Ligand Activation of Peroxisome Proliferator-Activated Receptor-β/δ (PPARβ/δ) Inhibits Cell Proliferation in Human HaCaT Keratinocytes

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Text Pages: 33

Tables: 2

Figures: 12

References: 53

Abstract: 250

Introduction: 857

Discussion: 1403

Nonstandard Abbreviations: 9cRA, 9-cis retinoic acid; ADRP, adipocyte differentiation-related protein; ANOVA, analysis of variance; Angptl4, angiopoietin-like protein 4; Akt, protein kinase B; atRA, all-trans retinoic acid; CYP26A1, cytochrome P450 26A1; DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s Minimal Essential Medium; EMEM, Eagle’s Minimal Essential Medium; EtOH, ethanol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ILK, integrin-linked kinase; PDPK1, 3-phosphoinositide-dependent protein kinase 1; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PBS, phosphate buffered saline; PTEN, phosphatase and tensin homolog deleted on chromosome Ten; PARP, Poly (ADP-ribose) polymerase; PI, propidium iodine; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate; Tgm1, transglutaminase 1; TBST, Tris buffered saline Tween 20;
ABSTRACT

While there is strong evidence that ligand activation of PPARβ/δ induces terminal differentiation and attenuates cell growth, some studies suggest that PPARβ/δ actually enhances cell proliferation. For example, it was recently suggested that retinoic acid (RA) is a ligand for PPARβ/δ and potentiates cell proliferation by activating PPARβ/δ. The present study examined the effect of ligand activation of PPARβ/δ on cell proliferation, cell cycle kinetics and target gene expression in human HaCaT keratinocytes using two highly specific PPARβ/δ ligands (GW0742, GW501516) and RA. Both PPARβ/δ ligands and RA inhibited cell proliferation of HaCaT keratinocytes. GW0742 and GW501516 increased expression of known PPARβ/δ target genes while RA did not; RA increased expression of known RAR/RXR target genes while GW0742 did not affect these genes. GW0742, GW501516 and RA did not modulate expression of 3-phosphoinositide-dependent protein kinase (PDPK1) or alter Akt phosphorylation. GW0742 and RA increased annexin V staining as quantitatively determined by flow cytometry. The effects of GW0742 and RA were also examined in wild-type and PPARβ/δ-null primary mouse keratinocytes to determine the specific role of PPARβ/δ in modulating cell growth. While inhibition of keratinocyte proliferation by GW0742 was PPARβ/δ-dependent, inhibition of cell proliferation by RA occurred in both genotypes. Results from these studies demonstrate that ligand activation of PPARβ/δ inhibits keratinocyte proliferation through PPARβ/δ-dependent mechanisms. In contrast, the observed inhibition of cell proliferation in mouse and human keratinocytes by RA is mediated by PPARβ/δ-independent mechanisms and is inconsistent with the notion that RA potentiates cell proliferation by activating PPARβ/δ.
Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors and members of the nuclear hormone receptor superfamily. There are three PPAR isoforms, PPARα, PPARγ and PPARβ (also referred to as PPARδ and PPARβ/δ), and each regulates tissue-specific target genes involved in many biological processes (Lee et al., 2003; Peraza et al., 2006). For example, PPARα is the molecular target for the fibrate class of hypolipidemic drugs (Peters et al., 2005) and PPARγ is the molecular target of the thiazolidinedione class of insulin sensitizing drugs (Willson et al., 2000). While ligand activation of PPARβ/δ can increase serum HDL cholesterol, increase skeletal muscle fatty acid catabolism and improve insulin sensitivity (Grimaldi, 2007; Lee et al., 2006), considerably less is known about the biological role of PPARβ/δ. In particular, the role of PPARβ/δ in tumorigenesis, apoptosis, and cell proliferation remains controversial. Given the pharmacological potential of PPARβ/δ agonists which have been examined in clinical trials (Pelton, 2006), it is critical to determine the safety of this class of compounds in the appropriate model(s).

A number of independent laboratories have shown that ligand activation of PPARβ/δ can induce terminal differentiation of keratinocytes and epithelium (reviewed in (Burdick et al., 2006; Peters et al., 2008)). Consistent with these findings, many laboratories have also demonstrated that PPARβ/δ inhibits cell growth in epithelium and other cell types, including keratinocytes, colonocytes, cardiomyocytes, lung fibroblasts, and cancer cell lines (reviewed in (Burdick et al., 2006; Peters et al., 2008)). Despite a large body of literature demonstrating the induction of terminal differentiation and inhibition of cell growth that are mediated by PPARβ/δ, there are limited reports...
suggesting that ligand activation of PPARβ/δ can potentiate cell growth. For example, it was originally shown that PPARβ/δ can inhibit expression of phosphatase and tensin homolog deleted on chromosome Ten (PTEN) and increase expression of 3-phosphoinositide-dependent-protein kinase 1 (PDPK1) and integrin-linked kinase (ILK) expression in keratinocytes during wound healing (Di-Poi et al., 2002). The combined effect of this PPARβ/δ-dependent regulation is increased phosphorylation of protein kinase B (Akt) leading to cell survival via inhibition of apoptosis that may be important during wound healing (Di-Poi et al., 2002). Subsequent work by others suggests that the anti-apoptotic signaling mediated by PPARβ/δ during wound healing is also functional in colonic epithelium and human keratinocytes (Gupta et al., 2004; Schug et al., 2007; Wang et al., 2006). However, these changes in the PTEN/PDPK1/Akt pathway are not consistently observed in response to ligand activation of PPARβ/δ in mouse and human keratinocytes, colonic epithelium or human cancer cell lines (Burdick et al., 2007; Hollingshead et al., 2007; Kim et al., 2005a; Marin et al., 2006) and are in direct contrast to the large body of evidence showing that PPARβ/δ induces terminal differentiation and inhibits cell proliferation (reviewed in (Burdick et al., 2006; Peters et al., 2008)).

There are a number of reasons that might explain the differences in the reported effects of PPARβ/δ ligands on cell proliferation and apoptosis, including differences in ligands and/or differences in experimental models. For example, GW501516 and GW0742 are two high affinity ligands for PPARβ/δ (Berger et al., 1999; Sznaidman et al., 2003) that have similar molecular structure but are structurally dissimilar with retinoic acid (RA), which was recently described as a PPARβ/δ ligand (Shaw et al., 2003). Structural differences between the ligands could explain why some investigators
have reported that PPARβ/δ ligand potentiate cell growth while others have reported that PPARβ/δ ligands inhibit cell proliferation. Differences in the approaches used to culture and treat cells and cell lines could also contribute to some of the variability in the literature. For example, studies examining the potential of lipophilic agonists to modulate apoptosis often culture cells in medium without serum or in medium containing a low percentage of charcoal-stripped serum to remove the influence of growth factors or other lipophilic compounds, since these are known to regulate apoptosis. This model system may not be optimal since it is unlikely that endogenous cells typically encounter conditions in the absence of normal serum and/or growth factors. Thus, there is potential for differences in ligands and experimental models to influence the effects of PPARβ/δ ligands on cell proliferation.

It was originally shown that ligand activation of PPARβ/δ induces terminal differentiation and inhibits cell proliferation of human keratinocytes (Burdick et al., 2007), which was consistent with findings from four independent laboratories showing similar effects in mouse keratinocytes (Kim et al., 2005a; Schmuth et al., 2004; Tan et al., 2001; Westergaard et al., 2001). In contrast, others have recently suggested that all-trans retinoic acid (atRA) is a PPARβ/δ ligand and that retinoid-specific activation of PPARβ/δ promotes cell survival of human HaCaT keratinocytes by inducing expression of PDPK1 and anti-apoptotic signaling (Schug et al., 2007; Shaw et al., 2003). It was concluded from these studies that PPARβ/δ-specific activation by RA might explain the pro-proliferative and anti-apoptotic effects of retinoic acid. However, this idea is inconsistent with the well-established role for PPARβ/δ in promoting terminal differentiation. Thus, the present study critically evaluated the effect of two highly
specific PPARβ/δ ligands (GW0742 and GW501516), atRA and 9-cis retinoic acid (9cRA) on gene expression and modulation of cell proliferation in human and mouse keratinocytes.
Materials and Methods

Materials. GW0742 (Sznaidman et al., 2003) was synthesized by GlaxoSmithKline (Research Triangle Park, NC). GW501516 (Sznaidman et al., 2003) was synthesized as previously described (Girroir et al., 2008). ATRA and 9cRA were purchased from Sigma-Aldrich (St. Louis, MO). GW0742 and GW501516 were dissolved in dimethyl sulfoxide (DMSO) and ATRA and 9cRA were dissolved in ethanol (EtOH). Propidium iodine (PI) was purchased from Sigma-Aldrich (St. Louis, MO). The FITC-Annexin V antibody was purchased from Invitrogen (Carlsbad, CA). The Caspase 3/7 Glo reagent was purchased from Promega (Madison, WI).

Cell Culture. HaCaT human keratinocytes were kindly provided from Dr. Stuart Yuspa (NCI). These cells were maintained in Dulbecco’s Minimal Essential Medium (DMEM) with 5% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO₂. Primary mouse keratinocytes from wild-type and PPARβ/δ-null mice were isolated from 2 day old neonates as previously described (Kim et al., 2005a). Keratinocytes were cultured in low calcium (0.05 mM) Eagle’s Minimal Essential Medium (EMEM) with 8% chelexed fetal bovine serum at 37°C and 7% CO₂ (Kim et al., 2005a).

Cell Proliferation Analyses. HaCaT cells were plated on a 12-well plate at a density of 20,000 cells per well 24 hours prior to cell counting at time 0. Cell proliferation was determined using a Z1 Coulter particle counter® (Beckman Coulter, Hialeah, FL). Cells were then serum starved, or not, for 24 hour prior to ligand treatment. After this 24 hour period, cells were maintained in DMEM with or without serum and treated with control (DMSO or EtOH), GW0742, GW501516, ATRA, 9cRA, or combinations for 24, 48, or 72 hours. The concentration of GW0742 and GW501516 used for all experiments
ranged from 0.1 \(\mu M\) to 10.0 \(\mu M\), as these concentrations have been shown to specifically activate PPAR\(\beta/\delta\) (Kim et al., 2005a). The concentration of atRA and 9cRA used for all experiments ranged from 0.1 \(\mu M\) to 1.0 \(\mu M\). Cells were counted every 24 hours. Triplicate samples for each treatment were used for each time point, and each replicate was counted three times. For the mouse primary keratinocyte proliferation assay, equivalent numbers (300,000) of cells from both genotypes were plated in 12-well plates. Two days post seeding, the day 0 plates were removed and counted. The remaining plates were switched to new low calcium media until day 1. Following day 1 counts, the remaining plates were treated with control (DMSO or EtOH), GW0742, atRA, or 9cRA for 24, 48, and 72 hours in low calcium media. The concentration of agonists examined was either 0.1 \(\mu M\) or 1.0 \(\mu M\). Triplicate samples for each treatment were used for each time point, and replicates were counted three times.

**Western Blot Analyses.** HaCaT cells were cultured on 60-mm culture dishes. Cells were serum starved for 24 hours, or not, prior to ligand treatment. After this time, cells were maintained in DMEM with (5%) or without serum and treated with control (DMSO or EtOH), GW0742, GW501516, atRA, or 9cRA for 12 hours. After 12 hours of exposure, protein was isolated using a lysis buffer containing phosphatase and protease inhibitors (20 mM Tris/150 mM NaCl/1 mM EGTA/1 mM EDTA/1 mM \(\beta\)-glycerophosphate/2 mM sodium pyrophosphate/1% Triton X). For analyzing the expression of retinoic acid receptors (RARs), HaCaT cells and primary keratinocytes from wild-type and PPAR\(\beta/\delta\)-null mice were cultured on 100-mm culture dishes in triplicate. Soluble protein was isolated from confluent plates using MENG buffer containing 500 mM NaCl, 1% NP-40, and protease inhibitors.
Protein samples were isolated from control- and ligand-treated cells as described above. A total of 25 µg of protein per sample was resolved using sodium dodecyl sulfate (SDS)-polyacrylamide gels. The samples were transferred onto polyvinylidene fluoride membrane using an electroblotting method. The membranes were blocked with 5% dried milk in Tris Buffered Saline Tween 20 (TBST) and incubated at 4°C overnight with primary antibodies. Following incubation with biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), immunoreactive proteins on the membrane were detected after incubation with \(^{125}\text{I}\)-labeled streptavidin (Amersham Biosciences, Piscataway, NJ). Hybridization signals for specific proteins were normalized to the hybridization signal of the housekeeping gene lactate dehydrogenase (LDH) or \(\beta\)-actin. Independent triplicate samples were analyzed for each treatment group. The following antibodies were used: anti-Akt (Cell Signaling Technology, Beverly, MA), anti-phospho-Akt (Cell Signaling Technology, Beverly, MA), PARP (Cell Signaling Technology, Beverly, MA), and anti-LDH (Rockland, Gilbertsville, PA), RAR\(\alpha\), RAR\(\beta\), RAR\(\gamma\) and RXR\(\alpha\) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The cleavage ratio of PARP was determined by the average ratio of normalized cleaved PARP to normalized uncleaved PARP.

**Real-time PCR.** HaCaT cells were cultured on 6-well plates. Cells were serum starved for 24 hours, or not, prior to ligand treatment. After this time, cells were maintained in DMEM with or without serum and treated with control (DMSO or EtOH), GW0742, GW501516 (4, 8, or 24 hours), or atRA, 9cRA (8 or 24 hours). For isolation of mRNA from primary keratinocytes, a similar protocol was utilized. Keratinocytes were cultured to 90-95% confluence before treatment with control (DMSO or EtOH),
GW0742, atRA, 9cRA for 8 or 24 hours. Total RNA was isolated from cells using TRIzol reagent and the manufacturer’s recommended protocol. The mRNA encoding PPARβ/δ, adipose differentiation-related protein (ADRP), angiopoietin-like protein 4 (Angptl4), 3-phosphoinositide-dependent-protein kinase 1 (PDPK1), transglutaminase 1 (Tgm1), cytochrome P450 26A1 (CYP26A1), small proline-rich protein 1A (SPR1A) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured by quantitative real-time polymerase chain reaction (qPCR) analysis. cDNA was generated from 2.5 µg total RNA using MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Real-time PCR primers for the above genes were designed using Integrated DNA Technologies (Coralville, IA) SciTools. The qPCR analysis was carried out using SYBR Green PCR master mix (Finnzymes, Espoo, Finland) in the iCycler and detected using the MyiQ Realtime PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The following PCR reaction was used for all genes: 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, and repeated for 45 cycles. Each PCR reaction included a no template control reaction to control for contamination, and all real-time PCR reactions had greater than 85% efficiency. The relative mRNA value for each gene was normalized to the relative mRNA value for the housekeeping gene GAPDH. Statistical analysis of GAPDH expression for all treatment groups revealed no significant differences in expression allowing for normalization to this gene product (data not shown).

**Flow Cytometry.** HaCaT cells were plated on a 6-well plate at a density of 75,000 (with serum) or 250,000 (without serum) cells per well. Cells were then serum starved, or not, 24 hour prior to ligand treatment. After this 24 hour period, cells were maintained in DMEM with or without serum and treated with control (DMSO or EtOH),
GW0742, GW501516, atRA, 9cRA, or combinations for 24 and 48 hours (without serum) or 48 and 72 hours (with serum) with daily renewal of treatment. Independent triplicate samples for each treatment were used for each time point.

For Annexin V analysis of apoptosis, cells were trypsinized, washed in cold-phosphate buffered saline (PBS), and pelleted. The cells were then re-suspended in 100 µL of annexin V buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and 5 µL of FITC-annexin V antibody was incubated at room temperature for 15 minutes. Ice-cold annexin V buffer (450 µL) was added to the cells with 2 µg propidium iodine, and the cells were analyzed by flow cytometry. Approximately 10,000 cells/sample were analyzed using an EPICS-XL-MCL flow cytometer (Beckman Coulter Electronics, Hialeah, FL) fitted with a single 15 mW argon ion laser providing excitation at 488 nm. Cells stained with FITC were monitored through a 525-nm bandpass filter. Early apoptosis was defined as the percentage of cells that were annexin V positive and propidium iodide negative and late apoptosis/necrosis was defined as the percentage of cells that were annexin V negative and propidium iodide positive.

**Caspase 3/7 activity.** HaCaT keratinocytes were cultured as described above, with and without culture medium serum for up to 72 hours in the presence or absence of either GW0742 or retinoic acid. Caspase 3/7 activity was measured using the Caspase 3/7 Glo reagent using the manufacturer's recommended procedures. As a positive control, HaCaT keratinocytes were irradiated with 20,000 µJ/cm² UV light using the CL-1000 Ultra Violet Crosslinker (UVP, Upland, CA) and examined 12 hours post-irradiation. Activity was normalized to protein content. Five independent samples per treatment group were examined.
Statistical Analysis. All analyses were made using either a one-way (Western blots) or two-way (proliferation, mRNA, and flow cytometry) analysis of variance (ANOVA) with Bonferroni’s multiple comparison test as mentioned in the figure legends. All results are reported as mean ± S.E.M.
Results

Activation of PPARβ/δ by Specific Ligands Inhibits Cell Proliferation of HaCaT Keratinocytes. To examine the effect of synthetic PPARβ/δ ligands on cell growth, HaCaT keratinocyte cell proliferation was quantified in the presence of either GW501516 or GW0742, with or without serum withdrawal. In the presence of culture medium with serum, inhibition of HaCaT cell proliferation was observed with 1.0 and 10 µM GW0742 (Fig. 1A). GW501516 did not influence cell growth of HaCaT cells in the presence of serum in the culture medium (Fig. 1B). Since growth factors and/or potential PPAR ligands present in serum could prevent detection of significant changes in cell proliferation, these experiments were also performed in the absence of culture medium serum. When HaCaT cells were cultured in the absence of serum, both GW0742 and GW501516 inhibited cell growth (Figs. 1C-D). These data do not distinguish between inhibition of cell cycle progression and cell death, but subsequent analysis examined these ideas.

Activation of PPARβ/δ by Specific Ligands Increases Expression of ADRP and Angptl4 but not PDPK1. To verify that the inhibition of proliferation by GW0742 and GW501516 (Fig. 1) is associated with specific ligand activation of PPARβ/δ, expression of known and putative PPARβ/δ-dependent target genes was examined. The known PPARβ/δ-dependent target genes ADRP and Angptl4 were induced by GW0742 and GW501516 in a dose-dependent manner that was independent of culture medium serum (Figs. 2A-D). Since increased expression of ADRP is a marker of keratinocyte differentiation (Burdick et al., 2007; Kim et al., 2005a; Schmuth et al., 2004;
Westergaard et al., 2001), expression of another mRNA marker of differentiation was also examined. Indeed, expression of SPR1A was increased by ligand activation of GW0742 (Supplemental Fig. 1), consistent with previous work demonstrating that ligand activation of PPARβ/δ induces terminal differentiation of keratinocytes (Burdick et al., 2007; Kim et al., 2005a; Schmuth et al., 2004; Tan et al., 2001; Westergaard et al., 2001). In contrast, expression of the putative PPARβ/δ target gene PDPK1 was not altered by either PPARβ/δ ligand at either timepoint, in the presence or absence of serum (Figs. 2E-F). PDPK1 was examined because others have recently suggested that activating PPARβ/δ in keratinocytes causes increased expression of this mRNA (Schug et al., 2007). These data demonstrate that HaCaT keratinocytes are responsive to PPARβ/δ ligands, as shown by the induction of two known PPARβ/δ-dependent target genes within 4 hours of treatment. These data also suggest that PDPK1 is not a target of PPARβ/δ in human HaCaT keratinocytes, consistent with past studies (Burdick et al., 2007; Kim et al., 2005a; Marin et al., 2006).

**Activation of PPARβ/δ by Specific Ligands Does Not Lead to Phosphorylation of Akt or Alter PARP Cleavage.** Quantitative western blotting was performed using protein from HaCaT cells treated with GW0742 and GW501516 for 12 hours in the presence or absence of culture medium serum. This timepoint was examined because recent work by others suggested that ligand activation of PPARβ/δ leads to increased phosphorylation of Akt in HaCaT cells after 12 hours (Schug et al., 2007). Similarly, since phosphorylated Akt is known to cause anti-apoptotic activity, PARP cleavage was examined to determine if ligand activation of PPARβ/δ would modulate this marker of apoptosis, with particular interest in potential changes that
might occur following serum-withdrawal when increased PARP cleavage should occur. No change in the expression of Akt protein and no evidence of altered Akt phosphorylation were observed in response to ligand activation of PPARβ/δ in the presence or absence of culture medium serum (Figs. 3A-D). An increase in the average ratio of cleaved to uncleaved PARP was only observed in serum-deprived HaCaT keratinocytes compared to cells cultured in the presence of serum (Figs. 3C-D versus Figs. 3A-B). Neither PPARβ/δ ligand had any effect on PARP cleavage at any concentration, in the presence of absence of culture medium serum.

**Ligand Activation of PPARβ/δ Increases Annexin V Staining and Caspase 3/7 Activity.** The observed decrease in cell proliferation (Fig. 1) could be due to inhibition of cell cycle and/or modulation of apoptosis. Flow cyometric analysis using BrdU did not reveal significant differences in cell cycle progression (data not shown). Thus, flow cytometric analysis was performed to determine if the observed decreases in cell proliferation by ligand activation of PPARβ/δ (Fig. 1) was due to modulation of apoptosis. The timing of this analysis corresponded to the time points just preceding and including the timepoint when a significant decrease in cell proliferation was observed (e.g. 48 – 72 hours post-ligand treatment in the presence of serum (Fig. 1A) and 24 – 48 hours post-ligand treatment in the absence of serum (Fig. 1C). A dose-dependent increase in the percentage of cells undergoing early apoptosis (annexin V positive/propidium iodide negative) was observed 48 hours post-ligand treatment in the presence of serum (Table 1-A), but these changes were not observed 72 hours post-ligand treatment (Table 1-B). In the absence of culture medium serum, an increase in cells undergoing early apoptosis was observed 48 hours post-ligand treatment in cells
exposed to 10 µM GW0742 (Table 1-D). No changes in the percentage of cells undergoing late apoptosis (annexin V negative/propidium iodide positive) were observed for any treatment group. Consistent with the observed changes in annexin V staining, increased caspase 3/7 activity was also found following ligand activation of PPARβ/δ by GW0742 in HaCaT keratinocytes (Supplemental Fig. 2).

**Retinoic Acid Inhibits HaCaT Cell Proliferation.** To examine the effect of retinoic acid on cell growth, HaCaT keratinocyte proliferation was quantified in the presence of either atRA or 9cRA, with or without culture medium serum withdrawal. atRA and 9cRA inhibited HaCaT cell proliferation in the presence of culture medium serum. Cells were more sensitive to 9cRA than atRA as inhibition of cell growth occurred at a lower concentration (0.1 µM) (Figs. 4A-B). In the absence of culture medium serum, both 0.1 and 1.0 µM concentrations of atRA and 9cRA inhibited HaCaT cell proliferation with similar efficacy.

**Retinoic Acid Regulates RAR-dependent Target Genes, but Does Not Regulate PPARβ/δ-dependent Target Genes.** Expression of PPARβ/δ-dependent target genes and retinoic acid receptor (RAR)-dependent target genes was examined following exposure to retinoic acid. atRA and 9cRA did not increase expression of mRNA encoding the well-characterized PPARβ/δ-dependent target Angptl4 after either eight or twenty-four hours of treatment (Fig. 5A). In contrast, a marked increase in the expression of Angptl4 mRNA was found in response to 0.2 µM GW0742 after 8 and 24 hours of culture (Fig. 5A). Both atRA and 9cRA modulated expression of known RAR-dependent target genes; CYP26A1 was induced and transglutaminase 1 was repressed (Figs. 5B-C). These changes were not observed following exposure to the PPARβ/δ
ligand GW0742. Neither atRA, 9cRA nor GW0742 influenced expression of mRNA encoding PDPK1 (Fig. 5D).

**Retinoic Acid Does Not Lead to Phosphorylation of Akt or Alter PARP Cleavage.** Quantitative western blotting was performed on protein samples from HaCaT cells to determine if retinoic acid can modulate phosphorylation of Akt and/or PARP cleavage as markers of apoptotic signaling. No change in the expression or phosphorylation of Akt was found in response to either atRA or 9cRA, in the presence of absence of culture medium serum (Fig. 6). Similarly, no change in PARP cleavage was observed in response to retinoic acid in the presence or absence of culture medium serum (Fig. 6). The only significant change in PARP cleavage was observed in serum-deprived cells as compared to cells cultured in the presence of serum (Fig. 6).

**Retinoic Acid Increases Annexin V Staining and Caspase Activity.** Flow cytometric analysis was performed to determine if the observed decreases in cell proliferation by retinoic acid (Fig. 4) was due to modulation of apoptosis. The timing of this analysis corresponded to the time points just preceding and including the timepoint when a significant decrease in cell proliferation was observed (e.g. 48 – 72 hours post-retinoic acid treatment in the presence of serum (Figs. 4A-B) and 24 – 48 hours post-retinoic acid treatment in the absence of serum (Figs. 4C-D). In the presence of culture medium serum, atRA and 9cRA significantly increased the percentage of cells undergoing early apoptosis 48 hours post-retinoic acid treatment (Table 2). The percentage of cells undergoing late apoptosis was also significantly increased by atRA and 9cRA 48 and 72 hours post-retinoic acid treatment (Table 2). In the absence of culture medium serum, no significant changes in the percentage of cells undergoing
early or late apoptosis was observed at either timepoint. Interestingly, the percentage of cells undergoing apoptosis was higher in retinoic acid treated cells (Table 2) as compared to GW0742 treated cells (Table 1). Consistent with the observed changes in annexin V staining, increased caspase 3/7 activity was also found following exposure to retinoic acid in HaCaT keratinocytes (Supplemental Fig. 2).

**GW0742 and Retinoic Acid Decrease Mouse Primary Keratinocyte Cell Proliferation.** Primary mouse keratinocytes from wild-type and PPARβ/δ-null mice were used to assess the specific role of PPARβ/δ in modulating cell growth. Keratinocytes from PPARβ/δ-null mice proliferated much faster as compared to wild-type keratinocytes, consistent with previous studies (Kim et al., 2005a). Inhibition of cell proliferation was observed in wild-type mouse keratinocytes following exposure to GW0742 and this effect was not found in similarly treated PPARβ/δ-null keratinocytes (Fig. 7A). In contrast, atRA and 9cRA inhibited cell proliferation in both wild-type and PPARβ/δ-null keratinocytes (Figs. 7B-C).

**Retinoic Acid Increases Expression of a RAR-dependent Target Gene but Does Not Increase Expression of a PPARβ/δ Target Gene in Mouse Primary Keratinocytes.** Primary keratinocytes from wild-type and PPARβ/δ-null mice were used to examine changes in gene expression of RAR- and PPARβ/δ-dependent target genes. At a concentration that specifically activates PPARβ/δ (0.2 µM), GW0742 increased expression of Angptl4 mRNA in wild-type keratinocytes at both time points examined, and this increase was not observed in similarly treated PPARβ/δ-null keratinocytes (Figs. 8A-B). atRA did not increase Angptl4 mRNA expression (Figs. 8A-B), but 9cRA did cause an increase after 24 hours of treatment (Figs. 8A-B). This is of interest.
because the increase in Angptl4 mRNA expression did not occur in PPARβ/δ-null keratinocytes. This is consistent with previous work showing that 9cRA can activate PPAR/RXR heterodimers and increase expression of PPAR target genes (Mukherjee et al., 1997). AtRA and 9cRA both increased expression of the RAR-dependent target gene CYP26A1 in wild-type and PPARβ/δ-null keratinocytes, while GW0742 had no effect on CYP26A1 mRNA in either genotype (Figs. 8C-D).

**HaCaT and Mouse Primary Keratinocytes Differentially Expression RAR Isoforms.** To confirm that retinoic acid receptors (RARα, RARβ, RARγ and RXRα) are expressed in HaCaT keratinocytes and primary keratinocytes, quantitative western blotting was performed on soluble cellular lysates from HaCaT cells and wild-type and PPARβ/δ-null primary keratinocytes. Expression of all three RAR isoforms was detected in HaCaT keratinocytes; however, RARγ was only expressed in primary keratinocytes (Fig. 9). The expression of RXRα, the heterodimerization partner of PPARβ/δ and RARs was highly expressed in both HaCaT and primary mouse keratinocytes.

**Co-treatment of GW0742 and Retinoic Acid Decreases HaCaT Cell Proliferation and Independently Regulate Receptor-Specific Target Genes.** If retinoids and PPARβ/δ ligands were functioning to promote anti-apoptotic signaling, then combining the two ligands might allow us to observe this effect that we were not observing with only the ligand. Towards this goal, both cell proliferation and markers of gene expression were examining using combinations of ligands using concentrations that are known to specifically activate the respective receptor. As noted above, others have suggested that ligand activation of PPARβ/δ will lead to increased expression of PDPK1 and subsequent anti-apoptotic activity (Schug et al., 2007). To begin to examine
if inhibition of cell proliferation by retinoic acid and GW0742 could lead to additive or synergistic effects, HaCaT cell proliferation was examined following co-treatment with retinoic acid and GW0742. Similar to results described above, atRA and 9cRA significantly inhibited HaCaT cell proliferation (Fig. 10A-C). GW0742 did not inhibit cell proliferation, but this concentration (0.2 µM) was used because it specifically activates PPARβ/δ without inhibiting cell growth (Figs. 1A and 5A). Co-treatment of atRA or 9cRA with 0.2 µM GW0742 did not lead to enhanced inhibition of cell proliferation as compared to inhibition observed with atRA or 9cRA alone, in the presence or absence of culture medium serum (Fig. 10). However, combining atRA with 9cRA caused a significantly greater inhibition of cell proliferation in the absence of culture medium serum as compared to inhibition observed with atRA or 9cRA alone (Fig. 10).

Ligand activation of PPARβ/δ caused an increase in the expression of mRNA encoding Angptl4 in HaCaT cells, while atRA and 9cRA had no effect on this PPARβ/δ target gene (Fig. 11A-B). Combining atRA or 9cRA with GW0742 did not consistently alter induction of Angptl4, but a modest enhancement was observed after 8 hours of treatment with atRA and GW0742 (Fig. 11A). Increased expression of CYP26A1 mRNA was observed in atRA and 9cRA treated HaCaT cells, but this effect was not consistently altered by co-treatment with GW0742 (Fig. 11C-D). Expression of mRNA encoding PDPK1 was not altered by atRA, 9cRA or GW0742 (Fig. 11E-F). No consistent changes in PDPK1 mRNA were observed following co-treatment with either atRA or 9cRA with GW0742, but a decrease in PDPK1 mRNA was found following co-treatment of GW0742 and 9cRA or co-treatment of atRA and 9cRA (Fig. 11E-F).
Discussion

Results from the present study clearly indicate that ligand activation of PPARβ/δ inhibits cell proliferation in human HaCaT keratinocytes (Fig. 12). This observation is consistent with previous work showing PPARβ/δ-dependent inhibition of cell proliferation in keratinocytes (Burdick et al., 2007; Kim et al., 2004; Kim et al., 2005a; Kim et al., 2005b; Man et al., 2008; Michalik et al., 2001; Peters et al., 2000; Westergaard et al., 2001) and many other cell types (reviewed in (Burdick et al., 2006; Peters et al., 2008)). Since the observed inhibition of cell proliferation by ligand activation of PPARβ/δ is not found in mouse keratinocytes that do not express PPARβ/δ, this demonstrates that this effect requires a functional receptor. The specific mechanism(s) that lead to inhibition of cell proliferation in human HaCaT keratinocytes cannot be determined from the present studies. However, since inhibition of cell proliferation is typically associated with terminal differentiation, it is important to note that increased expression of known differentiation markers (e.g. ADRP and SPR1A) was observed in the present study and that PPARβ/δ has been linked with modulation of terminal differentiation in keratinocytes (Burdick et al., 2007; Kim et al., 2005a; Man et al., 2008; Matsuura et al., 1999; Schmuth et al., 2004; Tan et al., 2001) and other cell types including intestinal epithelium (reviewed in (Burdick et al., 2006; Peters et al., 2008)). Interestingly, the induction of terminal differentiation of keratinocytes is associated with increased activity of pro-apoptotic-like signaling (Weil et al., 1999). Thus, the increase in annexin V-positive cells and caspase 3/7 activity found in response to ligand activation of PPARβ/δ is also consistent with the idea that PPARβ/δ mediates terminal differentiation, and might explain in part the decreased cell proliferation.
proliferation observed following activation of PPARβ/δ in HaCaT keratinocytes. Given that HaCaT keratinocytes are relatively resistant to the induction of apoptosis (Henseleit et al., 1996), the observed increase in apoptosis with ligand activation of PPARβ/δ illustrates a unique function of PPARβ/δ in this cell type.

Previous studies by others suggested that ligand activation of PPARβ/δ in keratinocytes promotes cell survival by modulating PTEN/PDK1/ILK/Akt activity leading to anti-apoptotic signaling (Di-Poi et al., 2002). However, this signaling appears to be context-specific since changes in these signaling proteins may occur in keratinocytes during wound healing but are clearly not found in normal mouse or human keratinocytes, based on results reported from the present study and from previous work (Burdick et al., 2007; Kim et al., 2005a). This is also supported by the lack of changes in the PTEN/PDK1/ILK/Akt expression and/or activity following ligand activation of PPARβ/δ in colon and human colon cancer cell lines (Hollingshead et al., 2007; Marin et al., 2006). Collectively, earlier work and results from the present study strongly support the idea that ligand activation of PPARβ/δ inhibits cell proliferation by inducing terminal differentiation and apoptotic signaling. Further, these findings do not support the hypothesis that PPARβ/δ promotes cell survival of keratinocytes by modulating PTEN/PDK1/ILK/Akt activity leading to anti-apoptotic signaling as suggested by others (Di-Poi et al., 2002).

Since recent evidence suggests that retinoic acid is a ligand for PPARβ/δ (Shaw et al., 2003) the effect of retinoic acid on HaCaT cell proliferation was also examined. Results from the present study provide convincing evidence that atRA and 9cRA inhibit cell proliferation of both human HaCaT keratinocytes and mouse primary keratinocytes,
and that this effect is associated with an increase in apoptosis. Interestingly, the relative percentage of cells undergoing apoptosis in response to atRA and 9cRA is significantly greater than the percentage of cells undergoing apoptosis in response to a potent PPARβ/δ ligand. These observations are consistent with inhibition of cell proliferation found in HaCaT keratinocytes, other human keratinocyte cell lines, and various human cancers following administration of retinoic acid (Chen et al., 2000; Hansen et al., 2000; Kanekura et al., 2000; Klaassen et al., 2001; Memezawa et al., 2007). Retinoic acid also inhibits cell proliferation in mouse primary keratinocytes, which is consistent with previous studies (Tong et al., 1988) and with inhibition of skin cancer by retinoids observed in several mouse models (Chen et al., 1994a; Chen et al., 1994b; Tennenbaum et al., 1998; Verma, 1987; Verma, 1988; Verma et al., 1980; Xu et al., 2006). It is also worth noting that loss of RAR isoforms has been shown to enhance tumorigenesis (Chen et al., 2004; Darwiche et al., 1995; Darwiche et al., 1996).

Combined, results from the present studies demonstrate that retinoic acid inhibits cell proliferation in mouse primary keratinocytes and human HaCaT keratinocytes (Fig. 12).

In contrast with results from the present study and other published reports, it was recently suggested that retinoic acid acts as a PPARβ/δ ligand and promotes cell survival and increased cell growth of HaCaT keratinocytes (Schug et al., 2007). This was an attractive hypothesis to potentially explain the known differential effects of retinoic acid reported in the literature showing that retinoic acid inhibits cell proliferation in some models but increases cell proliferation in other models. However, the former analysis was limited in scope and only examined expression of an mRNA encoding a putative PPARβ/δ target gene (e.g. PDPK1) and did not critically evaluate cell
proliferation and apoptosis in HaCaT keratinocytes as performed in the present analysis. Given the significant weight of evidence from multiple laboratories demonstrating PPARβ/δ-dependent inhibition of cell proliferation in keratinocytes (Burdick et al., 2007; Kim et al., 2004; Kim et al., 2005a; Kim et al., 2005b; Man et al., 2008; Martinasso et al., 2006; Westergaard et al., 2001) and many other cell types (reviewed in (Burdick et al., 2006; Peters et al., 2008)), it is surprising that the studies by Schug et al did not address this inconsistency in their work. Indeed, the present study in which cell proliferation was examined with quantitative measures under several culture conditions revealed multiple inconsistencies with the hypothesis that ligand activation of PPARβ/δ by retinoic acid promotes cell survival. For example, increased expression of PDPK1 is not found in HaCaT keratinocytes cultured in the presence of retinoic acid, despite demonstration of increased expression of known RA-responsive genes (e.g. CYP26A1). Both atRA and 9cRA also failed to increase expression of known PPARβ/δ target genes in HaCaT keratinocytes, while PPARβ/δ ligands did activate expression of ADRP and Angptl4. More importantly, retinoic acid did not alter phosphorylation of Akt, inhibit serum withdrawal-induced cleavage of PARP or reduce annexin V-positive cells, and notably, there was no increase in cell proliferation. These observations demonstrate that retinoic acid does not potentiate cell proliferation of HaCaT keratinocytes. Since combining ligand activation did not counteract the growth inhibitory effects of retinoic acid in HaCaT keratinocytes, this provides more indirect support that retinoic acid does not function differentially through both PPARβ/δ and RAR/RXRs. Additionally, atRA and 9cRA inhibited cell proliferation in both wild-type and PPARβ/δ-null mouse primary keratinocytes. This demonstrates that retinoic acid inhibits
cell proliferation, and that the mechanisms underlying this inhibition do not require PPARβ/δ. These results are not surprising given recent studies demonstrating that atRA does not bind to or activate PPARβ/δ, and that atRA does not cause PPARβ/δ to interact with a co-activator peptide in a time resolved fluorescence resonance energy transfer assay (Rieck et al., 2008). Collectively, these findings are in contrast to studies previously reported by others (Schug et al., 2007). These results demonstrate that retinoic acid modulates HaCaT keratinocyte cell proliferation by increasing apoptosis thereby inhibiting growth, but provide no evidence that retinoic acid potentiates cell proliferation by activating PPARβ/δ as previously suggested (Schug et al., 2007).

In summary, the present findings provide additional observations to the increasing body of evidence demonstrating that ligand activation of PPARβ/δ inhibits cell proliferation. This conclusion is based on comprehensive analysis using two high affinity ligands and quantitative measures of cell proliferation, differentiation and apoptosis. It is of interest to note that inhibition of keratinocyte proliferation by PPARα and PPARγ agonists have also been observed (Demerjian et al., 2006; Hanley et al., 1998), suggesting that there may be come redundancy in the target genes modulated by PPARs in keratinocytes that mediate this effect. Results from the present study also clearly demonstrate that retinoic acid inhibits proliferation of mouse and human keratinocytes, but does not activate PPARβ/δ. These findings also strongly suggest that the mechanisms underlying the differential effects of retinoids on cell proliferation are not mediated by PPARβ/δ. Further studies will be necessary to determine how retinoids can increase cell growth in some models and inhibit cell growth in others.
Acknowledgements. The authors gratefully acknowledge Drs. Andrew N. Billin and Timothy M. Willson for providing GW0742, and Dr. Pengfei He and the Penn State University Flow Cytometry Core Facility (Elaine Kunze, Susan Magargee, and Nicole Bem) for their technical support with flow cytometry and data analysis.
References


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Figure Legends:

**Fig. 1.** Ligand activation of PPARβ/δ inhibits cell proliferation of HaCaT keratinocytes. HaCaT cells were treated with either GW0742 (A,C) or GW501516 (B,D) with the indicated concentration of ligand (arrow), in the presence (A,B) or absence (C,D) of culture medium with serum and cell number was quantified as described in *Materials and Methods*. Values represent the mean ± SEM. *significantly different values (P < 0.05) from vehicle (DMSO) at the particular time point, as determined by ANOVA and Bonferroni’s multiple comparison test.

**Fig. 2.** Modulation of gene expression by ligand activation of PPARβ/δ in HaCaT keratinocytes. HaCaT cells were treated for either 4 or 8 hours with the indicated concentration of GW0742 (A,C,E) or GW501516 (B,D,F) in the presence or absence of serum. Quantitative real-time PCR was performed as described in *Materials and Methods* to examine the expression of mRNA encoding (A,B) adipose differentiation-related protein (ADRP), (C,D) angiopoietin-like protein 4 (Angptl4), and (E,F) 3-phosphoinositide-dependent-protein kinase 1 (PDPK1) normalized to mRNA encoding glyceraldehyde dehydrogenase (GAPDH). Values are the average fold change compared to control treatment and represent the mean ± SEM. Values with different letters are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test.
Fig. 3. Phosphorylation of Akt and PARP cleavage are not influenced by ligand activation of PPARβ/δ in HaCaT cells. HaCaT cells were treated for 12 hours with either GW0742 (A,C) or GW501516 (B,D) with the indicated concentration of ligand in the presence (A,B) or absence (C,D) of serum as described in Materials and Methods to examine the quantitative expression of phosphorylated Akt and PARP cleavage. Values are the average fold change compared to control treatment and represent the mean ± SEM. Values with different letters are significantly different, P < 0.05, as determined by ANOVA and Bonferroni's multiple comparison test. The cleavage ratio is an indicator of apoptosis and is the average ratio of cleaved PARP to uncleaved PARP normalized values. (E) Western blot analysis demonstrating specificity of the phospho-Akt antibody. Rat cerebrum lysate (+) was used as a positive control and representative samples from HaCaT cells treated with either GW0742, atRA or 9cRA were used as comparison. Phospho-Akt has a molecular weight of 60 kDa.

Fig. 4. Effect of retinoic acid on HaCaT cell proliferation. HaCaT cells were treated with either atRA (A,C) or 9cRA (B,D) with the indicated concentration (arrow) in the presence (A,B) or absence (C,D) of culture medium serum, and cell number was quantified as described in Materials and Methods. Values represent the mean ± SEM. *significantly different values (P < 0.05) from vehicle, as determined by ANOVA and Bonferroni's multiple comparison test.

Fig. 5. Effect of retinoic acid on gene expression in HaCaT keratinocytes. HaCaT cells were treated for either 8 or 24 hours with either GW0742 (0.2 µM), atRA or 9cRA at the
indicated concentration in the presence of serum. Quantitative real-time PCR was performed as described in *Materials and Methods* to examine the expression of mRNA encoding (A) angiopoietin-like protein 4 (Angptl4), (B) cytochrome P450 26A1 (CYP26A1), (C) transglutaminase (TGM1) and (D) 3-phosphoinositide-dependent-protein kinase 1 (PDPK1) normalized to mRNA encoding glyceraldehyde dehydrogenase (GAPDH). Values are the average fold change compared to control treatment and represent the mean ± SEM. Values with different letters are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test.

**Fig. 6.** Phosphorylation of Akt and PARP cleavage are not influenced by retinoic acid in HaCaT cells. HaCaT cells were treated for 12 hours with either atRA or 9cRA at the indicated concentration in the presence (A) or absence (B) of culture medium serum as described in *Materials and Methods* to examine the quantitative expression of phosphorylated Akt and PARP cleavage. Values are the average fold change compared to control treatment and represent the mean ± SEM. Values with different letters are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test. The cleavage ratio is an indicator of apoptosis and is the average ratio of cleaved PARP to uncleaved PARP normalized values.

**Fig. 7.** Role of PPARβ/δ in the modulation of cell growth of mouse primary keratinocytes by GW0742 or retinoic acid. Primary keratinocytes from wild-type (+/+) and PPARβ/δ-null (−/−) mice were treated with the indicated concentration of either (A) GW0742, (B) atRA or (C) 9cRA (arrow) and cell number was quantified as described in *Materials and Methods*. Values are the average fold change compared to control treatment and represent the mean ± SEM. Values with different letters are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test.
MOL #50609

**Methods.** Values represent the mean ± SEM. *significantly different values (P < 0.05) from vehicle, as determined by ANOVA and Bonferroni’s multiple comparison test.

**Fig. 8.** Role of PPARβ/δ in the modulation of gene expression by GW0742 or retinoic acid in mouse primary keratinocytes. Primary keratinocytes from wild-type (+/+) and PPARβ/δ-null (−/−) mice were treated for 8 (left panels) or 24 (right panels) hours with either GW0742, atRA or 9cRA at the indicated concentration. Quantitative real-time PCR was performed as described in *Materials and Methods* to examine the expression of mRNA encoding (A,B) angiopoietin-like protein 4 (Angptl4) or (C,D) cytochrome P450 26A1 (CYP26A1) normalized to mRNA encoding glyceraldehyde dehydrogenase (GAPDH). Values are the average fold change compared to control treatment and represent the mean ± SEM. Values with different letters are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test.

**Fig. 9.** Expression of retinoid receptors in mouse keratinocytes and HaCaT keratinocytes. HaCaT and primary keratinocytes from wild-type (+/+) and PPARβ/δ-null (−/−) mice were cultured as described in *Materials and Methods* to examine the quantitative expression of RARα, RARβ, RARγ and RXRα.

**Fig. 10.** Effect of combining GW0742 with retinoic acid on cell proliferation of HaCaT keratinocytes. HaCaT cells were treated with either (A,C) GW0742 (0.2 µM), atRA (1.0 µM) or 9cRA (1.0 µM) or (B,D) combinations of GW0742, atRA and 9cRA (arrow), in the presence (A,B) or absence (C,D) of culture medium serum and cell number was...
quantified as described in *Materials and Methods*. Values represent the mean ± SEM.

*significantly different values (P < 0.05) from vehicle (DMSO) at the particular time point, as determined by ANOVA and Bonferroni’s multiple comparison test.

**Fig. 11.** Effect of combining GW0742 with retinoic acid on gene expression in HaCaT keratinocytes. HaCaT cells were treated for either 8 (left panels) or 24 (right panels) hours with either GW0742 (0.2 µM), atRA or 9cRA at the indicated concentration in the presence of serum. Quantitative real-time PCR was performed as described in *Materials and Methods* to examine the expression of mRNA encoding (A,B) angiopoietin-like protein 4 (Angptl4), (C,D) cytochrome P450 26A1 (CYP26A1), and (E,F) 3-phosphoinositide-dependent-protein kinase 1 (PDPK1) normalized to mRNA encoding glyceraldehyde dehydrogenase (GAPDH). Values are the average fold change compared to control treatment and represent the mean ± SEM. Values with different letters are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test.

**Fig. 12.** PPARβ/δ- and RAR/RXR-dependent modulation of keratinocyte cell growth. In response to ligand activation, PPARβ/δ heterodimerizes with RXR leading to up-regulation of target genes that cause terminal differentiation and apoptotic signaling culminating in inhibition of cell growth. 9cRA may also interact with this signaling by enhancing this effect. atRA or 9cRA activate RAR and RXR, respectively, and lead to either heterodimerization or homodimerization with RXR, respectively, and up-regulation...
MOL #50609

describe target genes that cause an increase in apoptotic signaling and inhibition of cell
growth.
TABLE 1: Flow cytometry analysis of annexin V/propidium iodide in HaCaT cells following ligand activation of PPARβ/δ. HaCaT cells were treated in triplicate for the indicated times with the indicated concentration of ligand in the presence (upper panels) or absence (lower panels) of culture medium serum. Values (mean ± SEM) with different letters in each column are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test.

<table>
<thead>
<tr>
<th></th>
<th>GW0742 [µM]</th>
<th>% Early Apoptosis</th>
<th>% Late Apoptosis</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 hr</td>
<td>2.6 ± 0.1a</td>
<td>0.4 ± 0.1a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 µM</td>
<td>4.5 ± 0.4b</td>
<td>0.3 ± 0.1a</td>
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<tr>
<td></td>
<td>1.0 µM</td>
<td>5.6 ± 0.2c</td>
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<td></td>
<td>10.0 µM</td>
<td>6.8 ± 0.3d</td>
<td>0.5 ± 0.0a</td>
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<tr>
<td>+ Serum</td>
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<tr>
<td>0.1 µM</td>
<td>4.5 ± 0.4b</td>
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<td>1.0 µM</td>
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<tr>
<td>10.0 µM</td>
<td>6.8 ± 0.3d</td>
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<tr>
<td>- Serum</td>
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<td>1.1 ± 0.2a</td>
<td>0.9 ± 0.3a</td>
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<td>10.0 µM</td>
<td>0.8 ± 0.2a</td>
<td>0.4 ± 0.2a</td>
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Early apoptosis was defined as the percentage of cells that were annexin V positive and propidium iodide negative and late apoptosis/necrosis was defined as the percentage of cells that were annexin V negative and propidium iodide positive.
Table 2: Flow cytometry analysis of annexin V/propidium iodide in HaCaT cells following atRA or 9cRA. HaCaT cells were treated in triplicate for the indicated times with the indicated concentration of RA in the presence (upper panels) or absence (lower panels) of culture medium serum. Values (mean ± SEM) with different letters in each column are significantly different, P < 0.05, as determined by ANOVA and Bonferroni’s multiple comparison test.

<table>
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<th></th>
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<th>% Late Apoptosis</th>
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<tr>
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<td>6.7 ± 1.0c</td>
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<td></td>
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<td>1.0 ± 0.1a</td>
</tr>
<tr>
<td>0.1 µM atRA</td>
<td>0.5 ± 0.1a</td>
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<td>0.4 ± 0.1a</td>
<td>1.0 ± 0.1a</td>
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<tr>
<td>0.1 µM 9cRA</td>
<td>0.4 ± 0.1a</td>
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<td>1.0 µM 9cRA</td>
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<tr>
<td><strong>48 hr</strong></td>
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<tr>
<td>Vehicle</td>
<td>1.0 ± 0.0a</td>
<td>0.4 ± 0.1a</td>
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<td>0.1 µM 9cRA</td>
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<td>1.0 µM 9cRA</td>
<td>1.1 ± 0.1a</td>
<td>0.4 ± 0.1a</td>
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</table>
Fig. 4

A. atRA + serum

B. 9cRA + serum

C. atRA - serum

D. 9cRA - serum

The graphs show the total number of cells (X 1000) over time (hours) for different treatments:

- **Vehicle**
- **0.1 μM**
- **1 μM**

Significance is indicated with asterisks (*) for specific time points.
Fig 6
Figure 1:

A. GW0742

B. atRA

C. 9cRA

Graphs showing the total number of cells (x1000) over time (hours) for different treatments and concentrations:

- (+/+): Vehicle
- (+/+): 0.1 μM
- (+/+): 1.0 μM
- (−/−): Vehicle
- (−/−): 0.1 μM
- (−/−): 1.0 μM

Statistical significance indicated by asterisks: * and **.
**FIG. 9**

- β-actin
- RXRα
- RARγ
- RARβ
- RARα

HaCaT
(+/+)
(-/-)
**Relative Angptl4 mRNA**

- Vehicle
- 1.0 μM atRA
- 0.2 μM GW0742
- GW + atRA
- GW + 9cRA
- GW + atRA + 9cRA

**Relative CYP26A1 mRNA**

- Vehicle
- 1.0 μM atRA
- 0.2 μM GW0742
- GW + atRA
- GW + 9cRA
- GW + atRA + 9cRA

**Relative PDKP1 mRNA**

- Vehicle
- 1.0 μM atRA
- 0.2 μM GW0742
- GW + atRA
- GW + 9cRA
- GW + atRA + 9cRA

**Figure 11**