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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

EFFECT OF PPARβ/δ AND PPARγ ON CELL PROLIFERATION AND GENE EXPRESSION IN THE A431 HUMAN SKIN CANCER CELL LINE

CHRISTINA LEE

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Reviewed and approved* by the following:

Jeffrey M. Peters, Ph.D.
Distinguished Professor of Molecular Toxicology
and Carcinogenesis
Thesis Supervisor

Joseph Reese, Ph.D.
Professor of Biochemistry and Molecular Biology
Honors Advisor

Wendy Hanna-Rose, Ph.D.
Associate Department Head for Undergraduate Studies
Department of Biochemistry and Molecular Biology

* Signatures are on file in the Schreyer Honors College.

Abstract

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptors that play distinct roles in the β-oxidation of fatty acids. These receptors regulate gene expression by heterodimerization with retinoid X receptor (RXR), binding to PPAR response elements (PPREs), and recruitment of coactivators. Of the three PPAR isoforms, PPARα, PPARβ/δ, and PPARγ, PPARβ/δ is the most prevalent in the skin, but PPARγ is also present. While much controversy exists as to whether PPARs, especially PPARβ/δ, either promote or inhibit cell proliferation, increasing evidence suggests that PPARβ/δ promotes differentiation and inhibits proliferation. The goal of this thesis was to characterize the roles of PPARβ/δ and PPARγ in the A431 human skin cancer cell line by stably overexpressing the receptors. A431 cells were stably infected with either empty Migr1 retroviral vector, Migr1-hPPARβ/δ, or Migr1-hPPARγ. The Migr1 vector allows for expression of a protein such as PPARβ/δ or PPARγ and enhanced green fluorescent protein (eGFP). After stable infection, cells were sorted using flow cytometry and then the presence of elevated levels of PPARβ/δ and PPARγ was confirmed through western blot analysis. To confirm that the protein being expressed was functional, qPCR was performed to examine the expression of a PPAR target gene upon receptor-specific ligand treatments. Two synthetic ligands were used: GW0742, a PPARβ/δ-specific agonist, and rosiglitazone, a PPARγ-specific agonist. Parent and Migr1 cell lines were treated with these ligands over a 1000-fold dose response range. Receptor function was determined by measuring induction of the PPAR target gene, angiopoietin-like protein 4 (*ANGPTL4*), which responds to either PPARβ/δ or PPARγ. The overexpressed PPARβ/δ and PPARγ proteins were found to be functional as indicated by a significant increase in the induction of *ANGPTL4* in the cells overexpressing PPARβ/δ or PPARγ. The effects of PPARβ/δ

and PPARγ overexpression on cell proliferation were then examined using direct cell counting. Cells were treated with 0.01, 0.1, 1.0, or 10 μM GW0742 or rosiglitazone and cell number quantified with a Coulter counter after 24, 48, and 72 hours of treatment. Ligand activation of PPARβ/δ modestly inhibited cell proliferation of A431 cells as compared to control. Overexpression of PPARβ/δ in the A431-Migr1-hPPARβ/δ cells only marginally influenced this effect. Ligand activation of PPARγ more strongly inhibited cell proliferation of A431 cells as compared to control, and overexpression of PPARγ in A431-Migr1-hPPARγ cells enhanced this effect. While the results provide further evidence for PPARβ/δ and PPARγ as inhibitors of cell proliferation, future experiments must be performed to determine the mechanisms underlying this effect. Additionally, the experiments described in this thesis do not give any indication of how PPAR activation affects cell differentiation, which provides another area where further experiments are needed. Overall, the results of this experiment suggest that ligand activation of PPARβ/δ and PPARγ have some role in decreasing cell proliferation in human A431 skin cancer cells.

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Acknowledgements

I am very grateful to Dr. Jeff Peters for giving me the opportunity throughout the past few years to be a member of his laboratory and learn about PPARs. Thanks to him, I have gained much valuable experience in the field of biochemical research. Funding for this project was made entirely possible due to the hard work of Dr. Peters. I am also extremely grateful for Dr. Mike Borland, who has guided me throughout the research process and has taught me many of the techniques and theories that are part of my thesis. Without his guidance and dedication to my education, this thesis would not be possible. Lastly, I would like to acknowledge the rest of the Peters laboratory for assisting me with any questions I had throughout the research process.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand activated nuclear receptors and transcription factors that regulate the expression of certain target genes by binding to the enhancer region of target genes as a heterodimer with retinoid X receptor (RXR)[1]. Three PPAR isoforms, termed PPARα, PPARβ/δ and PPARγ, have been identified to date. Activation of PPARs by isoform-specific ligands subsequently results in the regulation of target gene expression (Figure 1).

Figure 1: Target Gene Transcription by PPAR Activation

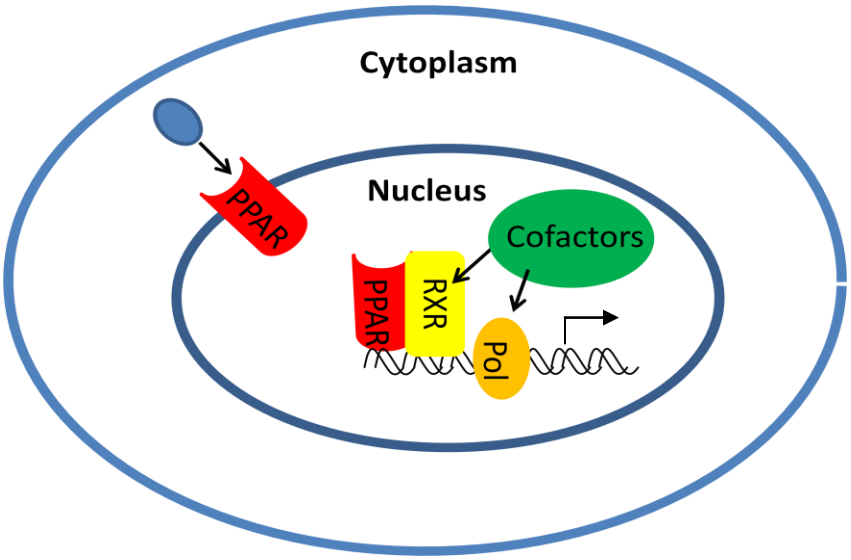

Figure 1: Ligand activation of PPAR causes it to dimerize with RXR in the nucleus. The heterodimer binds to DNA within target gene promoters. This results in the recruitment of cofactors that enhances the transcription of a downstream target gene.

To participate in the regulation of transcriptional activity, PPARs must be activated either by endogenous or exogenous ligands. Once activated by ligand, a PPAR heterodimerizes with RXR and undergoes a conformational change, displacing nuclear corepressors and forming a binding

cleft that allows the complex to bind to specific DNA sequences known as PPAR response elements (PPREs)[2]. This conformational change also recruits transcription coactivators, such as peroxisome proliferator-activated coactivator-1α (PGC1α) or other steroid receptor coactivators. The proteins interact with the coactivator domain of PPAR through an LXXLL motif. RNA polymerase is recruited and histones are remodeled due to acetylation near DNA regions of direct repeat motif 1 (AGGTCANAGGTCA), causing an increase in the transcription of target genes[2, 3, 4]. The resulting pre-mRNA can then be spliced by PGC1α, which contains an RNA processing motif in addition to its transcriptional activation domain. The processed mRNA is then available to be translated, resulting in functional protein and a biological effect[5].

Like other nuclear receptors, PPARs consist of various functional domains. The DNA binding domain is the most highly conserved region and consists of two zinc fingers that specifically bind to PPREs. The ligand binding domain is found at the C-terminus of the protein and only exhibits ~65% similarity among the three isoforms, accounting for the specificity of the different PPAR isoforms to different ligands[1, 2]. It consists of thirteen α-helices and a small four-stranded β-sheet. The ligand binding pocket of the PPARs is much larger as compared to other nuclear receptors, perhaps allowing a broad range of both natural and synthetic ligands to bind[2]. The C-terminus of the ligand binding domain contains a region known as the ligand-dependent activation function (AF-2), which is responsible for generating the coactivator binding pocket. Corepressors are able to bind in the AF-2 domain through the LXXLL motif, sterically inhibiting the ligand binding domain forming the active binding pocket. The ligand-independent activation function (AF-1), which also binds coactivators and corepressors, is found near the N terminus of the receptor and can be active in the absence of ligand[1, 6] (Figure 2).

Figure 2: Human PPARβ/δ Ligand Binding Domain Structure

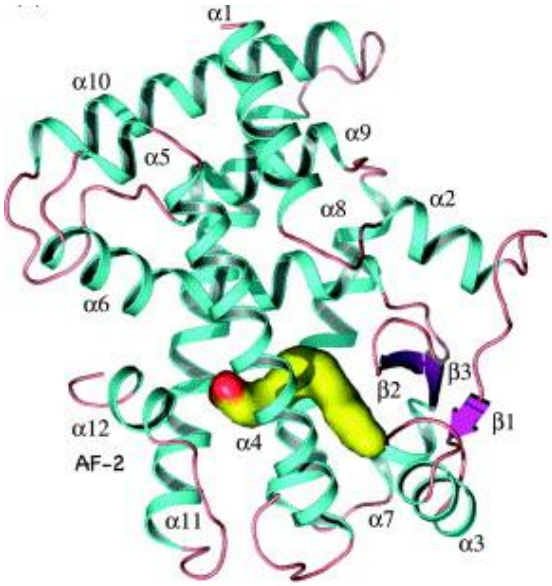

Figure 2: Ligand binding domain structure of activated human PPARβ/δ. The α helices and β sheets are labeled. The activation domain, α12, is also labeled AF-2. A fatty acid ligand is depicted as bound within the ligand binding domain[7].

Endogenous PPAR ligands are fatty acids, which is interesting because most of the PPAR target genes are involved in lipid transport and fatty acid metabolism. Some of the ligands are shared among the three isoforms, such as polyunsaturated fatty acids and eicosanoids[8]. Other ligands are specific to one isoform. This property is especially helpful in using synthetic ligands. For example, PPARα-specific agonists include WY 14643 as well as weaker agonists such as clofibrate, fenofibrate, and bezofibrate[9]. Rosiglitazone (Figure 3a), a thiazolidinedione (TZD) antidiabetic agent, has a very high affinity for PPARγ, as does other TZD family members, such as troglitazone and pioglitazone[10]. GW0742 (Figure 3b), on the other hand, is a strong agonist for PPARβ/δ that has been shown to inhibit pro-inflammatory cytokines, chemokines, and cell-

adhesion molecules as well as play roles in diabetes and β-oxidation[11, 12]. Other synthetic PPARβ/δ ligands include GW501516[13].

Figure 3: Structures of PPAR Ligands GW0742 and Rosiglitazone

a. b.

Figure 3: a) Structure of rosiglitazone, a high-affinity synthetic ligand of PPARγ in the thiazolidinedione family (CAS# 122320-73-4). **b)** Structure of GW0742, a high-affinity synthetic ligand of PPARβ/δ (CAS# 317318-84-6).

The ligands that bind to nuclear receptors are generally hydrophobic hormones that have the ability to traverse the cell membrane via simple or facilitated diffusion. The nuclear receptors then act as transcription factors by directly interacting with DNA. Two types of nuclear receptors exist: Type I and Type II. Type I receptors are localized in the cytoplasm and translocate to the nucleus after ligand binding. In their inactive form, they are usually associated with heat shock proteins. This class of receptors includes the classic steroid receptors such as glucocorticoid and androgen[14]. PPARs are considered Type II receptors, along with RXR, Vitamin D, and others. Unlike Type I receptors, Type II receptors reside in the nucleus and share the ability to bind to DNA without a ligand. While in the nucleus, they associate with other corepressors or coactivators on target gene promoters to induce the constitutive expression or repression of certain genes[15]. Ligand binding induces a conformational change in the protein and activates genes for transcription[14].

PPARs are so named because of their homology with PPARα, the first PPAR discovered that mediates peroxisome proliferation[16]. Peroxisomes are membrane-bound organelles that play key roles in lipid metabolism. They are able to β-oxidize long chain fatty acids to acetyl-coenzymeA (CoA), which is used in the citric acid cycle. They also produce and scavenge many reactions that produce dangerous reactive oxygen species (ROS), such as hydrogen peroxide, compartmentalizing those ROS to prevent them from damaging cellular components. Additionally, peroxisomes can function as signaling and organizing compartments during development, thereby maintaining lipid homeostasis[17].

The three PPAR isoforms are found in varying quantities throughout different tissues and play roles in different cellular functions. PPARα is found predominantly in the liver, kidneys, heart, skeletal muscle, and brown fat. It was named for its role in mediating increased peroxisome volume and density in hepatic tissue (peroxisome proliferation)[15]. PPARα plays a critical role in β-oxidation of fatty acids by regulating expression of proteins that catabolize fatty acids and inducing expression of the fatty acid transport protein (FATP)[2, 3, 14, 18]. Studies in PPARα deficient mice have also shown that PPARα is responsible for the triglyceride-lowering effect of fibrates[19]. PPARα has also been shown to have anti-inflammatory effects that are thought to be beneficial in reducing plaque size in atherosclerosis by disrupting the nuclear factor κB (NFκB) signaling pathway[20].

While PPARβ/δ and PPARγ share significant sequence homology with PPARα, these PPAR isoforms do not mediate peroxisome proliferation[15]. PPARγ exists as two different subtypes and is expressed at high levels in the adipocytes. Expression of PPARγ is linked to the differentiation of preadipocytes into adipocytes. It also regulates numerous genes involved in lipid metabolism and controls lipid uptake into adipocytes[2, 3, 18, 21, 22]. Additionally, ligand

activation of PPARγ interferes with proteins such as NFκB and mitogen-activated protein kinase (MAPK), causing a reduction in the expression of inflammatory cytokines[23]. PPARγ is also believed to be the insulin-sensitizing target of TZDs including rosiglitazone. Binding of TZDs to PPARγ leads to glucose-lowering effects *in vivo* through communication between adipocytes and the liver and kidneys, the main insulin-sensitive organs, and the absence of PPARγ creates a higher disposition for insulin desensitivity[24].

The biological functions of PPARβ/δ are less characterized than either PPARα or PPARγ. It is found in a wide array of tissue types, including the colon, small intestine, and liver, as well as in keratinocytes, a type of skin cell. Recent studies have elucidated the role of PPARβ/δ in regulating high and low density lipoprotein (HDL and LDL) levels[3]. Because of its roles in modulating lipid and glucose homeostasis and inflammation, PPARβ/δ is a prime target for antidiabetic research[4]. The fact that PPARβ/δ is expressed in many epithelial tissues, common sites of cancer, suggests that this PPAR isoform may be involved in tumorigenesis. While the collective literature has described a role for PPARβ/δ in tumorigenesis, it has not been clearly defined whether PPARβ/δ is inherently pro- or anti-tumorigenic. In particular, different studies have found conflicting evidence for the role of PPARβ/δ in skin cell proliferation. While many experiments agree that PPARβ/δ expression contributes to cell differentiation and apoptosis, others have found that an increase in PPARβ/δ expression leads to increased cell proliferation and inhibition of apoptotic factors[3, 25].

PPARβ/δ regulates cellular functions through mechanisms consistent with the previously described characteristics of Type II receptors. PPARβ/δ is known to predominantly reside in the nucleus in a complex with co-repressors and other proteins[26]. Once PPARβ/δ is activated by ligand binding, a conformational change causes the other proteins and co-repressors to be

released. Simultaneously, coactivators possessing histone acetylase activity are recruited to target gene promoters. This causes chromosome remodeling, recruitment of the transcription initiation complex, and target gene mRNA production[2, 18]. This main effect of transcription factor binding is upregulation of the target gene. Other mechanisms include transrepression of other signaling pathways, such as that of NFκB, or repression of gene expression[18].

As described previously, PPARs have significant functional roles in epidermal homeostasis. The skin is organized as two layers, termed dermis and the epidermis. The outer epidermis is further divided into four distinct layers – the basal layer, the spinous cell layer, the granular layer, and the outermost stratum corneum – with new cells constantly replacing old cells. The keratinocytes, which form the basal layer of stem cells at the bottom of the epidermis, are the only cells able to undergo mitosis. As cells progress upward from the basement of the epidermis, a biological process termed terminal differentiation leads to biochemical changes in the cells that create the water-insoluble properties of the skin. Differentiation of keratinocytes first leads to formation of spinous cells. These suprabasal cells are metabolically, though not mitotically, active[27]. As spinous cells continue to differentiate into the granular layer of the epidermis, they form an extensive network of crosslinking and packing that provides support for the outer cornified layer and protects the underlying layers[28]. The stratum corneum comprises the outermost layer of the epidermis. These cells have lost their nucleus and are encased in an extracellular matrix rich in lipids. At this last stage, all metabolic activity is ceased and the cells are flattened into squames of keratin filaments. This layer forms the waterproof barrier and protection against the environment[27, 28].

It has been hypothesized that anticancer effects of PPARs arise from a direct effect on the cancer cell itself, such as inhibiting cell cycle progression, activating cell differentiation or

inducing cell death (apoptosis) through cell signaling pathways, or by influencing the tumor environment by regulating inflammatory processes[29]. PPARα is thought to inhibit metastasis and anchorage-independent growth as well as increasing keratinoycte apoptosis. However, in the absence of PPARα, epidermal thickness and proliferation is not greatly altered, probably due to the presence of other PPARs or other factors that compensate for the absence of PPARα[28, 30].

PPARγ plays a role in mediating keratinocyte differentiation as well as differentiation of other epithelial cell types, including breast and colon tissue[1, 28]. PPARγ is necessary for differentiation of adipocytes; cells lacking PPARγ display no fat-cell markers[31]. Activation of PPARγ *in vitro* has been shown to cause antiproliferative effects. However, when applied topically in mice, PPARγ ligands induce both keratinocyte proliferation and keratinocyte apoptosis, resulting in no net change in epidermal thickness. It is believed that the effect of PPARγ on proliferation depends on the differentiation status of the keratinocytes. PPARγ causes a negative effect on proliferation in cases of rapid cellular proliferation[28]. While PPARγ is not found in markedly high levels in the skin, the inhibitory effects on cell proliferation are thought to be due to its role in mediating other mechanisms rather than a direct interaction[32].

The known high expression of PPARβ/δ in the skin and the ability of PPARs to modulate cell proliferation have led numerous investigations into the function of this receptor in skin carcinogenesis. Different models have been developed to study the effects of PPARβ/δ on cell proliferation. Mouse models are commonly used as an *in vivo* model, and the PPARβ/δ-null mouse model has been used to delineate the function of this receptor in the skin[33]. Many studies from independent laboratories have shown that PPARβ/δ inhibits skin cell proliferation in mouse models. In PPARβ/δ-null mice, the topical application of a tumor promoter such as 12-O-tetradecanoylphorbol 13-acetate (TPA) caused an increase in epithelial cell proliferation[2, 3, 29, 34].

PPARβ/δ-null mice are also more susceptible to tumors in skin carcinogenesis bioassays as compared to wildtype[4, 35, 36, 37]. In a two-stage skin cancer bioassay, ligand activation of PPARβ/δ attenuated cell proliferation and skin cancer in wild type mice but not in PPARβ/δ-null mice as compared to controls[28, 34]. These phenomena all suggest that PPARβ/δ attenuates epithelial cell proliferation and skin carcinogenesis[4, 34, 35, 36].

Studies on the effects of PPARβ/δ activation using *in vitro* cell culture models have also been performed. Primary keratinocytes isolated from mouse models have shown that the levels of proliferation markers are increased in keratinocytes isolated from PPARβ/δ-null mice but not in wild type keratinocytes[35]. In human keratinocytes treated with PPARβ/δ agonists such as GW0742, it was found that activation of PPARβ/δ in epidermal keratinocytes led to a decrease in cell proliferation[4, 22, 25, 38]. This is consistent with many studies showing that ligand activation of PPARβ/δ promotes terminal differentiation in keratinocytes since terminal differentiation is known to be associated with cell cycle withdrawal[4, 34, 39, 40]. In contrast, there is also evidence that PPARβ/δ may enhance cell proliferation. For example, PPARβ/δ activation causes hyperproliferation of keratinocytes from psoriasis patients and there is evidence that it may protect keratinocytes from apoptosis[41, 42]. However, despite the presence of contrasting studies, the majority of studies support the hypothesis that PPARβ/δ is a negative regulator of cell proliferation.

Very little is known about the effects of PPARβ/δ in humans as compared to mouse models. Many experiments to date use cultured keratinocytes and PPARβ/δ ligands to determine the effects of PPARβ/δ on cell survival[28, 34]. While it is difficult to obtain stable PPARβ/δ-null human models, it is possible to overexpress PPARβ/δ in human cell lines through viral transduction. In this experiment, a Migr1 viral vector containing the PPARβ/δ gene was used to

overexpress PPARβ/δ in A431 cells, a human skin cancer cell line. Additionally, PPARγ can also be overexpressed to characterize the effects of both receptors on cell proliferation and apoptosis in response to ligand activation. Since both PPARβ/δ and PPARγ may regulate keratinocyte function, it is hypothesized that overexpression of functional PPARs and subsequent ligand activation in A431 cells will alter the proliferative capacity of these cells. Data from these studies will help clarify the role of PPARs in skin cell proliferation and tumorigenesis. It is expected that cells overexpressing PPARβ/δ will exhibit less cell proliferation than the parent cells when induced by the GW0742 ligand, and that cells overexpressing PPARγ will also exhibit decreased cell proliferation when induced by the ligand rosiglitazone.

Materials and Methods

Cell Culture

A431 cells (American Type Culture Collection (ATCC), Manassas, VA) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The cells were propagated at 37°C and 5% CO_2. HEK293T cells were a gift from Dr. Yanming Wang and were grown in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% CO_2.

Establishment of Migr1 Stable Cell Lines

To facilitate stable receptor overexpression, the Migr1 retroviral system was utilized. The Migr1-hPPARβ/δ, and Migr1-hPPARγ vectors were created by subcloning the human PPARβ/δ and PPARγ cDNA sequences from pcDNA3.1-hPPARβ/δ and pcDNA3.1-hPPARγ into the

Migr1 vector. The Migr1 retroviral vector contains the mouse stem cell virus promoter to drive the expression of a bicistronic mRNA for the cDNA cloned into a cloning site and the sequence encoding enhanced green fluorescent protein (eGFP)[43]. An internal ribosome entry site (IRES) located between the cDNA and eGFP results in coexpression of two proteins, which facilitates identification and sorting of cells that have stably integrated the Migr1 retroviral vector. The coding sequence of the constructs was confirmed at the Penn State University Nucleic Acid Facility. The stable Migr1 (vector control), Migr1-hPPARβ/δ and Migr1-hPPARγ cell lines were established through retrovirus spinoculation **(**Pear, 1998 #3508). To summarize, each construct was cotransduced with pCL-Ampho plasmids into HEK293T cells to produce retrovirus using Lipofectamine® transfection reagent (Invitrogen) and the manufacturer's recommended protocol**.** Forty-eight hours after transduction, the cellular supernatant containing retrovirus was filtered using a 0.22 μm filter and used to spinoculate A431 cells. eGFP-positive cells were isolated by fluorescence-activated cell sorting using an InFlux V-GS Cytometry Workbench and the Spigot software (BD Biosciences, San Jose, CA). Forward-scatter and side-scatter dot plots gave cellular physical properties of size and granularity, allowing gating for live cells. Fluorescence was excited at 488 nm (eGFP) and emission was collected using a 525 nm band-pass filter. Collected eGFP cells possessed a minimum of 100-fold higher eGFP expression than non-eGFP cells. Fluorescence photomicrographs were collected with a SPOT SP100 cooled CCD camera fitted to Nikon EclipseTE300 upright microscope with EFD-3 episcopic fluorescence attachment. The presence of eGFP fluorescence was routinely checked using the Nikon fluorescence microscope.

Protein Isolation

Protein was isolated from 90-95% confluent 100-mm dishes of heterogenous A431 cell populations. Cells were trypsinized, then centrifuged into pellets at 900 rpm for 5 minutes. The pellets were resuspended in 1 mL DPBS and centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded and the pellets were resuspended in 1 mL DPBS and centrifuged again. The supernatant was discarded and the cells were resuspended in 300 µL lysis buffer, consisting of high-salt MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN3, and 10% glycerol, pH 7.5) containing 500 mM NaCl, 1% Nonidet P-40, and protease inhibitors. The cells were allowed to sit on ice for 1 hr, vortexing every 10-15 minutes, then centrifuged again at 14,000 rpm for 30 minutes at 4°C.

Western Blot Analysis

A total of 50 µg of the isolated protein per sample was resolved using SDS-polyacrylamide gels and transferred to a nitrocellulose membrane using an electroblotting method (90 V for 75 min). The membranes were blocked with 5% dried milk in Tris buffered saline/Tween-20 (TBST) and incubated overnight with primary antibodies. The membranes were then washed with TBST and incubated with biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). After washing with TBST, the membrane was incubated with ^{125}I-labeled streptavidin and immunoreactive proteins were detected. Hybridization signals for specific proteins were normalized to the hybridization signal for β-actin. The following antibodies were used: anti-human PPARβ/δ (Abcam – AB21209-100), anti-human PPARγ (Cell Signaling – #2430S), anti-RXRα (Santa Cruz – sc553), and anti-β-actin (Rockland – 600-401-886).

RNA Isolation

A431 cells were cultured on six plates and seeded one day before treatment. To characterize the functionality of PPARβ/δ over-expression, A431 and heterogenous Migr1 cell populations were treated for 8 hrs with vehicle (0.02% dimethylsulfoxide (DMSO)) or GW0742 (0.01 μM – 10.0 μM). To characterize the functionality of PPARγ over-expression, A431 and heterogenous Migr1 cell populations were treated for 24 hrs with vehicle (0.1% DMSO) or rosiglitazone (0.01 μM – 10.0 μM). All experiments were completed with independent triplicate samples. To isolate the RNA from the cells, RiboZol RNA Extraction Reagent (AMRESCO, Solon, OH) was added to the cells. The solution was transferred to Eppendorf tubes and 250 μL cold chloroform was added to each tube. The tubes were vortexed 10-15 s and centrifuged at 12,000 rpm/15 min/4°C. The transparent upper layer was carefully transferred to a clean Eppendorf tube and mixed with an equal volume cold isopropanol. This was stored at -20°C for at least 30 min. After refrigeration, the tubes were centrifuged again at 14,000 rpm/45 min/4°C. The supernatant was discarded and 500 μL 75% ethanol was added to each tube. The tubes were briefly vortexed and centrifuged at 14,000 rpm/5 min/4°C. The supernatant was then discarded and the tubes were placed upside-down on a drying rack until most moisture was gone. The pellet was then resuspended in 30 μL DEPC water and quantified using an ND-1000 Spectrophotometer (NanoDrop).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) analysis was used to measure the mRNA encoding angiopoietin-like protein 4 (*ANGPTL4*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) when cells were treated with either DMSO, 0.01, 0.1, 1.0, or 10 μM of

the PPARβ/δ ligand GW0742, or equal concentrations of the PPARγ ligand rosiglitazone. cDNA was generated using 2.5 µg of total RNA using the MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Real-time PCR primers for *GAPDH* and *ANGPTL4* were designed using the IDT SciTools (Coralville, IA). The primers for *GAPDH* were 5' TGCACCACCAACTGCTTAGC (forward) and 5' GGCATGGACTGTGGTCATGAG (reverse). The primers for *ANGPTL4* were 5' TCACAGCCTGCAGACACAACTCAA (forward) and 5' CCAAACTGGCTTTGCACATGCTGA (reverse). The qPCR was performed using SYBR Green PCR master mix (Finnzymes, Espoo, Finland) in the iCycler using the following protocol: 95°C for 10 s, 60°C for 30s, and 72°C for 30 s, repeated for 45 cycles. Each PCR reaction included a no-template control reaction to control for contamination. The final product was detected using the MyiQ Realtime PCR Detection System (Bio-Rad Laboratories, Hercules, CA). All real-time PCR reactions had efficiencies above 85%. The relative mRNA value for each gene was normalized to the relative mRNA value for the housekeeping gene *GAPDH*.

Cell Proliferation Analysis

A431 and Migr1 heterogeneous cells were plated on 12-well plates (50,000 cells/well) 24 hrs before cell counting time at time 0. Afterwards, the cells were treated with control (DMSO), GW0742 (0.01 µM – 10.0 µM), or rosiglitazone (0.01 µM – 10.0 µM).Each treatment was performed in triplicate. Cells were counted every 24 hrs using a Z1 Coulter particle counter (Beckman Coulter, Hialeah, FL) for 72 hrs after ligand treatment. Each replicate was counted three times.

Statistical Analysis

Statistical significance was determined using two-way analysis variance (ANOVA) and the Bonferroni's multiple comparison test (Prism 5.0 GraphPad Software Inc., La Jolla, CA). All data are presented as mean ± SEM.

Results

Establishment of Migr1 Stable Cell Lines

To overexpress the PPARβ/δ and PPARγ proteins in cells, the Migr1 vector was used to create a bicistronic gene expression system that contains eGFP and either hPPARβ/δ or hPPARγ. An empty Migr1 vector, which contained only the eGFP cDNA, was also transduced into cells to control for any effects potentially caused by the Migr1 vector. The Migr1, Migr1-hPPARβ/δ, and Migr1-hPPARγ vectors were transduced into A431 cells as previously described and compared to parent A431 cells. Stably infected cells were green due to the presence of eGFP when viewed under a fluorescence microscope (Figure 4). Fluorescent cells were isolated using fluorescence-activated cell sorting techniques. Heterogeneous populations of green cells were then used in subsequent studies.

Figure 4: Microscope Images of Parent and Migr1 Transduced A431 Cells

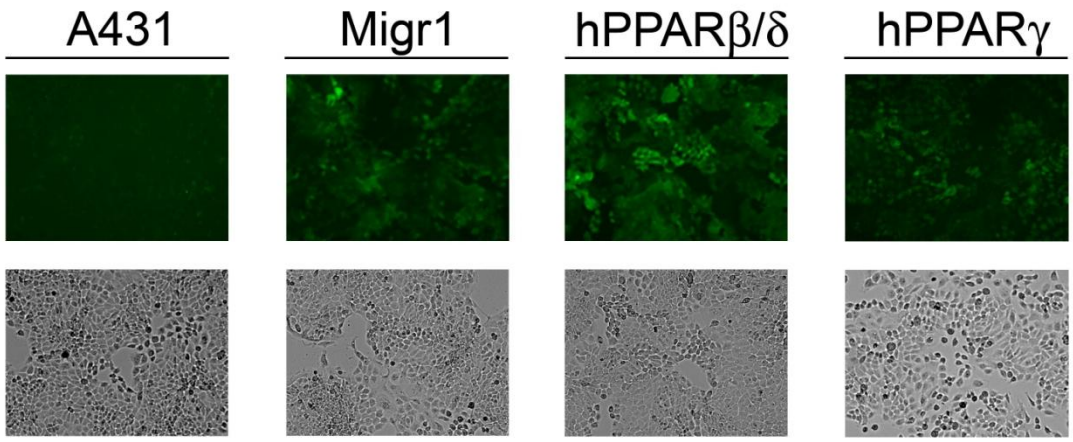

Figure 4: Parent A431 cells and A431 cells confluent and stably transduced with Migr1, Migr1-hPPARβ/δ, and Migr1-hPPARγ vectors viewed with light and fluorescence microscopes. Lipofectamine® was used to transfect HEK293T cells followed by spinoculation of A431 cells. Fluorescence was excited at 488 nm and collected at 525 nm, and cells with at least 100 fold eGFP expression were isolated by fluorescence-activated cell sorting.

Western Blot Analysis

To confirm that the cells infected with the Migr1 virus constructs were expressing higher levels of PPAR protein than the parent A431 cell line, western blot analysis was used. Protein was isolated from the Migr1-hPPARβ/δ transduced cells following the previously described protocol. The isolated protein was then used for a western blot. A PPARβ/δ antibody was used to confirm the presence of the proteins in the stable eGFP cells. β-actin expression was used for normalizing hybridization signals of PPARβ/δ (Figure 5).

Figure 5: Western Blot Analysis of PPARβ/δ in Parent and Transduced A431 Cells

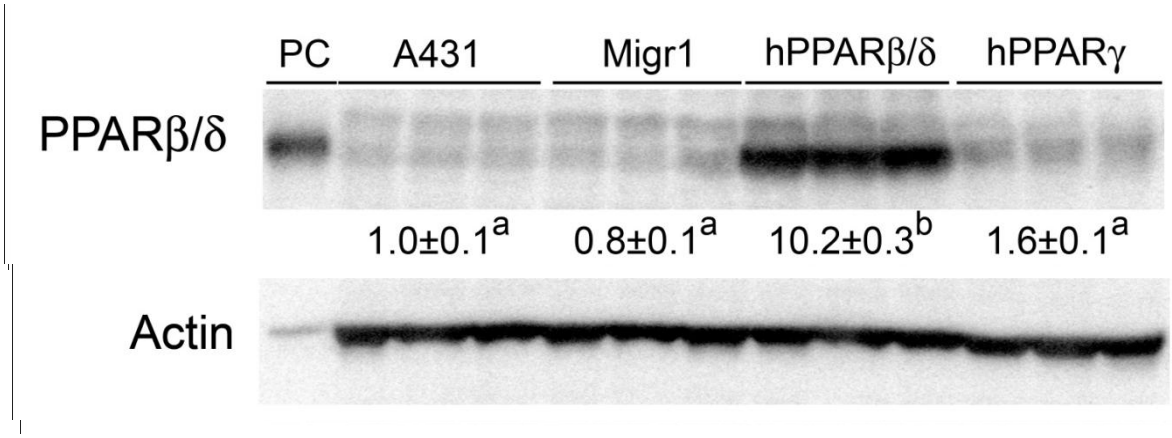

Figure 5: Western blot analysis of parent and transduced Migr1 cells probing for PPARβ/δ. Protein was isolated using high-salt MENG buffer. The signal was normalized to actin. There was significantly more PPARβ/δ in the Migr1-hPPARβ/δ cells. Anti-human PPARβ/δ and anti-β-actin antibodies were used followed by incubation with biotinylated secondary antibodies. The membrane was then probed with ^{125}I-labeled streptavidin. The numbers indicate quantified hybridization data, and the letters indicate any significant differences. PC is positive control.

The level of normalized PPARβ/δ protein was significantly higher in the Migr1-hPPARβ/δ cells compared to the other cell lines. The expression of PPARβ/δ was 10.2±0.3 fold higher than the parent A431 cells, thus indicating that the Migr1-hPPARβ/δ cells were overexpressing PPARβ/δ. PPARβ/δ expression was comparable between parent A431, Migr1, and Migr1-hPPARγ cell lines, thus showing that PPARγ overexpression does not alter PPARβ/δ protein expression. This also shows that the viral infection does not significantly alter PPAR expression unless specifically targeted by the construct.

The level of PPARγ in Migr1-PPARγ transduced cells was also examined by Western blot analysis. Presence of the protein was confirmed with an anti-PPARγ antibody. Hybridization signals of PPARγ were normalized to β-actin (Figure 6).

Figure 6: Western Blot Analysis of PPARγ in Parent and Transduced A431 Cells

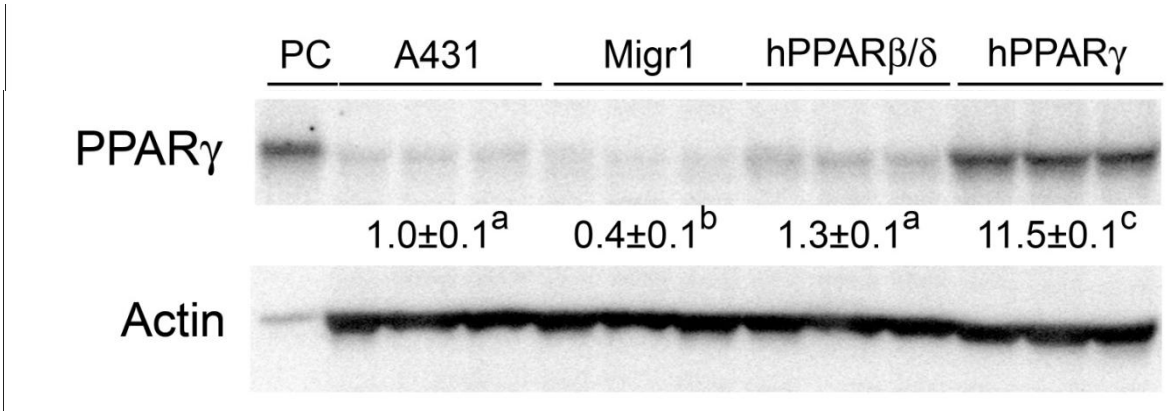

Figure 6: Western blot analysis of parent and transduced Migr1 cells probing for PPARγ. Protein was isolated using high-salt MENG buffer. The signal was normalized to actin. There was significantly more PPARγ in the Migr1-hPPARγ cells. Anti-human PPARγ and anti-β-actin antibodies were used followed by incubation with biotinylated secondary antibodies. The membrane was then probed with ^{125}I-labeled streptavidin. The numbers indicate quantified hybridization data, and the letters indicate any significant differences by analysis of variance ($p < 0.05$). PC is positive control.

The level of PPARγ protein expression was significantly higher in the Migr1-hPPARγ cells compared to the other cells. PPARγ expression in Migr1-hPPARγ cells was 11.5±0.1 fold higher than the parent A431 cell line, indicating that the Migr1-hPPARγ cells were overexpressing PPARγ. The expression level of PPARγ in the parent A431, Migr1, and Migr1-hPPARβ/δ cell lines was not significantly altered, indicating that PPARβ/δ expression does not alter PPARγ expression and that viral infection only alters PPAR expression when specifically targeted by the construct.

Quantitative Real-Time PCR Analysis

Next, to ensure that the increased level of PPAR protein was functional, RNA was isolated from the parent and transduced A431 cells that were treated with either the PPARβ/δ ligand GW0742 or the PPARγ ligand rosiglitazone. RNA was isolated and converted to cDNA using reverse transcription methods described previously. The cells were treated with ligands and the change in expression of the *ANGPTL4* gene, a direct target gene of PPARs, was measured and normalized to *GAPDH*, a housekeeping control gene. Because GW0742 is exclusively a PPARβ/δ ligand, the dose response was only performed on parent A431, Migr1, and Migr1-hPPARβ/δ cells (Figure 7):

Figure 7: GW0742 Ligand Dose Response Curve for PPARβ/δ from qPCR

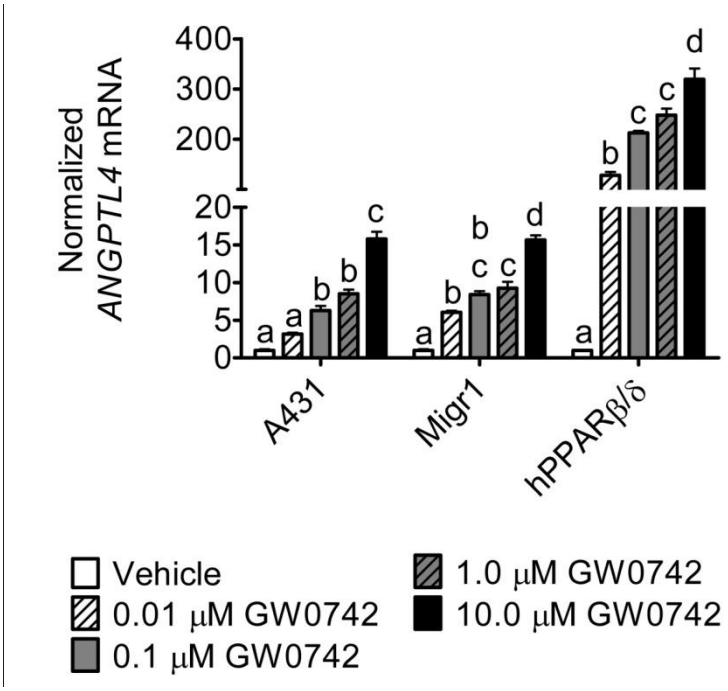

Figure 7: Dose response curve from qPCR of A431, Migr1, and Migr1-hPPARβ/δ cells using the PPARβ/δ ligand GW0742. Increased ligand dose is correlated with increased PPARβ/δ activity. Additionally, in the Migr1-hPPARβ/δ cells where PPARβ/δ is overexpressed, there was a higher level of *ANGPTL4* mRNA compared to cells with normal levels of PPARβ/δ. Letters indicate any significant differences in data by analysis of variance ($p < 0.05$).

As the concentration of ligand increased, mRNA levels of *ANGPTL4* increased as well. Because the *ANGPTL4* gene is a direct target of PPARs, this indicates that PPARβ/δ activity is also increased. In the Migr1-hPPARβ/δ cells, the induction of *ANGPTL4* mRNA levels was greatly enhanced as compared to the parent A431 or Migr1 cells treated with GW0742. Notably, even a dose of 0.01 μM was able to induce *ANGPTL4* mRNA expression, indicating that those cells are indeed functionally overexpressing PPARβ/δ and that this overexpression correlates with increased PPARβ/δ activity. In the parent and Migr1 A431 cell types, significant increases in *ANGPTL4* mRNA levels were only seen at 0.1, 1.0 and 10 μM.

RNA isolation and qPCR was also performed on Migr1-hPPARγ cells treated with DMSO, 0.01, 0.1, 1.0, and 10 μM rosiglitazone (Figure 8). Migr1-hPPARβ/δ cells were not used as rosiglitazone is exclusively a PPARγ ligand.

Figure 8: Rosiglitazone Ligand Dose Response Curve for PPARγ from qPCR

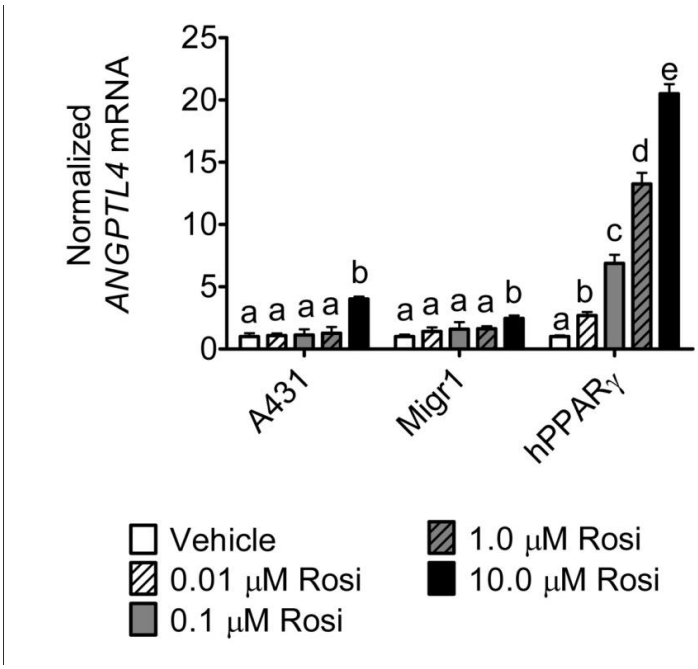

Figure 8: Dose response curve from qPCR of A431, Migr1, and Migr1-hPPARγ cells using the PPARγ ligand rosiglitazone. In the Migr1-hPPARγ cells where PPARγ is overexpressed, there is a higher level of *ANGPTL4* mRNA compared to cells with normal levels of PPARγ. The change in mRNA level as a result of ligand dose is also more marked in cells overexpressing PPARγ. Letters indicate any significant differences in data by analysis of variance ($p < 0.05$).

While there was not a very noticeable difference in *ANGPTL4* mRNA levels at the lower doses for the parent and Migr1 cells, a high dose (10 μM) of rosiglitazone led to a significant increase in *ANGPTL4* mRNA levels, indicating that perhaps PPARγ is less sensitive to its ligand than PPARβ/δ is to its ligand. However, when PPARγ was overexpressed in cells, there was not only a much higher overall level of *ANGPTL4* mRNA, but the mRNA levels in response to increasing concentration was also enhanced, indicating that the Migr1-hPPARγ cells are overexpressing functional PPARγ. These results show that Migr1-hPPARγ is also truly overexpressing PPARγ

and that these increased protein levels are correlated with increased expression of a PPAR target gene.

Assessment of Cell Proliferation

Since it was established that the stable cell lines were overexpressing functional PPARβ/δ and PPARγ protein, cell counting was performed to examine the effects of receptor overexpression and ligand activation on cell proliferation. Cells were grown as described previously and counted using a Coulter counter 0, 24, 48, and 72 hours after ligand treatment. All four cell lines were treated with either DMSO, 0.01, 0.1, 1.0, or 10 μM GW0742 at Day 0 to activate PPARβ/δ. No change in cell proliferation was found in response to ligand activation of PPARβ/δ in parent A431 cells as compared to control at any time point (Figure 9). Ligand activation of PPARβ/δ had no effect on cell proliferation in Migr1, Migr1-hPPARβ/δ or Migr1-hPPARγ cells with concentrations of GW0742 between 0.01 and 1.0 μM. However, inhibition of cell proliferation was observed in Migr1, Migr1-hPPARβ/δ and Migr1-hPPARγ cells treated with 10 μM GW0742 after 72 hours of treatment (Figure 9).

Figure 9: Effect of GW0742 on Cell Proliferation

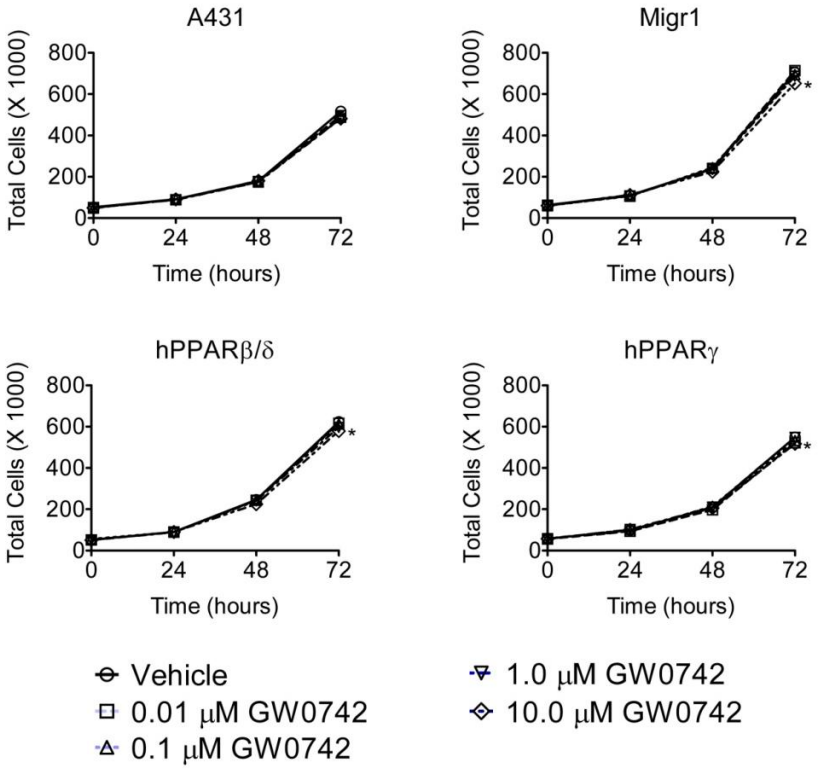

Figure 9: Effect of GW0742 treatment on A431, Migr1, Migr1-hPPARβ/δ, and Migr1-hPPARγ cell proliferation using a Coulter counter. Significant decreases in cell density were seen at 10 μM in the Migr1, Migr1-hPPARβ/δ, and Migr1-hPPARγ cell lines after 72 hrs. An asterisk, *, indicated statistically different values, as determined by analysis of variance ($p < 0.05$).

Overall, these results demonstrate that ligand activation of PPARβ/δ only marginally alters cell proliferation, and overexpression of PPARβ/δ does not markedly alter this effect.

The four A431 cell lines were also treated with either DMSO, 0.01, 0.1, 1.0, or 10 μM rosiglitazone to activate PPARγ and cell number quantified after 0, 24, 48, or 72 hours. In parent A431 cells, ligand activation of PPARγ with 0.1, 1.0 or 10 μM rosiglitazone inhibited cell proliferation after 72 hours. Cell proliferation was not changed by ligand activation of PPARγ in Migr1-control cells. In Migr1-hPPARβ/δ cells, ligand activation of PPARγ inhibited cell

proliferation following 10μM rosiglitazone after 72 hours, but this effect was not found with lower concentrations of rosiglitazone. Inhibition of cell proliferation after 72 hours of treatment was also found in Migr1-hPPARγ cells in response to either 1.0 or 10 μM rosiglitazone, but not with lower concentrations of rosiglitazone (Figure 10). The magnitude of the effect of ligand activation of PPARγ in Migr1-hPPARγ cells was greater than that found in the parent A431 cells.

Figure 10: Effect of Rosiglitazone on Cell Proliferation

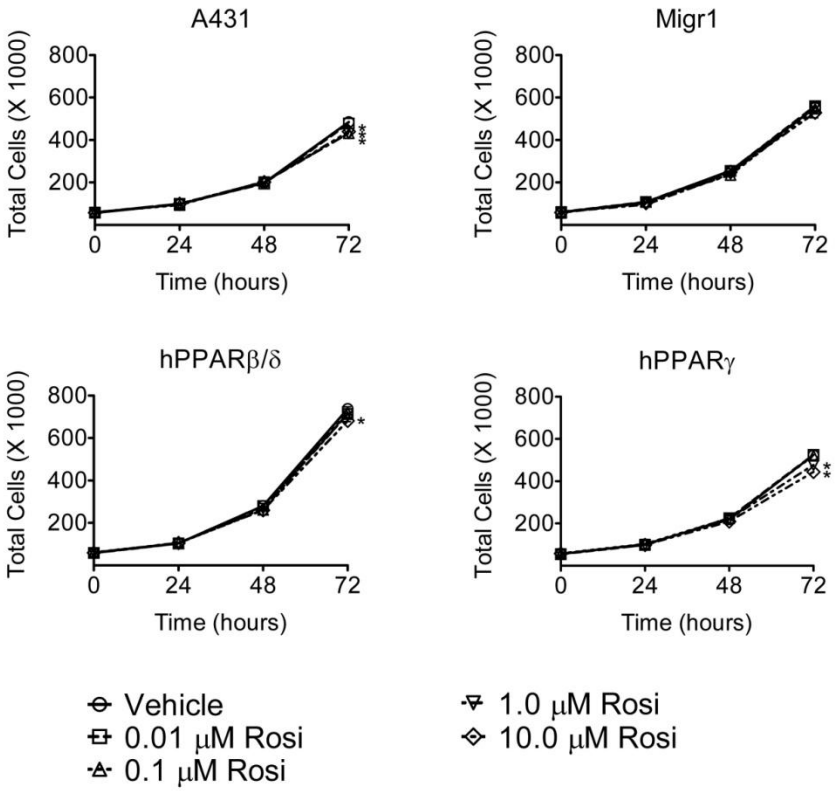

Figure 9: Effect of GW0742 treatment on A431, Migr1, Migr1-hPPARβ/δ, and Migr1-hPPARγ cell proliferation using a Coulter counter. Significant decreases in cell density were seen at 10 μM in the Migr1, Migr1-hPPARβ/δ, and Migr1-hPPARγ cell lines after 72 hrs. An asterisk, *, indicated statistically different values, as determined by analysis of variance ($p < 0.05$).

These results show that ligand activation of PPARγ causes a marginal effect on cell proliferation, and that increased PPARγ expression enhances the anti-proliferative effects of rosiglitazone in A431 cells.

Discussion

In recent years, increasing evidence shows that PPARβ/δ and PPARγ modulate keratinocyte proliferation and differentiation. However, the controversy surrounding the mechanisms by which PPARβ/δ influences cell proliferation remains unresolved[3, 25]. Null mouse models have provided enlightening results suggesting that PPARβ/δ causes a decrease in cell proliferation, but the lack of human null models leaves room for questions. This investigation used a different approach to examine the effects of PPARβ/δ and PPARγ on cell proliferation. Instead of using PPARβ/δ-null models, a human cell line model that overexpressed PPARβ/δ and PPARγ was used to examine the biological effects of receptor expression and subsequent ligand activation. Results show that these new models will be useful for studying the functional role of PPARβ/δ and PPARγ in a human skin cancer model. These models functionally overexpress the receptors, allowing high affinity ligands to target and induce PPARβ/δ or PPARγ target gene expression. This model provides information that cannot be obtained from null mouse models. Specifically, this model directly examines the effects of PPARs in the cell by overexpressing and inducing the receptors. This model is also a better model than siRNA or shRNA knockdown models, which may not be highly effective in regulating PPAR expression. This thesis posed the hypothesis that cells overexpressing functional PPARβ/δ and PPARγ proteins will exhibit decreased proliferation.

To achieve overexpression of PPARβ/δ and PPARγ in human cells, the respective cDNA was subcloned into a Migr1 retroviral vector coupled with eGFP. Specific ligands were used to activate the receptors – GW0742 for PPARβ/δ and rosiglitazone for PPARγ. Both these ligands are known agonists of their respective receptors[25, 44]. Because this thesis focuses specifically on the effects of PPARs in skin cells, A431 cells, a human skin cancer cell line, was chosen for transduction. The photomicrographs (Figure 4) show that the cells are morphologically similar, grow normally, and the fluorescence shows that a large portion of the stably growing cells had marked eGFP expression. Once a stable infection was achieved, it was necessary to ensure that the cells were indeed overexpressing functional copies of the genes. This was determined through western blot analysis and qPCR. Examination of cell proliferation was performed after establishing that the viral infection was successful and that the overexpressed proteins were fully functional in the respective cell lines.

Results show that ligand activation of PPARβ/δ only modestly inhibits cell proliferation (Figure 9). It is interesting to note that GW0742, which has a high affinity for PPARβ/δ, had no effect in A431 cells. This could be due to a number of reasons, such as the presence of endogenous high affinity ligands that may mask the potential effects of GW0742. Future experiments using this model should therefore address this possibility by using concentrations of GW0742 greater than 10 μM or by exploring the effects of different ligands or antagonists. It was also interesting to note that inhibition of cell proliferation was only observed in Migr1 and Migr1-hPPARβ/δ cells with 10 μM of GW0742, which is an effect that may have appeared due to the presence of the Migr1 virus and not as a direct effect of ligand activation of PPARβ/δ. Combined, while the effects of ligand activation are modest, they are consistent with different studies showing that activation of PPARβ/δ inhibits proliferation of keratinocytes[4, 34, 35, 39, 40], but

not with one study suggesting that activating PPARβ/δ promotes cell proliferation in human keratinocytes[41].

Results also show that ligand activation of PPARγ inhibited cell proliferation in A431 cells (Figure 10). Some differences were noted between the different cell lines. In parent A431 cells, a decrease in cell proliferation was found after 72 hours with 0.1, 1.0, and 10 μM rosiglitazone. Ligand activation by rosiglitazone did not have an effect on cell proliferation in Migr1 control cells at any time. In Migr1-hPPARβ/δ cells, a decrease in cell proliferation was found after 72 hours with 10 μM rosiglitazone while a decrease in cell proliferation was found in Migr1-hPPARγ cells after 72 hours with 1.0 and 10 μM rosiglitazone. Some of these differences observed may be due to changes caused by integration of Migr1 in the cells. However, the magnitude of the observed inhibition of cell proliferation was greater in Migr1-hPPARγ A431 cells as compared to the parent cells, showing that the efficacy of this effect is increased by overexpression of PPARγ. This shows that overexpression of PPARγ functionally increased the efficacy of inhibition of cell proliferation by ligand activation of PPARγ. These findings are consistent with many other studies showing that activating PPARγ in human cancer cell lines inhibits cell proliferation[32, 45, 46, 47].

While a connection between suppression of proliferation and activation of PPARs was observed in this experiment, the exact mechanism of this effect is not known. PPARβ/δ and PPARγ both modulate neural stem cell proliferation through slightly different pathways, suggesting that a similar effect may be seen in epithelial stem cells[48]. This, along with the slightly different results in the PPARβ/δ and PPARγ proliferation assays, suggest that different PPARs can induce similar effects through varying pathways. It is evident, therefore, that the anti-

proliferative effects of PPARs may have a promising future as agents in cancer treatments, specifically epithelial cancers, by potentially slowing the rate of cancerous cell proliferation.

It is important to note that the models used in this experiment presented challenges. One challenge of receptor overexpression is the presence of secondary effects. For example, such a drastic increase in PPAR expression could cause inhibition of other signaling pathways, such as the NFκB pathway[18]. A large amount of PPARs could also cause competition for the endogenous coactivators and corepressors available within the cell, causing a decrease in transcriptional activity efficiency of the receptor. Furthermore, PPARs modulate transcription in the nucleus. However, there is the possibility that with such a large pool of PPARs, some of the PPARs leached into the cytosol from the nucleus, which could cause completely different effects such as binding to other signaling pathway proteins, which may drastically alter those signaling pathways. Without verifying that all the PPARs are still contained within the nucleus, it cannot be determined whether the effects seen are produced by PPAR transcriptional activity.

The search for a role for PPARβ/δ and PPARγ has been investigated in past studies with varying success. Strong evidence shows that PPARγ inhibits growth in human cancer cells and inhibits tumorigenesis using mouse models supporting the targeting of various types of tumors[45]. The present study supports PPARγ as an anti-cancer treatment. Roles for PPARβ/δ in cancer have also been studied, although the role of this receptor in many cancers remains controversial[3]. However, it is clear that activating PPARβ/δ inhibits skin cancer, an effect that has not been disputed. Results from this study support these findings and the potential use of PPARβ/δ ligands as anti-cancer agents.

The scope of this experiment is limited only to cell proliferative effects of PPARs. Since PPARβ/δ and PPARγ have significant effects on cell proliferation, it is highly probable that they exert some influence on the cell cycle. Whether they act on cyclin/CDK complexes or some other mechanism remains unknown. This theory has been explored in past experiments[49]. However, the details of this mechanism cannot be confirmed by this experiment. Additionally, while it is thought that PPARs induce differentiation in the epidermis, this experiment provides no results on the effects of PPARs on differentiation[50]. Another limiting factor encountered in this experiment was the complete use of *in vitro* models. Because all the experiments were done in cell culture, there were no encounters with effects of an immune system or crosstalk between pathways at work in an animal.

Future Experiments

Further experiments must be conducted before PPARβ/δ and PPARγ can be fully characterized as modulators of cell proliferation. One drawback of the Coulter Counter cell proliferation assay is that dead cells may be counted as well as live cells. One way to bypass this is to perform a clonogenic assay. Cells can be plated onto small (60 mm) plates and treated with various concentrations of ligand. After a period of incubation, the cells are stained with a dye to visualize the cells that are attached to the plate. Dead cells do not adhere to the plate and are washed off. This method therefore gives a more accurate estimate of the number of cells that are present and serves as an easier method to visualize proliferation effects. Another way to bypass the problem of accidentally counting dead cells is to use flow cytometry to separate and count the cells. This method may be especially useful for those cells stably infected with the Migr1

vector because of the eGFP gene it contains. Separation of eGFP-positive versus eGFP-negative cells could then be obtained through flow cytometry and used to examine cell cycle progression.

To observe the effects that PPAR activation exerts on the cell cycle, a cell cycle analysis using flow cytometry techniques can be performed. Such a method can sort cells based on the stage of the cell cycle that they are in. Because a decrease in cell proliferation was seen in the proliferation assay, it is predicted that there will be a larger number of cells in the G1 and G2 phases of the cell cycle than the S phase in cells treated with ligand. Cells that are treated with DMSO should progress through the cell cycle normally while ligand treated cells remain in G1 cell cycle arrest. This effect has been observed in this laboratory in keratinocytes (unpublished results).

Once the mechanisms by which PPARs inhibit cell proliferation have been elucidated, it would be of interest to see whether PPARs affect transcription of target genes by interacting with other transcription factors and nuclear factors. A chromatin immunoprecipitation (ChIP) assay may be useful to see how PPARs affect proliferation at the transcriptional level by examining levels of transcription factors such as E2F, SP1, and AP-2. A ChIP assay is useful for isolating the target protein when it is bound to a gene promoter and any other proteins associated with it during transcription. Knowing the factors involved in transcription can help further determine the pathways by which PPARs inhibit cell proliferation.

One hypothesis for PPARs is that they also induce cell differentiation. To determine whether this is true, markers within the cell can be isolated and quantified. In the case of epidermal cells, as keratinocytes differentiate, different markers are found within the cell. The levels of these different markers enable the determination of what stage of differentiation the

cells are in and how ligand activation affects these levels of differentiation. Because PPARs, especially PPARβ/δ are found throughout the body in tissues other than just epidermal tissue, this experiment must be altered or different markers focused on in order to see differentiation in various types of tissues. If PPARs do induce differentiation, there should be increase in the levels of markers of later, more differentiated cells and a simultaneous decrease in the levels of markers for proliferating cells. This effect has been found in keratinocytes, but whether the increase in differentiation markers are true PPAR target genes is unknown[34, 37]. A ChIP assay is one method that could be used to resolve this question. By observing whether transcription factors and activators are bound to the genes of these differentiation markers, it is possible to begin to determine whether the expression of these markers is directly mediated by PPARs.

It would also be of interest to compare the effects of PPARs *in vitro* to effects *in vivo*. Past experiments have used PPAR-null mice to see how the absence of PPARβ/δ and PPARγ affect tumor progression and cell proliferation. An interesting experiment would be to see how overexpression of PPARβ/δ and PPARγ in mice compares to overexpression of those proteins in human cell lines and absence of those proteins in mice. This approach would also enable experiments focusing on PPARs and tumor progression in mice. Another way to observe tumor progression in mice is through a xenograft assay, in which the cells are grafted onto a mouse and tumor formation observed, which would further elucidate the link between PPARs and tumors. To observe how PPARs affect cancer and metastasis, a soft agar assay can be used to assess anchorage-independent growth, a common hallmark of metastatic cancer cells. Such experiments would give a much broader understanding of the effects of PPARβ/δ and PPARγ.

Finally, the Migr1 model could be further used to assess PPAR single nucleotide polymorphisms (SNPs) by specifically overexpressing PPAR mutants. These PPAR mutants

would differ by a single nucleotide mutation associated with common polymorphisms in the human population. By observing the biological effects of mutant PPAR proteins compared to wild type PPAR proteins, it would be possible to better understand how human variability in PPAR protein function could lead to biological or health effects.

References

1. Berger, J. and Moller, D. E. The Mechanisms of Action of PPARs. *Annu. Rev. Med.* 2002. **53**: 409-435.

2. Rieck, M., Meissner, W., Ries, S., Muller-Brusselbach, S., and Muller, R. Ligand-Mediated Regulation of Peroxisome Proliferator-Activated Receptor (PPAR) β/δ: A Comparative Analysis of PPAR-Selective Agonists and All-*trans* Retinoic Acid. *Molec. Pharm.* 2008. **74**(5): 1269-1277.

3. Peters, J. M. and Gonzalez, F. J. Sorting Out the Functional Role(s) of Peroxisome Proliferator-Activated Receptor-β/δ (PPARβ/δ) in Cell Proliferation and Cancer. *Biochim. Biophys. Acta*. 2009. **1796**: 230-241.

4. Bility, M. T., Zhu, B., Kang, B. H., Gonzalez, F. J., and Peters, J. M. Ligand Activation of Peroxisome Proliferator-Activated Receptor-β/δ and Inhibition of Cyclooxygenase-2 Enhances Inhibition of Skin Tumorigenesis. *Toxicol. Sci.* 2010. **113**(1): 27-36.

5. Puigserver, P. and Spiegelman, B. M. Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC1α): Transcriptional Coactivator and Metabolic Regulator. *Endocr. Rev.* 2003. **24**: 78-90.

6. Bain, D. L., Heneghan, A. F., Connaghan-Jones, K. D. and Miura, M. T. Nuclear Receptor Structure: Implications for Function. *Ann. Rev. Phys.* 2007. **69**: 201-220.

7. Fyffe, S. A., Alphey, M. S., Buetow, L., Smith, T. K., Ferguson, M. A. J., Sorensen, M. D., Bjorkling, F. and Hunter, W. N. Recombinant Human PPAR-β/δ Ligand-Binding Domain in an Activated Conformation by Endogenous Fatty Acids. *J. Molec. Biol.* 2006. **356**(4): 1005-1013.

8. Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M. G. and Wahli, W. Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay. *Molec. End.* 1997. **11**(6): 779-791.

9. Cuzzocrea, S. Di Paolo, R., Mazzon, E., Genovese, T., Muia, C. and Caputi, A. P. WY 14643, A Potent Exogenous PPAR-[alpha] Ligand, Reduces Intestinal Injury Associated with Splanchnic Artery Occlusion Shock. *Shock.* 2004. **22**(4): 340-346.

10. Kahn, C. R., Chen, L. and Cohen, S. E. Unraveling the Mechanism of Action of Thiazolidinediones. *J. Clin. Invest*. 2000. **106**(11): 1305-1307.

11. Kersten, S., Desvergene, B. and Wahli, W. Roles of PPARs in Health and Disease. *Nature*. 2000. **405**: 421-424.

12. Haskova, Z., Hoang, B., Luo, G., Morgan, L. A., Billin, A. N., Barone, F. C., Shearer, B. G., Barton, M. E. and Kilgore, K. S. Modulation of LPS-Induced Pulmonary Neutrophil Infiltration and Cytokine Production by the Selective PPARβ/δ Ligand GW0742. *Inflamm. Res.* 2008. **57**: 314-321.

13. Shearer, B. G., Steger, D. J., Way, J. M., Stanley, T. B., Lobe, D. C., Grillot, D. A., Iannone, M. A., Lazar, M. A., Willson, T. M. and Billin, A. N. Identification and Characterization of a Selective PPAR{beta}/{delta} (NR1C2) Antagonist. *Mol. Endocrinol*. 2008. **22**(2): 523-529.

14. Schoonjans, K., Staels, B., Auwerx, J. Role of the Peroxisome Proliferator-Activated Receptor (PPAR) in Mediating the Effects of Fibrates and Fatty Acids on Gene Expression. *J. Lipid Res*. 1996. **37**: 907-925.

15. Peraza, M. A., Burdick, A. D., Marin, H. E., Gonzalez, F. J., and Peters, J. M. The Toxicology of Ligands for Peroxisome Proliferator-Activated Receptors (PPAR). *Toxicol. Sci.* 2006. **90**(2): 269-295.

16. Issemann, I. and Green, S. Activation of a Member of the Steroid Hormone Receptor Superfamily by Peroxisome Proliferators. *Nature*. 1990. **347**: 645-650.

17. Titorenko, V. I. and Terlecky, S. R. Peroxisome Metabolism and Cellular Aging. *Traffic*. 2011. **12**(3): 252-259.

18. Peters, J. M., Hollingshead, H. E. and Gonzalez, F. J. Role of Peroxisome Proliferator-Activated Receptor β/δ (PPARβ/δ) in Gastrointestinal Tract Function and Disease. *Clin. Sci.* 2008. **115**: 107-127.

19. Ziouzenkova, O., Perrey, S., Marx, N., Bacqueville, D., and Plutzky, J. Peroxisome Proliferator-Activated Receptors. *Curr. Arth. Rep.* 2002. **4**: 59-64.

20. Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I. P., Delerive, P., Fadel, A., Chinetti, G., Fruchart, J., Najib, J., Maclouf, J. and Tedgui, A. Activation of Human

Aortic Smooth-Muscle Cells Is Inhibited by PPARα but Not PPARγ Activators. *Nature*. 1998. **393**: 790-793.

21. Mukherjee, R., Jow, L., Croston, G. E., Paterniti, Jr., J. R. Identification, Characterization, and Tissue Distribution of Human Peroxisome Proliferator-Activated Receptor (PPAR) Isoforms PPARγ2 *versus* PPARγ1 and Activation with Retinoid X Receptor Agonists and Antagonists. *J. Biol. Chem.* 1997. **272**: 8071-8076.

22. Burdick, A. D., Kim, D. J., Peraza, M. A., Gonzalez, F. J., and Peters, J. M. The Role of Peroxisome Proliferator-Activated Receptor-β/δ in Epithelial Cell Growth and Differentiation. *Cell. Signal.* 2006. **18**(1): 9-20.

23. Dworzanski, T., Celinski, K., Korolczuk, A., Slomka, M., Radej, S., Czechowska, G., Madro, A., and Cochoz-Lach, H. Influence of the Peroxisome-Proliferator Activated Receptor Gamma (PPAR-γ) Agonist, Rosiglitazone and Antagonist, Biphenol-A-Diglicydyl Ether (BADGE) on the Course of Inflammation in the Experimental Model of Colitis in Rats. *J. Phys. and Pharm.* 2010. **61**(6): 683-693.

24. Lehrke, M. and Lazar, M. A. The Many Faces of PPARγ. *Cell*. 2005. **123**(6): 993-999.

25. Burdick, A. D., Bility, M. T., Girroir, E. E., Billin, A. N., Willson, T. M., Gonzalez, F. J. and Peters, J. M. Ligand Activation of Peroxisome Proliferator-Activated Receptor-β/δ (PPARβ/δ) Inhibits Cell Growth of Human N/TERT-1 Keratinocytes. *Cell. Signal.* 2007. **19**(6): 1163-1171.

26. Girroir, E. E., Hollingshead, H. E., Billin, A. N., Willson, T. M., Robertson, G. P., Sharma, A. K., Amin, S., Gonzalez, F. J. and Peters, J. M. Peroxisome Proliferator-Activated Receptor-β/δ (PPARβ/δ) Ligands Inhibit Growth of UACC903 and MCF7 Human Cancer Cell Lines. *Toxicology*. 2008. **243**(1-2): 236-243.

27. Fuchs, E. Epidermal Differentiation: The Bare Essentials. *J. Cell Biol.* 1990. **111**: 2807-2814.

28. Schmuth, M., Jiang, Y. J., Dubrac, S., Elias, P. M., and Feingold, K. R. Peroxisome Proliferator-Activated Receptors and Liver X Receptors in Epidermal Biology. *J. Lipid Res*. 2008. **49**: 499-509.

29. Michalik, L. and Wahli, W. PPARs Mediate Lipid Signaling in Cell Inflammation and Cancer. *PPAR Res*. 2008; **2008**: 134059.

30. Schadendorf, D. Peroxisome Proliferator-Activating Receptors: A New Way to Treat Melanoma? *J. Invest. Derm.* 2009. **129**: 1061-1063.

31. Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., Spiegelman, B. M. and Mortensen, R. M. PPARγ Is Required for the Differentiation of Adipose Tissue In Vivo and In Vitro. *Molec. Cell*. 1999. **4**(4): 611-617.

32. Nicol, C. J., Yoon, M., Ward, J. M., Yamashita, M., Fukamachi, K., Peters, J. M., and Gonzalez, F. J. PPARγ Influences Susceptibility to DMBA-Induced Mammary, Ovarian, and Skin Carcinogenesis. *Carcinogenesis*. 2004. **25**(9): 1747-1755.

33. Peters, J. M. Lee, S. S. T., Li, W., Ward, J. M., Gavrilova, O., Everett, C., Reitman, M. L., Hudson, L. D. and Gonzalez, F. J. Growth, Adipose, Brain, and Skin Alterations Resulting from Targeted Disruption of the Mouse Peroxisome Proliferator-Activated Receptor β(δ). *Mol. Cell. Biol.* 2000. **20**(14): 5119-5128.

34. Kim, D. J., Bility, M. T., Billin, A. N., Willson, T. M., Gonzalez, F. J., and Peters, J. M. PPARβ/δ Selectively Induces Differentiation and Inhibits Cell Proliferation. *Cell Death and Diff.* 2006. **13**: 53-60.

35. Kim, D. J., Murray, I. A., Burns, A. M., Gonzalez, F. J., Perdew, G. H. and Peters, J. M. Peroxisome Proliferator-Activated Receptor-β/δ Inhibits Epidermal Cell Proliferation by Down-Regulation of Kinase Activity. *J. Biol. Chem.* 2005. **280**: 9519-9527.

36. Zhu, B., Lai, R., Kennett, M. J., Kang, B., Gonzalez, F. J. and Peters, J. M. Chemoprevention of Chemically Induced Skin Tumorigenesis by Ligand Activation of Peroxisome Proliferator-Activated Receptor-β/δ and Inhibition of Cyclooxygenase 2. *Mol. Cancer Ther.* 2010. **9**: 3267.

37. Bility, M. T., Devlin-Durante, M. K., Blazanin, N., Glick, A. B., Ward, J. M., Kang, B. H., Kennett, M. J., Gonzalez, F. J. and Peters, J. M. Ligand Activation of Peroxisome Proliferator-Activated Receptor β/δ (PPARβ/δ) Inhibits Chemically Induced Skin Tumorigenesis. *Carcinogenesis*. 2008. **29**(12): 2406-2414.

38. Borland, M. G., Foreman, J. E., Girroir, E. E., Zolfaghari, R., Sharma, A. K., Amin, S., Gonzalez, F. J., Ross, A. C. and Peters, J. M. Ligand Activation of Peroxisome-Proliferator Activated Receptor-β/δ Inhibits Cell Proliferation in Human HaCaT Keratinocytes. *Molec. Pharm.* 2008. **74**(5): 1429-1442.

39. Schmuth, M., Haqq, C. M., Cairns, W. J., Holder, J. C., Dorsam, S., Chang, S., Lau, P., Fowler, A. J., Chuang, G., Moser, A. H., Brown, B. E., Mao-Qiang, M., Uchida, Y., Schoonjans, K., Auwerx, J., Chambon, P., Willson, T. M., Elias, P. M. and Fengold, K. R. Peroxisome Proliferator-Activated Receptor (PPAR)-β/δ Stimulates Differentiation and Lipid Accumulation in Keratinocytes. *J. Invest. Derm.* 2004. **122**: 971-983.

40. Westergaard, M., Henningsen, J., Johansen, C., Rasmussen, S., Svendsen, M. L., Jensen, U. B., Schroder, H. D., Staels, B., Iversen, L., Bolund, L, Kragballe, K. and Kristiansen, K. Expression and Localization of Peroxisome Proliferator-Activated Receptors and Nuclear Factor κB in Normal and Lesional Psoriatic Skin. *J. Invest. Derm.* 2003. **121**: 1104-1117.

41. Romanowska, M., al Yacoub, N., Seidel, H., Donandt, S., Gerken, H., Phillip, S., Haritonova, N., Artuc, M., Schweiger, S., Sterry, W., and Foerster, J. PPARδ Enhances Keratinocyte Proliferation in Psoriasis and Induces Heparin-Binding EGF-Like Growth Factor. *J. Invest. Derm.* 2008. **128**: 110-124.

42. Di-Poi, N., Michalik, L., Tan, N. S., Desvergne, B., and Wahli, W. The Anti-apoptotic Role of PPARβ Contributes to Efficient Skin Wound Healing. *J. Steroid Biochem. And Molec. Biol.* 2003. **85**: 257-265

43. Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L. and Baltimore, D. Efficient and Rapid Induction of a Chronic Myelogenous Leukemia-Like Myeloproliferative Disease in Mice Receiving P210 bcr/abl-Transduced Bone Marrow. *Blood*. 1998. **92**(10): 3780-3792.

44. Oberfield, J. L., Collins, J. L., Holmes, C. P., Goreham, D. M., Cooper, J. P., Cobb, J. E., Lenhard, J. M., Hull-Ryde, E. A., Mohr, C. P., Blanchard, S. G., Parks, D. J., Moore, L. B., Lehmann, J. M., Plunket, K., Miller, A. B., Milburn, M. V., Kliewer, S. A. and Willson, T. M. A Peroxisome-Proliferator Activated Receptor γ Ligand Inhibits Adipocyte Differentiation. *Proc. Natl. Acad. Sci.* 1999. **96**(11): 6102-6106.

45. Han, S. and Roman, J. Peroxisome Proliferator-Activated Receptor [gamma]: A Novel Target for Cancer Therapeutics? *Anti-Cancer Drugs*. 2007. **18**(3): 237-244.

46. Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M., Fletcher, C., Singer, S. and Spiegelman, B. M. Terminal Differentiation of Human Breast Cancer Through PPARγ. *Molec. Cell*. 1998. **1**(3): 465-470.

47. Brockman, J. A., Gupta, R. A. and DuBois, R. N. Activation of PPARγ Leads to Inhibition of Anchorage-Independent Growth of Human Colorectal Cancer Cells. *Gastroenterol.* 1998. **115**(5): 1049-1055.

48. Cimini, A. and Ceru, M. P. Emerging Roles of Peroxisome Proliferator-Activated Receptors (PPARs) in the Regulation of Neural Stem Cells Proliferation and Differentiation. *Stem Cell Rev.* 2008. **4**: 293-303.

49. Wang, C., Fu, M., D'Amico, M., Albanese, C., Zhou, J., Brownlee, M., Lisanti, M. P., Chatterjee, V. K. K., Lazar, M. A. and Pestell, R. G. Inhibition of Cellular Proliferation Through IκB Kinase-Independent and Peroxisome Proliferator-Activated Receptor γ-Dependent Repression of Cyclin D1. *Mol. Cell. Biol.* 2001. **21**(9): 3057-3070.

50. Heikkinen, S., Auwerx, J. and Argmann, C. A. PPARγ in Human and Mouse Physiology. *BBA – Molec. and Cell Biol. Lipids*. 2007. **1771**(8): 999-1013.

Curriculum Vitae

Christina Lee
5 Purdue Dr.
Richboro, PA 18954
czl5050@psu.edu

EDUCATION

The Pennsylvania State University **University Park, PA**

- Bachelor of Science, Biochemistry and Molecular Biology (2011)
- Schreyer Honors College Scholar
- Minor in Music Performance

RESEARCH EXPERIENCE

Peters Research Group, The Pennsylvania State University **University Park, PA**
Undergraduate Researcher *Fall 2008 – Spring 2011*

- Conduct *in vitro* research to determine the role of PPARβ/δ in PPARγ in human skin cell proliferation

Wang Laboratory, Roswell Park Cancer Institute **Buffalo, NY**
Undergraduate Research Intern *Summer 2010*

- Perform *in vivo* and *in vitro* experiments to identify novel components of ubiquitin-dependent proteasome pathways and the roles of these factors in human cancer

PUBLICATIONS

Palkar, P. S. Borland, M. G., Naruhn, S., Ferry, C. **Lee, C.**, Sk, U. H., Sharma, A. K., Amin, S., Murray, I. A., Anderson, C. R., Perdew, G. H., Gonzalez, F. J. and Peters, J. M. Cellular and Pharmacology Selectivity of the Peroxisome Proliferator-Activated Receptor-β/δ Antagonist GSK3787. *Molec. Pharm.* 2010. **78**(3): 419-430.

Manuscript in preparation
Borland, M. G., **Lee, C.**, Zhu, B., Gonzalez, F. J. and Peters, J. M. Characterization of Genome-Wide Peroxisome Proliferator-Activated Receptor-β/δ (PPARβ/δ) Binding and Transcriptional Regulation.

Manuscript in preparation
Borland, M. G., Bility, M. T., Palkar, P.S., **Lee, C.**, Marcus, C. B., Gonzalez, F. J., Perdew, G. H. and Peters, J. M. Modulation of AHR-dependent Signaling by PPARβ/δ in Murine and Human Keratinocytes.

Manuscript in preparation
Borland, M. G., Khozoie, C., Palkar, P.S., **Lee, C.**, Balandaram, G., Lahoti, T., Zhu, B., Gonzalez, F. J. and Peters, J. M. Stable Over-expression of PPARβ/δ and PPARγ to Examine Receptor Signaling in HaCaT Keratinocytes.

ABSTRACTS

Palkar, P.S., Borland, M. G., Khozoie, C., Zhu, B., **Lee, C.**, Gonzalez, F. J. and Peters, J. M. Stable Over-expression of PPARβ/δ and PPARγ to Examine Receptor Signaling in Human HaCaT Keratinocytes. *The Toxicologist*. **120**(1): 145, Abstract 354. A Poster presentation at the 2011 SOT Annual Meeting.

Morales, J. L., Borland, M. G., **Lee, C.**, Gonzalez, F. J. and Peters, J. M. PPARβ/δ Modulates AHR Signaling in Mouse and Human Keratinocytes. *The Toxicologist*. **120**(1): 145, Abstract 355. A poster presentation at the 2011 SOT Annual Meeting.

EMPLOYMENT

The Pennsylvania Centre Orchestra **State College, PA**
Substitute Violinist *Fall 2010 – Spring 2011*

- Substitute as a violinist in the Pennsylvania Centre Orchestra on an as-needed basis

The Settlement Music School **Jenkintown, PA**
String Teacher *Summer 2009*

- Instruct children ages 7-14 in orchestral and string chamber music performance

ACTIVITIES

Schreyer Honors College Orientation **University Park, PA**
First Year Academics Co-Team Leader, Mentor *Fall 2008 – Fall 2010*

- Plan and lead informational workshops and activities for the Schreyer Honors College incoming freshman orientation in the area of first-year academics
- Lead groups of 12-15 first years incoming Schreyer Scholars in a weeklong orientation

The Penn State Dance MaraTHON **University Park, PA**
Organization Member, Dancer *Fall 2008 – Spring 2011*

- Provide financial and emotional support to three adoptive families as a member of Springfield THON, an independent organization
- Support families fighting pediatric cancer by not sitting or sleeping for 46 hours

Nittany Divers **University Park, PA**
Member *Fall 2010 – Spring 2011*

- Plan and participate in scuba diving trips throughout Pennsylvania and the United States

PROFESSIONAL MEMBERSHIPS

Society of Toxicology
American String Teacher Association