and the medium was replenished every 3rd day. The relative cell numbers were measured by a colorimetric assay for endogenous hexosaminidase activity (17). For cell proliferation assays, the endothelial cells were seeded in phenol red-free large-vessel endothelial cell-growth medium, containing 2% DCFCS, human epidermal growth factor, fibroblast growth factor with heparin, hydrocortisone, gentamicin (25 μg/ml), and amphotericin B (50 ng/ml) in 96-well plates coated with 30 μg/ml human plasma fibronectin (Sigma). Cells were treated with recombinant 20 ng/ml vascular endothelial growth factor α (VEGFα)-165 (R&D systems), 1, 10, 20, 50, and 100 nM GW501516, or vehicle for up to 14 days. Cells were replenished with fresh medium every 3rd day, and relative cell numbers were quantified as above.

Western Blot Analysis. Cell cultures that were 80–90% confluent growing in regular medium were lysed in SDS/PAGE loading buffer and were analyzed by Western blot using standard procedures (18). The PPARδ antiserum was raised against bacterially expressed full-length hPPARδ and was a gift from Dr. David Bell, Nottingham University (Nottingham, United Kingdom). This serum was used at a dilution of 1:2000. A peroxidase-conjugated, mouse antirabbit IgG antiserum (Sigma) was used as a secondary detection reagent at a dilution of 1:3000, and the results were visualized using enhanced chemiluminescence (ECL+) as described by the manufacturer (Amersham).

Immunohistochemistry. Cultures were treated with 2 μg/ml doxycycline or DMSO for 48 h and then were fixed in fresh 4% paraformaldehyde. Immunostaining was performed using standard procedures (18) with an antiserum raised against the AB domain (amino acids 1–100) of hPPARδ. This serum was used at a dilution of 1:500. A FITC-conjugated mouse antirabbit IgG antiserum (Sigma) was used as a secondary detection reagent at a dilution of 1:320. Chromatin counterstaining was performed using 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes).

Transient Transfection and Reporter Assays. For transient transfection, cells were seeded at a density of 8 × 10^5 cells/well in 6 well plates in RPMI
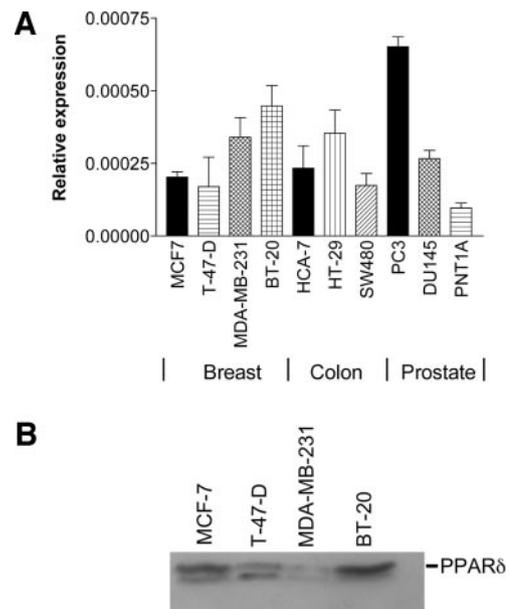

Fig. 1. Peroxisome proliferator-activated receptor δ (*PPARδ*) expression in breast, colon, and prostate cancer cell lines. *A*, the steady-state levels of mRNA encoding PPARδ in a panel of human epithelial cancer cell lines was quantified by quantitative real-time PCR and normalized to 18S rRNA levels. *B*, PPARδ protein levels in human breast cancer cells were determined by Western blotting using a polyclonal antiserum raised against the full-length human PPARδ. Shown are the signals obtained with MCF7, T47D, MDA-MB-231, and BT-20 cells. Protein levels do not correlate well with mRNA levels, because MDA-MB-231 have consistently lower levels of PPARδ protein compared with the other cell lines; however they express similar amounts of PPARδ mRNA. Shown is a representative blot of three independent experiments.

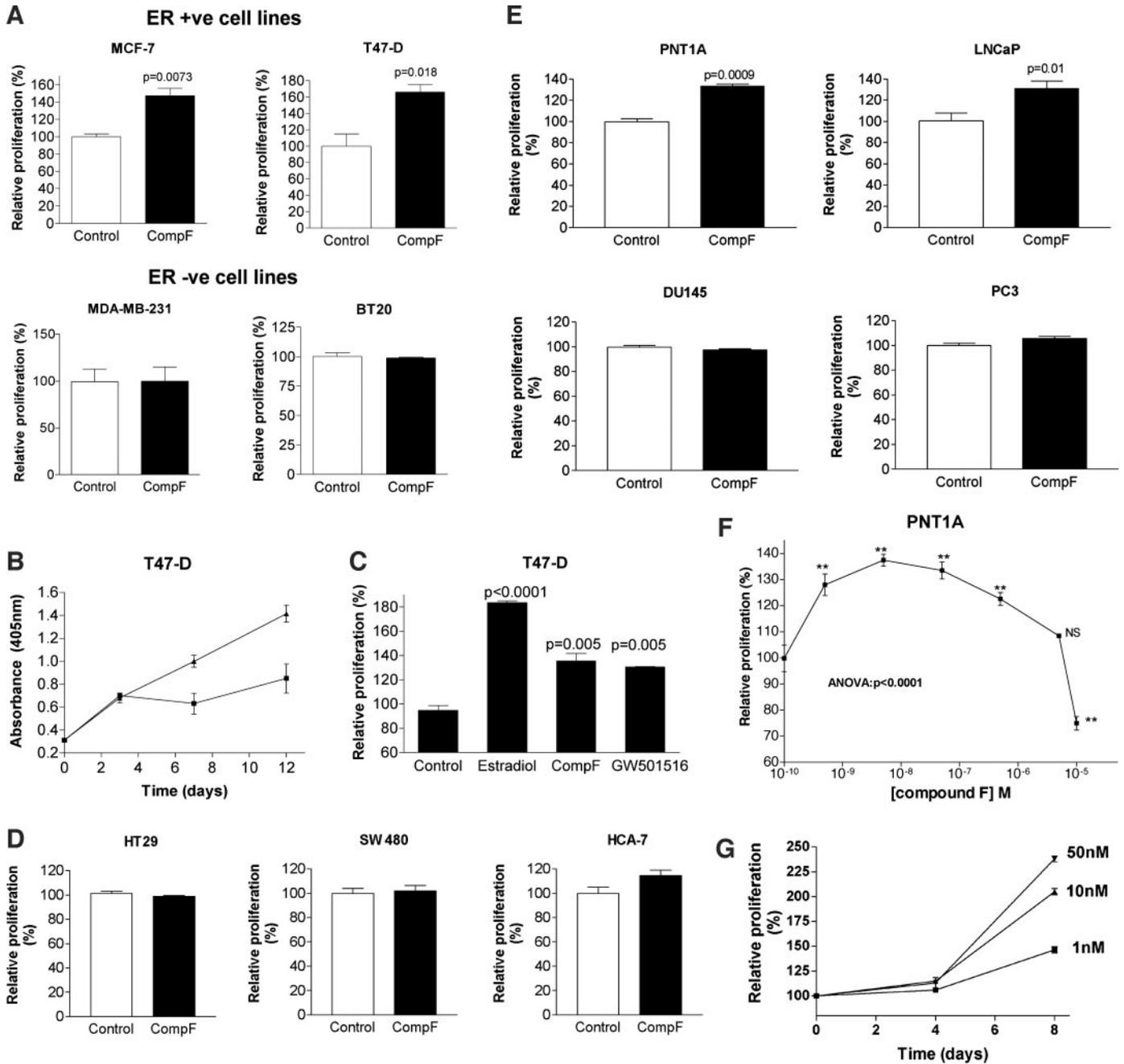[4] M. Gossen, unpublished data; GenBank accession no. U89931.

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Fig. 2. Activation of peroxisome proliferator-activated receptor δ (PPARδ) stimulates proliferation of a subset of epithelial cell lines. *A,* effect of compound F on cell proliferation in breast cancer cells. The figure shows growth stimulation of estrogen receptor-positive (*ER +ve*) breast cancer cells by compound F (*CompF*). Human breast cancer cells were grown in phenol red-free medium with 5% dextran charcoal-stripped FCS (DCFCS) and were treated with 10 nM compound F; relative cell numbers were determined at day 12. Relative proliferation at the final time point are expressed in percentage control ± SE (*n* = 3–6); *ER −ve,* estrogen receptor negative. *B,* shown is a time course of T47D cells growing with (*triangles*) and without (*squares*) 10 nM compound F. *C,* shown is the relative stimulation of proliferation of T47D cells by estradiol compared with compound F (*CompF*; 10 nM) and an additional PPARδ agonist, GW501516 (20 nM) for 14 days. *D,* effect of compound F (*CompF*; 10 nM) on cell proliferation in human colon cancer cell lines. Relative proliferation at day 12 is shown. *E,* effect of compound F (*CompF*; 10 nM) on proliferation in prostate cell lines. The figure shows that compound F stimulates proliferation in androgen-sensitive PNT1A and LNCAP cells, but not in androgen-insensitive DU145 and PC3 cells. Relative proliferation at day 12 is shown. *F,* a dose response to compound F treatment of PNT1A cells for 6 days is shown with the maximum growth stimulation being observed at 10 nM. **, *P* < 0.001, in a Dunnet's post-test relative to the control cultures. *G,* an additional PPARδ ligand, GW501516, also stimulates the proliferation of PNT1A cells in a dose- and time-responsive fashion. The proliferation of the drug-treated cultures is expressed as a percentage of the value obtained with the control cultures. *Error bars,* SE from three triplicate cultures. Each experiment was repeated three times.

1640 containing 5% DCFCS. The following day the medium was removed, replenished with serum free medium and transfected using LipofectAMINE Plus (Life Technologies, Inc.) with pFABPLuc reporter vector (1) and the internal control pSVβgal (Promega). Drug treatments were started the day after transfection and were performed in RPMI 1640 containing 5% DCFCS. For the estrogen response element (ERE) reporter experiments, cells were transfected with pERELuc (19) and the internal control pSVβgal. The cells were harvested 48 h posttransfection. Luciferase and β-galactosidase activity was measured in

the resulting lysates using the appropriate assay kits (Promega, Roche). The results are expressed as luciferase/β-galactosidase ratios.

Cell Cycle Analysis. T47D cells were plated out at 1×10^6 cells/dish in RPMI 1640, 10% FBS in 10 cm dishes and allowed to attach for 24 h. The medium was then changed to phenol red free RPMI 1640, 0.1% FBS containing GW501516 (50 nM), Compound F (50 nM), estradiol (10 nM) or DMSO for 72 h. Cells were labeled with 30 μM bromodeoxyuridine (BrdUrd, Sigma Aldrich) in tissue culture medium at 37°C for 15 min. Cells were then rinsed

Fig. 3. Effect of compound F on peroxisome proliferator response element driven luciferase reporter in human breast cancer cells. The pFAB-PLuc reporter construct was transfected into T47D (*A*), MCF7 (*B*); and MDAMB-231 (*C*) cells and was assessed for a response to 10 nM compound F (*CompF*). *Error bars,* the SE of 3–6 replicate cultures, and each experiment was repeated twice or more. The results shown for MDA-MB-231 (*C*) are pooled from three separate experiments to test the significance of the marginal induction observed.
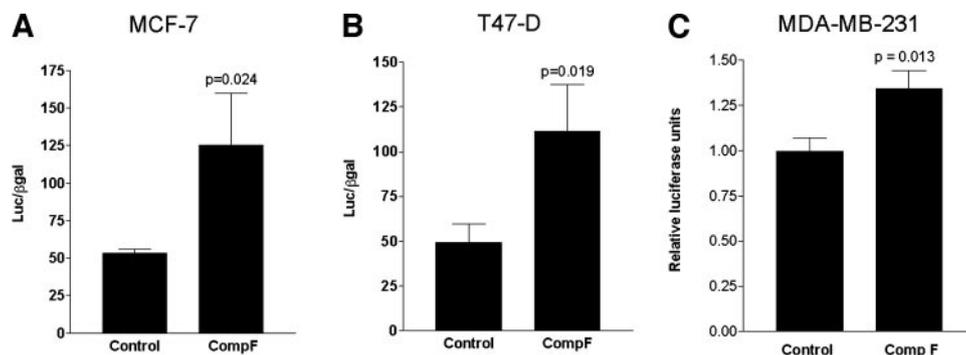
with PBS, trypsinized, added into medium, pelleted by centrifugation at 1000 rpm for 5 min, and resuspended in 1 ml PBS. The cell suspension was added dropwise to 3 ml of ethanol while gently vortexing. Cells were fixed at 4°C for a minimum of 2 h, after which, cells were centrifuged at 2500 rpm for 5 min and 2 ml of prewarmed pepsin (1 mg/ml, pH 1.5) was added. Samples were mixed for 30 min at 37°C and pelleted at 2500 rpm for 5 min. 1 ml of 2M HCl was added and samples incubated for 15 min at room temperature. The sample was then diluted in 5 ml of PBS before centrifuging at 2500 rpm for 5 min, this step was repeated with a final wash of antibody buffer (PBS, 0.5% BSA, and 0.5% Tween 20) and samples were centrifuged. Pellets were resuspended in 200 μl antibody buffer containing a 1:50 dilution of anti-bromodeoxyuridine monoclonal antibody (Becton-Dickinson) and incubated for 1 h at room temperature. 5 ml of PBS was added and samples pelleted by centrifugation. FITC conjugated antimouse antibody (diluted to 20 μg/ml in antibody buffer) was added and pellets resuspended and left at room temperature for 30 min. The sample was then diluted to 5 ml with PBS and centrifuged as before. The final pellet was resuspended in PBS containing 25 μg/ml propidium iodide and sorted on a Becton Dickinson FACScan Flow Cytometer. The resulting dual propidium iodide and FITC fluorescence profiles were analyzed using Cell Quest software (Becton Dickinson).

RNA Analysis. For RNA analysis, 8×10^5 cells were plated in 6-well plates in phenol red-free RPMI containing 5% DCFCS and 2 mM glutamine. The following day the medium was changed and replenished with estradiol or compound F, or with DMSO. Experiments were performed with triplicate cultures and repeated three times. The cells were treated for 22 h, cells were harvested and, RNA extractions were performed as per the manufacturer's protocol (RNA-Bee; Tel-Test Inc). The concentration of RNA was determined by absorbance at 260 nm. The RNA was then subjected to DNase treatment and cDNA was synthesized. TAQMAN quantitative real-time PCR analysis was carried out on the cDNA using reagents from Applied Biosystems. Relative levels of mRNA were calculated using the values obtained for each target gene compared with the value obtained for 18S rRNA (Applied Biosystems). The probes and primers used in the assays are described in Table 1.

Gene Expression Analysis of Human Endothelial Cell Cultures. HUVECs for RNA analysis were plated at 8×10^5 cells in 6 well dishes in phenol red free large-vessel endothelial cell-growth medium supplemented with 2% DCFCS, epidermal growth factor, fibroblast growth factor, heparin, hydrocor-

tisone, and gentamicin. The following day, the medium was changed and supplemented with DMSO, 10 nM GW501516, or 25 nM GW501516 for 22 h. RNA was extracted from these cultures as described above.

Statistical Analysis. Graphpad Prism Version 3 for Macintosh was used for all statistical tests (GraphPad Software, SanDiego, CA). Standard Student *t* tests were used to test differences between samples. One-way ANOVA was used to examine the significance of the dose response in Fig. 2*F* with a Dunnet's post-test to examine the relationship between treatment groups and the control group. Two-way ANOVA was used to test the relationship between doxycycline treatment and GW501516 treatment on the 15-day proliferation of MCF7 Tet-On cells overexpressing PPARδ in Fig. 7*F*.

RESULTS

PPARδ Expression in Cancer Cell Lines. PPARδ mRNA levels were compared between four breast cancer cell lines (MCF7, T47D, MDAMB-231, BT-20), three colon cancer cell lines (HCA-7, HT29, SW480), two prostate cancer cell lines (PC3, DU145), and one immortalized prostate epithelial cell line (PNT1A). The level of PPARδ mRNA varied less than 5-fold among the cancer lines, with most cell lines expressing within a 2-fold range (Fig. 1*A*). Examination of PPARδ protein expression in breast cancer cell lines by Western blot analysis demonstrated that PPARδ protein levels do not totally correlate with mRNA levels (Fig. 1*B*). In particular, PPARδ protein was barely detectable in MDA–MB-231 cells, which displayed similar levels of mRNA to the other cell lines. This observation was confirmed with another antibody raised against the AB domain of PPARδ (data not shown).

PPARδ Agonists Are Pro-Proliferative Agents in Hormone-Responsive Cell Lines. Because all of these cancer cell lines express PPARδ, we proceeded to investigate the effects of compound F, a PPARδ-selective agonist, on cell proliferation in each of these cell lines. Under normal culture conditions (high plating density, 10% FCS) no effects of compound F could be observed in any cell line (data not shown). However, we observed that compound F has the potential to stimulate proliferation on hormone deprivation of low-density cultures. Both T47D and MCF7 human breast cancer cells were growth stimulated by 10 nM compound F (Fig. 2*A*). In contrast, compound F did not have a proliferative effect on MDA-MB-231 or BT-20 cell lines. Importantly, all of these cell lines were growth stimulated by the addition of 10% FCS and only the known estrogen receptor (ER)-positive cell lines responded to added estrogen (data not shown). At the level of plating and serum used in these assays, the untreated T47D cells grew very poorly over a 2-week period; however, growth stimulation by compound F was evident after 3–7 days of culture (Fig. 2*B*). The magnitude of the growth stimulation by compound F represented ∼20–40% of the magnitude of the maximal growth stimulation by estradiol (Fig. 2*C*). The hypothesis that the pro-proliferative responses to compound F represent a PPARδ-specific phenomenon was supported by the observation that an unre-
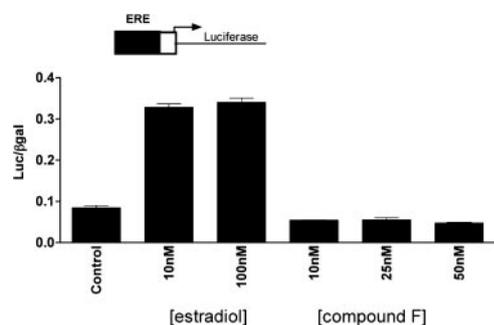

Fig. 4. Compound F cannot activate an estrogen response element (*ERE*). The pERE-Luc reporter plasmid was transfected into T47D cells, and the cells were assessed for a response to 10 nM compound F. *Error bars,* the SE of three replicate cultures. This experiment was performed twice.
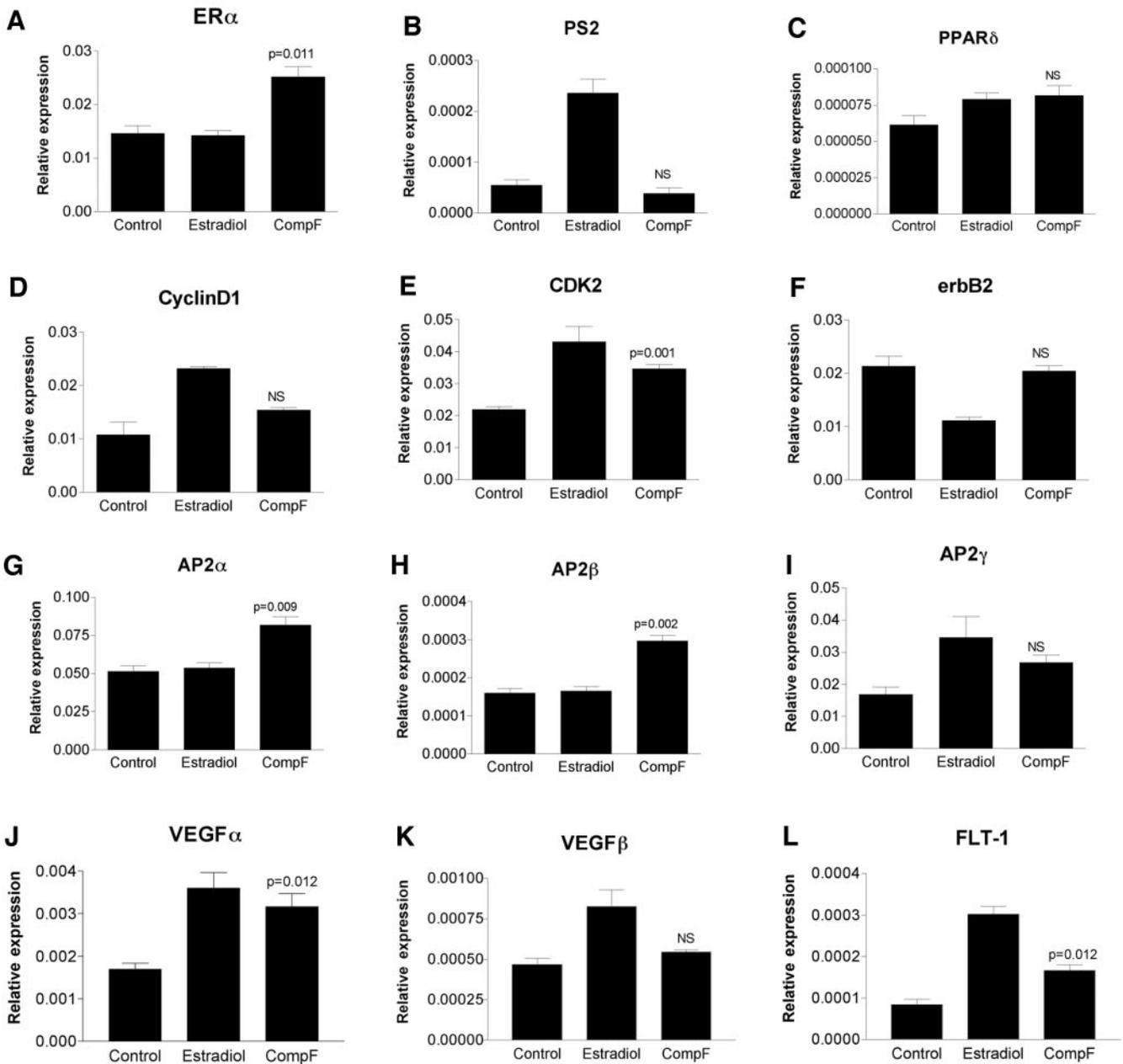
Fig. 5. Compound F modulates distinct growth regulation genes compared with estrogen. T47D cells were treated with DMSO (*Control*), 10 nM estradiol (*Estradiol*), or 10 nM compound F (*CompF*) for 22 h, and RNA was prepared from the cells. The expression of putative target genes was measured using quantitative real-time PCR. *Error bars,* SE of three replicate cultures. The experiment was repeated three times. *ER*, estrogen receptor; *PPAR*, peroxisome proliferator-activated receptor; *VEGF*, vascular endothelial growth factor.

lated, highly selective PPARδ agonist, GW501516 (EC$_{50}$ for PPARδ = 1 nM) stimulated the proliferation of T47D cells to a similar extent as was observed with compound F (Fig. 2*C*).

We also examined the effect of compound F on the proliferation of a number of colon cancer cell lines and did not observe any effects on the proliferation of these cell lines in various experiments in which we modulated both the plating density and the serum concentrations. Shown are the negative results obtained using 5000 cells/well and RPMI containing 5% DCFCS (Fig. 2*D*). Two of the prostate cell lines did, however, respond to compound F, and these cell lines were also the prostate cell lines, known to be responsive to sex hormones (Fig. 2*E*). Indeed, the proliferation of the immortalized normal prostate epithelial (PNT1A) cells was stimulated by compound F, as was LnCaP, which is an androgen-responsive, poorly tumorigenic cancer cell line. This is in contrast to the steroid-insensitive and highly

tumorigenic cell lines, DU145 and PC3, which did not display increased proliferation in the presence of compound F.

The concentrations at which compound F stimulated proliferation were well correlated with the known activity for PPARδ activation (EC$_{50}$ = 2 nM), with a maximal stimulation at 10 nM. This is shown for the PNT1A cell line in Fig. 2*F*. A bell-shaped activity curve was observed with higher concentrations leading to decreased proliferation. Decreased proliferation only occurred at concentrations above 100 nM, at which concentrations the drugs may have PPARδ-independent effects, including the activation of PPARγ (1). An inhibition of growth at high concentrations was observed for all of the responsive cell lines studied (data not shown). In agreement with our studies of the T47D cells, GW501516 also stimulated proliferation of PNT1A cells at 1, 10, and 50 nM consistent with the activation of PPARδ (Fig. 2*G*).

Effect of a PPARδ Ligand on a Synthetic Peroxisome Proliferator Response Element-Driven Luciferase Reporter in Breast Cancer Cell Lines. To further assess the ability of exogenous ligand to activate endogenous PPARδ in MCF7, T47D, and MDA-MB-231 cells, these cell lines were transiently transfected with the reporter construct, pFABPLuc. After transfection, the cells were treated with either vehicle or compound F (10 nM). In the MCF7 and T47D cell lines (Fig. 3, *A* and *B*), compound F significantly increased peroxisome proliferator response element-driven luciferase reporter activity (2-fold). The MDA-MB-231 cells displayed a marginal (1.3-fold), but reproducible, response to compound F (10 nM; Fig. 3*C*). This diminished peroxisome proliferator response element activation supports the observation that the MDA-MB-231 cells express less PPARδ protein (Fig. 1*B*) and do not display a proliferative response to compound F.

Compound F Does Not Activate Transcription from an ERE. To investigate the possibility that compound F could directly activate the ER, T47D human breast cancer cells were transiently transfected with the pERELuc construct (20) and treated with compound F and estradiol. Estradiol increased the transcription from the ERE, whereas compound F did not have any effect (Fig. 4).

Activation of PPARδ Modulates ER Expression Levels, but Is Not Sufficient to Activate ER Signaling. Quantitative real-time PCR analysis of RNA from T47D cells revealed that compound F regulates the expression of ERα. In cells treated with compound F (10 nM) the levels of mRNA encoding ERα increased 1.72-fold ($P = 0.011$; Fig. 5*A*). We then investigated whether compound F could regulate the expression of an archetypical estrogen-regulated gene, PS2. PS2 is regulated by estrogen by direct binding of the ER to EREs in the vicinity of the gene (19). Estradiol increased PS2 expression 4.3-fold ($P = 0.004$), whereas compound F did not have any effect (Fig. 5*B*).

PPARδ Expression Is Not Regulated by Estradiol or Compound F. We, and others have observed that PPARδ gene expression can be induced in different cell lines by a variety of stimuli such as phorbol esters and tumor necrosis factor α (1, 21). We, therefore, examined the effect of estradiol and compound F on levels of PPARδ mRNA. Neither estradiol nor compound F had any effect on PPARδ expression (Fig. 5*C*).

Compound F-Induced Proliferation Is Associated with Altered Expression of Cell Cycle Genes. Because compound F increases proliferation in T47D cells, the expression of selected genes involved in cell cycle was investigated. Estrogen has been shown to up-regulate the transcription of the gene encoding cyclin D1 in human breast cancer cell lines (22–24), and PPARδ agonists have recently been shown to increase Cdk2 protein levels in vascular smooth muscle cells (25). In our experiments, compound F resulted in a 1.57-fold increase in the expression of Cdk2 ($P = 0.001$; Fig. 5*E*) and a slightly greater increase by estradiol (fold induction = 1.925, $P = 0.012$). However, although estradiol clearly increased cyclin D1 expression, compound F did not have any significant effect (Fig. 5*D*).

Compound F Regulates the Expression of Other Transcription Factors. In addition to the regulation of ER, compound F regulates the expression of several non-ligand-activated transcription factors. AP2α and γ have been shown to be up-regulated in breast tumor specimens compared with benign breast epithelia (26). AP2 expression has also been shown to be regulated by the activation of PPARδ during development (27). Treatment of T47D cells with compound F resulted in a 1.58-fold increase of AP2α expression ($P = 0.009$; Fig. 5*G*).

Compound F also increased the expression of AP2β resulting in a 1.85-fold increase ($P = 0.002$; Fig. 5*H*), whereas estradiol did not have any effect. AP2γ expression was increased 1.58-fold by estradiol
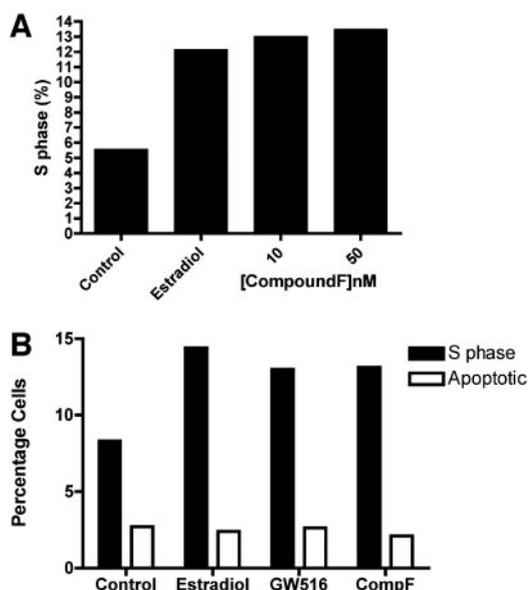

Fig. 6. Compound F and GW501516 increase the number of cells in active cell division. *A*, T47-D cells were grown in 5% dextran charcoal-stripped FCS with solvent alone (*Control*), or estradiol (*Estradiol*, 10 nM), or compound F (*CompoundF*, 10 nM) for 7 days. The cells were then labeled with bromodeoxyuridine and propidium iodide and were subjected to fluorometric cell-sorting analysis. The percentage of cells in S phase is shown. *B*, T47-D cells were grown in 0.1% FCS with solvent alone (*Control*), estradiol (*Estradiol*, 10 nM), GW510516 (*GW516*, 25 nM), or compound F (*CompF*, 10 nM). *Black bars*, the percentage of cells in S phase; *white bars*, the percentage of cells in apoptosis.

($P = 0.035$; Fig. 5*I*), and there was only a marginal increase of AP2γ by compound F ($P = 0.065$). The up-regulation of the gene expression of the AP2 family members prompted the examination of the effects on ErbB2 gene expression. ErbB2 expression has been shown to be regulated by the AP2 family and the ER. However compound F had no effect on ErbB2 expression (Fig. 5*F*), whereas estradiol treatment resulted in a 52% decrease in expression ($P = 0.007$).

Effect on Other Genes That Regulate Growth through Autocrine and Paracrine Pathways. VEGFα has been shown to be regulated by estrogen in breast cancer cells (28). In this study, we have investigated whether PPARδ could regulate the levels of VEGFα mRNA. We observed a 2.11-fold increase in VEGFα mRNA levels ($P = 0.008$) on stimulation by estrogen. Compound F treatment also increased VEGFα mRNA levels (Fig. 5*J*; 1.86-fold; $P = 0.012$). The expression of VEGFβ was also explored. Treatment with estrogen resulted in a 1.76-fold increase ($P = 0.032$) in VEGFβ mRNA levels, whereas compound F exerted no effect (Fig. 5*K*). KDR and FLT1 are the two known receptors for VEGFs. T47D cells expressed FLT1 but not KDR (data not shown), and treatment with compound F resulted in increased expression of FLT1 mRNA (1.96-fold; $P = 0.012$). Treatment with estrogen also increased the levels of FLT1 mRNA (3.5-fold; $P < 0.001$; Fig. 5*L*).

PPARδ Agonists Increase the Number of Cells in the Cell Cycle. The above results suggested that the PPAR agonists were acting as mitogens and may modulate progression through the cell cycle. Fluorometric cell-sorting analysis (FACS) was used to determine the cell cycle status of cell populations. T47-D cells were grown in 5% DCFCS as used in the proliferation experiments shown in Fig. 2. The cells were then labeled with bromodeoxyuridine and propidium iodide and subjected to fluorometric cell-sorting analysis. Both compound F and estradiol increased the proportion of the cells that were observed in S phase to a similar extent (Fig. 6*A*), indicating a stimulation of the cell cycle with these compounds. We also performed the experiment under very low serum conditions (0.1% FCS) as described for the stimulation of the proliferation of HepG2 cells (12). Treatment

Fig. 7. Doxycycline (DOX)-regulated peroxisome proliferator-activated receptor δ (PPARδ) expression increases the proliferative response to GW501516. MCF7 Tet-On human PPARδ (hPPARδ) and MCF7 Tet-On empty vector cells were treated with 2 μg/ml DOX (PLUS DOX) or solvent alone (No DOX) for 48 h. A, the steady-state levels of mRNA encoding PPARδ were measured by quantitative real-time-PCR. B, PPARδ protein levels were visualized by fluorescence-based immunohistochemistry. PPARδ was visualized using FITC (green), and the chromatin was counterstained with 4′,6-diamidino-2-phenylindole (blue). C, MCF7 and MCF7 Tet-On hPPARδ cells were cultured with different concentrations of estradiol. D, MCF7 Tet-On hPPARδ cells and MCF7 Tet-On empty vector cells were grown for 15 days in the presence or absence of DOX and/or GW501516 (GW516, 50 nM). The relative cell proliferation was determined. Est, estradiol. E, the time dependency of this experiment is shown. VA-DMSO, vector alone + vehicle; VA-DOX, vector alone + DOX; VA-GW515+DOX, vector alone +GW501516+DOX; δ-DMSO, PPARδ+vehicle; δ-DOX, PPARδ+DOX; δ-GW516+DOX, PPARδ+GW501516+DOX. F, the effects of GW501516 and DOX were tested by two-way ANOVA, and the resulting Ps are shown. Error bars, the SE of three replicate cultures. The experiment was repeated four times.
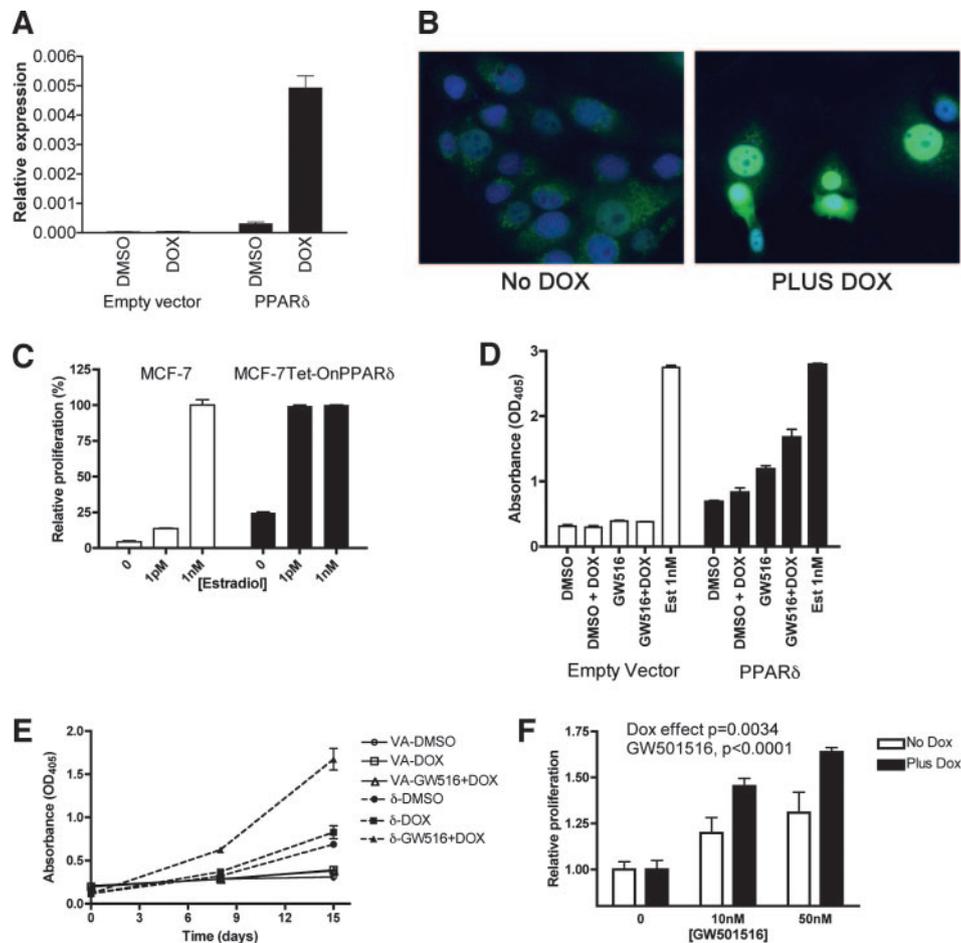
of the cells with estradiol, compound F, and GW501516, all stimulated S phase to a similar extent (Fig. 6B). The levels of sub-G_0 DNA content indicative of apoptosis did not alter under any of the conditions examined.

Regulatable Overexpression of PPARδ in MCF7 Tet-On Cells Increases the Proliferative Effect of PPAR Agonists. To test the role of PPARδ in the proliferative response to the drugs, a MCF7 Tet-On cell line bearing a construct mediating the doxycycline regulation of the PPARδ cDNA was characterized. The regulation of PPARδ at the mRNA level was assessed by quantitative real-time PCR and found to be ~18-fold inducible by doxycycline (Fig. 7A). Doxycycline-dependent PPARδ protein expression was also observed by fluorescence-based immunohistochemistry (Fig. 7B). From the RNA levels it was apparent that PPARδ was overexpressed constitutively in this cell line, albeit at much lower levels than the doxycycline-treated cells. These cells displayed much higher growth rates at lower concentrations of estradiol when compared with the MCF7 cell line. Therefore, proliferation experiments with this cell line had to be carried out in the absence of any estradiol (The proliferation experiments using the MCF7 cells in Fig. 2 all required the presence of 1 pM estradiol). Under completely estradiol-free conditions, neither the MCF7 nor the MCF7 Tet-On empty vector control lines would grow to any appreciable extent (Fig. 7, C–E) and cannot be growth stimulated by GW501516 or doxycycline. However, the PPARδ-overexpressing cell line demonstrated a doxycycline- and GW501516-dependent increase in proliferation under these conditions (Fig. 7, D–F). In the complete absence of estradiol, it was possible to stimulate this cell line with doxycycline and GW501516 to 60% of the growth rate of the cells that have been fully stimulated by 1 nM estradiol. Impor-

tantly, the growth rate of all of the cell lines was stimulated to the same maximal rate in the presence of 1 nM estradiol (Fig. 7, C and D).

PPARδ Activation Also Stimulates VEGF Receptor Expression and Proliferation of Human Endothelial Cells. Regulation of the VEGF pathway by PPARδ agonists invoked a model in which PPARδ agonists may affect both cancer cell proliferation and the concomitant angiogenesis that is required for tumor growth. We, therefore, examined the effect of PPARδ activation on primary cultures of HUVECs. HUVECs that were treated with various concentrations of the PPARδ agonist GW501516 showed a dose-dependent increase in proliferation. The magnitude of the stimulation of proliferation by GW501516 was similar to that observed with the addition of recombinant VEGFα (Fig. 8A). Treatment of HUVECs with GW501516 also resulted in increased expression of both VEGFα and FLT-1 mRNA similar to that observed in the breast cancer cell line (Fig. 8, B and C).

DISCUSSION

In this study, we have provided the first pharmacological evidence for a role of PPARδ in the regulation of proliferation of breast and prostate cancer cells. Our study demonstrates that the highly selective agonist for PPARδ, compound F, can increase proliferation in some prostate and breast cancer cell lines. We have reproduced this observation using a second PPARδ selective agonist, GW501516, to stimulate the growth of T47D and PNT1A cells and have used a conditional expression system to validate PPARδ as the mediator of these effects. Our observation that growth stimulation occurs only under hormone-deprived conditions may be because, under regular growth conditions, i.e., in the presence of serum, the effect of PPARδ agonists may be masked because of the
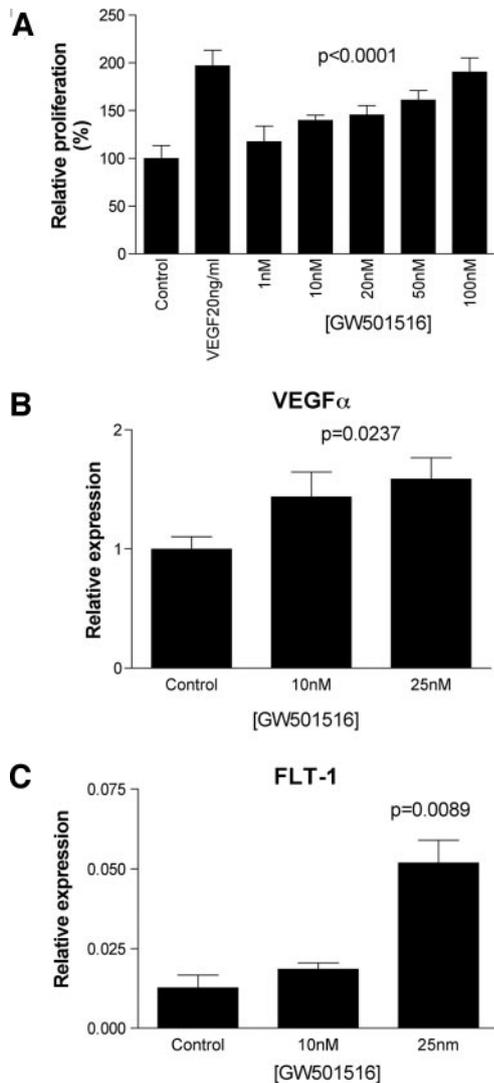
Fig. 8. GW501516 stimulates proliferation of human umbilical vein endothelial cells (HUVECs). *A,* HUVECs were treated with vehicle (Control), 20 ng/ml human recombinant vascular endothelial growth factor (*VEGF*)α-165 (*VEGFα*) or 1, 10, 20, 50, and 100 nM GW501516 for up to 14 days. Shown is the relative proliferation. *B,* peroxisome proliferator-activated receptor δ (PPARδ) activation is associated with increased expression of VEGFα mRNA. Shown here is the relative mRNA levels of VEGFα in response to treatment of HUVECs with 0, 10, and 25 nM GW501516 for 22 h. *C,* PPARδ activation is associated with increased expression of FLT-1 mRNA. Shown is the relative mRNA levels of FLT-1 in response to treatment of HUVEC with 0, 10 and 25 nM GW501516 for 22 h. The dose response was analyzed using one-way ANOVA, with a post-test for trend. The *P* is shown for the post test. The *error bars* represent SE of three triplicate cultures. Each experiment was performed twice.

overriding activation of other signaling pathways (9) or may be caused by the endogenous production of ligands for PPARδ (8, 29). These ligands may include prostacyclin and also other unknown ligands. It has been shown in many previous studies that estrogen can stimulate the synthesis of prostaglandins that may act as potential ligands for PPARs (30–32). This may help explain the finding that we were able to demonstrate the PPARδ effects on growth in the sex hormone-sensitive lines, in which this stimulus can be removed experimentally, but not in colon cancer cell lines, which are devoid of sex-hormonal control of cell growth. Interestingly, it has recently been shown that GW501516 can prevent apoptosis stimulated by complete growth factor withdrawal in the human colorectal cancer cell line HCT116. This response to GW501516 is absent in cells that have had the PPARδ gene deleted (13).

PPARδ activation was associated with increased expression of ERα. This suggested that PPARδ agonists may promote proliferation by acting as a direct or indirect activator of ER signaling.

Further experimentation suggested that this was not the case. First, PPARδ activation by compound F was not associated with increased transcription of the typical estrogen-regulated gene PS2, and, secondly, transient transfection studies demonstrated that compound F cannot transactivate an ERE reporter. This indicates that compound F treatment does not appear to be sufficient for the activation of the ER in these cells. In addition, PPARα has previously been shown to bind directly to the particular ERE used in this study (33); however, PPARδ does not appear to be able to signal through this particular enhancer. Additional studies are in progress to assess the role of PPARδ in regulating cellular responses to estrogens.

Although both estrogen and compound F elicit a growth response, there are some gene targets that are ligand selective and other targets that are promiscuous. Cdk2 represents a common gene target that was increased by both estrogen and compound F. In contrast, selectivity was observed in the regulation of the genes encoding the AP2 family of transcription factors. Expression of the genes encoding AP2α and AP2β was regulated by compound F, whereas estrogen selectively regulated AP2γ. The AP2 family of transcription factors has been reported to regulate the expression of both ERα and ErbB2 in breast cancer (34, 35). Interestingly, in our system PPARδ activation did not have any effect on ErbB2 expression. This could be because the transcription of ErbB2 is under the control of a complex range of transcription factors (34). In contrast, estrogen resulted in down-regulation of ErbB2 in the T47D cells as reported previously (36). The mechanism of the repression of ErbB2 gene expression by estradiol has recently been shown to be due to coactivator competition on a non-ER binding site of the ErbB2 promoter (34).

The observation that PPARδ activation can result in increased expression of VEGFα and its receptor FLT-1, suggests a novel autocrine mode to regulate growth in breast cancer (Fig. 9). This agrees with recent observations that VEGFα gene expression is increased in response to PPARδ agonists in human bladder cancer (37). Regulation of VEGF expression by PPAR ligands has also been observed in macrophages and vascular smooth muscle cells (38, 39). In contrast, the genes encoding FLT-1 and FLK-1 are repressed by PPARγ ligands in endothelial cells (40). Another interesting observation was that PPARδ activation in HUVECs was associated with increased proliferation. Interestingly, the extent to which GW501516 stimulated
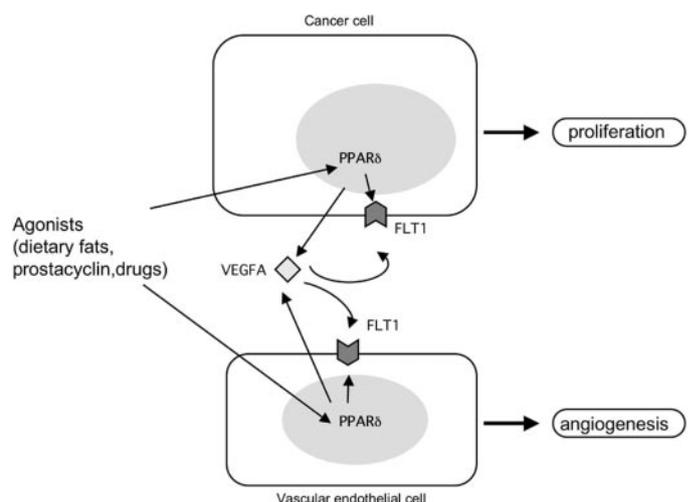

Fig. 9. A proposed model of the action of peroxisome proliferator-activated receptor δ (PPARδ) in tumor growth and angiogenesis. Activation of PPARδ is associated with increased expression of vascular endothelial growth factor α (*VEGFα*) and FLT-1. Locally produced VEGFα can result in the activation of FLT-1 receptor. PPARδ may also have paracrine effects whereby VEGFα that is produced in the cancer cells can bind to FLK1 and FLT-1 receptors expressed on endothelial cells. This effect may be further augmented by the PPARδ-mediated up-regulation of FLT-1 in endothelial cells.

growth was similar to that exerted by VEGFα. Furthermore, PPARδ activation in HUVECs resulted in increased expression of FLT-1, in a manner similar to that observed in T47D cells, which suggests that PPARδ may play a role in tumor angiogenesis. This hypothesis will have to be tested in *in vivo* models of cancer growth.

In summary, this study provides the first evidence that activation of PPARδ by an agonist ligand can result in increased proliferation of breast and prostrate cancer cell lines, as well as endothelial cells, and supports the hypothesis that PPARδ antagonists might be of therapeutic value in the management of common epithelial cancers.

ACKNOWLEDGMENTS

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