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The Graduate School
The Huck Institutes for Life Sciences

MODULATION OF SKIN CANCER BY PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR BETA/Delta

A Dissertation in
Integrative Biosciences
by
Moses Turkle Bility

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Doctor of Philosophy

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The dissertation of Moses Turkle Bility was reviewed and approved* by the following:

Jeffrey M. Peters
Professor of Veterinary and Biomedical Sciences
Chair of Intercollege Graduate Degree Program in Molecular Toxicology
Dissertation Advisor
Chair of Committee

Adam B. Glick
Associate Professor of Veterinary and Biomedical Sciences

Gary H. Perdew
Smith Professor of Veterinary and Biomedical Sciences

Curtis Omiecinski
Professor of Veterinary and Biomedical Sciences

Andrea M. Mastro
Professor of Biochemistry and Molecular Biology

Peter Hudson
Willaman Professor of Biology
Director, Huck Institutes of the Life Sciences

*Signatures are on file in the Graduate School
ABSTRACT

*Pparb/d*-null mice exhibit enhanced tumorigenesis in a two-stage carcinogenesis bioassay model when compared to wild-type mice, which is likely due in part to enhanced epidermal hyperplasia and decreased apoptosis following treatment with phorbol ester. Previous work also showed that ligand activation of PPARβ/δ induces terminal differentiation and inhibits proliferation in primary keratinocytes. In the present studies, the effect of ligand activation of PPARβ/δ on skin carcinogenesis was examined using both *in vivo* and *ex vivo* skin carcinogenesis models. Inhibition of chemically-induced skin tumorigenesis was observed in wild-type mice administered the synthetic PPARβ/δ ligand GW0742, and this effect was likely the result of PPARβ/δ activation induced terminal differentiation. These effects were not found in similarly treated *Pparb/d*-null mice. Ligand activation of PPARβ/δ also inhibited cell proliferation and induced terminal differentiation in neoplastic keratinocyte lines that represent different stages of skin carcinogenesis. The initiation stage of the chemically-induced skin cancer model can be replaced by introduction of the ras oncogene. Mutation of the Ha-ras allele by an initiator is a critical event in the initiation stage of the chemically-induced skin cancer model. To further characterize the molecular mechanisms by which PPARβ/δ inhibited skin carcinogenesis, a ras oncogene-induced neoplastic keratinocyte model was utilized.
v-ras\textsuperscript{Ha}-induced neoplastic/malignant transformation was exacerbated in \textit{Pparb/d}\textsuperscript{−}\textsuperscript{null} keratinocytes when compared to wild-type keratinocytes, and this effect was likely the result of PPAR\(\beta/\delta\) mediated induction of senescence and terminal differentiation and concomitant inhibition of cell proliferation. PPAR\(\beta/\delta\) can also inhibit cell proliferation by modulating the mitogen activated protein kinase (MAPK) signaling cascade, which is induced in the oncogenic \textit{ras} model. Members of the MAPK signaling cascade are critical downstream effectors of Ras signaling. v-ras\textsuperscript{Ha}-induced neoplastic transformation of primary keratinocytes resulted in an enhanced activation of MAPK proteins in \textit{Pparb/d}\textsuperscript{−}\textsuperscript{null} keratinocytes when compared to wild-type keratinocytes. Oncogenic \textit{ras} exerts its tumorigenic effects via several different downstream effectors. A major downstream effector of oncogenic \textit{ras} signaling is COX2. To further characterize the chemotherapeutic efficacy of PPAR\(\beta/\delta\) activation on skin carcinogenesis, the effect of PPAR\(\beta/\delta\) ligand coupled with COX2 inhibitor on skin carcinogenesis was examined using both \textit{in vivo} and \textit{ex vivo} skin carcinogenesis models. Results from these studies suggest that combining PPAR\(\beta/\delta\) ligands with other chemotherapeutic agents such as COX2 inhibitors could serve as powerful chemopreventive and/or chemotherapeutic tools against skin cancer. Overall, this dissertation provides evidence that targeting ligand activation of PPAR\(\beta/\delta\) could improve the efficacy of current chemopreventive/chemotherapeutic strategies for skin cancer.
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Chapter 1

Introduction

1.1 Nuclear Receptors

1.1.1 An overview of the nuclear hormone receptors

Disruption of cellular homeostasis and signaling by genetic defects or environmental factors is the underlying cause of most human diseases. Cellular homeostasis and signaling are governed by the regulation of genes and genetic products, which allows for normal development and physiology. Lipophilic hormones such as steroids were identified as major regulators of development and normal physiology; however at the time of their discovery the mechanisms by which these hormones exert their effects remained elusive. Over the past three decades, significant efforts have been made in the discovery, identification and characterization of nuclear hormone receptors and their endogenous ligands. The concept that nuclear receptors mediate the physiological effects of hormones was first proposed in the mid 1960s [1]. The development of radiolabeled ligands allowed the identification and molecular characterization of hormone binding proteins, which where shown to undergo nuclear translocation upon binding hormones; thus providing a link between transcriptional control and physiology [1].
The concept of a link between transcriptional control and physiology, as the mechanism by which hormones and their binding proteins mediate their effects, was further demonstrated by studies done in the mid-1970s. Ashburner et al. demonstrated the ecdysteroids, metamorphic hormones of insects caused puffing in certain areas of drosophila polytene chromosome; thus providing direct evidence that hormone alters the state of a chromosome [2]. In the mid-1970s, steroids were shown to be targeted to responsive tissues by the presence of high affinity receptors, which were postulated to regulate target gene transcription in hormone responsive cells [3-6]. The identification of hormonally responsive target genes completed the initial characterization of the signaling mechanism by which hormones and hormone binding receptors exert their physiological effect in target tissues.

The characterization of nuclear hormone receptors was further expanded, following the cloning of the cDNA encoding the glucocorticoid and estrogen receptors in the mid-1980s [7-11]. The homology of these receptors to the v-erbA oncogene led to the cloning of the thyroid hormone receptor [12, 13]. The cloning of the glucocorticoid and estrogen receptors ushered in the era of reverse endocrinology. The concept of reverse endocrinology is based on the high degree of sequence homology between nuclear hormone receptors; this allows characterization of receptor prior to the identification and characterization of its endogenous ligands and physiological functions.
These novel nuclear receptors were classified as orphan receptors, which describes nuclear receptors whose endogenous ligands have not been identified. The discovery of the retinoid X receptor (RXR) family and their 9-cis retinoic acid endogenous ligand further solidified the concept of a nuclear receptor superfamily [14-16]. Following the discovery of the RXR family, several different orphan receptors were discovered using reverse endocrinology; this dramatically broadens the scope of the nuclear receptor superfamily.

Nuclear receptors are a large superfamily of proteins that serve as mediators of nuclear hormones; they provide a direct link between transcriptional regulation and physiological effect. Nuclear hormones are small lipophilic molecules, which diffuse through the lipid bilayer and binds to nuclear hormone receptors. Nuclear receptors are classified as ligand-activated transcription factors due to the mechanism by which they exert their physiological effect. In the classical mechanism of transcriptional regulation, nuclear hormone receptors bind to specific DNA sequences known as hormone response elements (HREs) or DNA response elements (DREs) that are primarily located in or around the promoter region of target genes upon ligand binding. Ligand binding is followed by co-repressor disassociation, co-activator recruitment and subsequent chromatin remodeling, which leads to transcriptional regulation of target genes. Nuclear receptors bind to HREs or DREs of target genes as monomers or hetero or homo-dimers. Recent evidence suggests that nuclear receptors can also regulate gene transcription by directly binding to other proteins; this mechanism is referred to as a “transrepression” [17, 18].
Nuclear receptors modulate gene expression via transrepression through several mechanisms including competition for a limiting pool of coactivators, direct interaction with transcription factors such as p65 and p50 subunits and c-Jun, modulation of mitogen-activated protein kinase (MAPK) activity, and inhibition of corepressors clearance [17-19]

1.1.2 Structure of nuclear hormone receptors

Despite the differences in their endogenous ligands, target genes, cellular functions; nuclear hormone receptors share a well-conserved DNA and protein structure. The modular structure of nuclear receptors consists of 4 functional domains, which includes the NH$_2$-terminal region (A/B), the DNA binding domain (DBD or C), the hinge domain (D) and the ligand binding domain (LBD or E/F) [20] (Figure 1-1). The NH$_2$-terminal region or A/B domain is highly variable in both size and sequence. This region also contains a ligand independent activation domain, known as the AF1 domain, which is regulated by phosphorylation [21]. AF1 domain activation shows promoter and cell-specificity, suggesting that it contributes to the cell-specific functions of different nuclear receptor isoforms [20]. The DNA binding domain is the most conserved domain of the nuclear hormone receptor superfamily, despite its ability to recognize and bind specific DNA sequences (DREs or HREs) of target genes.
The DNA binding domain contains two zinc fingers and a COOH-terminal extension (CTE) that is responsible for DNA binding [20, 22]. The first zinc finger contains the “P box” which is responsible discrimination of DNA recognition motifs, and the second zinc finger contains the “D box” which is responsible for dimerization of the receptors with other proteins. The COOH-terminal extension contains the T and A boxes, which are responsible for monomeric DNA binding. The D or hinge domain is located between the DBD and the LBD, and facilitates rotation of the DBD, which contributes to receptor dimerization and DNA binding. Additionally, the hinge domain has been implicated in nuclear translocation and the interaction with co-repressors [20]. The LBD is a multifunctional domain (LBD), which is critical for ligand-dependent transcriptional activation (AF2), located in the COOH-terminal motif. The LBD is also critical for dimerization and co-regulator association [22-24].
**Figure 1-1:** Schematic of the modular structure of nuclear receptors. The A/B domain contains the ligand-independent AF1 transactivation domain. The C domain is the highly conserved DNA-binding domain. The D domain contains the hinge region, which allows the receptor to undergo conformational change upon ligand binding. The ligand-binding domain (E/F region) facilitates ligand-dependent transactivation and dimerization.
1.1.3 Classification of nuclear hormone receptors

There are approximately 48 human nuclear receptors identified to date, however endogenous ligands and physiological functions have been elucidated for only half of these receptors (Table 1-1). The nuclear hormone receptor superfamily can be divided into 6 subfamilies based on their ability to bind to DNA as homo- or heterodimers [25]. However, evolutionary studies showed that nuclear receptors are derived from a common ancestor [25]. The first nuclear receptor subfamily consists of the thyroid hormone receptors (TRs), retinoic acid receptors (RARs), peroxisome proliferator-activated receptors (PPARs), vitamin D receptors (VDRs), ecdysone receptors (EcRs), liver X receptors (LXRs), constitutive androstane receptor (CAR), Farnesoid X receptor (FAR) and orphan receptors that can form heterodimers with retinoid X receptors (RXRs). The second nuclear receptor subfamily consists of the retinoid X receptors (RXRs), chicken ovalbumin upstream stimulators (COUPs), hepatocyte nuclear factor 4 (HNF4) and testis receptors (TR2). RXRs are very important in nuclear receptor signaling, because they serve as heterodimer partners of other nuclear hormone receptors, in addition to their own signaling via activation by 9-cis-retinoic acid [26]. The third nuclear receptor subfamily consists of the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER), progesterone (PR), and androgen receptor (AR), which bind to DNA as homodimers.
The fourth and fifth nuclear receptor subfamily consists of the orphan receptor NGFI-B and Fushi Tarazu factor-1/steroidogenic factor-1 (FTZ-1/SF-1) respectively. Lastly, the sixth nuclear receptor subfamily consists of the germ cell nuclear factor (GCNF), an orphan receptor.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Subtype</th>
<th>Denomination</th>
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</thead>
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<tr>
<td>TR</td>
<td>α,β</td>
<td>Thyroid hormone receptor</td>
<td>Thyroid hormone (T3)</td>
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<td>RAR</td>
<td>α,β/δ,γ</td>
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<td></td>
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<tr>
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<td>Vitamin D receptor</td>
<td>1-25(OH)2 vitamin D3</td>
</tr>
<tr>
<td>PXR</td>
<td></td>
<td>Pregnan X receptor</td>
<td>Pregnanes, C21 steroids</td>
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<tr>
<td>DAX</td>
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<td>Dosage-sensitive sex reversal receptor</td>
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</table>
1.2 Peroxisome proliferator-activated receptors (PPARs)

1.2.1 An overview of PPARs

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. PPARs regulate the expression of genes that are involved in a myriad of biological functions, including cell proliferation, immune response, differentiation, fatty acid metabolism and energy homeostasis (Table 1-2) [27-29]. PPARs generally modulate gene expression by undergoing conformational change upon ligand binding; this allows the release of co-repressors, recruitment of co-activators and the subsequent heterodimerization with the retinoid X receptor-α (RXRα) (Figure 1-2). The ligand bound PPAR-RXRα heterodimer complex binds specific peroxisome proliferator response element (PPRE) in the promoter region of target genes and recruits cellular transcriptional machinery, which results in upregulation of gene expression (Figure 1-2)[30]. Additionally, PPARs have also been shown to inhibit gene expression by various mechanisms. PPARs have been shown to inhibit gene transcription by binding to PPREs in the absence of ligands and recruiting corepressor complexes that mediate gene repression [17, 18].
In recent studies, PPARs have been shown to inhibit gene expression by antagonizing the actions of other transcription factors, such as nuclear factor-κB (NF-κB), and activator protein-1 (AP-1) upon activation by PPAR agonists; this mechanism is known as transrepression (Figure 1-2) [31]. PPARs inhibit the actions of these transcription factors by physical interacting with them, via protein-protein interactions, which prevent binding to their response elements. (Figure 1-2) [18]. In addition, activation of PPARs also inhibits MAPK activity (c-Jun N-terminal kinase (JNK) and p38), thus inhibiting the expression of MAPK target genes [18, 32, 33]. PPARs also modulate gene expression via transrepression by competing for a limiting pool of coactivators, such as CREB-binding protein [34-36]. Additionally, PPARs can inhibit gene expression by blocking the signal-dependent clearance of corepressor complexes such as NcoR complexes [18, 37, 38].
The modular structure of PPARs consists of 4 functional domains (Figure 1-2) [30]. The A/B domain is located in the N-terminus and contains the first activation domain, which is involved in ligand-independent activation. The C domain or DNA binding domain (DBD) is a highly conserved domain across the PPAR isoforms and consists of two zinc fingers that bind to the PPRE(s) of target genes. The D domain or hinge domain allows the molecule to undergo conformational change. The E/F domain consists of the ligand-binding domain and the ligand-dependent activation domain (AF2), which enables co-activators recruitment and dimerization with RXRα.

### Table 1-2: A summary of PPAR isoforms, ligands, tissue distribution and physiological roles.

<table>
<thead>
<tr>
<th></th>
<th>PPARα</th>
<th>PPARβ/δ</th>
<th>PPARγ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ligands</strong></td>
<td>Fatty acids</td>
<td>Fatty acids</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td></td>
<td>Fibrates</td>
<td>L-165041</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td></td>
<td>Eicosanoids</td>
<td>GW0742</td>
<td>Eicosanoids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GW501516</td>
<td></td>
</tr>
<tr>
<td><strong>Tissue distribution</strong></td>
<td>Liver, Heart, Kidney, Adipose tissue, Muscle</td>
<td>Ubiquitous, Highly expressed in skin, Brain, Adipose tissue</td>
<td>Adipose tissue, Macrophages, Kidney, Pancreas, Spleen, Colon and large intestine, Heart, Muscle</td>
</tr>
<tr>
<td><strong>Physiological role</strong></td>
<td>Fatty acid catabolism, Hepatocarcinogenesis (Rodent), Lipid homeostasis</td>
<td>Fatty acid catabolism, Glucose homeostasis, Adipocyte differentiation, Anti-inflammatory, Carcinogenesis (?)</td>
<td>Glucose homeostasis, Insulin homeostasis, Adipocyte differentiation, Macrophage Function</td>
</tr>
</tbody>
</table>
The PPAR nuclear receptor subfamily consists of three distinct isoforms, PPAR\(\alpha\), PPAR\(\beta/\delta\) and PPAR\(\gamma\). The biological roles of PPAR\(\alpha\) and PPAR\(\gamma\) have been extensively characterized; however less is known about PPAR\(\beta/\delta\) when compared to the other PPARs.

**Figure 1-2: Regulation of gene expression by PPARs.** A). The classic mechanism of PPARs regulation of gene transcription is initiated by ligand activation, which is followed by conformational change in the receptor that enables the release of co-repressors, recruitment of co-activators and heterodimerization with RXR\(\alpha\). The activated heterodimer complex binds to PPREs and recruits cellular transcriptional machinery, which results in upregulation of gene expression. B). PPAR/co-repressor complexes have also been reported to repress gene transcription; ligand activation relieves this repression. C). PPARs can also modulate gene transcription by directly interacting with other transcription factors.
1.2.2 An overview of PPARα

PPARα was the first PPAR to be cloned and identified [39]. PPARα was originally cloned from murine liver, and since then have been subsequently clone from various species including humans [40, 41]. Mouse and human PPARα share a very high sequence homology, with 85% homology at the nucleotide level and 91% homology at the amino acid level [42]. As expected of nuclear receptors, the sequence homology of the DBD is highly conserved between species. However, the homology of the LBD between different species is less conserved, which might reflect evolutionary adaptation to dietary fatty acids by different species. PPARα is activated by structurally diverse ligands, which include both endogenous ligands and synthetic ligands. Endogeneous ligands of PPARα include saturated fatty acid and fatty acid derivatives [42]. PPARα is activated by 8(S)-HETE, an eicosanoid metabolite and by the pro-inflammatory eicosanoid Leukotriene B4 (LTB4) [43, 44]. Synthetic ligands of PPARα include hypolipidemic drugs (fibrates) and phthalate monoesters [45-47].

Activation of PPARα by hypolipidemic drugs such as Wy-14,643 has been shown to induce peroxisome proliferation, hepatomegaly and ultimately hepatic cancer in rodents [48, 49]. However, these effects are not found in primates or humans despite activation of PPARα by these chemicals [50-52]. Long-term administration of peroxisome proliferators to Syrian hamsters does not result in hepatocarcinogenesis [53].
In addition, non-human primates exhibit refractory response to PPARα agonist-induced hepatic peroxisome proliferation, hepatomegaly and hepatocarcinogenesis [54-56]. Examination of liver samples from individuals treated with fibrates showed no evidence of peroxisome proliferation or hepatocarcinogenesis [57]. Recent evidence obtained using humanized-Ppara mice showed that humanized mice were resistant to PPARα agonist mediated hepatocellular proliferation, hepatomegaly, and hepatic cancer, despite induction of lipid metabolism target genes [58, 59]. This discrepancy between mice and human responsiveness to PPARα activation may be explained by differences in hepatic expression of PPARα, ligand affinity and differential transcriptional regulation of target genes [50, 60]. Mice have much higher levels of PPARα expression in the liver than do humans, which could account for differences in species responsiveness [60]. However, there is also evidence that levels of PPARα expression do not appear to be a significant factor in the differential response between species, as studies utilizing humanized-Ppara mice where expression of human PPARα protein in humanized-Ppara mice was similar to wild-type mouse PPARα levels showed differential effects in liver hyperplasia [59, 61]. Additionally, differences in ligand affinity could account for differential species responsiveness. In vitro transactivation studies have shown that PPARα agonists have a higher affinity for mouse PPARα than human PPARα, and thus could explain why human PPARα does not mediate significant induction of target genes that promote hepatocyte proliferation and carcinogenesis [47, 62].
However, studies using humanized-\textit{Ppara} mice show that PPAR\(\alpha\) agonists were capable of inducing several known PPAR\(\alpha\) target genes involved in fatty acid catabolism at comparable levels to wild-type mice, thus indicating that ligand affinity differences between mouse and human PPAR\(\alpha\) may not have a significant impact on differential species responsiveness [59, 61]. Results from studies utilizing humanized-\textit{Ppara} mice suggests that differences in species responsiveness to PPAR\(\alpha\)-mediated hepatocarcinogenesis is due to mouse PPAR\(\alpha\) preferential activation of cell cycle genes required for cell proliferation and hepatocarcinogenesis [59, 61]. Cyclins and cyclin dependent kinases (CDKs) regulate the transition of cells through cell cycle, and overexpression of these proteins, as well as proliferating cell nuclear antigen (PCNA) and \textit{c-myc}, can lead to increase cell cycle progression, uncontrolled cell proliferation and ultimately carcinogenesis. Previous reports showed that cell cycle regulators are markedly up-regulated in wild-type mice fed Wy-14,643, and this phenotype is lost in the absence of PPAR\(\alpha\) expression [49]. Consistent with this finding, Wy-14,643 treatment was shown to cause an induction in the expression of various genes involved in cell cycle control (PCNA, \textit{c-myc}, CDK1, CDK4, and cyclins A2, D1, and E) in the livers of wild-type mice; however, the expression of these genes was unaffected in Wy-14,643 treated humanized-PPAR\(\alpha\) mice [59, 61].
Additionally, the tumor suppressor gene *p53*, was markedly induced in Wy-14,643 treated humanized-PPARα mice, but was comparatively lower in similarly treated murine-PPARα mice; this finding suggests that human PPARα activation could lead to induction of apoptosis and ultimately resistance to hepatocarcinogenesis [59, 61].

Despite the refractory response of primates to PPARα mediated hepatocarcinogenesis, PPARα has a critical role in the regulation of hepatocarcinogenesis in rodents. Peroxisome proliferator-induced hepatocarcinogenesis is hypothesized to be mediated through several mechanisms including oxidative damage to macromolecules, increased cell proliferation and inhibition of apoptosis. PPARα is hypothesized to induce oxidative damage via the induction of peroxisomal β-oxidation, which increases the intracellular concentration of hydrogen peroxide [63-65]. Activation of PPARα results in increased levels of enzymes associated with the peroxisomal fatty acid β-oxidation including acyl-CoA oxidase and to a lesser extent microsomal CYP4A subfamily of enzymes involved in fatty acids ω-oxidation, leading to an increased in hydrogen peroxide (H₂O₂) levels in the absence of a concomitant increase in catalase activity (H₂O₂-degrading enzyme) [66]. Elevated levels of H₂O₂ could potentially react with metals and generate highly reactive hydroxyl radicals, or react with lipid resulting in lipid peroxides, ultimately elevating reactive oxygen species (ROS) and oxidative stress that can cause damage to DNA, protein, lipids and other macromolecules, which ultimately contribute to hepatocarcinogenesis in rodents.
Activation of PPARα is also hypothesized to induce hepatocarcinogenesis via induction of cell cycle regulators, which results in increased cell cycle progression and proliferation. Treatment of rodents with peroxisome proliferators results in elevated expression of several cell cycle control genes and oncogenes including cyclins, CDKs, c-Ha-ras, jun and c-myc, which results in elevated DNA replication and cell proliferation, which ultimately contribute to hepatocarcinogenesis [64, 68-71]. Additionally, PPARα have also been shown to repress apoptosis, which could result in proliferation of damage cells and ultimately hepatocarcinogenesis [72]. Recent studies suggest that PPARα is a major regulator of hepatic miRNA expression, which plays critical role in the regulation of biological processes including metabolism, embryogenesis, and oncogenesis [73-75]. Activation of PPARα was shown to inhibit let-7C, a miRNA important in the modulation of cell growth and carcinogenesis [75]. Let-7C was shown to target c-myc for degradation via direct interaction with the 3' untranslated region of c-myc [75]. Activation of PPARα was shown to induce c-myc expression via inhibition of let-7C [75]. Additionally, studies utilizing a human PPARα-mouse model, which is responsive to Wy-14,643 effects on β-oxidation and serum triglycerides but resistant to hepatocellular proliferation and tumorigenesis, demonstrated a critical role for let-7C in inducing hepatocarcinogenesis via activation of murine PPARα [75].
Wy-14,643 treatment did not inhibit let-7C or induce c-myc and mir-17 expression in humanized PPARα mice; this mechanism could also account for differences between mice and human responsiveness to PPARα agonist induced hepatocarcinogenesis [75].

Despite the adverse effect of hepatocarcinogenesis induction upon PPARα activation, this nuclear receptor has beneficial roles in the regulation of inflammation, fatty acid and lipid homeostasis. PPARα is highly expressed in metabolically active tissues such as liver, kidney, heart, brown adipose tissue and skeleton muscle [76, 77]. PPARα has critical physiological role in the regulation of cellular uptake, activation and metabolism of lipids. PPARα is involved in the regulation of fatty acid transport proteins such as fatty-acid transport protein (FATP) and fatty acid transferase (FAT) [78, 79]. Additionally, PPARα also regulates the expression of fatty acid transport proteins such as carnitine palmitoyl transferase I (CPTI), which is involved in the translocation of activated fatty acids into the mitochondria [80]. In addition to regulating the expression of fatty acid transport proteins, PPARα has critical roles in the transcriptional regulation of genes involve in fatty acid metabolism. PPARα regulates the expression of enzymes involved in the β-oxidation pathway, which includes acetyl-CoA synthetase, acyl-CoA oxidase (ACO), enoyl-CoA hydratase/dehydrogenase and keto-acyl-CoA thiolase [81-87]. PPARα also regulates the expression of the CYP4A subclass of cytochrome P450s, which are involved in microsomal ω-oxidation [88, 89].
PPARα acts as an extracellular lipid sensor involved in the control of high-density lipoprotein (HDL) levels and the lowering of triglyceride levels. PPARα modulation of lipid levels involves the regulation of apolipoproteins including apolipoprotein A-I (apoA-I) [90]. Apolipoproteins are lipid-binding proteins that are important regulators involved in elevating HDL levels. Administration of Wy-14,643 to rodents increased hepatic apoA-I mRNA and lowered serum triglycerides levels in a PPARα dependent manner [91]. Administration of PPARα agonists to wild-type animals have also been shown to suppress hepatic apo-CIII expression, while increasing lipoprotein lipase (LPL) gene expression, leading to increased HDL levels and decreased levels of very-low-density lipoprotein (VLDL) [92, 93]. These results showed a definitive role of PPARα in the modulation of lipid homeostasis. In addition to being a major regulator of fatty acid homeostasis, PPARα has also been implicated in the regulation of inflammation. Activation of PPARα by leukotriene B4 (LTB4) inhibits expression of hepatic enzymes involve in eicosanoid metabolism, thus promoting an anti-inflammatory response [44]. Additionally, Ppara-null mice had extended inflammatory responses when compared to wild-type littermates in response to LTB4 or arachidonic acid [44]. PPARα also exerts anti-inflammatory effects by inhibiting nuclear factor-κB (NFκB) and AP1 signaling pathways and reducing IL-6 plasma levels [94].
PPARα inhibits NFκB signaling by interacting with the Rel homology domain of the p65 subunit of NFκB. PPARα also inhibits the NFκB pathway by inducing the expression of IκB, the key inhibitor of NFκB signaling. Additionally, PPARα have also been shown to attenuate lipopolysaccharide-induced inflammation [95]. These studies suggest that PPARα elicits anti-inflammatory action via multiple mechanisms and could play a critical role in the attenuation of inflammatory and lipid associated diseases such as artherosclerosis.

In summary, PPARα plays critical roles in the modulation of multiple physiological pathways, including the induction of fatty acid catabolism, inhibition of inflammatory signaling, promotion of cholesterol homeostasis and promotion of hepatocarcinogenesis in rodents. However, primates including humans are refractory to the hepatocarcinogenic effects of PPARα, thus suggesting that PPARα agonists could be powerful chemopreventive/chemotherapeutic tools against several metabolic diseases (Figure 1.3).
Activated PPARα

(Rodent and Human)  (Rodent only)

- Increased FA catabolism
- Increased HDL
- Decreased Triglycerides

- Decreased IL-6
- Decreased NFκB
- Increased IκB

Modulate the following events in a rodent specific manner
- Increased ROS
- Increased cell cycle progression genes
- Decreased apoptotic genes
- Increased oncogene levels via the downregulation of Let7c miRNA expression

Attenuation of metabolic diseases
- Diabetes, Dyslipidemia
- Atherosclerosis.

Promotes rodent hepatocarcinogenesis

**Figure 1-3: An overview of PPARα physiological and pathological functions.**

PPARα is actively involved in fatty acid catabolism, anti-inflammatory responses, cholesterol homeostasis, which contributes to the attenuation lipid-associated pathology, such as dyslipidemia, type II diabetes and other cardiovascular diseases. Additionally, activation of PPARα results in the promotion of hepatocarcinogenesis in rodents but not in primates including humans.
1.2.3 An overview of PPARγ

Mammalian PPARγ was discovered from studies examining the enhancer region of the adipocyte fatty acid binding protein (aP2) gene, which stimulates adipose-specific gene expression [96]. Transcriptional regulation studies showed that the nuclear factor, ARF6 is a key regulator of aP2 gene expression [96, 97]. ARF6 was shown to regulate aP2 gene expression through the ARE6 and ARE7 cis-acting elements [96, 97]. Further analysis of ARF6 showed that this nuclear receptor was part of the PPAR superfamily and was subsequently classified as PPARγ [98]. PPARγ has been identified in several different species including human, mouse and xenopus [82, 99-102]. PPARγ is preferentially expressed in adipose tissue and at lower levels in other tissues including the heart, colon, intestines, kidneys, pancreas, and spleen [99, 103]. Genomic structure analysis showed that three Pparg mRNA transcripts (Pparg1, Pparg2 and Pparg3) are expressed in humans; a result of differential promoter usage [103]. The three Pparg isoforms display differential tissue expression and distribution with Pparg1 exhibiting broad distribution, while Pparg2 is preferentially expressed in the adipose tissue and Pparg3 is expressed in the adipose tissue, macrophages and colon epithelium [103, 104]. Analysis of sequence homology across different species showed that PPARγ is highly conserved, with 95% amino acid homology between mice and humans [42]. The high sequence homology across species suggests that PPARγ may have critical physiological functions.
PPAR\(\gamma\) is activated by endogeneous ligands such as polyunsaturated fatty acids including arachidonic acid, linoleic acid and eicosapentanoic acid [105]. Additionally, lipoxygenase products of arachidonic acid, 9-HODE and 13-HODE also activate PPAR\(\gamma\) [106]. Exogenous ligands of PPAR\(\gamma\) include insulin-sensitizing thiazolidinediones (TZD) compounds, troglitazone, rosiglitazone, and pioglitazone [105, 107]. Activation of PPAR\(\gamma\) by the thiazolidinedione class of anti-diabetic drugs has been shown to improve insulin sensitivity and reduce plasma glucose and serum triglyceride levels in both animals and humans exhibiting hyperglycemia and obesity, however the exact mechanism(s) by which PPAR\(\gamma\) exerts these effects is still unknown [108]. TZDs have been shown to induce adipocyte differentiation via modulation of adipogenic proteins expression, and this effect is mediated via increase transcriptional activation of PPAR\(\gamma\) [109-112]. Furthermore previous studies show that activation of PPAR\(\gamma\) is both necessary and sufficient to induced differentiation of fibroblasts into mature adipocytes [98]. The extremely low body fat depots in mice deficient in PPAR\(\gamma\) expression further demonstrated the significance of PPAR\(\gamma\) in adipocyte differentiation [113, 114]. Activation of PPAR\(\gamma\) by TZDs was first shown to regulate expression of the fatty acid-binding protein aP2, which is critical in adipocyte differentiation [115]. Identification of thiazolidinediones as PPAR\(\gamma\) agonists, also suggested PPAR\(\gamma\) plays critical roles in the modulation of insulin signaling [109].
TZDs have been shown to repress gene expression of resistin, insulin receptor substrate-2 (IRS-2), tumor necrosis factor-α (TNFα) and IL-6, all of which have been implicated in the modulation of insulin resistance [116-120]. PPARγ agonists have been shown to inhibit TNFα expression in the adipose tissue of obese rodents; furthermore, PPARγ agonists inhibited TNFα-induced insulin resistance [121, 122]. PPARγ ligands have also been shown to induce the expression of adiponectin, an insulin-sensitizing adipocyte hormone [123]. PPARγ ligands downregulate 11β−hydroxysteroid dehydrogenase 1 (11β-HSD1), an enzyme that generates the active cortisol and promotes insulin resistance in adipocytes, suggesting an additional mechanism by which PPARγ promotes insulin sensitivity [124, 125]. Furthermore, TZDs decrease adipocyte secretion of PAI-1, a pro-thrombotic that is increased in obesity [126, 127]. In addition to directly affecting the expression of adipocyte hormones and genes that are involved in adipocyte fatty acid metabolism; PPARγ ligands also regulate the expression of several other genes that enhance glucose homeostasis in adipocytes, including those that encode the insulin-responsive glucose transporter GLUT4 and c-Cbl associating protein (CAP) [128]. CAP is essential for GLUT4 translocation to the cell surface [128].
Administration of PPAR\(\gamma\) agonists to diabetic mice have been shown to induce the expression of adipocyte-related complement protein (Acrp30), a protein involved in decreasing glucose, triglycerides, and free fatty acids [129, 130]. Increased glucose uptake into adipocytes directly improves systemic glucose homeostasis, and also contributes to fatty acid incorporation into triglycerides in adipocytes [131]. Further analysis showed that PPAR\(\gamma\) regulate the expression of other genes involve in lipid metabolism and uptake including PEPCK, acyl-CoA synthase, LPL, FATP-1 and CD36 [78, 132-135]. In addition to regulating genes involve in lipid metabolism and uptake, PPAR\(\gamma\) also regulates genes involve in cellular energy homeostasis. Activation of PPAR\(\gamma\) has been shown to increase the expression of the mitochondrial uncoupling proteins, UCP-1, UCP-2, and UCP-3 [136]. Additionally, PPAR\(\gamma\) has also been shown to downregulate leptin, an adipocyte-selective protein that inhibits feeding [137].

In addition to inhibiting TNF\(\alpha\)-mediated inflammation, PPAR\(\gamma\) agonists also inhibit other pro-inflammatory cytokines. PPAR\(\gamma\) agonists were shown to downregulate nitric oxide (NO) production in macrophages [138]. Additionally, PPAR\(\gamma\) agonists were also shown to inhibit phorbol 12-myristate 13-acetate (PMA) - induced syntheses of Interleukin 6 (IL6) and TNF\(\alpha\) in monocytes [139]. Similar to the anti-inflammatory effects of PPAR\(\alpha\), activation of PPAR\(\gamma\) have also been shown to inhibit NF-\(\kappa\)B signaling in macrophages and other cells via trans-repression [140]. PPAR\(\gamma\) inhibits NF-\(\kappa\)B signaling by directly interacting with the p50 and p65 subunits [141]. Additionally, recent reports suggest that PPAR\(\gamma\)-
mediated anti-inflammatory effects are regulated by SUMOylation [37, 142]. Results from Pascual et al., 2005, suggest that the SUMO E3 ligase, Pias1, is involved in the modulation of PPARγ-mediated anti-inflammatory effects via the PPARγ K77 and K365 SUMOylation sites [37, 142]. Ligand-dependent SUMOylation of PPARγ directs the receptor to the promoters of inflammatory genes where it inhibits transcription by stabilizing corepressor complexes [37, 142]. Ligand-activated PPARγ inhibited LPS-induce inflammation in macrophages by preventing the clearance of NCoR and HDAC3 co-repressors; this mechanism could also be involved in modulating the anti-inflammatory effects of other PPARs [37, 142].

In contrast to the beneficial effects of PPARγ as therapeutics for diabetics, inflammation and obesity, the role of PPARγ in tumor development was initially controversial. Initial reports using adenomatous polyposis coli (APC)-deficient mice model, which mimics human familial adenomatous polyposis (FAP) suggested that ligand activation of PPARγ promotes colon polyp formation [143, 144]. Neither report showed any increase colon tumor formation in genetically unsusceptible mice following PPARγ agonist administration, which suggest that promotion of colon tumor development by PPARγ activation depends on the presence of APC mutations. In addition, PPARγ was shown to be elevated in human colon tumors and colon cancer cell lines [145-147]. However, the bulk of the subsequent literature suggests that PPARγ inhibit carcinogenesis. Genetic studies show that PPARγ heterozygous mice have an increased
susceptibility to develop colon tumors [148]. Activation of PPARγ in human colon
cancer cell lines has been shown to inhibit anchorage-independent cell growth
cell growth and promote cell cycle arrest and differentiation [145]. Treatment of
human colorectal cancer cells transplanted into nude mice, with troglitazone
resulted in a significant reduction of tumor growth [146]. Additionally, treatment of
rodents carrying an APC mutation, with troglitazone was shown to inhibit the
early stages of colon carcinogenesis [149, 150]. Examination of human colorectal
carcinomas suggests that somatic mutations resulting in loss of PPARγ function
promotes carcinogenesis [146]. Furthermore, it has been shown that PPARγ-
heterozygous mice treated with the colon specific carcinogen azoxymethane,
have high levels of β-catenin and develop colon cancer with higher incidence
when compared to similarly treated wild-type mice [148]. However, comparision of
PPARγ/APC heterozygous mice to APC-heterozygous mice shows no difference
in colon tumor formation, suggesting that PPARγ is not a very potent tumor
suppressor [148]. In addition, PPARγ heterozygous mice have also been shown
to exhibit an increased susceptibility to tumor development in other tissues
including mammary, ovarian and skin [148]. Activation of PPARγ has been shown
to promote cell cycle arrest in several neoplastic cells including liposarcoma cells
and malignant adipogenic HIB-1B cells [151, 152]. Activation of PPARγ in the
NMU-induced rat model of mammary carcinogenesis has been shown to inhibit
tumor growth, alter cell morphology and promote differentiation; furthermore,
tumorigenesis of MCF-7 cells breast cancer cell line was also inhibited by PPARγ
agonists in nude mice graft models [153-155]. Activation of PPARγ has also been shown to inhibit MCF-7 breast cancer cell growth via proteasome-dependent degradation of cyclin D1 and estrogen receptor alpha [156].

The bulk of the reports examining the role of PPARγ in carcinogenesis suggest a tumor suppressor role, however a better understanding of the mechanism by which PPARγ activation inhibits carcinogenesis is required, and discrepancies in the literature need to be addressed. Overall, there is good evidence that PPARγ agonists have the potential to serve as therapeutic agents in a number of different diseases including Type II diabetes, atherosclerosis and inflammatory disorders, however renewed effort is needed to delineate the role of this receptor in cancers (Figure 1-4).
Activated PPARγ

- Induction of adipocyte differentiation
- Promotes insulin sensitivity and glucose homeostasis
- Promotes lipid and cholesterol homeostasis
- Inhibits inflammation
- Decreased cell cycle progression genes
- Induction of apoptotic genes

Attenuation of metabolic and inflammatory diseases
- Diabetes, Atherosclerosis.

Inhibits carcinogenesis

**Figure 1-4: An overview of PPARγ physiological functions.** PPARγ is actively involved in adipocyte differentiation, insulin sensitivity, cholesterol homeostasis and anti-inflammatory responses, which contributes to the attenuation lipid-associated pathology, such as type II diabetes and other cardiovascular diseases. Additionally, activation of PPARγ results in the inhibition of carcinogenesis.
1.2.4 An overview of PPARβ/δ

PPARβ was first cloned from *Xenopus laevis* in the early 1990s and classified as a novel receptor because of sequence homology to several nuclear receptors including thyroid hormone, retinoids and vitamin D3 receptors [82]. PPARβ was subsequently cloned from a human osteosarcoma cell cDNA library and designated NUCI [157]. Several different research groups cloned PPARβ from mice and termed it, PPARδ, NUCI, and fatty acid activated receptor (FAAR) [101, 158, 159]. Human PPARβ/δ has been mapped to chromosome 6p21.1-p21.2 and mice PPARβ/δ has been mapped to chromosome 17 [160, 161]. Comparison of PPARβ/δ between human and mouse shows high degree of sequence homology, with approximately 90% homology between the LBD [42].

PPARβ/δ is activated by fatty acids and their derivatives. Naturally occurring fatty acids such as linoleic acid are endogenous agonists for PPARβ/δ; both saturated and unsaturated fatty acids have been shown to bind PPARβ/δ, albeit at lower affinity than other PPARs [101, 162, 163]. Arachidonic acid, dihomo-γ-linoleic acid and eicosapentanoic acid are polyunsaturated fatty acids that have been shown to activate PPARβ/δ [162, 164]. Additionally, several eicosanoids including carbaprostacycllin (prostacyclin), prostaglandin A1 and prostaglandin D2 have been shown to activate PPARβ/δ; however, recent studies showed that prostacyclin does not activate PPARβ/δ [164, 165]. Retinoic acids, the well-known activator of retinoic acid receptors, have also been shown to activate PPARβ/δ with high affinity, however recent studies disputed those
reports [163, 166, 167]. Potent activators of PPARβ/δ include the exogenous ligands, phthalate monoesters, tetradecyl-thioacetic acid (TTA), GW501516, GW0742, and L-165041 [47]. L-165041 is a fatty acid-like compound, which shows high affinity for PPARβ/δ compared to the other PPARs [168]. L-165041 was identified as a PPARβ/δ agonist because screening studies show 10-fold selectivity for human PPARβ/δ activation over other PPARs; however selectivity for mouse PPARβ/δ was much less when compared to human PPARβ/δ [168]. Recent studies using high-throughput screening and optimization of chemicals for activation identified very potent synthetic agonists for PPARβ/δ, GW501516 and GW0742 [169]. Ligand activation studies using GW501516 and GW0742 show approximately 1000-fold selectivity for human PPARβ/δ over the other human PPAR isoforms [169]. GW501516 and GW0742 have an EC_{50} of 1.1 nM for human PPARβ/δ and an EC_{50} of 20 nM for mouse PPARβ/δ.
The recent development of *Pparb/d*-null mouse models and potent ligands (Table 1-3), which are crucial tools in the characterization of biological functions of nuclear receptors, have enabled the delineation of PPARβ/δ physiological functions [27, 169-171]. PPARβ/δ is expressed very early in embryogenesis and exhibits ubiquitous tissue distribution, with relatively high expression in adipose tissue, brain, skin and colon, which suggest a physiological role of this receptor in these tissues [77, 172].
Disruption of the PPAR\(\beta/\delta\) gene is lethal during embryonic development due to placental defects [170]. However, backcrossing of surviving \(Pparb/d\)-null mice exhibit no placental defects, suggesting that embryonic lethality might be due to targeting strategies [27]. \(Pparb/d\)-null animals are smaller than control littermates and exhibit reduction of fat mass, skin defects and alteration of myelination [27] (Table 1-3).

The reduction in fat mass as a result of disruption of PPAR\(\beta/\delta\) suggests that PPAR\(\beta/\delta\) plays a critical role in the regulation of metabolic processes including fatty acid metabolism, glucose homeostasis and lipid homeostasis. Activation of PPAR\(\beta/\delta\) has been shown to induce the transcription of the genes encoding fatty acid transporter (FAT), adipocyte lipid-binding protein, and PPAR\(\gamma\), which play critical roles lipid metabolism and adipocyte differentiation [173]. Activation of either PPAR\(\beta/\delta\) or PPAR\(\gamma\) has been shown to enhanced adipocyte differentiation and lipid accumulation in adipocytes [174]. Additionally, the absence of PPAR\(\beta/\delta\) expression results in reduced adipocyte differentiation following treatment with either PPAR\(\beta/\delta\) or PPAR\(\gamma\) ligands; this suggest that both PPAR\(\beta/\delta\) and PPAR\(\gamma\) are essential for adipocyte differentiation and lipid accumulation, and that PPAR\(\beta/\delta\) potentiates adipocyte differentiation mediated by PPAR\(\gamma\) [174]. In addition to promoting lipid homeostasis and adipocytes differentiation in adipocytes, a report by Wang et al. provided evidence about the role PPAR\(\beta/\delta\) in the regulation of fatty acid metabolism and energy utilization in brown adipose tissue \textit{in vivo}. Mice expressing a constitutively active VP-16-
PPARβ/δ transgene (ligand-independent active form of PPARβ/δ, is specifically expressed in white and brown adipose tissues) under the control of the aP2 promoter were lean and had reduced serum triglycerides under obesity and hyperlipidemia inducing conditions; further more, administration of the potent PPARβ/δ agonist GW501516 was shown to promote fat burning and protection against obesity [175]. VP-16-PPARβ/δ transgenic animals exhibited significant induction in genes involved in fatty acid metabolism in brown adipose tissue, including LCAD and CPT-1; however the effects were moderate in the white adipose tissue. In addition to regulating fatty acid and lipid metabolism in adipose tissues, several reports have suggested critical roles for PPARβ/δ in regulation of fatty acid and lipid metabolism in the skeletal muscle. Fatty acid catabolism is very active in muscle and utilization of lipids is enhanced under physiological conditions such as fasting. Gene expression analysis of PPARs show that PPARβ/δ is the predominant isoform expressed in skeletal muscle [176]. In addition, fasting has been shown to upregulate PPARβ/δ expression in mouse skeletal muscles [28]. Treatment of L6 myotubes with GW0742 have been shown to increase fatty acid oxidation [177]. In addition, treatment of muscle C2C12 cells overexpressing PPARβ/δ with PPARβ/δ synthetic agonist or long chain fatty acids results in induction of genes involved in lipid metabolism, including heart-fatty acid binding protein (h-FABP), fatty acid translocase (FAT/CD36), lipoprotein lipase and carnitine palmitoyl transferase I [28].
The induction of lipid metabolism genes by activation of PPARβ/δ, ultimately resulted in the induction of long chain fatty acid oxidation, and these effects were abolished in muscle C2C12 cells overexpressing dominant negative forms of PPARβ/δ [28]. These observations were confirmed by transcriptomic wide analysis of L6 myotubes, which show that PPARβ/δ controls fatty acid catabolism by regulating expression of a large panel of genes involved in fatty acid transport, β-oxidation and mitochondrial respiration [178]. Furthermore, another study showed that in myotubes, fatty acid oxidation is specifically controlled by PPARβ/δ while PPARα and PPARγ regulate glycogen synthesis and lipogenesis, respectively [179]. Examination of muscles overexpressing PPARβ/δ shows an increase in the number of oxidative myofibers, including succinate dehydrogenase (SDH) positive fibers, when compared to control animals. Muscle remodeling involving increase oxidative myofibers involves hyperplasia, with a specific increase in SDH-positive myofibers, in soleus and tibialis anterior muscles and conversion of SDH-negative to SDH-positive fibers. Furthermore, enzymatic and gene expression analyses confirmed that muscle-specific PPARβ/δ overexpression results in increase oxidative enzymatic activities, such as citrate synthase or β-hydroxyacyl-CoA dehydrogenase, and increase expression of genes implicated in fatty acid metabolism [180]. Additionally, muscle PPARβ/δ-overexpressing animals exhibited significant reduction of fat mass including periovarian and peridorsal fat depot weight, due to PPARβ/δ mediated fat burning [180].
These observations were supported by results from Wang et al, 2004, which show that targeted overexpression of activated PPAR\(\beta/\delta\) in the skeletal muscle resulted in induction type I fibers \[181\]. Type I fibers are mitochondria-rich and use mainly oxidative metabolism for energy production, which provides a stable and long-lasting supply of ATP, and thus are fatigue-resistant \[181\]. Additionally, over-expression of activated PPAR\(\beta/\delta\) resulted in significant induction of oxidation enzymes, mitochondrial biogenesis, and production of specialized type I fiber contractile proteins that are essential for muscle fiber type switching \[181\]. Targeted over-expression of activated PPAR\(\beta/\delta\) in the skeletal muscle enhanced animal endurance, and confers resistance to obesity and improved metabolic profiles in animals under obesity inducing conditions (high-fat diet) \[181\]. Additionally, treatment of wild-type mice with PPAR\(\beta/\delta\) agonist also elicited a similar type I fiber gene expression profile in muscle and protected animals from obesity \[181\]. In addition to regulation fatty acid metabolism in adipose tissue and skeleton muscle, PPAR\(\beta/\delta\) is has also been implicated in the regulation of fatty acid metabolism in the heart. Recent reports showed that PPAR\(\beta/\delta\) is highly expressed in the heart and treatment of cardiomyocytes with PPAR\(\beta/\delta\) agonist results in upregulation of genes involve in fatty acid catabolism and enhanced fatty acid oxidation \[182, 183\]. Additionally, heart-specific disruption of PPAR\(\beta/\delta\) gene resulted in cardiac dysfunctions, progressive cardiac lipotoxicity, cardiac hypertrophy and heart failure with increased mortality \[182, 183\].
PPARβ/δ has also been implicated in the regulation of lipid metabolism in the brain and activation of PPARβ/δ has been shown to regulate the expression ACS2, which plays a critical role for PPARβ/δ in brain lipid metabolism [184].

PPARβ/δ regulation of fatty acid metabolism and lipid homeostasis in metabolic tissues suggests a critical role for PPARβ/δ in glucose homeostasis. Recently it was reported that Pparb/d-null mice are metabolically less active, glucose intolerant, and that administration of GW501516 improved insulin sensitivity in high fat diet feed mice that carry functional PPARβ/δ gene but not in Pparb/d−null mice [185]. Additionally, administration GW501516 also improves glucose intolerance, normalized the glucose and insulin levels, and increased the serum high-density lipoprotein (HDL) cholesterol levels in monosodium L-glutamate-induced metabolic syndrome mice [186]. Furthermore, gene expression analysis show that the above phenotypes might be due to enhancement of fatty acid oxidation in muscle, adipose tissue and the liver, improvement of insulin-stimulated glucose transportation in skeletal muscle and adipose tissue [186]. In 2004, Akiyama et al. showed that Pparb/d-null mice fed with a high fat diet exhibit increased adiposity, increased levels of serum triglycerides, increased rate of hepatic very low density lipoprotein (VLDL) synthesis when compared to wild-type mice, and suggested that this effect this was due to elevation of the very low density lipoprotein (VLDL) and reduction of lipoprotein lipase [187].
Furthermore, administration of the PPAR\(\beta/\delta\) agonist GW501516 to obese mice resulted in reduction of adiposity and improvement of insulin sensitivity [175, 178]. Additionally, administration of GW501516 decreased glucose and insulin levels in obese rhesus monkeys [188]. Administration of GW501516 has also been shown to markedly increase serum high-density lipoproteins (HDL) levels, while lowering serum triglyceride (TG) levels in insulin-resistant obese rhesus monkeys [188]. Previous studies also show that treatment of db/db (obese) mice with L-165041 raised HDL levels without changes in LDL [189]. These effects were suggested to be mediated by PPAR\(\beta/\delta\) dependent induction of ABCA1, which functions to reverse cholesterol transport.

Chronic inflammation is closely associated with the progression of a variety of metabolic diseases, such as atherosclerosis and diabetes. Emerging evidence in literature suggests that PPAR\(\beta/\delta\) exerts anti-inflammatory effects in multiple tissues. Several reports have suggested a role for PPAR \(\beta/\delta\) in the modulation of arteriosclerosis, a metabolic and inflammation associated disease. In atherosclerotic lesions within the artery of \(Ldlr^{-/-}\) mice fed a high-fat diet, activation of PPAR\(\beta/\delta\) with GW0742 inhibited the expression of pro-inflammatory molecules TNF\(\alpha\), IFN\(\gamma\), monocyte chemotactic protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) [190]. Additionally, GW0742 has been shown to effectively inhibit LPS-induced iNOS and COX2 expression in macrophages [191].
In human endothelial cells, PPARβ/δ agonist L-165041 significantly down-regulated VCAM-1 and MCP-1 expression, and also caused an inhibition of NFκB nuclear translocation [192]. Lipid-accumulating macrophages, also referred to as foam cells, are critical component in the development of atherosclerotic lesions. PPARβ/δ agonist promotes macrophage activation and induces the expression of scavenger receptor A (SRA) and scavenger receptor B (SRB), which are involved in lipid uptake in macrophages [193]. Additionally, PPARβ/δ induces transcriptional expression of adipose differentiation-related protein (ADRP) in response to oxidized LDL products in macrophages, which results in increased triglycerides levels in these immune cells [194]. Ironically, PPARβ/δ agonist increases the expression of the cholesterol efflux mediator ATP-binding cassette transporter (ABCA1) in macrophages [188]. PPARβ/δ modulates inflammation and atherosclerosis via transcriptional repression. Lee et al. demonstrated that dissociation of PPARβ/δ with the transcriptional repressor B-cell CLL/lymphoma 6 (BCL-6) following ligand binding, results in suppression of cytokines and chemokines synthesis by BCL-6, which lead to the attenuation of inflammation [17]. In addition, mutant PPARβ/δ receptor carrying a deficiency in the ligand-induced coregulator release is a potent inducer of the monocyte chemoattractant protein MCP-1 and IL-1β but exhibits loss of function for ligand-induced suppression of MCP-1 and IL-1β expression [17].
This report suggests that ligand activation of PPAR β/δ can repress the expression of MCP-1 and IL-1β by modulating the exchange between corepressor and coactivator association with PPARβ/δ transcriptional complex. Inconsistency concerning the role of PPARβ/δ in atherosclerosis in the previously mentioned studies may be due to the strain differences of animals, cell lines and/or receptor-independent effects of the PPARβ/δ synthetic ligands used in the studies; however more research is needed to determine the specific mechanisms underlying these disparities. In rat cardiomyocytes, activation of PPARβ/δ resulted in the inhibition of lipopolysaccharide (LPS)-induced TNFα production by interfering with NF-κB signaling by the mechanism known as transrepression [195]. This is consistent with previous studies showing PPARβ/δ inhibition of NF-κB transcriptional activity by physically interacting with the N-terminal Rel homology domain of p65 as a mechanism for PPARβ/δ anti-inflammatory effects [94, 196, 197]. Additionally, activation of PPARβ/δ inhibited NF-κB nuclear translocation in human endothelial cells [192]. Shan et al, also showed that PPARβ/δ attenuates hepatic toxicity by inhibiting the expression of several NFκB proinflammatory target genes in a chemically-induced liver toxicity model [198, 199]. Expression of TNF-α was higher in Pparb/d-null primary hepatocytes in response to interleukin-1β treatment compared to similarly treated wild-type hepatocytes, however agonist treatment did not affect TNFα expression in either genotype [199].
Furthermore, Peters et al. reported that Pparb/d-null mice exhibited refractory response to the anti-inflammatory drug sulindac in the 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced inflammation model [27]. In a recent study, it was also shown that treatment with PPAR β/δ agonist ameliorates TPA-induced inflammation [200]. Crosstalk between the PPARβ/δ and other nuclear receptors has also become intriguing topic of late. In 2005, Moulin et al. reported that the PPARγ agonist rosiglitazone stimulates interleukin-1 receptor antagonist (IL-1Ra), a natural inhibitor of IL-1β signaling, in synovial fibroblasts in a PPAR β/δ-dependent manner [201]. Several studies have shown that SUMOylation is involved in the modulation of PPARγ-mediated anti-inflammatory effects; this regulatory mechanism could also be involved the regulation of other PPARs including PPARβ/δ [37, 142]. A recent report by Oishi et al., suggests that SUMOylation indirectly regulates PPARβ/δ-mediated transrepression by modulating the activity of Krüppel-like transcription factor 5 (KLF5) [202]. These studies suggest that PPAR β/δ agonists can be use as therapeutics for several metabolic diseases, including obesity and diabetes, as well as certain inflammatory diseases including atherosclerosis.

As mentioned earlier, PPARβ/δ is highly expressed in the skin, this suggest a role for this receptor in maintaining normal skin physiology. Additionally, PPARβ/δ expression is rapidly upregulated following cutaneous injury or irritation. Wound repair involves the covering of the wounded area with a newly differentiated protective epidermal layer. Wound repair requires the
integration of interdependent processes and signals that involve, among others, inflammatory cytokines produced by a variety of cell types, cell proliferation and migration, cell differentiation, and production of extracellular matrix components. The initial inflammatory stage of wound repair is followed by the proliferation and migration of keratinocytes to wound site, a process called re-epithelialization. Concomitant dermal repair involves the recruitment and proliferation of fibroblasts and angiogenesis. Several studies show skin wound-healing delay in PPAR\(\beta/\delta\)-heterozygous mice and attribute this phenotype to a disruption of the balance between proliferation and apoptosis, as well as to defects in migration and cell adhesion of PPAR\(\beta/\delta\)-heterozygous keratinocytes [203-205]. Additionally, skin healing is delayed in the Pparb/d-null mice [206]. Recent studies showed that interplay between PPAR\(\beta/\delta\) and the C/EBP transcription factors controls proliferation and differentiation in keratinocytes [207]. C/EBP\(\alpha\) and C/EBP\(\beta\), members of the C/EBP family of transcription factors were shown to inhibit the basal expression of PPAR\(\beta/\delta\) in mouse keratinocytes, through its recruitment of a complex containing histone deacetylase-1 (HDAC-1) to the PPAR\(\beta/\delta\) promoter. Additionally, the expression patterns of PPAR\(\beta/\delta\) and C/EBPs are mutually exclusive in keratinocytes of the interfollicular epidermis and hair follicles in the skin. PPAR\(\beta/\delta\) expression is undetectable in the interfollicular epidermis of healthy rodent skin but upregulated during the entire wound healing process. The expression of PPAR\(\beta/\delta\) is increased via binding of the AP-1 transcription factor complex to its promoter, which is triggered by the
activation of the stress associated protein kinase pathway by pro-inflammatory cytokines, such as TNFα [204].

In addition to the induction of pro-inflammatory cytokines during wound healing, production of endogenous PPARβ/δ ligand is also induced during wound healing, and results in maximal PPARβ/δ activation [204]. Following epithelialization of wound site, TNFα-induced PPARβ/δ expression is repressed by TGFβ-1 signaling, which inhibits AP-1 binding to the PPARβ/δ promoter [208]. Furthermore, prolonged expression of PPARβ/δ is obtained through down-regulation of TGFβ-1 activity [209]. PPARβ/δ regulation of keratinocyte survival in injured epidermis is mediated by the transcriptional regulation of genes coding for integrin-linked kinase (ILK) and 3-phosphoinositide-dependent kinase-1 (PDK1), which are critical anti-apoptotic signaling proteins [210]. Additionally, exogenous application of TGFβ-1 on a skin wound at day 2 following the injury resulted in the downregulation of PPARβ/δ expression and PKBα/Akt1 activity and a transient delay in wound closure [211]. PPARβ/δ expression has also reported to be induced in hyperproliferative psoriatic skin lesions [197, 212]. As in the case with injured skin, increased PPARβ/δ expression in psoriatic lesions is most probably due to pro-inflammatory signals. Furthermore, numerous lipid molecules, such as lipoxygenase products, which are potent activators of PPARβ/δ in human keratinocytes, have been reported to accumulate in the psoriatic lesions [197].
Despite the potential therapeutic role for PPARβ/δ agonists in the modulation of various metabolic syndromes, its role in carcinogenesis is very controversial. Some reports suggest that PPARβ/δ promotes epithelial carcinogenesis, while others suggest that PPARβ/δ inhibits epithelial carcinogenesis. He et al., 1999 reported that adenoma polyposis colitis (APC) tumor suppressor gene negatively regulates PPARβ/δ expression in colorectal tumor cell lines [213]. Mutation of the APC allele results in the abrogation of APC mediated suppression of the β-catenin/TCF4 regulatory pathway, causing an up-regulation of oncogenes including cyclin D1, c-myc and ultimately uncontrolled proliferation of colon cells. Additionally, this report suggested that PPARβ/δ is upregulated by the β-catenin/transcription factor 4 (TCF4) cell proliferation pathway, and that activation of PPARβ/δ by cyclooxygenase (COX) derived metabolites promotes colon carcinogenesis [213]. β-catenin/TCF4 upregulation of PPARβ/δ was suggested to be a possible mechanism by which β-catenin potentiate colon carcinogenesis. Furthermore, inhibition of COX metabolism is known to inhibit colon cancer, and it was suggested that the mechanism underlying this inhibition of carcinogenesis was that COX-derived metabolites function as PPARβ/δ ligands that stimulate cell proliferation. Subsequent reports also suggested that PPARβ/δ promotes colon cancer; Gupta et al. reported that increased PPARβ/δ expression correlates with elevated levels of COX-2 in colon cancer [214].
Park et al. also reported that PPARβ/δ deficient colorectal tumor lines exhibited decreased tumorigenesis in xenograft cancer model [214, 215]. Recently it was reported that treatment with PPARβ/δ agonist enhances small intestine tumorigenesis in the APC\textsuperscript{min} colon cancer model [216]. Additionally, Shao et al. show that PPARβ/δ is up-regulated and highly activated in K-Ras-transformed rat intestinal epithelial cells due to increased mitogen-activated protein kinase activity and receptor activation by endogenous production of prostacyclin via the cyclooxygenase-2 pathway [217]. Furthermore, administration of PGE\textsubscript{2} to APC\textsuperscript{min} mice has been shown to exacerbate intestinal tumorigenesis, and these effects were lacking in APC\textsuperscript{min} mice on a Ppar\textsubscript{b/d}.null background [218]. Similar to the role of PPARβ/δ in wound healing model, administration of PPARβ/δ agonists is reported to suppress apoptosis in colon cancer cell lines [216, 218]. Di-Poi et al., 2002 suggested that PPARβ/δ inhibits stress-induced apoptosis via the upregulation of anti-apoptotic signaling proteins phosphoinoside-dependent kinase 1 (PDK1) and integrin-linked kinase (ILK) and the down-regulation of the pro-apoptotic signaling protein (phosphatase and tensin homolog deleted on chromosome 10 (PTEN)) [210, 219]. A subsequent study suggested that the anti-apoptotic signaling mediated by PPARβ/δ promotes efficient wound healing in a disease model [210, 219].
Although these reports seem to contradict the findings that PPARβ/δ promotes apoptosis, it is possible that PPARβ/δ exhibits differential response in the regulation of apoptosis under different physiological/pathological conditions. In contrast to the above reports, there is a large body of literature suggesting that PPARβ/δ inhibits epithelial cancers. Genetic mutation of the APC allele in mouse colon was found to cause decreased, not increased, expression of Pparb/d mRNA and protein [220]. Additionally, gene expression analysis of cells expressing a dominant negative TCF-4 revealed no significant differences in PPARβ/δ expression [221]. Analysis of PPARβ/δ expression in intestinal polyps or normal intestinal tissues of APC\textsuperscript{min} mice showed that PPARβ/δ expression is either unchanged or decreased as compared to controls [220, 222-224]. Additionally, gene expression analysis of human tumor samples found that PPARβ/δ expression was decreased in tumor samples when compared to matched normal control tissue [222, 225]. Collectively, these reports raise doubts regarding the role of PPARβ/δ in colon carcinogenesis and suggest that decreased expression of PPARβ/δ could be causally linked to the progression of colon tumorigenesis. In a recent study, it was reported that colon carcinogenesis is exacerbated in Pparb/d-null mice in both APC\textsuperscript{min} and chemical (azoxymethane)-induced colon cancer models [223].
A subsequent report suggested that ligand activation of PPAR\(\beta/\delta\) attenuates chemically-induced colon carcinogenesis [226]. Additionally, treatment of human colonocytes with PPAR\(\beta/\delta\) agonist results in decreased cell growth and does not support the notion that ligand activation of PPAR\(\beta/\delta\) potentiates colon cancer cell growth [227]. Lastly, administration of the pan PPAR agonist bezafibrate was shown to decrease aberrant crypt formation and decrease the number of intestinal polyps in \(\text{APC}^{\text{min}}\) mice [150, 228]. However, bezafibrate not only effectively activate PPAR\(\beta/\delta\), but also activates PPAR\(\alpha\) and PPAR\(\gamma\) with an EC\(_{50}\) ranging from 55 to 110 \(\mu\text{M}\) [229]. This suggests that inhibition of intestinal tumorigenesis by bezafibrate could be mediated by PPAR\(\beta/\delta\) and other PPARs. Overall, considerable controversy remains regarding the role of PPAR\(\beta/\delta\) in colon cancer, however the bulk of the literature suggest the possibility that PPAR\(\beta/\delta\) ligands could actually be utilized as a chemopreventive strategy. Examination of the effect of PPAR\(\beta/\delta\) on carcinogenesis in other cell types show that non-small-cell lung cancer (NSCLC) cells express PPAR\(\beta/\delta\) protein and that treatment with the selective PPAR\(\beta/\delta\) agonist (GW501516) increases EP4 expression and promotes cell proliferation in a PPAR\(\beta/\delta\) dependent manner [230]. Treatment of NSCLC cells with GW501516 was shown to increase phosphorylation of Akt and decreased PTEN expression [230].
Additionally, pretreatment of NSCLC cells with GW501516 further increased NSCLC cell proliferation in response to exogenous dimethyl-prostaglandin E2 (PGE$_2$) and this effect was abrogated in the absence of PPAR$\beta/\delta$ expression [230]. In a similar study, activation of PPAR$\beta/\delta$ was also shown to stimulate NSCLC cell proliferation and anchorage-independent cell growth and inhibits apoptosis in a PPAR$\beta/\delta$ dependent manner [231]. Furthermore, the PPAR$\beta/\delta$ mediated induction Akt phosphorylation correlated with upregulation of PDK1, downregulation of PTEN, and increased expression of Bcl-xL and COX-2. However, more recent studies raised serious concern since ligand activation of PPAR$\beta/\delta$ had no influence on PTEN or PDK in the same cell line [230]. These studies suggest that PPAR$\beta/\delta$ exerts proliferative and anti-apoptotic effect via PI3K/Akt1 and COX-2 pathways. Furthermore, Müller-Brüsselbach et al. showed impaired growth of syngeneic wild-type tumors in $Pparb/d$-null mice, with a concomitant inhibition of blood flow and an abundance of hyperplastic microvascular structures [232]. However, matrigel plugs containing pro-angiogenic growth factors harbor increased numbers of morphologically immature, proliferating endothelial cells in $Pparb/d$-null mice, and retroviral transduction of PPAR$\beta/\delta$ triggers microvessel maturation; suggesting that the absence of PPAR$\beta/\delta$ dependent regulation of angiogenesis might be responsible for the inhibition of tumor growth in $Pparb/d$–null animals [232].
Indeed, the Cdkn1c gene encoding the cell cycle inhibitor p57 (Kip2) was identified as a PPARβ/δ target gene and as the mediator of the PPARβ/δ-mediated inhibition of cell proliferation, which provides a possible explanation for the observed tumor endothelial hyperplasia and deregulation of tumor angiogenesis in the absence expression [232]. Contrary to the above report that shows PPARβ/δ inhibits endothelial cell proliferation, treatment of several endothelial cells with GW501516 increased human endothelial cell proliferation and endothelial cell outgrowth from murine aortic vessels in vitro, and angiogenesis in a murine matrigel plug assay in vivo [233]. Additionally, GW501516 induced vascular endothelial cell growth factor mRNA and peptide release, as well as adipose differentiation-related protein (ADRP), a PPARβ/δ target gene [233]. GW501516-induced proliferation, morphogenesis, vascular endothelial growth factor (VEGF), and ADRP were absent in endothelial cells transfected with dominant-negative PPARβ/δ [233]. Overall, the reports suggest that PPARβ/δ promotes carcinogenesis, however examination of the effect of PPARβ/δ on other cancer cell type, shows a tumor suppressor role for PPARβ/δ. Evidence supporting a tumor suppressor role for PPARβ/δ was reported by Girroir et al. showing inhibition of cell growth in both MCF7 and UACC903 cell lines cultured in the presence of either GW0742 or GW501516, and the presence or absence of serum had little influence on this inhibition [234].
Additionally, both tumor cell lines exhibited an upregulation of the known PPARβ/δ target gene angiopoietin-like protein 4 (ANGPTL4) following treatment with either GW0742 or GW501516 [234]. Furthermore, treatment of various human cancer cell lines (HT29, HCT116, LS-174T, HepG2 and HuH7) with the GW0742 and GW501516 in the presence or absence of serum inhibited cell growth, but had no effect on phosphorylation of Akt, or expression of VEGF, COX2 [235]. Similarly, liver, colon and colon polyps from mice administered PPARβ/δ agonists in vivo did not exhibit changes in those similar markers [235]. These reports suggest that PPARβ/δ inhibits carcinogenesis, and that the PPARβ/δ dependent promotion of carcinogenesis observed in lung cancer cell may be cell type and/or culture condition dependent. In 2004, Maggiora et al., provided evidence to support the hypothesis that PPARβ/δ activation might have contradictory effect on tumor cells based on cell type and/or tissue culture condition [236]. Treatment of bladder, hepatic, prostate and mammary human tumor cell lines with linoleic acid, an endogenous PPARβ/δ agonist exerted different effects, ranging from inhibitory to neutral, even promoting growth; while conjugated linoleic acid, another PPARβ/δ agonist inhibited growth in all lines examined and was particularly effective against the more malignant cells, with the exception of mammary tumor cells [236]. In addition to the above studies, examination of the role of PPARβ/δ in skin carcinogenesis also suggests a tumor suppressor role.
The first evidence to suggest that PPARβ/δ inhibit skin carcinogenesis was the observation that animals lacking PPARβ/δ exhibited enhanced epidermal hyperplasia in response to the tumor promoter TPA [219]. Recent evidence showed that mice lacking PPARβ/δ exhibited enhanced skin carcinogenesis in the 7,12-dimethylbenz anthracene (DMBA)/12-O tetra-decanoylphorbol-13-acetate (TPA) (two-stage) chemical carcinogenesis bioassay [237]. Pparb/d-null mice exhibited early onset of tumor formation, increased tumor incidence, and increased tumor size when compared to wild-type mice [237]. Pparb/d-null mice also exhibited enhanced epidermal hyperplasia and cell proliferation, and reduced caspase 3 activity and apoptosis in response to topical treatment with TPA [237]. Results from Kim et al., 2004 suggested that PPARβ/δ dependent transcriptional upregulation of ubiquitin C (UbC) was partially responsible for the attenuation of skin carcinogenesis [237]. In another report, Kim et al. suggested that PPARβ/δ inhibits epidermal cell proliferation through the inhibition of kinase (protein kinase C-α(PKCα/Raf/MEK/ERK)) activity via the ubiquitin-mediated turnover of the upstream target PKCα [238]. Additionally, several reports show that activation of PPARβ/δ inhibits proliferation in various rodent and human cancer cell lines, and also inhibited the MAPK signaling cascade [167, 219, 234, 239]. Furthermore, subsequent studies showed that PPARβ/δ agonist selectively inhibits epidermal cell proliferation and induced differentiation in a PPARβ/δ dependent manner [46].
The first suggestion that PPARβ/δ influences epidermal differentiation were the observations that Pparb/d mRNA expression is increased in phorbol ester treated cultured human keratinocytes and mouse skin, and that this increase is correlated with increased expression of markers for keratinocyte differentiation [240]. The number of cornified envelopes in neonatal skin of Pparb/d-null mice is significantly lower as compared to wild-type mice [238]. Despite the induction of cornified envelope formation by PPARβ/δ, the expression of mRNA markers of differentiation including small proline-rich proteins (SPRs), transglutaminase-1 (TG1) and involucrin is comparable between wild-type and Pparb/d-null mouse skin following administration of phorbol ester, suggesting that PPARβ/δ is dispensable in phorbol ester signaling [27]. Furthermore, induction of terminal differentiation marker mRNAs is similar in cultured primary keratinocytes from both wild-type and Pparb/d-null mice treated with either phorbol ester or high culture medium calcium, despite significantly increased PPARβ/δ expression [238]. These findings suggest that activation of PPARβ/δ could potentiate terminal differentiation, but is dispensable in the presence of more potent differentiation inducers. Indeed, several recent reports showed that ligand activation of PPARβ/δ can result in terminal differentiation in skin [200, 204, 238, 241]. In addition to inducing terminal differentiation in normal skin, the PPARβ/δ agonist GW501516 has also been shown to stimulate terminal differentiation in a hyperplasia disease model [200].
Additionally, ligand activation of PPARβ/δ induces increased expression of proteins required for terminal differentiation, including TG1, SPRs and involucrin and ultimately induced cornified envelope formation [238]. PPARβ/δ agonists have also been shown to induce terminal differentiation in other cell types [242-244]. These reports demonstrate that PPARβ/δ promotes terminal differentiation, and that this could be a mechanism by which PPARβ/δ exert its anti-carcinogenic effects, since induction of keratinocyte terminal differentiation is known to be associated with the induction of an apoptotic-like pathway [245]. Attenuation of epithelial carcinogenesis usually involves the inhibition of proliferation, induction of differentiation and subsequent induction of apoptosis. In support of the hypothesis that PPARβ/δ attenuates skin carcinogenesis, reports have demonstrated that activation of PPARβ/δ induces apoptotic signaling [237, 246].

Many well established drugs use in the treatment of dyslipidemias and type 2 diabetes have been shown to exert their biological effects via PPARα and PPARγ respectively [178, 179, 185, 247-249]. Furthermore, PPARβ/δ has recently been implicated in the physiological modulation of obesity, hyperlipidemia, cholesterol homeostasis and type 2 diabetes [178, 179, 185, 247-249]. These findings have lead to the development of therapeutics that target PPARβ/δ for activation, despite the controversy surrounding the role of PPARβ/δ in epithelial cancers; thus understanding the role of PPARβ/δ in carcinogenesis is of critical importance [250, 251].
Activated PPARβ/δ

- Increased FA catabolism
- Induction of adipocyte differentiation
- Promotes insulin sensitivity and glucose homeostasis
- Promotes lipid and cholesterol homeostasis

- Inhibits inflammation
- Induces/inhibits differentiation (?)
- Promotes/inhibits proliferation (?)
- Promotes/inhibits apoptosis (?)

Attenuation of metabolic and inflammatory diseases
- Diabetes, Atherosclerosis.

Inhibits/promotes carcinogenesis (?)

**Figure 1-5: An overview of PPARβ/δ physiological functions.** PPARβ/δ is actively involved in adipocyte differentiation, insulin sensitivity, cholesterol homeostasis and anti-inflammatory responses, which contributes to the attenuation lipid-associated pathology, such as type II diabetes and other cardiovascular diseases. However, there is controversy surrounding the role of PPARβ/δ in carcinogenesis.
1.3 Skin Carcinogenesis

1.3.1 Structure and physiology of the skin

The skin is the largest organ of the human body, accounting for 16% of total body weight [252-255]. The skin is the body first line of defense against external threats. The skin protects the body against pathogenic agents, environmental threats, toxic chemicals and loss of body fluids [255]. The skin also plays critical role in the regulation of body temperature, energy storage, insulation, sensation, synthesis of vitamin D and the protection of vitamin B folates [255]. The skin can be divided into three primary layers, namely hypodermis, dermis and epidermis [256].

The hypodermis is the lower most layer of the skin and is made of loose connective tissue containing subcutaneous fat. The dermis is the layer sandwich between the hypodermis and the epidermis layer. The dermis is tightly connected to the epidermis by a basement membrane, and contains nerve endings that enable the sense of touch and heat/cold (temperature). The dermis is made of connective tissue, blood vessels, nerve endings, hair follicles, sebaceous glands, apocrine glands and sweat glands. The epidermis is the outermost layer of the skin, and provides the first line of defense against the external environment [253, 254].
The epidermis is composed of five layers, namely stratum germinativum (basal), stratum spinosum (spinous), stratum granulosum (granular), stratum lucidum, and stratum corneum (cornified) [253, 254, 257, 258]. The basal layer is the innermost layer of the epidermis and is composed a distinct population of keratinocyte stem cells that inherently has a potential for self-renewal, and provides a continuous supply of progenitor cells to repopulate the epidermis. Additionally, the basal layer is also composed of melanocytes, which make approximately 25% of the cells in this layer. Melanocytes produce melanin, which is a pigment found in skin, eyes, and hair. Terminal differentiation of keratinocytes begins when stem cells in this basal layer divide and generate daughter cells that no longer retain their self-renewal ability. These differentiated keratinocytes progress up through the epidermis to the spinous layer. The spinous layer is the second layer of the epidermis and is made of cuboidal cells joined by desmosomes, which are intercellular adhesion complexes. The cells of the spinous layer synthesize cytokeratins, which are intermediate filaments composed of keratins. Cytokeratins are anchored to the desmosomes, joining adjacent cells to provide structural support. As keratinocytes become further differentiated, they progressed to the next upper layer, the granular layer. Additionally, the spinous layer is also composed of Langerhans cells, which are dendritic cells containing large granules.
The granular layer contains a few rows of squamous cells, which contains many small basophilic granules in their cytoplasm. The granules are composed of proteins formed during differentiation and are assembled into various structures. The stratum lucidum also refer to as the clear layer, is a thin layer of dead skin cells in the epidermis, and is named for its translucent appearance when view using microscopy. This layer contains a clear substance called eleidin, which is very similar to keratin.

The cornified layer is the outermost layer of the epidermis and is composed mainly of dead cells that lack nuclei and other cellular organelles. The cells in the cornified enveloped are embedded within an intracellular matrix of lipid that is deposited through secretion by lamellar bodies. The dead keratinocytes in the cornified layer are surrounded by an insoluble protein envelope, known as the cornified envelope. The cornified envelope provides a unique and efficient protective surface against outside environment. Additionally, the lipid layer is also an important barrier of the cornified layer, as it prevents the loss of fluids and ions. As these dead cells slough off, they are continuously replaced by new cells from the basal layer [253, 254].

The structural integrity and differentiation state of epidermal keratinocytes is maintained primarily by keratins. Keratins are intermediate filament proteins that are tough and insoluble and form an extensive cytoskeletal network that form hard but non-mineralized structures found in many animals including reptiles, mammals, birds [257-259].
Keratins can be divided into two major groups on the basis of amino acid sequence. Type I keratins are acidic keratins with molecular masses of 40-60 kDa and type II keratins are basic keratins with molecular masses of 50-70 kDa.

Keratin proteins are assembled into filaments by forming heterodimers composing of an acidic keratin and a basic keratin [253, 254, 259]. Expression of specific keratins in epidermal keratinocyte is tightly regulated and serves as markers for keratinocyte differentiation. The type II keratin K5 and type I keratin K14 are expressed and constitute about 30% of the protein expressed in keratinocytes in the basal layer of the epidermis. The type II keratin K1 and type I keratin K10 are highly expressed in keratinocytes in suprabasal layers, and are critical markers for the induction of differentiation in epidermal keratinocytes [259].

In addition to the induction of K1 and K10, several different structural proteins are induced in keratinocytes in the suprabsal layer as these cells commit to terminal differentiation. Involucrin is induced as keratinocytes commit to terminal differentiation and is a major component of the cornified envelope. Involucrin is cross-linked to almost all other proteins in the cornified envelope by transglutaminase [260, 261]. Transglutaminases are a family of 8 enzymes that catalyzes the formation of covalent bond between a free amine group and the gamma-carboxamid group of protein- or peptide-bound glutamine of structural proteins. Transglutaminase-I also referred to as keratinocyte transglutaminase is highly expressed in the skin epithelia and is the major cross-linking enzyme in keratinocytes [262, 263].
Other structural proteins expressed during keratinocyte differentiation include small proline-rich proteins (SPR), loricrin, and filaggrin. SPRs are differentiation proteins with high proline contents and range in molecular masses of 8-18 kDa. Loricrin is a major component of the cornified envelope in terminally differentiated epidermal cells. Loricrins are cross-linked together with SPRs by transglutaminase to form the cornified envelope reinforcement complex in epidermal cells [264]. The progression of keratinocytes through the epidermis and the concomitant induction of terminal differentiation is control by the combined expression of various structural proteins and transglutaminases and the cross-linking of these proteins into an insoluble protective complex, the cornified envelope [253, 254]. These dead-cornified cells (cornified envelopes) make up the outermost layer in the epidermis and functions as a protective barrier for the skin [253, 254, 265].

Keratinocyte differentiation and proliferation is regulated by several biological factors. Epidermal growth factor (EGF) and transforming growth factor β (TGFβ) are critical for keratinocyte migration and proliferation [266-270]. Additionally, TGFβ has opposing role in keratinocyte differentiation and proliferation that is concentration dependent; it inhibits keratinocyte growth at low concentrations, but it inhibits normal differentiation at high concentration [271]. Another critical regulator of keratinocyte differentiation and proliferation is calcium. Primary keratinocytes proliferate rapidly under low calcium condition (0.05 mM) but undergo terminal differentiation under higher concentration levels (0.12–1.2 mM) [272, 273].
Under normal healthy conditions the tightly control regulation of proliferation and differentiation is necessary for maintaining normal skin physiology and structure; however the emergence of pathological condition(s) in the skin require alteration in the regulation of epidermal proliferation and differentiation. Keratinocyte proliferation and differentiation is regulated by an alternate process called activation during wound healing and other pathological conditions [274]. During activation, keratinocytes become migratory and hyperproliferative and produce cytokines, chemokines, keratins K6, K16, and K17. Activated keratinocytes undergo an alteration their cytoskeleton and become contractile. Additionally, activated keratinocytes augment the levels of cell surface receptors, and produce components of the basement membrane, which results in re-epithelialization of the wound-site. Activated keratinocytes at the injury site also alter signaling in surrounding cell types by producing autocrine and paracrine signals, which aids in the recruitment of additional cells in the wound healing process [274]. The balance between keratinocyte proliferation and differentiation is critical for normal skin physiology, and the perturbation of this balance by exogenous factors and defects in critical regulators can lead to pathological conditions such as psoriasis and skin cancer [275].
Figure 1-6: Skin structure and physiology. Adapted from http://www.mydr.com.au
1.3.2 Mechanisms of Skin Carcinogenesis

Cancer is a disease caused by the deregulation of cell proliferation, apoptosis and differentiation, which leads to uncontrolled cell growth, tumor formation and progression. Carcinogenesis is caused by a myriad of factors including viruses, industrial/environmental toxicants, genomic instability; which can act independently or in complex combinations [276-280]. Cancer is a major public health concern, with skin cancer having the highest incidence in the United States and most developed countries.

A major cause of skin cancer in humans is ultraviolet radiation (UV) radiation, which produces DNA lesions in affected skin. UV radiation produces DNA mutations in critical proto-oncogenes and tumor suppressor genes which could lead to skin cancer development and progression [281, 282]. Additionally, industrial chemicals have been linked to the increased in skin cancer in humans due to the ubiquitous nature of some of these toxicants. Skin tumors can be divided into two broad types based on the ability of the tumor to metastasize [283]. Benign skin tumor is characterized by the inability of the tumor to invade surrounding tissue or metastasize, and includes papillomas and keratochanomas. Malignant skin tumor is characterized by the ability of the tumor to invade surrounding tissue or metastasize, and includes basal cell carcinomas (BCC), squamous cell carcinomas (SCC), and melanomas [283].
Basal cell carcinomas develop in the basal cell layer of the skin, while squamous cell carcinomas originate from squamous cells in the epidermis (Mukhtar et al., 1995). BCC and SCC readily invade surrounding tissues, but their propensity to metastasize is far lower than melanomas [283]. Furthermore, BCC and SCC are the most common form of human skin cancer but rarely result in fatality. Melanoma is a malignant tumor of melanocytes, which are found predominantly in basal layer of the epidermis [280, 283]. Melanoma is a rare type of human skin cancer but causes the majority of skin cancer related deaths due to its ability to readily metastasize. Recent evidence suggests that malignant melanoma stem cells (MMSC) may be responsible for high degree of tumor invasiveness, neoplastic progression and resistance to cytotoxic agents observed in melanomas [280, 283]. Normal adult stem cells reside in most somatic tissues, where they maintain tissue homeostasis and repair damage tissue, for example, in the skin [284]. These relatively rare, tissue-specific stem cells are defined by their ability for self-renewal, and by their ability to give rise to progenitor, which further differentiates into the mature cell types that constitute the tissue of origin; however they provide ideal candidates for tumor initiation [285]. Cancer stem cells (CSC) have been identified in hematopoietic cancer and several solid cancers including breast cancers, medulloblastoma, glioblastoma, and colon cancer [286-288].
Similar to normal stem cells, CSC are capable of self-renewal and differentiation of progenitor cells and have the potential for indefinite proliferation, a characteristic through which they may cause tumor growth and malignant conversion. The transition of epidermal cells from a normal cellular state to that of a tumorigenic or malignant state involves genetic and/or epigenetic alterations. In order to study the molecular mechanisms of skin carcinogenesis several animal models have been developed.

The chemically (two-stage)-induced skin carcinogenesis model is a widely used model, which provided insight into the multistage nature of epithelial carcinogenesis [289-294]. The development of cancer in the two-stage skin carcinogenesis model involves three stages; namely initiation, promotion and progression. Initiation is caused by an exposure to a subthreshold dose of a genotoxic carcinogen that causes mutation(s) in critical gene(s) in a relatively small population of cells, without causing tumor development. Widely used initiators in the chemically-induced skin carcinogenesis model include 7, 12 dimethylbenz- anthracene (DMBA), benzopyrene, urethane (ethyl carbamate), and N-methyl-N’-nitro-N-nitrosoguanidine (MNNG). Initiators chemically bind to cellular DNA and cause alteration to bases, which results in mutation(s) following replication or transcription in the absence of DNA repair. Polyaromatic hydrocarbons (PAHs), which include DMBA, are biologically inert in skin, and require bio-activation into electrophilic metabolites.
PAHs are metabolized by cytochrome P450 monooxygenases and microsomal epoxide hydrolase that convert PAHs into highly reactive diol epoxides [295-297]. PAH diol epoxides are highly electrophilic metabolites, which covalently binds to exocyclic amino groups of guanine and adenine, forming stable adducts within DNA [295-297]. In the absence of DNA repair, the diol epoxide-DNA adducts may become permanent mutations following DNA replication or transcription [298]. Many genes may be mutated due to initiation with a genotoxic carcinogen; however the H-ras oncogene is the most critical and commonly mutated gene in chemically-induced skin carcinogenesis [299, 300]. Several studies show that the H-ras gene contained an activating mutation, with an A to T transversion in codon 61 in more than 90% of the papillomas induced by initiation with DMBA or urethane in the chemically-induced skin carcinogenesis model [300]. Additionally, MNNG, an alkylating agent and carcinogen, also causes an activating mutation in the H-ras gene in half of the papillomas in the skin carcinogenesis model [301]. Permanent and irreversible mutations in critical genes such as H-ras result in preneoplastic lesion that can progress to a neoplastic lesion following tumor promotion [302]. Tumor promotion is accomplished through repeated application of a nonmutagenic agent that alters epigenetic processes, gene expression, and stimulates proliferation.
Tumor promotion of initiated animal results in clonal selection and expansion of initiated cells and subsequent tumor development [302]. Tumor promoters for mouse skin carcinogenesis bioassay model include 12-O-tetradecanoylphorbol-13-acetate (TPA), benzoyl peroxide, croton oil, and mezerein [302, 303]. TPA, one of the most potent and commonly used tumor promoter is a phorbol ester which is functionally analogous to diacylglycerol (DAG) and thus able to activate the protein kinase C (PKC) pathway. Additionally, TPA is a strong pro-inflammatory and hyperplasiogenic agent. TPA induces epidermal ornithine decarboxylase activity, which catalyzes the biosynthesis of polyamines, and has important roles in tumor promotion [276]. Additionally, most tumor promoters including TPA induce synthesis of DNA, RNA, protein and prostaglandins [276, 303]. Tumor promotion is a reversible process and requires repeated application of tumor promoter over a prolonged period [276, 303]. A relatively small percentage of the papillomas that developed following tumor promotion do progress to become malignant skin tumors.

Tumor progression of benign papilloma to a malignant carcinoma is characterized by increased genetic instability in tumor cells. Tumor progression is initiated by further genetic alterations, and an increased frequency in genetic changes in additional critical genes including proto-oncogenes, tumor suppressor genes within the expanded population of initiated cells [303].
Clonal selection and expansion of initiated cells in the tumor mass with additional mutations in critical tumor suppressor genes and proto-oncogenes are critical for malignant conversion. Malignant conversion is an irreversible process, which can be induced by prolonged treatment of papillomas with a tumor promoter. Additionally, treating papilloma-bearing animals with a mutagenic agent can also significantly increase the rate of malignant conversion [303]. Different mouse strains have been shown to display varying degree of sensitivity to the two-stage skin carcinogenesis bioassay. Sencar mice have been shown to be the most sensitive to the chemically-induced skin carcinogenesis model [289, 303]. C57BL/6 mice have a medium sensitivity among mice strains and show a resistance to malignant conversion of benign tumors [278, 304-306].
Figure 1-7: An overview of multistage skin carcinogenesis. Initiation is induced by a single exposure to a genotoxic carcinogen that causes a genetic mutation(s) in critical proto-oncogene or tumor suppressor genes, such as H-ras or p53. Promotion is performed by repeated and sustained application of a non-mutagenic tumor promoter, which stimulates cell proliferation, resulting in clonal expansion of initiated cells and the development of benign papillomas. Progression is initiated by additional genetic changes and genomic instability in initiated cells, which converts a benign papilloma into a malignant carcinoma.
In addition to studying the mechanisms of skin carcinogenesis using the chemically-induced carcinogenesis model, the ultraviolet (UV) radiation-induced carcinogenesis model is also widely used. Unlike the chemically-induced multistage carcinogenesis model, UV radiation is a complete carcinogen and therefore distinct stages of carcinogenesis cannot be discerned with repeated exposure to UV radiation. However, a single UV radiation exposure can be used as an initiator in a multi-stage carcinogenesis model and promotion is accomplished by prolong application of a tumor promoter such as TPA. UV radiation causes photochemical cellular damage, with UVB (280-315 nm) being the most carcinogenic radiation. In addition to inducing DNA damage, UVB radiation has also been implicated in the disruption of tumor suppressor pro-apoptotic signaling pathways and the suppression of immune responses [307, 308]. UVB radiation causes cellular DNA damage in the form of cyclobutane-type pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts [309, 310]. UV radiation induced DNA damage results in mutations through semiconservative DNA replication, in which DNA polymerase misincorporates an A residue in the unreadable lesion; thus, a cytosine dimer gives rise to a CC→TT transition [311]. Most skin cancers in humans are caused by UV radiation from sunlight and previous studies have shown that human skin cancers contained p53 mutations as the predominant mutation when induced by UV radiation [312].
Previous reports have also shown that roughly over half of human squamous and basal cell carcinomas have p53 mutations [311, 313, 314]. Additionally, exposure of mice to UV radiation also results in tumors carrying p53 mutations [312]. In addition to inducing mutations in the p53 tumor suppressor gene, UV radiation also induced mutations in the ras proto-oncogene in certain rodent strains [312, 313]. Approximately 20% of the tumors in C3H mice carry N-ras mutation in UV radiation-induced skin carcinogenesis model [313].

Cell proliferation, differentiation and apoptosis are critical processes in the modulation of skin homeostasis and hence skin carcinogenesis. Major hallmarks of neoplastic/malignant keratinocyte transformation include their high rate of proliferation and resistance to apoptotic and differentiation signals [315, 316]. Differentiation signals such as high calcium induces terminal differentiation and ultimately apoptosis in normal primary keratinocytes, however neoplastic/malignant keratinocytes are un-responsive to high calcium mediated differentiation and apoptosis. In addition to studying the mechanisms of skin carcinogenesis using animal bioassay, several neoplastic/ malignant epidermal cell lines that represent different stages of skin carcinogenesis have been utilized in examining the mechanisms of skin carcinogenesis. The 308-keratinocyte line is associated with the initiation stage of skin carcinogenesis because of its development from DMBA-initiated skin [317, 318].
308 keratinocytes developed into tumors when grafted onto athymic nude mice and treated with the tumor promoter TPA [317, 318]. The SP1 keratinocyte line is associated with the promotion stage of skin carcinogenesis because of its development from DMBA/TPA induced papilloma [317, 318]. SP1 keratinocytes also developed into tumors when grafted onto athymic nude mice [302]. The Pam212 keratinocyte line was generated by spontaneous transformation of normal keratinocytes, but since it forms carcinomas when transplanted in animal models it has been characterized as a carcinoma cell line which represents the malignant progression state [319]. These neoplastic/malignant epidermal cell lines and many other neoplastic/malignant epidermal cell lines provide easily manipulated and very cost effective models for studying the effect of various genes and chemicals on skin carcinogenesis.
1.3.3 Ras-induced Skin Carcinogenesis

Induction of tumor in the chemically-induced skin carcinogenesis bioassay model is a multistage process, which proceeds through the formation of benign tumors prior to malignancy. The isolation of a c-ras\(^{\text{Ha}}\) oncogene from DMBA and other PAH-initiated skin papillomas provided evidence that this proto-oncogene might be a critical target for initiation mutation in the chemically-induced skin carcinogenesis bioassay model [300]. In vitro studies show that introduction of v-ras\(^{\text{Ha}}\) gene of the Harvey murine sarcoma virus (Ha-MSV) into normal culture keratinocytes transformed these normal keratinocytes into initiated keratinocytes by blocking their ability to undergo differentiation [320-322]. Additionally, introduction of v-ras\(^{\text{Ha}}\) into normal cultured keratinocytes and subsequent skin grafts of these initiated keratinocytes produces papillomas in recipient mice [315, 316, 323, 324]. v-ras\(^{\text{Ha}}\) initiated keratinocytes were mixed with normal dermal fibroblasts at approximately 1:3 ratio and grafted onto the dorsum of athymic nude mice and papillomas were produced within 6 weeks [315, 316, 323, 324]. On the contrary, Dotto et al, reported that co-grafting of v-ras\(^{\text{Ha}}\) initiated keratinocytes with normal dermal fibroblasts inhibits malignant transformation in syngenic graft experiments [325]. Co-grafting of normal fibroblasts and established cancer cell lines such as 308, LC 14 and SP1 keratinocyte lines, which carry activating ras mutation(s) also produces papillomas and carcinomas in vivo [318].
The 308, SP1, and LC 14 keratinocyte lines have activating ras mutation at codon 61, which is similar to activating mutation found in tumor cell derived from the two-stage skin carcinogenesis bioassay model. Further evidence for role of ras in skin carcinogenesis, was found from the induction of papillomas in mice overexpressing oncogenic ras following treatment with the tumor promoter TPA. Additionally, several reports show induction of tumors by ras oncogenes expressed in a tissue specific manner. The MMTV-v-Hras mouse has oncogenic Hras expression under the MMTV promoter and exhibits hyperplasia, mammary tumors, and other malignancies [326]. The Elastase-I-Hras mouse has oncogenic Hras expression under the elastase-I-regulating elements and exhibits pancreatic neoplasia [327]. The Tyr (Tet)-ras mice has oncogenic Hras expression under the control of the tyrosinase promoter and tetracycline regulatory sequences and exhibits melanoma development [328, 329]. The CCSP (Tet)-Kras G12D mice have Tet regulated oncogenic Kras expression under the Clara cell-secretory protein promoter and produces adenocarcinomas of the lung [330]. The rasH2 transgenic mouse model, which carries the human c-Ha-ras oncogene in addition to endogeneous murine Ha-ras gene, exhibits elevated level of tumor formation following exposure to carcinogenic and tumor promoting agents [331]. Additionally, Hras knockout mice exhibit decreased tumor formation following carcinogenic treatments [332, 333].
The compartmentalized and tightly regulated expression of differentiation markers in the epidermis provides a useful analysis of phenotypic changes associated with neoplastic and malignant transformation. Benign papillomas carrying a mutated Ha-ras gene usually expressed reduced levels of spinous cell keratins, while expression of late-stage markers is concurrently increased [334]. These alterations in differentiation gene expression in tumors consisting of v-ras\textsuperscript{Ha} keratinocytes serve as a marker for malignant progression.

Introduction of ras oncogene into normal keratinocytes triggers the induction of cellular senescence; however under in vivo conditions v-ras\textsuperscript{Ha} transformed keratinocytes develop into tumors. Induction of cellular senescence in v-ras\textsuperscript{Ha} transformed keratinocytes is accompanied by upregulation of p53, p19ARF, P21Waf1 and P16ink4a [335-337]. TGFβ1 signaling is critical for v-ras\textsuperscript{Ha}-induced cellular senescence in keratinocytes [338]. TGFβ1 regulates gene expression and cell proliferation via intracellular signaling proteins SMAD 4 and SMAD 2/3, which are transcriptional transactivators [339]. The absence of Smad3 expression in v-ras\textsuperscript{Ha} keratinocytes promotes rapid conversion from benign papilloma to malignant carcinoma.
The genesis of *Ras* research can be traced to a report by Jennifer Harvey, which showed that a preparation of a murine leukemia virus - Harvey-MSV retrovirus (HA-MSV) was capable of inducing sarcomas in newborn mice [340]. Following this discovery, three additional retroviruses were shown to carry ras oncogenes. The Kirsten-MSV retrovirus (Ki-MSV) was discovered in 1967 by serial passage of murine leukemia viruses in rats [341]. BALB-MSV retrovirus was discovered in 1974 and the Rasheed strain of rat sarcoma virus was discovered in 1978 [342, 343]. In 1981, Ellis RW et al reported that the HA-MSV and Ki-MSV were triple recombinants between the replication competent retrovirus, rat cellular sequences of retroviral origins and rat genes *Hras* and *Kras* in HA-MSV and Ki-MSV respectively [344]. In 1982, Der CJ et al reported that DNAs isolated from NIH-3T3 cells that had been transformed EJ bladder (EJ) and lung (LX-1) carcinoma cells DNAs had sequences homologous to ras oncogenes [345]. Identification of human oncogenic homologs of *ras*, laid the groundwork for identification of *ras* oncogenes in human cancers. *Nras*, the third member of the *ras* gene family was discovered in 1983 [346]. Molecular cloning of normal human *Hras* gene and its oncogenic allele allowed the identification of a single point mutation as the cause of the differences in oncogenicity between the two alleles [347].
The ras proto-oncogene is a key regulator of cell growth in all eukaryotic cells. Ras mediated signal transduction pathways are critical for the respond to diverse extracellular stimuli, including cytokines, growth factors and hormones. Ras proteins are GTPases, and their activation/deactivation is regulated by a Guanosine diphosphate (GDP)/Guanosine triphosphate (GTP) cycle [348, 349]. Guanine nucleotide exchange factors including GEFs, RasGRF1/2, and Sos1/2 promote the formation of GTP-bound Ras (active form) [350]. GTPase-activating proteins (GAPs), including p120 GAP and NF1 increase the intrinsic GTP hydrolytic activity of Ras, which results in the formation of GDP-bound Ras (inactive form) [350]. Oncogenic activation of Ras proto-oncogene involves mutations at amino acids 12, 13, or 61, which renders Ras resistant to GAP action and hence constitutively active in transformed cells [351]. Activating mutations of Ras involving GAP resistance are present in many types of human cancers and it is estimated that 30% of human tumors have activated Ras [352]. Additionally, chronic up-regulation of the Ras pathway, in the absence of activating ras mutations have been shown to stimulate aberrant Ras signaling in human cancers [353].

Ras regulates cell proliferation by activating several kinase signaling pathways that are involve in cell proliferation. Activated Ras activates the Raf (c-Raf, A-Raf, and B-Raf) and Mitogen-activated protein kinases (MAPK) signaling pathways [354, 355]. Ras activates the MAPK signaling cascade by directly binding to and activating Raf [356].
MAPKs are a family of serine/threonine kinases that coordinate the transmission of various types of signals such as growth factors to specific cellular targets [357, 358]. Upon activation MAPKs translocate from the cytoplasm to the nucleus and phosphorylate specific transcription factors, which results in the modulation gene expression [357, 358]. MAPKs regulate a wide variety of transcription factors that are critical for tumor development and malignant transformation including activator protein-1 (AP-1) [359-361]. Major members of the MAPK cascade include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 [357, 358]. Mitogenic agents typically activate ERK, while stress-inducing agents typically activate JNK and p38. Upon activation, ERKs phosphorylate cytoplasmic targets and translocate to the nucleus, where they activate various transcription factors including AP-1 and ELK-1 [357, 358]. AP-1 is a transcription factor which is a heterodimeric protein and is composed of proteins belonging to the c-Fos, c-Jun, activating transcription factor (ATF) and Jun dimerization protein (JDP) families [359-361]. AP-1 upregulates transcription of genes containing the TPA response element and has critical roles in tumor development and malignant transformation [359-361]. Cooperation between ras oncogene and PKCα, an upstream modulators of AP1 signaling, has been observed in the transformation of epidermal cells [362]. Additionally, AP1 transcription factors are essential for v-ras^{Ha}-induced transformation of epidermal cells [363].
In addition to inducing ERKs activity through activation of Raf, Ras also activates other MAPK (JNK, p38) cascades independent of Raf activation [364]. Additional effectors of Ras include p120 Ras GAP, GEFs for Ral, AF6/Canoe, RIN1, and phosphatidylinositol 3-kinase (PI3K) [352]. Activated Ras recognizes these structurally and functionally distinct proteins through its switch I (30-37) and II (59-76) residues [365]. Small GTPase Ral, a target of GEFs has been implicated in Ras mediated transformation [366, 367]. Ral has been implicated in the regulation of phospholipase D and actin cytoskeleton rearrangements via interaction with RalBP1 [368]. RIN1 interacts directly with activated Ras and has been shown to enhance the transforming activity of Bcr/Abl [352]. PI3K interacts directly with Ras and is involved in Ras mediated actin cytoskeletal rearrangements in transformed cells [352]. PI3K is a lipid kinase, which is activated by a variety of extracellular stimuli leading to the accumulation of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP₃). Downstream effectors of PIP₃ include the serine/threonine kinase Akt/Protein kinase B (PKB). Upon activation, Akt/PKB phosphorylates and inactivates the pro-apoptotic protein BAD and suppresses other pro-apoptotic signaling, thus promoting cell survival [369].
Ras signaling pathways. Ras modulate many signaling pathways in response to activation by cell surface receptors that serve as sensors to external stimuli such as growth factors and hormones. Ras promotes cell proliferation via the MAP kinase signaling cascade which upregulate AP1 family genes. Ras also promotes cell survival by activating the PI3K anti-apoptotic signaling pathway.
1.3.4 The Role of Cyclooxygenase Signaling in Epithelial Carcinogenesis

Prostaglandins are derivatives of arachidonic acid, and act as autocrine and paracrine lipid mediators in many biological processes [370, 371]. Prostaglandins have very short half-lives and are synthesized \textit{de novo} from membrane-released arachidonic acid when cells are activated by various stimuli including cytokines, growth factors mechanical stress [370, 371]. Arachidonic acid released from the plasma membrane is regulated by phospholipase, with type IV cytosolic PLA2 being the key enzyme involved [370, 371]. Arachidonic acid is metabolized to an intermediate prostaglandin PGH$_2$ by cyclooxygenase (COX), which exists as two isoforms referred to as COX-1 and COX-2 [372]. PGH$_2$ is then metabolized into various prostaglandins including PGD$_2$, PGE$_2$, PGF$_2$, thromboxane A$_2$ (TXA$_2$), and prostacyclin (PGI$_2$) by downstream enzyme in a cell specific manner [370-372]. Prostaglandins have critical role in various physiological functions including blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, and wound healing [373] [372]. PGF$_2$ is involved in the regulation of smooth muscle contractility and PGE$_2$ is involved in maintaining gastric mucosa physiology [374]. TXA$_2$ is involved in the regulation of blood pressure and platelet aggregation [375]. Prostaglandins also play critical role in the regulation of inflammatory responses and carcinogenesis.
COX-1 is constitutively expressed under normal conditions and is involved in maintaining the basal level of prostaglandins necessary for tissue homeostasis. On the contrary, COX-2 is undetectable in normal tissues and is induced by growth factors, cytokines, and tumor promoters under pathological conditions including inflammation and cancer. Additionally, increased COX-2 mediated prostaglandins synthesis has been associated with inflammatory diseases like arthritis and various cancers including colon cancer [376]. COX-2 expression is undetectable in normal intestinal mucosa, but highly expressed in both human and animal colorectal tumors [377]. Previous reports have shown a 40-50% decrease in relative risk for colorectal cancer in individuals who regularly use aspirin and other non–steroidal anti-inflammatory drugs (NSAIDs), which are potent inhibitors of COX. NSAIDs are potent inhibitors of COX activity, thus prostaglandins synthesis and are widely used as therapeutic agents to treat various pathophysiological diseases including pain, osteoarthritis [378-382]. Recent studies have also shown that NSAIDs and COX-2 specific inhibitors can significantly inhibit tumor incidence and progression in both animal models and in treatment of cancer patients [378-382]. Many epithelial cancers and tumors including colon and skin exhibit increased production of prostaglandins [383]. Increased prostaglandin activity in cancers and tumors enhances cell proliferation and inhibits apoptosis [383]. Examination of the role of COX-2 in carcinogenesis using an animal model for familial adenomatous polyposis showed that the number and size of intestinal polyps are greatly reduced by the absence of COX-2 expression [384-386].
Examination of the role of COX in skin carcinogenesis using the two stage skin carcinogenesis bioassay showed that tumorigenesis is reduced by approximately 75% in Cox-2 or Cox-1-null mice when compared to wild-type mice, indicating its role in tumor initiation and promotion [383]. Papillomas from Cox-2 or Cox-1-null mice exhibited reduced levels of PGE$_2$ when compared to papillomas from wild-type mice [383]. Additionally, induction terminal differentiation in DMBA-initiated keratinocytes was higher in Cox-2 or Cox-1-null mice, with high expression of K1 and K10 when compared to wild-type mice, suggesting critical roles for COX in inhibiting epidermal differentiation [383]. COX deficient mice also exhibited reduced epidermal hyperplasia and BrdUrd-labeling of epidermal cells in response to treatment with the tumor promoter TPA, suggesting a role for COX in promoting cell proliferation and skin hyperplasia [383]. In addition to inhibiting skin tumorigenesis in the chemical carcinogenesis model, NSAIDs and celecoxib, a selective COX-2 inhibitor have been shown to attenuate tumor formation in UVB-induced skin carcinogenesis [387]. Furthermore, topical treatment with the chemotherapeutic drug 5-Fluorouracil (5-FU), and the COX inhibitor celecoxib was up to 70% more effective in reducing the number of UVB-induced skin tumors than 5-FU treatment alone [388]. These studies show critical roles for COX, especially COX-2 in the regulation of epidermal differentiation and proliferation and its potential as a therapeutic target in the prevention and treatment of skin cancer.
Overexpression of COX-2 in skin has been reported to protect mice from skin tumor development; however this report is contrary to the bulk of the literature, which suggests that COX promotes skin carcinogenesis [389].

Recent reports have shown the critical role of PPARs in the regulation of carcinogenesis [390]. Prostaglandins, which are fatty acid derivatives have been identified as endogenous PPAR ligands [214]. Early studies examining the role of PPARβ/δ in epithelial carcinogenesis suggested that adenoma polyposis colitis (APC) tumor suppressor gene negatively regulates PPARβ/δ expression in colorectal tumor cell lines and that PPARβ/δ is activated by prostacyclin (PGI$_2$) [213, 214]. These report suggested that PPARβ/δ is upregulated by the β-catenin/TCF4 cell proliferation pathway and that activation of PPARβ/δ by cyclooxygenase (COX) derived metabolites promotes colon carcinogenesis [213, 214]. Contrary to reports that suggest that PGI$_2$ promotes carcinogenesis through activation of PPARβ/δ, PGI$_2$ have been shown to promote apoptosis by activating PPARβ/δ [391]. However, in vivo studies have shown that COX-2 inhibitors attenuate skin and colon carcinogenesis independent of PPARβ/δ [392, 393]. Additionally, several reports suggest that the absence of PPARβ/δ promotes skin and colon cancers and that ligand activation of PPARβ/δ attenuates colon carcinogenesis [223, 226, 237]. Furthermore, Induction of skin Inflammation by TPA was significantly lower in wild-type mice on NSAID (sulindac) diet when compared to Pparb/d-null mice [27].
Figure 1-9: COX mediated prostaglandin production and its physiological effects. Prostaglandins are synthesized from arachidonic acid by Cyclooxygenase 1/2 (COX1/2). Prostaglandins affect various biological processes that are involved in normal physiological functions including reproduction, inflammation and wound healing. Prostaglandins also play critical roles in cancer formation. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit COX1/2 mediated prostaglandin synthesis.
1.4 Hypothesis

PPARβ/δ is a ligand-activated nuclear receptor that is ubiquitously expressed in many tissues, with relatively high expression observed in the skin. Previous studies have shown that the epidermal hyperplastic response induced by the tumor promoter TPA is greatly increased in Pparβ/d-null mice, and that tumor incidence, multiplicity and size in the two-stage bioassay is significantly higher in Pparβ/d-null mice when compared to similarly treated wild-type mice [27, 237]. Furthermore, activation of PPARβ/δ has also been shown to inhibit cell proliferation and induce terminal differentiation in normal rodent and human keratinocytes [238, 239]. Activation of PPARβ/δ has also been shown to attenuate colon tumorigenesis and inhibit proliferation of various tumor cell types [223, 234, 239, 392, 394]. Additionally, TPA-induced inflammation in skin is lower in wild-type mice fed sulindac than in similarly treated Pparβ/d-null mice, suggesting a role for PPARβ/δ in the attenuation of TPA induced inflammation [27]. Based on these observations, the hypothesis of this study is that activation of PPARβ/δ inhibits epidermal cell proliferation and skin carcinogenesis via inhibition of oncogenic Ras signaling and induction of differentiation signaling. Furthermore, combining PPARβ/δ agonists with chemotherapeutic agents could enhance attenuation of skin carcinogenesis. The focus of this study is to develop model systems to test this hypothesis, and to elucidate the molecular signaling in order to determine the functional roles of PPARβ/δ in epithelial cell proliferation and skin carcinogenesis.
The first approach is to use the two-stage carcinogenesis bioassay to determine if activation of PPARβ/δ with potent synthetic ligands attenuates chemically-induced skin carcinogenesis. After initiation with 7,12-dimethylbenzanthracene (DMBA), wild-type and Pparb/d-null mice will be treated topically with TPA followed by GW0742 or vehicle three times per week for 48 weeks. The onset of papilloma formation, and the size and number of papillomas will be assessed in both groups of mice over the duration of the experiment. Results from this work will show whether or not the activation of PPARβ/δ can functionally alter chemically-induced skin carcinogenesis. If skin carcinogenesis is attenuated in wild-type mice, but not in Pparb/d-null following treatment with GW0742, these results will demonstrate that activation of PPARβ/δ attenuates skin carcinogenesis. Furthermore, the effect of PPARβ/δ activation on proliferation, differentiation and apoptosis will be examined under in vivo and ex vivo conditions. Additionally, epidermal tumor cell lines of various degrees of malignancy will be treated with PPARβ/δ agonists to determine the effect of PPARβ/δ activation on proliferation, differentiation and apoptosis in neoplastic cell lines.

Secondly, retroviral infection of wild-type and Pparb/d-null keratinocytes with ras oncogene, the rate limiting mutation in the chemically-induced skin cancer model will be use to determine if activation of PPARβ/δ attenuates chemically-induced skin carcinogenesis via inhibition of the oncogenic ras signaling pathway.
v-ras\textsuperscript{Ha}-infected primary keratinocytes will be use to mechanistically evaluate the direct role of PPAR\(\beta/\delta\) and ligand activation in modulating cell growth, differentiation and senescence, which are critical factors affecting malignant transformation of normal keratinocytes. v-ras\textsuperscript{Ha}-infected primary keratinocytes will also be use to evaluate the role of PPAR\(\beta/\delta\) and ligand activation in malignant transformation in cell culture.

Thirdly, the two-stage carcinogenesis bioassay will be performed using wild-type and \textit{Pparb/d}-null mice to determine if combining the COX inhibitor nimesulide and the PPAR\(\beta/\delta\) agonist GW0743 can cause regression of skin tumorigenesis. COX-mediated prostaglandin signaling is a major downstream effector of oncogenic \textit{ras}-mediated malignant transformation; thus attenuation of skin carcinogenesis by a combination of PPAR\(\beta/\delta\) activation and COX inhibition will provide further evidence for a tumor suppressor role for PPAR\(\beta/\delta\). Additionally, various epidermal tumor cell lines of varying degree of malignancy will also be treated with PPAR\(\beta/\delta\) agonists and nimesulide to determine if combining PPAR\(\beta/\delta\) activation and COX inhibition increases the efficacy of COX inhibitor chemotherapeutic potential. The combined purpose of this study is to determine the effects of PPAR\(\beta/\delta\) activation on skin carcinogenesis and the mechanisms by which PPAR\(\beta/\delta\) exerts those effects.
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Chapter 2

Ligand activation of peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) inhibits chemically-induced skin tumorigenesis.

2.1 Abstract

*Pparb/d*–null mice exhibit enhanced tumorigenesis in a two-stage chemical carcinogenesis model as compared to wild-type mice. Previous work showed that ligand activation of PPARβ/δ induces terminal differentiation and inhibits proliferation of primary keratinocytes, and this effect does not occur in the absence of PPARβ/δ expression. In the present studies, the effect of ligand activation of PPARβ/δ on skin tumorigenesis was examined using both *in vivo* and *ex vivo* skin carcinogenesis models. Inhibition of chemically-induced skin tumorigenesis was observed in wild-type mice administered GW0742, and this effect was likely the result of ligand-induced terminal differentiation and inhibition of replicative DNA synthesis. These effects were not found in similarly treated *Pparb/d*–null mice. Ligand activation of PPARβ/δ also inhibited cell proliferation and induced terminal differentiation in initiated/neoplastic keratinocyte cell lines representing different stages of skin carcinogenesis. Furthermore, contrary to other studies, ligand activation of PPARβ/δ does not inhibits apoptosis.
These studies suggest that topical administration of PPARβ/δ ligands may be useful as both a chemopreventive and/or chemotherapeutic approach to inhibit skin cancer.

2.2 Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and part of the nuclear hormone receptor superfamily. PPARs regulate expression of genes involved in a myriad of biological functions, including cell proliferation, immune responses, differentiation, fatty acid metabolism and energy homeostasis [1-6]. PPARs modulate gene expression by undergoing conformational change after ligand binding allowing release of co-repressors, recruitment of co-activators and subsequent heterodimerization with the retinoid X receptor-α (RXRα). The ligand bound PPAR-RXRα heterodimer complex binds specific peroxisome proliferator response elements (PPRE) in target genes and increases their expression [1-6]. The PPAR subfamily consists of three distinct members, PPARα, PPARβ/δ and PPARγ. The biological roles of PPARα and PPARγ have been extensively characterized, whereas the physiological function of PPARβ/δ remains less clear.
PPARβ/δ is the most ubiquitously expressed PPAR isotype [7,8] with particularly high expression in the intestine, liver and keratinocytes [9]. PPARβ/δ is activated by fatty acids [10], tetradecylthioacetic acid (TTA) [11], GW501516 [12], GW0742 [12], and L-165041 [13]. PPARβ/δ agonists are being evaluated as therapeutic agents as there is strong evidence that ligand activation of this receptor can modulate lipid and glucose homeostasis, making this PPAR an excellent target for treating diseases including diabetes, dyslipidemias, metabolic syndrome and obesity. For example, the PPARβ/δ agonist GW501516 markedly increases serum high-density lipoproteins (HDL) levels, while lowering serum triglyceride levels in obese animals [14,15]. Ligand activation of PPARβ/δ also increases fatty acid oxidation in skeletal muscle by upregulation of fatty acid metabolizing enzymes [16-19]. In addition to modulating lipid homeostasis, ligand activation of PPARβ/δ can improve insulin sensitivity through a mechanism that requires PPARβ/δ [20-22]. Recent evidence also suggests that PPARβ/δ may be constitutively active since it can be co-immunoprecipitated with its obligatory heterodimerization partner in nuclear extracts [9]. It is also worth noting that there is evidence that PPARβ/δ has anti-inflammatory activities in a number of different models systems (reviewed in [23]). Thus, there is good reason for targeting PPARβ/δ to prevent/treat metabolic diseases.
Despite the high value of targeting PPAR\(\beta/\delta\) for treatment of various metabolic disorders, there remains a concern about the role of PPAR\(\beta/\delta\) in carcinogenesis due to considerable controversy (reviewed in [2,24]). Despite some reports indicating that ligand activation of PPAR\(\beta/\delta\) potentiates tumorigenesis (reviewed in [2,24]), there is a large body of evidence that do not support this view including the observations made by a number of independent laboratories that PPAR\(\beta/\delta\) mediates terminal differentiation and/or inhibition of cell proliferation (reviewed in [2,24]), and that PPAR\(\beta/\delta\) has anti-inflammatory activities (reviewed in [23]). Definitive evidence that PPAR\(\beta/\delta\) ligands fail to increase cell growth or inhibit apoptotic signaling was also recently reported in seven different human cancer cell lines [25,26]. Collectively, these and many other related observations (reviewed in [2,24]) suggest that ligand activation of PPAR\(\beta/\delta\) attenuates tumorigenesis, although this hypothesis must be examined critically.

The first evidence suggesting that PPAR\(\beta/\delta\) inhibits skin tumorigenesis was the observation that mice lacking PPAR\(\beta/\delta\) expression exhibited enhanced epidermal hyperplasia in response to the tumor promoter TPA [27,28]. It is worth noting that these observations were made in two distinct null mouse models generated by different laboratories and gene targeting strategies. Consistent with these observations, mice lacking PPAR\(\beta/\delta\) expression exhibit enhanced skin tumorigenesis in the two-stage [7,12-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)] chemical carcinogenesis bioassay [29].
An earlier onset of tumor formation, increased tumor incidence, and increased tumor size is found in \textit{Pparb/d}–null mice as compared to wild-type mice [29]. \textit{Pparb/d}–null mice also exhibit enhanced epidermal hyperplasia and cell proliferation, and reduced caspase 3 activity and apoptosis in response to topical treatment with TPA [29]. These findings demonstrate that PPAR$\beta/\delta$ protects against chemically-induced skin tumorigenesis, through mechanisms that include attenuation of cell proliferation and/or modulation of apoptosis. These findings also suggest that ligand activation of PPAR$\beta/\delta$ could inhibit chemically-induced skin tumorigenesis. Indeed, reports from four independent laboratories show that ligand activation of PPAR$\beta/\delta$ results in the induction of terminal differentiation of keratinocytes and/or inhibition of cell proliferation [11,30-32], and that this effect is mediated by PPAR$\beta/\delta$ [30]. Thus, the present studies examined the hypothesis that ligand activation of PPAR$\beta/\delta$ inhibits skin tumorigenesis through the induction of terminal differentiation using both \textit{in vivo} and \textit{ex vivo} model systems.
2.3 Materials and Methods

2.3.1 Two-stage chemical carcinogenesis bioassay

Female, wild-type and *Pparb/d*–null mice, in the resting phase of the hair cycle (6-8 weeks of age) were initiated with 50 µg of DMBA dissolved in 200 µL acetone. One week after initiation, wild-type and *Pparb/d*–null mice were treated 3 days/week for 48 weeks with topical application of 5 µg of TPA followed an hour later with either acetone or GW0742 (200 µL of either a 1 or 5 µM stock solution). These concentrations of ligand were chosen based on previous work showing PPARβ/δ-dependent induction of terminal differentiation in mouse skin within this concentration range [30]. The onset of tumor formation, tumor size and tumor number was assessed weekly. After 48 weeks of TPA and vehicle/ligand treatment, mice were euthanized by overexposure to carbon dioxide. Skin and tumor samples from each mouse were fixed in 10% neutral-buffered formalin or 70% ethanol and then paraffin embedded, sectioned and stained with H&E. A pathologist examined H&E-stained sections of suspected carcinomas.
2.3.2 Short-term analysis of epidermal cell proliferation and terminal differentiation

Wild-type or Pparb/d-null mice were treated topically with vehicle control (acetone) or TPA (5 µg/mouse) followed an hour later with either vehicle control (acetone) or GW0742 (200 µL of 5 µM solution). Skin samples were collected either 6 or 24 hours after this last treatment. Replicative DNA synthesis was examined in mice 25 hours post-TPA treatment as described above. Mice were injected intraperitoneally with BrdU (100 mg/kg) at the time TPA was applied, and 8 hours post-TPA application. Skin samples were obtained and fixed in 10% neutral buffered formalin. Representative skin samples were embedded in paraffin and sections (4-6 µm) and prepared for immunohistochemical analysis of BrdU incorporation. Detection of BrdU-labeled keratinocytes was performed using an immunohistochemical kit (Exalpha Biologicals, Inc., Watertown, MA) following the manufacturer’s recommended procedures. BrdU-labeled keratinocytes were quantified using light microscopy by counting the number of BrdU-labeled and unlabeled keratinocytes in a given 40X frame. Representative slides from each treatment group were examined with a minimum of two different 40X frame per slide being scored for labeling analysis for each mouse skin sample. Keratins mRNA and protein expression were examined using quantitative real-time PCR (qPCR) analysis and immunohistochemistry, respectively. Total RNA was also isolated from skin samples using TRIZOL reagent (Invitrogen, Carlsbad, CA).
For qPCR analysis, cDNA was generated using 2.5 µg total RNA with M-MLV Reverse Transcriptase (Promega, Madison, WI). Primers were designed for qPCR using PrimerQuest™ software (Integrated DNA Technologies, Coralville, IA). qPCR reactions were performed using SYBR green PCR master mix (Finnzymes, Espoo, Finland) in the iCycler and detected using the MyiQ™ Real-time PCR Detection System (BioRad, Hercules, CA). The following conditions were used for PCR: 95 ºC for 15 sec, 94 ºC for 10 sec, 60 ºC for 30 sec, and 72 ºC for 30 sec, and repeated for 45 cycles. The PCR included a no template control reaction to control for contamination and/or genomic amplification. All reactions had >90% efficiency. Five representative samples from independently treated mice were used for each group and timepoint. Relative expression levels of mRNA were normalized to GAPDH and analyzed for statistical significance using one-way ANOVA (Prism 4.0). For immunohistochemistry, antigens were retrieved using the citrate buffer epitope retrieval method and keratin proteins were detected using mouse keratin polyclonal antibodies (Covance, Berkeley, CA) and a peroxidase detection system (Vector Laboratories, Burlingame, CA). Five representative samples from independently treated mice were examined for each treatment group at each time point.
2.3.3 Analysis of keratinocyte apoptosis

In order to delineate the role of PPARβ/δ in apoptosis, the effect of ligand activation of PPARβ/δ on expression of the PTEN/PDK1/ILK1/Akt cell survival pathway was examined in wild-type and Pparb/d−null primary keratinocytes and mice. Additionally, the effect of ligand activation of PPARβ/δ on serum deprivation and UV-induced apoptosis was also examined in wild-type and Pparb/d−null primary keratinocytes. mRNA and protein expression analysis of GW0742 treated primary keratinocytes and mice were performed using quantitative northern and western blot analysis, respectively. Keratinocytes samples were obtained after 8 or 12 h of treatment with 0.2 µM GW0742, while animals were treated with 50 µM GW0742 for 8 hrs.

For cell count assay, keratinocytes were maintained in low calcium medium (0.05 mM) with or without 8% serum in the presence or absence of 0.5 µM GW0742. The number of cells was quantified 24 and 72 h after serum removal using a Z1 coulter particle counter® (Beckman Counter, Inc., Hialeah, FL). This approach allowed for examination of serum deprivation-induced apoptosis, and whether ligand activation of PPARβ/δ modified this effect through a PPARβ/δ-dependent mechanism. As an alternative to this approach, primary keratinocytes from wild-type and Pparb/d−null mice were also irradiated with 20,000 µJ/cm² UV light using the CL-1000 Ultra Violet Crosslinker, and caspase 3 activity was measured 9 or 18 h post-irradiation using the Caspase 3 Glo reagent (Promega) following manufacturer's recommended procedures.
2.3.4 Keratinocyte-ex vivo cancer models

To examine the hypothesis that ligand activation of PPARβ/δ can cause initiated or neoplastic keratinocytes to differentiate and inhibit cell proliferation, several keratinocyte cell lines were used: 1) the 308 keratinocyte cell line derived from DMBA-treated mouse skin [33,34], 2) the SP1 keratinocyte cell line derived from a DMBA/TPA-treated mouse skin papilloma [33,34], and 3) the Pam212 keratinocyte cell line derived from spontaneously transformed neonatal keratinocytes [35]. Expression of Pparb/d mRNA in these cell lines was examined using qPCR analysis as described previously. Analysis of neoplastic keratinocyte proliferation was performed using a colony formation assay and cell counting.

For the colony formation assay, 308 keratinocytes were seeded at low density (100 cells/well in a 6 well plate) and treated with PPARβ/δ ligands under high calcium (1.4 mM) conditions in the presence or absence of 32 nM TPA for 2 weeks. This assay is based on the observations that initiated keratinocytes will form foci when exposed to tumor promoters (e.g. TPA) in the presence of differentiation-inducing conditions (e.g. high calcium) due to their relative inability to undergo terminal differentiation [36,37]. Foci number and size were quantified using the binary color counting procedure from NIH image J software (version 1.37).
To determine the effect of ligand activation of PPARβ/δ on cell proliferation, 308, SP1 and Pam212 initiated/neoplastic keratinocytes were cultured and treated with PPAR ligands. Cell number was quantified over time in triplicate, independent samples using a Z1 Coulter® particle counter (Beckman Coulter, Fullerton, CA).

Analysis of neoplastic keratinocyte differentiation was performed by examining differentiation mRNA marker expression and cornified envelope formation. For analysis of differentiation marker mRNA, 24 hours after treatment with PPAR ligands under high calcium (1.4 mM) conditions, total RNA was isolated from 308 keratinocytes using TRIZOL reagent (Invitrogen, Carlsbad, CA), and the manufacturer’s recommended procedures. Ten micrograms of total RNA was analyzed using Northern blot analysis. The following previously described cDNAs were used for random primed ³²P-labeled probes: ADRP, SPR1A and SPR2H [30]. qPCR analysis was performed using RNA from ligand treated 308 cells as described above. To quantify cornified envelope formation, 308 or Pam212 keratinocytes were seeded at approximately 90% confluence in 6-well plates and treated with either GW0742 or L-165041 in low calcium (0.05 mM) or a terminal-differentiation inducing condition (1.4 mM calcium). After this treatment period, cells were collected and treated with lysis solution containing 2% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol for 5 min. Undissolved cornified envelopes observed under a hemocytometer were quantified as previously described [38].
Analysis of neoplastic keratinocyte apoptosis was performed by examining caspase 3/7 activity. Neoplastic keratinocytes at approximately 50% confluency were treated with or without 1 µM GW0742 for 12 hours. Following initial 12 h treatment, keratinocytes were treated with or without UV light (20,000 µJ/cm²) and maintained under prior treatment condition. Caspase 3/7 activity was measured 12 hours post-irradiation using the Caspase 3/7 Glo reagent (Promega, Madison, WI) following manufacturer's recommended procedures.

2.4 Results

2.4.1 Ligand activation of PPARβ/δ inhibits chemically-induced skin tumorigenesis

To determine if ligand activation of PPARβ/δ inhibits skin carcinogenesis, a two-stage bioassay was performed using wild-type and Pparb/d−null mice treated topically with the highly specific PPARβ/δ ligand GW0742 at concentrations previously shown to specifically activate PPARβ/δ [30]. Consistent with past studies [29], the onset of papilloma formation was earlier after initiation in the absence of PPARβ/δ expression (Figure 2-1A,D). Topical administration of GW0742 did not influence the onset of papilloma formation in either genotype at a concentration of 1 µM (Figure 2-1A) but significantly (P ≤ 0.05) delayed papilloma formation by five weeks in the wild-type mice at a concentration of 5 µM (Figure 2-1D).
This effect was not found in similarly treated \( Pparb/d\)–null mice (Figure 2-1D). The average number of wild-type mice with tumors was significantly less in response to topical application of 1 \( \mu \text{M} \) GW0742 from weeks 13 through 29 (\( P \leq 0.05 \)), and this was not found in similarly treated \( Pparb/d\)–null mice (Figure 2-1A). This PPAR\( \beta/\delta\)-dependent effect was not observed in response to 5 \( \mu \text{M} \) GW0742 (Figure 2-1D). There was no significant effect observed with tumor multiplicity following topical application of 1 \( \mu \text{M} \) GW0742 in either genotype (Figure 2-1B). However, tumor multiplicity was decreased in wild-type mice by topical application of 5 \( \mu \text{M} \) GW0742 (Figure 2-1E) during early (weeks 12-23) and later stages (weeks 36-48) of the bioassay (\( P \leq 0.05 \)). This effect was not found in similarly treated \( Pparb/d\)–null mice up to week 42, but after week 42, tumor multiplicity was lower in \( Pparb/d\)–null mice treated topically with 5 \( \mu \text{M} \) GW0742 (Figure 2-1E). The average tumor size was not affected by ligand treatment in either genotype (Figure 2-1C, F), but the average size of skin tumors was greater in \( Pparb/d\)–null mice as compared to wild-type mice during the later stages of the bioassay (\( P \leq 0.05 \)). Lesions suspected of being squamous cell carcinomas were examined for histopathology.
Figure 2-1: Ligand activation of PPARβ/δ inhibits chemically-induced skin tumorigenesis. Two-stage chemical carcinogen testing was performed in wild-type (+/+ or 0)/0/0) or Ppad/δ-null (−/−) mice as described in Materials and Methods. Mice were treated with or without the PPARβ/δ ligand GW0742 (1 or 5 µM) during tumor promotion. The incidence and onset of lesion (lesions include papillomas, keratocanths and carcinomas) formation in mice treated with and without 1.0 µM GW0742 (A) or 5.0 µM GW0742 (D). Skin lesion multiplicity in mice treated with and without 1.0 µM GW0742 (B) or 5.0 µM GW0742 (E). Average skin lesion size in mice treated with and without 1.0 µM GW0742 (C) or 5.0 µM GW0742 (F).
The majority of lesions suspected of being squamous cell carcinomas were later classified as keratocanthomas (Figure 2.2). The incidence of keratocanthomas was significantly greater in $Pparb/d$–null mice as compared to wildtype mice (Figure 2.2A-B). Topical administration of GW0742 decreased the incidence of keratocanthoma in wild-type mice at both 1 and 5µM GW0742 with greater inhibition being observed with 5 µM GW0742. This effect was not found in similarly treated $Pparb/d$–null mice (Figure 2.2A-B). Squamous cell carcinomas were only observed in $Pparb/d$–null mice but not in wild-type mice (Figure 2.2A-B).
Figure 2-2: Ligand activation of PPARβ/δ inhibits tumor progression. Suspected carcinomas were examined microscopically and classified as either keratocantheta or carcinomas by a pathologist. (A) Incidence of keratocanthomas and carcinomas in mice treated with and without 1 µM GW0742 (left panel) or 5 µM GW0742 (right panel). Values represent the percentage of mice with keratocanths or carcinomas. (B) Tumor multiplicity in mice treated with and without 1 µM GW0742 (left panel) or 5 µM GW0742 (right panel). Values represent the average number of lesions per mouse with the lesion ± S.E.M.
2.4.2 PPARβ/δ-dependent inhibition of replicative DNA synthesis and modulation of differentiation signaling

To determine if ligand activation of PPARβ/δ can modulate epidermal cell proliferation and differentiation, mice were treated topically with the PPARβ/δ ligand, GW0742, after a single application of TPA. Topical administration of 5 µM GW0742 significantly decreased the number of BrdU-labeled keratinocytes in TPA-treated wildtype mice and this effect was not observed in similarly treated Pparb/d–null mice (Figure 2-3). Pparb/d–null mice treated with TPA had a higher BrdU labeling index as compared to similarly treated wild-type mice (Figure 2-3). To examine the effect of ligand activation of PPARβ/δ on differentiation signaling, expression of early and late markers of keratinocyte terminal differentiation was examined, at both the mRNA and protein level.
Expression of mRNA encoding *keratin 1* was significantly reduced by topical administration of TPA and co-administration of TPA and GW0742 in both genotypes, six hours post-application (Figure 2-4A). No changes in the expression of *keratin 1* mRNA were observed with any treatment in either genotype twenty-four hours post-application (Figure 2-4A). Changes in the expression of mRNA encoding *keratin 10* were similar between both genotypes at both timepoints (Figure 2-4B).

**Figure 2-3**: Ligand activation of PPARβ/δ inhibits TPA induced replicative DNA synthesis. Wild-type (+/+) and *Pparb/d*−/− (−/−) mice injected with BrdU were topically treated with acetone or TPA (5 µg/mouse) followed by topical application of 5 µM GW0742. BrdU labeling index was determined as described in Materials and Methods. Values represent the mean ± S.E.M.. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, *P* ≤ 0.05.
Decreased expression of keratin 10 mRNA was observed following TPA and co-administration of TPA and GW0742, and no change was found after GW0742 treatment in both genotypes (Figure 2-4B). While expression of mRNA encoding keratin 5 was modestly lower in response to both TPA application and co-administration of TPA and GW0742 after six hours, this effect was not statistically significant (Figure 2-4C). No change in the expression of mRNA encoding keratin 5 was found with any treatment in wild-type mice after twenty-four hours, but was higher in Pparb/d−null mouse skin after topical application of either TPA or co-administration of GW742 with TPA (Figure 2-4C). Expression of mRNA encoding keratin 14 was not changed by GW0742, TPA or co-administration of TPA and GW0742 in wild-type mouse skin after six hours, but was higher in Pparb/d−null mouse skin after TPA or TPA and GW0742 co-treatment at this timepoint (Figure 2-4D). By twenty-four hours post-application of either TPA or TPA and GW0742, increased expression of keratin 14 was similar between genotypes (Figure 2-4D). Expression of mRNA encoding involucrin was similar between genotypes after six hours and was not markedly changed by any treatment (Figure 2-4E). However, co-administration of TPA with GW0742 resulted in a PPARδ-dependent increase in involucrin, as this effect was not found in similarly treated Pparb/d−null mice (Figure 2-4E). In addition to examining expression of mRNA markers of keratinocyte differentiation, immunohistochemical analysis of these proteins was also performed.
Figure 2-4: The effect of ligand activation of PPARβ/δ on expression of mRNAs encoding differentiation markers. Wild-type (+/+) and Pparb/d−null (−/−) mice were topically treated with acetone or TPA (5 µg/mouse) followed by topical administration of vehicle or 5 µM GW0742. RNA was isolated from skin samples obtained after 6 or 24 hours and mRNA encoding markers of terminal differentiation was examined using quantitative real-time PCR analysis. Gene expression profile of (A) keratin 1, (B) keratin 10, (C) keratin 5, (D) keratin 14 and (E) involucrin. Values represent the mean ± S.E.M.. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, \( P \leq 0.05 \).
Topical application of TPA caused a reduction in keratin 1 protein, in particular after 24 hours in the wild-type mice (Figure 2-5A). Expression of keratin 1 was higher in wild-type mouse skin following co-administration of GW0742 and TPA after 24 hours, and this effect was not observed in Pparb/d–null mice (Figure 2-5A). Expression of keratin 1 was higher in wild-type mice in all treatment groups at both the 6 and 24 hour time points as compared to similarly treated Pparb/d–null mice (Figure 2-5A). Expression of keratin 10 was higher in wild-type mice as compared to Pparb/d–null mice for all treatment groups at both the 6 and 24 hour time points and topical application of GW0742 had no effect in either genotype (Figure 2-5B). Immunohistochemical analysis of keratin 5 showed higher staining in Pparb/d–null mice as compared to wild-type mice for all treatment groups at the 6 hour time point (Figure 2-6A). Relative expression of keratin 5 was higher 24 hours post-TPA as compared to 6 hours post-TPA, although expression was similar for all treatment groups in both genotypes (Figure 2-6A). Six hours post-TPA application, no difference in immunoreactive keratin 14 was observed between treatments or genotype (Figure 2-6B). However, expression of keratin 14 was markedly higher in Pparb/d–null mice 24 hours post-TPA as compared to similarly treated wild-type mice (Figure 2-6B).
Figure 2-5: Immunohistochemical analysis of keratin expression in response to ligand activation of PPARβ/δ. Wild-type (+/+) and Pparb/d-null (−/−) mice were topically treated with acetone or TPA (5 µg/mouse) followed by topical administration of vehicle or 5 µM GW0742. Skin samples were obtained after 6 or 24 hours, and keratin expression was examined using immunohistochemistry. Representative photomicrographs of skin samples for (A) keratin 1 and (B) keratin 10.
Figure 2-6: Immunohistochemical analysis of keratin expression in response to ligand activation of PPARβ/δ. Wild-type (+/+) and Pparb/d–null (−/−) mice were topically treated with acetone or TPA (5 µg/mouse) followed by topical administration of vehicle or 5 µM GW0742. Skin samples were obtained after 6 or 24 hours, and keratin expression was examined using immunohistochemistry. Representative photomicrographs of skin samples for (A) keratin 5 and (B) keratin 14.
2.4.3 Ligand activation of PPARβ/δ by GW0742 does not alter expression of the PTEN/PDK1/ILK1/Akt cell survival pathway and apoptosis

To examine the effect of PPARβ/δ activation on the PTEN/PDK1/ILK1/Akt cell survival pathway, primary keratinocytes from wild-type and Pparb/d−null mice were cultured in the presence of GW0742 and used for analysis of mRNA expression by northern blotting and protein expression by quantitative western blotting. A significant increase in the expression of ADRP mRNA was observed in wild-type keratinocytes after 8 and 12 h of culture in 0.2 µM GW0742, and this effect was not observed in similarly treated Pparb/d−null keratinocytes (Figure 2-7A). These results demonstrate a PPARβ/δ-dependent increase of ADRP mRNA expression by GW0742. However, in contrast to a previous report [57], ligand activation of PPARβ/δ led to an increase in the mRNA encoding PTEN, and no significant changes in the expression of mRNA encoding PDK1 or ILK1 in wild-type keratinocytes (Figure 2-7A). Interestingly, constitutive expression of mRNA encoding PDK1 and ILK1 was significantly higher in Pparb/d−null keratinocytes as compared to wild-type keratinocytes (Figure 2-7A). Quantitative protein analysis showed no significant differences in the level of PTEN, PDK1, ILK1, phospho-Akt or Akt by GW0742 in wild-type or Pparb/d−null keratinocytes (Figure 2-7B). However, constitutive expression of ILK1 was significantly higher in Pparb/d−null keratinocytes (Figure 2-7B), consistent with mRNA analysis (Figure 2-7A).
Furthermore, *in vivo* quantitative protein analysis showed no significant differences in the level of PTEN, ILK1, phospho-Akt or Akt by GW0742 in wild-type or *Pparb/d*-null mice (Figure 2.7C). However, expression of PDK1 was significantly higher in *Pparb/d*-null keratinocytes (Figure 2.7C). These results obtained from animal skin are similar to results from keratinocyte expression analysis (Figure 2.7).
Figure 2-7: Effect of PPARβ/δ activation on the PTEN/PDK1/ILK1/Akt cell survival pathway. (A) Expression of mRNA encoding PTEN, PDK1, ILK1 or the PPARβ/δ target gene ADRP was determined by Northern blot analysis as described in Materials and methods. Normalized hybridization values are presented as the fold change relative to control and represent the mean ± S.E.M. Significantly (*) different than (+/+) control at 0 h, $P \leq 0.05$. (B) Protein was isolated from (+/+ and (−/−) keratinocytes cultured in the presence of absence of 0.2 µM GW0742 for up to 12 h and separated by SDS-PAGE. Quantitative western blot analysis was performed using radioactive detection methods as described in Materials and methods. Normalized hybridization values are presented as the fold change relative to control and represent the mean ± S.E.M. Significantly (*) different than control (+/+ at 0 h, $P \leq 0.05$. (C). Mice were treated topically with 50 µM GW0742 for 8 hours, fold-induction of normalized Protein levels were determined relative to αLDH using Western Blot analysis. Significantly (*) different than controls, $P \leq 0.05$. 
These results suggest that ligand activation of PPARβ/δ in keratinocytes would not lead to inhibition of the apoptotic pathway as previously suggested by others [55-57]. Indeed, when apoptosis was induced in primary keratinocytes using serum withdrawal, similar decreases in cell number were observed in both genotypes (Figure 2-8A). Further, treatment with 0.5 µM GW0742 did not alter the decrease in average cell number caused by serum withdrawal in either genotype at either timepoint (Figure 2-8A). Consistent with previous reports, treatment of wild-type keratinocytes with 0.5 µM GW0742 resulted in a significant decrease in the average number of cells after three days of culture in the presence of serum, and this effect was not found in similarly treated Pparb/d−null keratinocytes (Figure 2-8A). Additionally, average cell number was significantly greater in Pparb/d−null keratinocytes cultured in the presence of serum after three days as compared to wild-type keratinocytes (Figure 2-8A). These results demonstrate that while ligand activation of PPARβ/δ can inhibit cell growth of primary keratinocytes, GW0742 does not exacerbate or attenuate the decrease in cell number resulting from serum withdrawal. Since serum withdrawal can reduce cell number through mechanisms of growth inhibition including necrosis and apoptosis, the effect of UV irradiation on apoptosis was examined in mouse primary keratinocytes using caspase-3 activity as a marker. Consistent with the analysis of serum withdrawal, UV-induced caspase-3 activity was not different between wild-type and Pparb/d−null keratinocytes (Figure 2-8B).
**Figure 2-8**: Effect of PPARβ/δ on serum deprivation and UV-induced changes in cell growth in wild-type (+/+) and *Pparb/d*–null (−/−) primary keratinocytes. Primary keratinocytes from (+/+) and (−/−) mice were treated with and without 8% serum in the presence or absence of 0.5 µM GW0742. Cell number was measured using a Coulter counter after 1 day of culture (left panel) and 3 days of culture (right panel). Values represent the mean ± S.E.M. (*) Significantly less than (+/+) control cultured in the presence of 8% serum. (†) Significantly greater than (+/+) control cultured in the presence of 8% serum. (B) UV-induced caspase 3 activity was measured in (+/+) and (−/−) keratinocytes after 9 h (left panel) and 18 h (right panel) Values represent the mean ± S.E.M. Significantly (*) different than control, *P* < 0.05.
2.4.4 Ligand activation of PPARβ/δ inhibits mouse papilloma and carcinoma cell line growth

The observed inhibition of replicative DNA synthesis and chemopreventive effects in the chemically-induced skin carcinogenesis suggests that ligand activation of PPARβ/δ could inhibit proliferation of cells in varying stages of skin carcinogenesis (e.g. initiated cells, benign papillomas and pre- and post-malignant carcinomas). Thus, the effect of ligand activation in three mouse keratinocyte tumor cell lines was examined. The 308 keratinocyte cell line has a ras mutation and was derived from mouse skin following initiation with DMBA [33,34]. 308 cells can form papillomas with and without tumor promotion when grafted onto mouse skin in vivo [33,34]. The SP1 keratinocyte cell line is a papilloma-like cell line derived from DMBA/TPA treated animals with a ras mutation and produces papillomas in vivo when grafted onto mouse skin [33,34]. The Pam212 keratinocyte cell line is a carcinoma-like cell line derived from spontaneous transformation of neonatal keratinocytes in culture condition, and produces squamous cell carcinoma in vivo when grafted onto mouse skin [35]. All three of these keratinocyte cancer lines are resistant to calcium-induced terminal differentiation [39]. PPARβ/δ is expressed in the three keratinocyte cancer lines, but is noticeably lower as compared to control keratinocytes (Figure 2-9). Inhibition of cell proliferation is observed in all three keratinocyte cancer cell lines in response to ligand activation of PPARβ/δ (Figure 2-10).
Figure 2-9: PPARβ/δ expression in neoplastic keratinocyte cancer cell lines. (A) RNA was isolated from primary keratinocytes (Primary) or neoplastic keratinocytes (308 cells, SP1 cells or Pam212 cells) and Pparb/d mRNA was measured using quantitative real-time PCR analysis. (B) Protein was isolated from mouse primary keratinocytes (Kera) or neoplastic keratinocytes (308 cells, SP1 cells or Pam212 cells) and PPARβ/δ protein quantified using quantitative western blot analysis. Values represent the fold change relative to keratinocytes.
Figure 2-10: Ligand activation of PPARβ/δ inhibits cell proliferation in neoplastic keratinocyte cancer cell lines. Neoplastic keratinocytes were cultured in low (0.05 mM) calcium culture medium containing either GW0742 (0.5 µM) or L165041 (2.5 µM) for 6 days prior to cell proliferation assay and treatment was continue during the assay (n=4 replicates per treatment group). Cell number was quantified using a Coulter counter. Cell proliferation kinetics in (A) 308 keratinocytes, (B) SP1 keratinocytes, and (C) Pam212 keratinocytes. Values represent the mean ± S.E.M. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, $P \leq 0.05$. 
As indirect measures of the ability of PPAR\(_{\beta/\delta}\) to modulate events that promote conversion of an initiated cell into a cancerous lesion, a colony formation assay was performed using the 308 keratinocyte cell line. Unlike primary keratinocytes that undergo terminal differentiation in high (1.4 mM) calcium medium, 308 keratinocytes form proliferative foci when seeded at low density in this high calcium medium [33,34]. Treatment with the PPAR\(_{\beta/\delta}\) ligand GW0742 or TTA inhibited foci formation and caused a significant reduction in the average size of foci in both the presence and absence of TPA (Figure 2-11A-C). For example, GW0742 and TTA caused a 47% and 99% decrease, respectively, in the average number of colonies as compared control DMSO cells (Figure 2-11B). Similarly, GW0742 and TTA caused a 16% and 76% in average colony size as compared to control DMSO cells (Figure 2-11B). Culturing cells in the presence of the PPAR\(_{\gamma}\) agonist troglitazone also inhibited colony formation and average size, while the PPAR\(_{\alpha}\) agonist WY-14,643 had no effect (Figure 2-11A-C).
Figure 2-11: Ligand activation of PPARβ/δ inhibits colony/foci formation in an initiated keratinocyte cancer cell line. 308 keratinocytes were cultured in the presence of PPAR ligands (PPARα - 50 µM Wy-14,643; PPARβ/δ-1 µM GW0742 or 10 µM TTA; PPARγ – 30 µM troglitazone) under high (1.4 mM) calcium conditions in the presence or absence of 32 nM TPA. Foci were stained with rhodamine dye at the end of treatment period. (A) Representative photomicrographs of stained foci from each group. The average number of foci (B) and the average size of foci (C) in each group. Values represent the mean ± S.E.M. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, $P \leq 0.05$. 
2.4.5 Ligand activation of PPARβ/δ induces differentiation of mouse papilloma and carcinoma cell lines

To determine if ligand activation of PPARβ/δ induces terminal differentiation in neoplastic keratinocyte cell lines, cornified envelope formation was examined in 308 and Pam212 cells. Indeed, treatment of 308 keratinocytes and Pam212 keratinocytes with PPARβ/δ ligands increased cornified envelope formation (Figure 2-12). To further examine the effect of ligand activation on terminal differentiation, expression of mRNA markers of differentiation was examined in the 308 keratinocyte cell line. Increased expression of mRNA encoding ADRP and SPRs was observed after treatment with PPARβ/δ ligands (Figure 2-13A). Additionally, expression of mRNA encoding keratin 5, keratin 6A and keratin 14 were decreased, and mRNA encoding keratin 10 was increased by GW0742 treatment in 308 cells (Figure 2-13B). Similarly, expression of mRNA encoding keratin 5 and keratin 14 were decreased, and mRNA encoding involucrin was increased by L165041 treatment in 308 cells (Figure 2-13B). Both GW0742 and L165041 increased expression of the known PPARβ/δ target gene angiopoietin-like protein 4 (Figure 2-13B).
Figure 2-12: Ligand activation of PPARβ/δ induces terminal differentiation in 308 and Pam212 keratinocyte cancer cell lines. Cells were seeded at approximately 90% confluency and cultured in low calcium (0.05 mM) medium, or high calcium (1.4 mM) medium to induce terminal differentiation, in the presence or absence of either GW0742 (0.5 µM) or L165041 (5.0 µM). The average number of cornified envelopes was determined in (A) 308 keratinocytes and (B) Pam212 keratinocytes. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, $P \leq 0.05$. 
Figure 2-13: Ligand activation of PPARβ/δ increases mRNA markers of terminal differentiation in 308-initiated keratinocytes. (A) Northern blot analysis of a PPARβ/δ target gene (ADRP) and differentiation-associated genes (SPR1A and SPR2H) using RNA from 308 keratinocytes after treatment with PPAR ligands (PPARα – 25 µM Wy-14,643; PPARβ/δ – 0.5 µM GW0742, 10 µM TTA, 5 µM L165041; PPARγ – 10 µM troglitazone) for 24 hours using culture medium containing high calcium (1.4 mM). Significantly (*) different than controls, P ≤ 0.05. (B) Quantitative real-time PCR analysis of a PPARβ/δ target gene (ANGPTL4) and differentiation-associated genes (keratin 5 [K5], keratin 6A [K6A], keratin 10 [K10], keratin 14 [K14], involucrin) using RNA from 308 keratinocytes after treatment with either 0.5 µM GW0742 or 5 µM L-165041 under culture conditions as described above. Values represent the mean ± S.E.M. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, P ≤ 0.05.
Since the induction of terminal differentiation can be associated with an increase in apoptotic or apoptotic-like signaling, the effect of ligand activation of PPARβ/δ on neoplastic keratinocyte apoptosis was also examined. There was no change in caspase 3/7 activity in 308, SP1 or Pam212 neoplastic keratinocytes in response to GW0742 (Figure 2-14). Following exposure to UV radiation, caspase 3/7 activity increased in all three neoplastic keratinocyte cell lines although Pam212 cell line keratinocyte was less responsive as compared to 308 or SP1 keratinocyte cell lines (Figure 2-14). Further, while UV irradiation caused a significant increase in caspase 3/7 activity, the presence of GW0742 did not significantly modulate this increase in any of the three neoplastic keratinocyte cell lines (Figure 2-14).

![Figure 2-14](image)

**Figure 2-14**: Ligand activation of PPARβ/δ does not influence caspase 3/7 activity in neoplastic keratinocyte cancer cell lines. Caspase 3/7 activity was examined in neoplastic keratinocytes treated with or without 1 µM GW0742 and either with or without UV irradiation. Values represent the mean ± S.E.M. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, \( P \leq 0.05 \).
2.5 Discussion

Two-stage chemically-induced skin tumorigenesis is exacerbated in Pparb/d−null mice [29] and ligand activation of PPARβ/δ induces terminal differentiation and inhibits cell growth in keratinocytes [11,30-32,40,41]. These observations support the hypothesis that ligand activation of PPARβ/δ could be chemopreventive for chemically-induced skin cancer. Results from the present study showed that ligand activation of PPARβ/δ during the promotion phase of chemically-induced skin cancer inhibited the onset of tumor formation, the incidence of tumors, and tumor multiplicity. This clearly demonstrates that ligand activation of PPARβ/δ is chemopreventive in a chemically-induced skin carcinogenesis model. Since none of the tumors examined in the wild-type mice were squamous cell carcinomas, it cannot be determined whether ligand activation of PPARβ/δ can inhibit malignant conversion. However, it is of interest to note that the average number of keratocanthomas was significantly reduced by GW0742 in the wild-type mice and this effect was not found in Pparb/d−null mice. While keratocanthomas are benign lesions in humans, they can progress to malignant carcinomas in mice [42]. This suggests that ligand activation of PPARβ/δ could potentially inhibit malignant conversion. Consistent with this idea, squamous cell carcinomas were only found in Pparb/d−null mice, and administration of GW0742 had no influence on this endpoint. Further research is needed to determine whether malignant conversion can be inhibited by ligand activation of PPARβ/δ.
Since there are a number of PPARβ/δ ligands that have been and continue to be developed, it will also be of interest to determine if all PPARβ/δ ligands will be suitable for chemoprevention and/or chemotherapy of skin cancer. Ultraviolet (UV) radiation contributes significantly to the incidence of human skin cancer, whether ligand activation of PPARβ/δ will be useful for preventing or treating UV-induced skin cancers should be evaluated. Interestingly, contrary to previous studies showing inhibition of apoptosis by PPARβ/δ activation [55, 56, 57]; ligand activation of PPARβ/δ does not influence the PTEN/PDK1/ILK1/Akt cell survival pathway or serum deprivation-induced apoptosis, consistent with reports by Burdick et al., Borland et al., and Marin et al. [40, 24, 25, 58, 59]. Additionally, ligand activation of PPARβ/δ does not influence UV-induced caspase 3 activity in primary or neoplastic keratinocytes in the present study, which suggest that modulation of apoptotic signaling as a mechanism for skin cancer prevention or treatment may not be central in the PPARβ/δ-UV-induced skin cancer model. However, it remains possible that ligand activation of PPARβ/δ may effectively induce terminal differentiation in response to UV-induced skin lesions and this idea should be examined in greater detail.

As an alternative to examining malignant conversion, the effect of ligand activation in keratinocyte cell lines representing different stages of neoplasia was also examined. Despite their known resistance to calcium-induced terminal differentiation, induction of mRNA markers of terminal differentiation was found in 308 cells and Pam212 cells in response to ligand activation of PPARβ/δ.
Further, an increase in cornified envelopes was also observed in neoplastic keratinocyte cell lines after ligand activation of PPARβ/δ. This is also consistent with inhibition of cell proliferation found in 308 and Pam212 cells, the inhibition of foci number and size observed with the colony formation assay, and the inhibition of chemically-induced skin tumorigenesis in response to ligand activation of PPARβ/δ. This clearly demonstrates that ligand activation of PPARβ/δ can induce terminal differentiation in cell types that have DNA damage and are predisposed to develop into skin tumors and another cell type that models squamous cell carcinomas. Collectively, these findings suggest that ligand activation of PPARβ/δ can target specific cell lineages critical for the progression of chemically-induced skin cancer and inhibit cell growth by inducing terminal differentiation. In particular, these findings also suggest that ligand activation of PPARβ/δ can be both chemopreventive and chemotherapeutic.

Based on results from the present studies and findings from previous work as well, the mechanism by which ligand activation of PPARβ/δ inhibits chemically-induced skin tumorigenesis is likely through the induction of terminal differentiation with a concomitant inhibition of cell growth of tumor cells. For example, there is strong evidence from a number of independent laboratories supporting a role for PPARβ/δ in mediating terminal differentiation and/or inhibition of cell growth in a variety of cell types (reviewed in [2,24]).
In contrast to work by others showing increased expression of terminal differentiation markers preceding the chemopreventive influence of deleting cyclooxygenase during chemical carcinogenesis [43], earlier than normal expression of keratin 1 and keratin 10 was not found in response to ligand activation of PPARβ/δ, following acute exposure to the tumor promoter TPA in vivo. However, discordant expression of keratin 5 and keratin 14 at the mRNA level were observed in the absence of PPARβ/δ expression following TPA administration, consistent with the previously observed hyperplastic phenotype in Pparb/d−null mice treated with TPA [27-29,44].

There is an interesting balance between differentiation and apoptosis in keratinocytes that could be influenced by PPARβ/δ and impact skin tumorigenesis. There is evidence that when keratinocytes undergo terminal differentiation to ultimately form a cornified cell, that this process is associated with increased activity of caspases including caspase 3 and caspase 14 [45,46]. While caspase 3 is known to be central in the process of apoptosis and increased caspase 3 activity occurs during keratinocyte terminal differentiation [11], differentiating and differentiated keratinocytes (e.g. cornified cells) do not exhibit shrinkage, DNA fragmentation and are not phagocytosed [47,48]. Additionally, caspase 14 does not participate in apoptotic signaling but rather modulates terminal differentiation [45]. Thus, apoptotic signaling observed in differentiating keratinocytes is relatively unique.
While there is strong evidence that ligand activation of PPAR\(\beta/\delta\) induces terminal differentiation in keratinocytes, this process likely involves apoptotic-like signaling as well. Since it is known that increasing apoptosis by chemicals such as silymarin can inhibit skin carcinogenesis [49], it is possible that the observed inhibition of skin carcinogenesis by ligand activation of PPAR\(\beta/\delta\), which could be due in part to increased terminal differentiation and inhibition of cell growth, could also be enhanced by modulating apoptotic signaling as well. This idea should be examined in greater detail.

While one mechanism by which ligand activation of PPAR\(\beta/\delta\) can inhibit tumorigenesis is through the induction of terminal differentiation and inhibition of cell growth, it is important to note that other mechanisms may also be involved. For example, there is good evidence that PPAR\(\beta/\delta\) mediates anti-inflammatory activities in a number of cell types including colon epithelium, macrophages, cardiomyocytes, immune cells, keratinocytes, myoblasts, endothelial cells, nervous tissue and hepatocytes (reviewed in [5]). Additionally, recent evidence indicates a novel role for PPAR\(\beta/\delta\) agonists in suppressing expression of tissue factor, which is central in initiating thrombosis [50]. This is of interest because inhibition of thrombosis is a strategy for chemoprevention and/or chemoprevention [51-54]. Whether the anti-inflammatory activities or anti-thrombotic activity of PPAR\(\beta/\delta\) ligands contributes to the observed inhibition of chemically-induced skin tumorigenesis in the present studies should be further examined.
Additionally, whether combining ligand activation of PPARβ/δ with other potential therapeutics that target other major pathways (e.g. kinases, growth factor receptors, etc) can increase the efficacy of chemoprevention and/or chemotherapy deserves further investigation.
2.6 Bibliography


activated receptor (PPAR)\(\beta/\delta\) stimulates differentiation and lipid accumulation in keratinocytes. *J Invest Dermatol*, **122**, 971-83.


Chapter 3

Peroxisome proliferator-activated receptor-beta/delta (PPARβ/δ) inhibits v-ras\textsuperscript{Ha}-induced malignant transformation of primary keratinocytes

3.1 Abstract

Mice lacking functional PPARβ/δ exhibit enhanced tumorigenesis in the two-stage chemical carcinogenesis model. Additionally, ligand activation of PPARβ/δ inhibits skin carcinogenesis in both in vivo and ex vivo skin carcinogenesis models and these effects are not found in the absence of PPARβ/δ expression. Ligand activation of PPARβ/δ induces terminal differentiation and inhibits proliferation of in both primary and neoplastic keratinocytes carrying activating ras mutation. Mutation of the Ha-ras allele is a critical event in the initiation stage in the two-stage chemical carcinogenesis model. In the present studies, the effect of PPARβ/δ on v-ras\textsuperscript{Ha}-induced neoplastic/malignant transformation of primary keratinocytes was examined using ex vivo models. v-ras\textsuperscript{Ha}-infected keratinocytes exhibited increased mitogen-activated protein kinase (MAPK) signaling, cell proliferation and decreased ras-induced cellular senescence and growth arrest in the absence of PPARβ/δ expression.
Furthermore, ligand activation of PPARβ/δ induced terminal differentiation in v-ras\(^{Ha}\)-infected wild-type keratinocytes and this effect was not seen in v-ras\(^{Ha}\)-infected Pparb/d-null keratinocytes. Additionally, v-ras\(^{Ha}\)-infected wild-type keratinocytes were protected from malignant transformation, while v-ras\(^{Ha}\)-infected Pparb/d-null keratinocytes underwent rapid malignant transformation. These studies suggest that PPARβ/δ attenuates skin carcinogenesis via inhibition of oncogenic ras signaling.

### 3.2 Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. PPARs regulate expression of genes involved in a myriad of biological functions, including cell proliferation, immune response, differentiation, fatty acid metabolism and energy homeostasis [1-4]. The PPAR subfamily consists of three distinct isoforms, PPARα, PPARβ/δ and PPARγ. The physiological roles of PPARα and PPARγ have been extensively characterized, whereas the role of PPARβ/δ is only beginning to emerge. PPARβ/δ is the most ubiquitously expressed PPAR isotype, and exhibits high level of expression in the skin [5,6]. PPARβ/δ is activated by natural ligands such as fatty acids and their derivatives and by synthetic agonists such as tetradecylthioacetic acid (TTA), GW501516, GW0742, and L-165041.
PPARβ/δ agonists are being evaluated as therapeutic agents for the treatment of diabetes, dyslipidemia and other metabolic syndromes, as there is strong evidence that PPARβ/δ promotes lipid and glucose homeostasis. Recent reports showed that the PPARβ/δ agonist GW501516 markedly increases serum high-density lipoproteins (HDL) levels, while lowering serum triglyceride levels in obese animals [7]. Ligand activation of PPARβ/δ also increases fatty acid oxidation in skeletal muscle by upregulation of fatty acid metabolizing enzymes [8-11]. In addition to modulating lipid homeostasis, ligand activation of PPARβ/δ also improves insulin sensitivity in diabetic animals [12-14]. Furthermore, PPARβ/δ exhibits anti-inflammatory activity in a number of different models systems, and this could be a mechanism by which PPARβ/δ attenuate inflammation-associated metabolic disease such as diabetes [15-22]. These results demonstrate the potential of PPARβ/δ agonists as therapeutics for the prevention/treatment of various metabolic diseases.

Despite the potential of PPARβ/δ agonists as therapeutics for various metabolic syndromes, the role of PPARβ/δ in carcinogenesis remains controversial [23,24]. The first evidence to suggest that PPARβ/δ modulate skin carcinogenesis was the observation that animals lacking PPARβ/δ exhibited enhanced epidermal hyperplasia in response to the tumor promoter TPA [23]. Recent evidence showed that mice lacking PPARβ/δ exhibited enhanced skin carcinogenesis in the two-stage chemical carcinogenesis model [25].
*Pparb/d*-null mice exhibited earlier onset of tumor formation, increased tumor incidence, and increased tumor size compared to similarly treated wild-type mice [25]. *Pparb/d*-null mice also exhibited enhanced epidermal hyperplasia and cell proliferation, and reduced caspase 3 activity and apoptosis in response to topical treatment with TPA [25]. Results from Kim et al., 2004 suggested that PPARβ/δ dependent transcriptional regulation of ubiquitin C (UbC) was partially responsible for the attenuation of skin carcinogenesis [25]. In another report, Kim et al. suggested that PPARβ/δ inhibits epidermal cell proliferation through the inhibition of PKCα/Raf/MEK/ERK activity (MAP kinase cascade) via the ubiquitin-mediated turnover of the upstream target PKCα [26]. Treatment with PPARβ/δ agonist (GW501516) has also been shown to stimulate differentiation *in vivo* and in a hyperplasia disease model [20]. Subsequent studies showed that PPARβ/δ agonist selectively inhibits epidermal cell proliferation and induced differentiation in a PPARβ/δ dependent manner [27]. In addition, recent data showed that ligand activation of PPARβ/δ inhibits chemically-induced skin carcinogenesis in both *in vivo* and *ex vivo* skin cancer models [28]. These reports support the hypothesis that PPARβ/δ inhibits skin carcinogenesis; however, the mechanisms mediating this effect remained uncertain.
Examination of tumors produced by the dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) (two-stage) skin carcinogenesis model reveals that nearly all tumors contained an activated c-ras"Ha oncogene [29,30]. In addition, malignant progression of benign tumors has been associated with over-expression, amplification, and homozygosity of oncogenic ras [31]. Targeted introduction of oncogenic ras into the epidermis of animals can replace the initiation step in the two-stage chemical carcinogenesis model [32,33]. Introduction of ras oncogene into normal keratinocytes can also produce tumors when grafted onto nude mice [29,30]. Ras proteins modulate varied signal transduction pathways in response to activation by cell surface proteins that mediate cellular responses to various extracellular stimuli [34,35]. Ras promotes cell growth and survival in response to various external stimuli including hormones, growth factors, and cytokines [35-37]. Ras promotes cell proliferation via the mitogen-activated protein kinase (MAPK) signaling cascade, which upregulate several growth genes including activator protein 1 (AP1) family genes [38-40]. Activator protein 1 (AP1) transcription factors are essential for v-ras"Ha-induced transformation of epidermal cells [41]. Additionally, ras has been shown to promote cell survival via activation of the PI3K/AKT cell survival pathway [42-44]. Previous reports showed that activation of PPARβ/δ attenuated skin carcinogenesis in ex vivo and in vivo models involving activating ras mutation(s). This suggests that PPARβ/δ could mediate this effect by inhibition of oncogenic ras signaling, which was examined in these studies.
3.3 Materials and Methods

3.3.1 Virus Production

The v-ras\textsuperscript{Ha} retrovirus was generated from ψ2 producer cells as described previously [45]. Virus titer was determined to be between 1–2 x 10\textsuperscript{7} virus/ml using a NIH3T3 focus-forming assay.

3.3.2 Cell Culture

Primary keratinocytes from newborn C57BL/6 mice were prepared and cultured according to established methods [46]. Keratinocytes were infected with the v-ras\textsuperscript{Ha} retrovirus on day 3 of culture for 3 days at a MOI of 1–3 to ensure infection of all cells in culture.

3.3.3 Cell Counting

For growth curves, v-ras\textsuperscript{Ha}-infected keratinocytes were seeded on 12-well tissue culture trays and treated in low calcium (0.05 mM) or high calcium (0.50 mM) medium in the presence or absence of 1 μM GW0742 [47]. Duplicate samples for each treatment group were counted twice at indicated timepoints using a using a Z1 Coulter particle counter® (Beckman Coulter, Hialeah, FL).
3.3.4 Cell Cycle Analysis

Subconfluent cells were pulsed with 10 µM bromodeoxyuridine (BrdUrd) (Sigma) for 1hr, trypsinized, fixed in 70% ethanol, and then stained with either a FITC-labeled anti-BrdUrd monoclonal antibody and propidium iodide or propidium iodide alone, according to the manufacturer's specifications (Phoenix Flow Systems). DNA content of stained cells were analyzed by flow cytometry. Duplicate samples were analyzed for each treatment group. Approximately 10,000 cells/sample were analyzed using an EPICS-XL-MCL flow cytometer (Beckman Coulter Electronics, Hialeah, FL). The percentage of cells at each phase of the cell cycle ± S.D. was determined with MultiCycle® analysis software.
3.3.5 SA-β-galactosidase Assay

v-ras<sup>Ha</sup>-infected keratinocytes were treated with DMSO or 0.5 µM GW0742 [47] three days post-retroviral infection. Following an additional seven days in culture, cells were washed in PBS, fixed for 3-5 min (room temperature) in 0.5% glutaraldehyde (pH 7.2), washed, and incubated at 37°C (no CO<sub>2</sub>) with fresh senescence-associated β-galactosidase (SA-β-Gal) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal) per ml (stock = 20 mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5mM potassium ferricyanide/ 150 mM NaCl/2 mM MgCl2. To detect lysosomal β-Gal, the citric acid/sodium phosphate was pH 4.0. Staining was evident by 2-4 hr and maximal after 12-16 hr. The percentages of β-galactosidase-positive cells were quantified using a Nikon inverted microscope (20X microscope frame). β-galactosidase-positive cells were expressed as a percentage of total cells for five different quadrants of each well and averaged to represent the percentage of β-galactosidase-positive cells per well. Triplicate samples were analyzed for each treatment group.
3.3.6 In vitro Malignant Transformation Assay

Primary keratinocytes were plated in 6-well culture dishes at high density and retrovirally infected on day 3. Infected keratinocytes were cultured for 15 days in Eagle’s Minimal Essential Medium containing 0.05 mM calcium and 8% FBS. The infected keratinocytes at 100% confluency were then switched to 0.5 mM calcium medium for 6 weeks, and colonies of keratinocytes resistant to calcium-induced differentiation were identified by staining the cells with 0.35% rhodamine/10% formalin. Rhodamine stained-colonies were counted with a dissecting microscope and colony diameter was measured using a ruler.

3.3.7 Neoplastic Keratinocyte Differentiation Assay

To quantify cornified envelope formation, neoplastic keratinocytes were seeded at approximately 90% confluency in 6-well plates and treated with either GW0742 under low calcium (0.05 mM) or a terminal-differentiation inducing condition (1.4 mM calcium). After this treatment period, cells were collected and treated with lysis solution containing 2% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol for 5 min. Undissolved cornified envelopes were quantified with a hemocytometer. Triplicate samples were analyzed for each treatment group.
3.3.8 Protein analysis

Following treatment, cells were washed twice in ice-cold PBS, and then lysed in cold lysis buffer (20 mM Tris pH 7, 2mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 M leupeptin, 5 M pepstatin A, 0.5 % Triton X-100) containing 1% protease inhibitor cocktail and 1% NP40. Insoluble material was removed by centrifugation and protein concentration quantified from the supernatants (Pierce-BCA). Equal amounts of protein were separated using SDS-PAGE, and transferred to a PVDF membrane. Transfer efficiency and equal protein loading was verified with Ponceau-S staining. Following blocking for 1 h in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20, immunoblots were incubated with primary antibodies. The primary antibodies for ERK1/2, phospho-ERK1/2, retinoblastoma (Rb), phospho-Rb (S780, S807/S811), Elk-1, phospho-Elk-1 (S383), MEK1/2, phosphor-MEK1/2, p21, CDK2, CDK4, cyclin D1 were obtained from Cell Signaling (Beverly, MA) and the antibody for lactic dehydrogenase (LDH) was from Jackson Immunoresearch (West Grove, PA). After incubation with primary antibodies, membranes were washed and then incubated with biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA). Subsequently, membranes were washed, incubated in 125I-streptavidin, and exposed to phosphorimager plates. Hybridization signals were detected using a phosphorimager and normalized to the signal detected for αLDH, which was used as a loading control.
3.4 Results

3.4.1 PPARβ/δ promotes cellular senescence in v-ras\textsuperscript{Ha}-infected keratinocytes

Introduction of oncogene(s) or mutation of proto-oncogene(s) in normal cells usually results in induction of cellular senescence and growth arrest as a negative feedback mechanism under cell culture conditions [48,49]. To determine if PPARβ/δ promotes senescence in oncogenic v-ras\textsuperscript{Ha}-transformed keratinocytes, an SA-β-Galactosidase assay was performed in v-ras\textsuperscript{Ha}-infected wild-type and Pparb/d-null keratinocytes grown in low calcium medium in the presence or absence of 0.5 μM GW0742, following 10 days in culture. The absence of PPARβ/δ expression in v-ras\textsuperscript{Ha}-infected keratinocytes significantly inhibited v-ras\textsuperscript{Ha}-induced senescence; however treatment of v-ras\textsuperscript{Ha}-infected keratinocytes with GW0742 did not significantly enhance induction of cellular senescence in wild-type keratinocytes (Figure 3-1).
Figure 3-1: **PPARβ/δ** promotes cellular senescence in v-ras\textsuperscript{Ha}-infected keratinocytes. v-ras\textsuperscript{Ha}-infected keratinocytes at equal density were treated with DMSO or GW0742, 3 days post-retroviral infection and fixed following an additional 7 days of culture and stained for SA-β-galactosidase. The percentage of β-galactosidase-positive cells was quantified using a Nikon inverted microscope. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, $P \leq 0.05$. 

\begin{figure}[h]
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\caption{PPARβ/δ promotes cellular senescence in v-ras\textsuperscript{Ha}-infected keratinocytes.}
\end{figure}
Induction of cellular senescence by the \( v-ras^{Ha} \) oncogene involves the promotion of G1 phase cell cycle arrest [48,49]. To examine the effect of PPAR\( \beta/\delta \) on cell cycle in \( v-ras^{Ha} \)-infected keratinocytes, cell cycle and proliferation analyses were performed in \( v-ras^{Ha} \)-infected wild-type and \( Pparb/d \)-null keratinocytes grown in low and high calcium medium in the presence or absence of GW0742 following 6 days in culture. The percentage of \( v-ras^{Ha} \)-infected keratinocytes in the G1, S or G2/M phase was similar for both wild-type and \( Pparb/d \)-null keratinocytes in both low and high calcium medium and in the absence or presence of GW0742 (Figure 3-2A-B). Despite the similarity in cell cycle profile between both \( v-ras^{Ha} \)-infected wild-type and \( Pparb/d \)-null keratinocytes, analysis of cell proliferation in both low and high calcium medium showed that the absence of PPAR\( \beta/\delta \) significantly promoted proliferation in \( v-ras^{Ha} \)-infected keratinocytes; however treatment with GW0742 did not inhibit proliferation in \( v-ras^{Ha} \)-infected wild-type keratinocytes when compared to DMSO (vehicle)-treated controls (Figure 3-2C-D).
Figure 3-2: The effect of PPARβ/δ on cell cycle and growth arrest in v-ras$^{Ha}$-infected keratinocytes. v-ras$^{Ha}$-infected keratinocytes were seeded at equal density and treated with DMSO or GW0742 in low (0.05 mM) or high (1.4 mM) calcium medium 3 days post-retroviral infection and (A,B) cell cycle and (C,D) proliferation analyses were performed following additional 4 days in culture. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, $P \leq 0.05$. 
Several cell cycle regulatory proteins tightly control cell cycle progression and growth arrest [50-54]. The effect of PPARβ/δ on the expression of cell cycle proteins was examined in \( v-ras^{\text{Ha}} \)-infected wild-type and \( Pparb/d \)-null keratinocytes grown in low calcium medium following 6 days in culture. Consistent with the cell cycle analysis data, the expression of cyclin D1, CDK2 and CDK4, and phosphorylation of Rb were similar in both \( v-ras^{\text{Ha}} \)-infected wild-type and \( Pparb/d \)-null keratinocytes in the presence or absence of GW0742. (Figure 3). All \( v-ras^{\text{Ha}} \)-infected keratinocytes expressed high level of \( v-ras^{\text{Ha}}\cdot\text{p21} \) protein (Figure 3-3).
Figure 3-3: The effect of PPARβ/δ on cell cycle regulators expression in v-ras\(^{Ha}\)-infected keratinocytes. v-ras\(^{Ha}\)-infected keratinocytes were treated with DMSO or GW0742 in low (0.05 mM) calcium medium 3 days post-retroviral infection and western blot analysis of cyclin D1, CDK2, CDK4, pRb, Ras and \(\alpha\)LDH expression were performed following an additional 4 days in culture.
3.4.2 PPAR\(\beta/\delta\) promotes growth arrest and inhibits proliferation in v-ras\(^{Ha}\)-infected keratinocytes

To examine the effect of PPAR\(\beta/\delta\) on v-ras\(^{Ha}\)-induced keratinocyte growth arrest, a cell proliferation assay was performed in v-ras\(^{Ha}\)-infected wild-type and Pparb/d\(^{-}\)-null keratinocytes in both low and high calcium medium in the presence or absence of GW0742 over a period of 96 hours. The absence of PPAR\(\beta/\delta\) expression significantly inhibited v-ras\(^{Ha}\)-induced growth arrest in low calcium medium (Figure 3-4). Culturing of v-ras\(^{Ha}\)-infected keratinocytes in high calcium medium inhibited v-ras\(^{Ha}\)-induced growth arrest in both v-ras\(^{Ha}\)-infected wild-type and Pparb/d\(^{-}\)-null keratinocytes, however the rate of proliferation was significantly higher in Pparb/d\(^{-}\)-null keratinocytes as compared to similarly treated wild-type keratinocytes (Figure 3-4). Treatment with GW0742 had no significant effect on keratinocyte growth arrest or proliferation in either genotype (Figure 3-4).
Figure 3-4: PPARβ/δ promotes growth arrest and inhibits proliferation in v-ras\textsuperscript{Ha}-infected keratinocytes. v-ras\textsuperscript{Ha}-infected keratinocytes were seeded at equal density and treated with DMSO or GW0742 in (A) low (0.05 mM) or (B) high (1.4 mM) calcium medium 6 days post-retroviral infection and cell proliferation analysis were performed over a period of 4 days. Significantly (*) different than controls, $P\leq 0.05$. 
3.4.3 PPARβ/δ inhibits MAPK signaling pathways in v-ras<sup>Ha</sup>-infected keratinocytes

The MAPK signaling cascade is a major downstream target in oncogenic ras signaling, and its activation of AP1 signaling is critical for ras-induced malignant transformation of primary keratinocytes [38-40]. To examine the mechanism by which PPARβ/δ promotes growth arrest and inhibits proliferation in v-ras<sup>Ha</sup>-infected keratinocytes, the expression and phosphorylation of proteins of the MAPK signaling cascade were examined using western blot analysis. The levels of phospho-MEK and phospho-ERK1/2 were significantly higher in v-ras<sup>Ha</sup>-infected Pparb/d−null keratinocytes when compared to similarly treated v-ras<sup>Ha</sup>-infected wild-type keratinocytes in both low and high calcium medium; with the exception of vehicle treated v-ras<sup>Ha</sup>-infected Pparb/d−null keratinocytes in low calcium medium, which was similar to v-ras<sup>Ha</sup>-infected wild-type keratinocytes (Figure 3-5). Additionally, treatment with GW0742 significantly inhibited phosphorylation of MEK in v-ras<sup>Ha</sup>-infected keratinocytes cultured in high calcium medium and phosphorylation of ERK1/2 in v-ras<sup>Ha</sup>-infected keratinocytes cultured in low calcium medium, in a PPARβ/δ-dependent manner (Figure 3-5). v-ras<sup>Ha</sup>-infected keratinocytes cultured in high calcium medium exhibited increased phosphorylation of both MEK1/2 and ERK1/2 in the absence PPARβ/δ expression (Figure 3-5).
The expression of MEK and ERK1/2 was similar in both wild-type and
Pparb/d-null v-ras$^{Ha}$-infected keratinocytes cultured in both low and high calcium
medium; however, the expression of p44 ERK1/2 was lower in cells cultured in
high calcium medium as compared to cells cultured in low calcium medium
(Figure 3-5).

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Table: PPARβ/δ inhibits MAPK signaling pathways in v-ras$^{Ha}$-infected keratinocytes.

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Figure 3-5: PPARβ/δ inhibits MAPK signaling pathways in v-ras$^{Ha}$-infected keratinocytes. v-ras$^{Ha}$-infected keratinocytes at equal density were treated with
DMSO or GW0742 in low (0.05 mM) or high (1.4 mM) calcium medium 5 days
post-retroviral infection and western blot analysis of MAPK proteins was performed
following 24 hrs of culture. Significantly (*) different than controls, $P\leq0.05$. 
3.4.4 Ligand activation of PPARβ/δ promotes terminal differentiation in v-ras\textsuperscript{Ha}-infected keratinocytes

Primary keratinocytes rapidly undergo terminal differentiation in high calcium medium. In contrast, introduction of v-ras\textsuperscript{Ha} oncogene into primary keratinocytes confers resistance to high calcium induce differentiation [45,55-57]. v-ras\textsuperscript{Ha}-infected wild-type and Pparb/d-null keratinocytes exhibited similar level of cornified envelope formation in the absence of GW0742 in both low and high calcium medium; however treatment of v-ras\textsuperscript{Ha}-infected wild-type keratinocytes with GW0742 resulted in a significantly higher level of cornified envelope formation, an effect not found in similarly treated Pparb/d-null keratinocytes (Figure 3.6). Culturing of v-ras\textsuperscript{Ha}-infected keratinocytes in high calcium medium resulted in a significantly higher level of cornified envelope formation as compared to keratinocytes cultured in low calcium medium, however the percentage of keratinocytes forming cornified envelopes remained extremely low (~1%)(Figure 3-6). Introduction of v-ras\textsuperscript{Ha} oncogene into primary keratinocytes results into neoplastic transformation, which induces negative feedback mechanisms including induction of cellular senescence and growth arrest, which prevents malignant transformation [48,49,55-57]. Malignant transformation of neoplastic keratinocytes can be measured by the ability of the keratinocyte to form foci under differentiation inducing (high calcium medium) conditions [55-57].
Figure 3-6: Ligand activation of PPARβ/δ promotes terminal differentiation in v-ras$^{Ha}$-infected keratinocytes. v-ras$^{Ha}$-infected keratinocytes seeded at equal density were treated with DMSO or GW0742 in low (0.05 mM) or high (1.4 mM) calcium medium 5 days post-retroviral infection and cornified envelope formation was examined following 24 hrs under indicated treatment conditions. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, $P \leq 0.05$. 
3.4.5 PPARβ/δ blocks malignant transformation of v-ras\textsuperscript{Ha}-infected keratinocytes

Examination of the effect of PPARβ/δ on malignant transformation in v-ras\textsuperscript{Ha}-infected keratinocytes showed that malignant transformation of neoplastic keratinocytes is completely inhibited in the presence of PPARβ/δ expression. Ligand activation of PPARβ/δ had no effect on this inhibition (Figure 3-7).

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**Figure 3-7:** PPARβ/δ blocks malignant transformation of v-ras\textsuperscript{Ha}-infected keratinocytes. v-ras\textsuperscript{Ha}-infected keratinocytes at equal density were treated with DMSO or GW0742 in high (1.4 mM) calcium medium 15 days post-retroviral infection and malignant transformation was examined following 5 weeks under indicated treatment condition. Significantly (*) different than control, \( P < 0.05 \).
3.5 Discussion

There is considerable controversy regarding the effect of ligand activation of PPARβ/δ in epithelial carcinogenesis with some studies showing enhanced cell growth, while others show no influence or inhibited cell growth (reviewed in [23,24]). There are a number of possible explanations that could account for the disparity among the various studies, including species-specific effects, cell type-specific effects, ligand-specific effects, differences in experimental design and experimental error. Given the potential beneficial use of PPARβ/δ ligands for the treatment of human diseases, it is essential to precisely determine the effect of ligand activation on epithelial carcinogenesis with specific emphasis on neoplastic and malignant transformation of normal and initiated epithelial cells. For this reason, the present studies examined the effects of PPARβ/δ in v-rasHa-infected primary keratinocytes. Ras proto-oncogene mutation is a rate-limiting factor for tumor initiation in the chemically-induced skin carcinogenesis model, which is exacerbated in the absence of functional PPARβ/δ expression [58]. Results from the present investigation demonstrated that PPARβ/δ promotes cellular senescence and inhibits cell proliferation in ras oncogene-transformed primary keratinocytes.
However, no significant difference in cell cycle or the expression of cell cycle regulators was observed between v-ras\textsuperscript{Ha}-infected wild-type and Pparb/d-null keratinocytes. Furthermore, it should be noted that the majority (~75%) of the v-ras\textsuperscript{Ha}-infected keratinocytes of either genotype are in G1 phase arrest, suggesting that PPAR\(\beta/\delta\) does not play a critical role in cell cycle arrest in the presence of potent cell cycle arrest factors such as the \textit{ras} oncogene. Overall, these findings are consistent with previous studies showing PPAR\(\beta/\delta\) dependent inhibition of cell growth in mouse and human keratinocytes (reviewed in [23,24]); however, these finding suggest that signaling pathways other than cell cycle arrest might be responsible for PPAR\(\beta/\delta\) dependent inhibition of epithelial carcinogenesis [24,28,58-61]. To begin to understand how PPAR\(\beta/\delta\) promotes cellular senescence and inhibition of cell growth, other possible mechanisms were examined.

To examine the mechanisms underlying the inhibition of cell growth by PPAR\(\beta/\delta\) in v-ras\textsuperscript{Ha}-infected keratinocytes, the expression and phosphorylation of proteins of the MAPK signaling cascade, that have critical roles in neoplastic and malignant transformation were examined. Interestingly, v-ras\textsuperscript{Ha}-infected Pparb/d-null keratinocytes exhibited increased levels of phospho-MEK1/2 and phospho-ERK1/2 expression compared to similarly treated v-ras\textsuperscript{Ha} infected wild-type keratinocytes. Whether this effect contributes directly to the PPAR\(\beta/\delta\) mediated inhibition of neoplastic cell proliferation cannot be determined from the present study, but this possibility should be examined in future studies.
The most consistent finding from these studies is the induction of terminal differentiation by GW0742 in v-ras$^{Ha}$-infected wild-type keratinocytes; this finding was not observed in the absence of PPAR$\beta/\delta$ expression. A number of independent laboratories have provided strong evidence that PPAR$\beta/\delta$ can mediate the terminal differentiation of keratinocytes and other cell types [28,62,63]. Findings from the present studies support these observations as induction of cornified envelope formation was also found in v-ras$^{Ha}$-infected wild-type keratinocytes treated with GW0742. Since it is known that the induction of terminal differentiation in keratinocytes is associated with a concomitant decrease in cell growth, this suggests that induction of terminal differentiation is one mechanism by which PPAR$\beta/\delta$ promotes inhibition of cell growth in neoplastic keratinocytes, which are characterized by their resistance to terminal differentiation (reviewed in [23]).

Finally, the findings demonstrating that PPAR$\beta/\delta$ promotes senescence and differentiation and inhibits proliferation suggest that the absence of PPAR$\beta/\delta$ expression could promote malignant conversion of v-ras$^{Ha}$-infected keratinocytes, since it is known that the induction of senescence in oncogene-transformed keratinocytes is associated with a concomitant abrogation of malignant conversion in cell culture [55-57,64]. Indeed, the absence of functional PPAR$\beta/\delta$ expression in v-ras$^{Ha}$-infected keratinocytes resulted in the formation of calcium resistant foci in an ex vivo malignant conversion assay.
It must be noted that senescence induced inhibition of malignant conversion of v-ras$^{Ha}$-infected keratinocytes is overcome \textit{in vivo}. Whether PPAR$\beta/\delta$ dependent promotion of senescence in cell culture attenuates malignant conversion \textit{in vivo} cannot be determined from the present study, but should be examined in future studies. Additionally, future studies should examine the effect PPAR$\beta/\delta$ on v-ras$^{Ha}$-induced skin tumorigenesis and malignant progression \textit{in vivo}, both from an initiated cell and stroma perspective. In summary, results from the current studies showed that PPAR$\beta/\delta$ promotes senescence and differentiation and inhibits proliferation, which ultimately results in the inhibition of malignant conversion in cell culture. Further work is still necessary to definitively determine the molecular mechanisms underlying these effects.
3.6 Bibliography


Chapter 4

Combining Ligand Activation of Peroxisome Proliferator-Activated Receptor-\(\beta/\delta\) (PPAR\(\beta/\delta\)) and Inhibition of Cyclooxygenase 2 (COX2) Enhances Inhibition Skin Tumorigenesis

4.1 Abstract

Ligand activation of peroxisome proliferator-activated receptor-\(\beta/\delta\) (PPAR\(\beta/\delta\)) and inhibition of cyclooxygenase-2 (COX2) activity by non-steroidal anti-inflammatory drugs (NSAID) can both attenuate skin tumorigenesis. There is also evidence that attenuation of skin tumorigenesis by inhibition of COX2 activity occurs through PPAR\(\beta/\delta\) independent mechanisms. The present study examined the hypothesis that combining ligand activation of PPAR\(\beta/\delta\) with inhibition of COX2 activity will increase the efficacy of inhibition of chemically-induced skin tumor progression using both \textit{in vivo} and \textit{ex vivo} models. A two-stage chemical carcinogenesis approach was used to generate wild-type and \textit{Pparb/d–null} mice with papillomas. After twenty-two weeks when a similar incidence of papillomas were present in both genotypes, cohorts of mice were divided into experimental groups and began to be treated with topical application of the PPAR\(\beta/\delta\) ligand GW0742, dietary administration of the COX2 inhibitor nimesulide, or both GW0742 and nimesulide. Ligand activation of PPAR\(\beta/\delta\) did not influence skin tumor progression while a modest decrease in skin tumor multiplicity was observed with dietary nimesulide.
Interestingly, the combined treatment of GW0742 and nimesulide increased the efficacy of the decrease in papilloma multiplicity for six weeks in wild-type mice, but this effect was not found in later timepoints and was not found in similarly treated Pparb/d-null mice. Neoplastic keratinocyte lines cultured with GW0742 and nimesulide also exhibited enhanced inhibition of cell proliferation. Results from these studies support the hypothesis that combining ligand activation of PPARβ/δ with inhibition of COX2 activity can inhibit chemically-induced skin tumor progression.

4.2 Introduction

Peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) is a ligand-activated transcription factor that is related to PPARα, PPARγ and other members of the nuclear hormone receptor superfamily. In response to ligand activation, PPARβ/δ can regulate homeostasis by direct transcriptional upregulation of target genes that modulate physiological functions ranging from fatty acid catabolism, glucose homeostasis and inflammation (reviewed in [1-3]). However, there is also evidence that PPARβ/δ can also down-regulate transcription of target genes, most notably those associated with inflammation (e.g. TNFα, MCP), and in doing so mediate anti-inflammatory activities via interfering with other transcription factors such as NF-κB [4-6].
Because ligand activation of PPARβ/δ can modulate lipid and glucose homeostasis, and inflammation, targeting PPARβ/δ for the treatment and prevention of diabetes, obesity and dyslipidemias is of current interest.

There is also compelling evidence from many laboratories demonstrating that ligand activation of PPARβ/δ promotes terminal differentiation and is associated with inhibition of cell proliferation in a number of cell types (reviewed in [7-9]). These collective observations strongly support the hypothesis that PPARβ/δ can inhibit tumorigenesis by promoting terminal differentiation and/or inhibiting cell growth. Consistent with this idea, it was originally shown that deletion of PPARβ/δ in skin caused enhanced cell proliferation in response to tumor promotion using two different Pparb/d−null mouse models [10,11], suggesting that PPARβ/δ attenuates cell proliferation in skin. Subsequent studies established that chemically-induced skin cancer is exacerbated in the absence of PPARβ/δ expression [12], and that ligand activation of PPARβ/δ can attenuate chemically-induced skin tumorigenesis [7]. The mechanism underlying the observed inhibition of skin tumorigenesis by ligand activation of PPARβ/δ is likely due to the induction of terminal differentiation and associated inhibition of cell growth [13], but could also be due in part to PPARβ/δ attenuation of kinase signaling [14]. Combined, these findings suggest that ligand activation of PPARβ/δ may be suitable for chemoprevention and/or chemotherapy of skin tumorigenesis.
There is also a large body of evidence showing that inhibition of cyclooxygenase (COX) can prevent a number of cancers (reviewed in [15]). In particular, previous studies using genetic and pharmacological approaches suggest that inhibition of COX2 activity can inhibit both chemically-induced and UV-induced skin tumorigenesis [16-19]. The mechanisms by which COX-derived prostaglandins exert their pro-neoplastic effects are not completely understood but include both receptor-dependent and independent activities. The mechanisms underlying the chemopreventive effects of COX inhibitors are largely thought to be due to down-regulation of prostaglandin production by COX, which in turn leads to reduced activities of prostaglandins that promote cell growth and cell survival. While targeting COX for chemoprevention and chemotherapy using specific pharmacological inhibitors has recently been hampered due to significant toxicities associated with cardiovascular function, pharmacological inhibitors of COX and related signaling molecules (e.g. prostaglandin receptors) remains of interest.

Given the reported chemopreventive effect of PPARβ/δ agonists and COX2 inhibitors and the recent finding that chemoprevention of chemically-induced skin tumorigenesis occurs through mechanisms that are independent of PPARβ/δ [20], the present study examined the hypothesis that combining ligand activation of PPARβ/δ with inhibition of COX2 activity will increase the efficacy of inhibition of chemically-induced skin tumor progression.
4.3 Material and method

4.3.1 Two-stage chemical carcinogenesis bioassay

Wild-type and \textit{Pparb/d}-null mice on a C57BL/6 genetic background [11] were initiated with 50 µg of 7,12-dimethylbenz(a)anthracene (DMBA) dissolved in 200 µL acetone. One week after initiation, mice were treated topically with 5 µg of 12-O-tetradecanoylphorbol-13-acetate (TPA), 3 days/week. After 22 weeks, the percentage of mice with papillomas was similar between genotypes. At this time, to determine the effect of combining ligand activation of PPARβ/δ and inhibition of COX2 activity on skin tumor progression, four cohorts of mice from both genotypes were divided into one of the following groups: control diet and topical application of acetone, control diet and topical application of the PPARβ/δ ligand GW0742 (5 µM), nimesulide diet (400 mg/kg) and topical application of acetone, or nimesulide diet (400 mg/kg) and topical application of GW0742 (5 µM). The concentration of topical GW0742 was used because previous work has demonstrated that this is within the concentration range that will specifically activate PPARβ/δ in skin [7,13]. The concentration of nimesulide was used because previous work has demonstrated that this concentration can inhibit chemically-induced colon cancer [21]. After a total of 43 weeks, mice were euthanized by overexposure to carbon dioxide.
Skin and tumor samples from each mouse were fixed in 10% neutral-buffered formalin or 70% ethanol and then paraffin embedded, sectioned and stained with H&E. H&E-stained sections of suspected carcinomas were scored for benign or malignant pathology.

4.3.2 Keratinocyte-ex-vivo cancer models

To examine the hypothesis that combining ligand activation of PPARβ/δ with inhibition of COX2 activity can inhibit cell proliferation of initiated or neoplastic keratinocytes, the following keratinocyte cell lines were used: 1) 308 keratinocyte cell line, derived from DMBA-treated mouse epidermis [22,23], 2) SP1 keratinocyte cell line, derived from a DMBA/TPA induced papilloma [22,23], 3) Pam212 keratinocyte cell line, derived from spontaneous transformation of neonatal keratinocytes [24] and 4) Wild-type and Pparb/d−null v-ras<sup>Ha</sup> transformed primary keratinocytes, derived by infecting primary keratinocytes with the v-ras<sup>Ha</sup> retrovirus for 5 days in culture [25].
4.3.3 **Cell Proliferation assay**

308, SP1 and Pam212 neoplastic keratinocytes were seeded at equal density and treated with either vehicle control, GW0742 (1 µM), nimesulide (100 µM) or GW0742 (1 µM) and nimesulide (100 µM) for up to 96 hours. Cell number was quantified over time in triplicate, independent samples using a Z1 Coulter particle counter (Beckman Coulter, Fullerton, CA).

4.3.4 **Measurement of Prostaglandins in keratinocytes**

Neoplastic keratinocyte cell lines were cultured with either GW0742, nimesulide or both as described above. Twenty-four hours after treatment, the cellular concentrations of PGE$_2$, PGF$_{2\alpha}$ and PGI$_2$ (as assessed by 6-keto PGF$_{1\alpha}$) were determined using enzyme-linked immunoassays (Assay designs, Ann Arbor, MI; Cayman Chemical, Ann Arbor, MI).

4.3.5 **Western blot analysis**

Keratinocytes were lysed in 1X RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with supplemental protease and phosphatase inhibitors. Samples were centrifuged at 14,000 rpm at 4 °C for 30 minutes and the supernatant obtained. Twenty micrograms of protein from each sample was resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
The samples were transferred onto a polyvinylidene difluoride membrane using an electroblotting method. Membranes were blocked in 5% milk in Tris buffered saline Tween-20 and incubated overnight at 4 °C with the primary antibody. The following antibodies were used: anti-COX2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PPARβ/δ [26] or anti-PPARγ (Affinity BioReagents, Golden, CO). After washing, membranes were incubated with biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. Immunoreactive proteins were detected by incubating membranes with (1^{125})-labeled streptavidin followed by exposure to phosphorimager plates and the level of radioactivity quantified with a Packard phosphorimager. Hybridization signals were normalized to the hybridization signals of lactate dehydrogenase (Rockland, Gilbertsville, PA).
4.4 Results

4.4.1 The combination of ligand activation of PPARβ/δ and inhibition of COX2 inhibits chemically-induced skin tumorigenesis

To determine if combining ligand activation of PPARβ/δ with inhibition of COX2 activity can inhibit skin tumor progression, wild-type and Pparb/d–null mice with pre-existing tumors were treated with the highly specific PPARβ/δ ligand GW0742 and the COX2 inhibitor nimesulide. Consistent with previous reports [7,12], the incidence of papilloma formation was higher in Pparb/d–null mice as compared to similarly treated wild-type mice (Figure 4.1a). Topical application of the PPARβ/δ ligand GW0742 alone did not significantly affect skin tumor progression in either genotype as shown by a lack of change in tumor multiplicity and average tumor size (Figures 4.1b and 4.1c). Dietary nimesulide significantly decreased tumor multiplicity in wild-type mice between week 28 and week 31, and this effect was surprisingly not found in similarly treated Pparb/d–null mice (Figure 4.1b). The combination of dietary nimesulide and topical application of GW0742 resulted in a decrease in tumor multiplicity between week 25 and 31 in wild-type mice and this effect was greater as compared to that observed with dietary nimesulide alone (Figure 4.1b). While tumor multiplicity was modestly lower in Pparb/d–null mice treated with both dietary nimesulide and topical GW0742, this effect was not statistically significant (Figure 4.1b).
Average tumor size was modestly greater in the \textit{Pparb/d}–null mice as compared to wild-type mice, but this difference was not statistically significant (Figure 4-1c). None of the treatment regimes caused a significant change in average tumor size in either genotype (Figure 4-1c).
Figure 4-1: The combination of ligand activation of PPARβ/δ and inhibition of COX2 inhibits chemically-induced skin tumorigenesis. Wild-type (+/+) and Pparβ/d-null (−/−) mice with skin tumors were treated with GW0742 (GW), nimesulide (NIM) or the combination of GW0742 and nimesulide beginning 22 weeks after initiating a 2-stage chemical carcinogenesis bioassay (arrow), and continuing for 21 weeks. (a) The incidence and onset of tumor formation. (b) Tumor multiplicity. (c) Average tumor size per mouse.
Topical GW0742 caused a decrease in the incidence of keratocanthomas in wild-type mice not found in Pparb/d–null mice, but the average number of keratocanthoma per mouse did not exhibit a PPARβ/δ-dependent decrease (Figures 4-2a and 4-2c). The incidence of keratocanthomas and the average number of keratocanthoma per mouse were not influenced by dietary nimesulide (Figures 4-2a and 4-2c). The lower incidence of keratocanthomas was similar as a result of combining GW0742 with nimesulide as compared to GW0742 alone (Figure 4-2a) and the average number of keratocanthoma per mouse was lower in response to the combined treatment of both GW0742 with nimesulide in both wild-type and Pparb/d–null mice (Figure 4-2c). Squamous cell carcinomas were only found in Pparb/d–null mice and not in wild-type mice (Figures 4-2b and 4-2d).
Figure 4-2: The combination of ligand activation of PPARβ/δ and inhibition of COX2 inhibits tumor progression. Incidence and average number of keratoacanthoma and squamous cell carcinomas in wild-type (+/+) and Pparb/d−null (−/−) mice in response to ligand activation of PPARβ/δ and inhibition of COX2. Suspected keratoacanthomas and squamous cell carcinomas were examined microscopically and classified as either keratoacanthomas or squamous cell carcinomas by an expert pathologist. (a) Incidence of keratoacanthomas or carcinomas. (b) Multiplicity of keratoacanthoma or squamous cell carcinomas. Values represent the average number of lesions per mouse with lesion.
4.4.2 The combination of ligand activation of PPARβ/δ and inhibition of COX2 inhibits cell proliferation in keratinocyte cancer lines

The observed inhibition of chemically-induced skin tumor progression by ligand activation of GW0742 and inhibition of COX2 activity suggests that this combined treatment paradigm could also inhibit proliferation during early and later stages of tumor progression. To begin to examine this idea, the effect of combining GW0742 with nimesulide on neoplastic keratinocyte proliferation was examined in neoplastic keratinocytes in varying stages of skin carcinogenesis (e.g. initiated cells, benign papillomas and pre- and post-malignant carcinomas). Thus, the effect of ligand activation in three mouse keratinocyte tumor cell lines was examined. The 308 keratinocyte cell line has a ras mutation and was derived from mouse skin following initiation with DMBA [22,23]. 308 cells can form papillomas with and without tumor promotion when grafted onto mouse skin \textit{in vivo} [22,23]. The SP1 keratinocyte cell line is a papilloma-like cell line derived from DMBA/TPA treated animals with a ras mutation and produces papillomas \textit{in vivo} when grafted onto mouse skin [22,23]. The Pam212 keratinocyte cell line is a carcinoma-like cell line derived from spontaneous transformation of neonatal keratinocytes in culture condition, and produces squamous cell carcinoma \textit{in vivo} when grafted onto mouse skin [24]. These three keratinocyte cancer lines are resistant to calcium-induced terminal differentiation [27].
In the 308 keratinocyte cell line, inhibition of cell proliferation was not observed by day two of exposure but by day four, GW0742, nimesulide and the combined treatment of GW0742 and nimesulide caused a significant decrease in cell proliferation as compared to controls (Figure 4-3a). In the SP1 keratinocyte cell line, inhibition of cell proliferation was observed by day two and day four in response to GW0742 and nimesulide, and this effect was more pronounced by day four following the combined exposure to GW0742 and nimesulide (Figure 4-3b). In the Pam212 keratinocyte cell line, treatment of these keratinocyte cancer lines with GW0742 or nimesulide significantly inhibited proliferation, and this inhibition was further enhanced by treatment with a combination of both GW0742 and nimesulide (Figure 4-3).
Figure 4-3: The combination of ligand activation of PPARβ/δ and inhibition of COX2 inhibits cell proliferation in keratinocyte cancer lines. 308, SP1 and Pam212 keratinocyte cancer lines were cultured in low (0.05 mM) calcium keratinocyte culture medium containing GW0742 (1 µM), nimesulide (100 µM) or the combination of both (n=3 replicates per treatment group). Cell number for (A) 308 keratinocytes, (B) SP1 keratinocytes, and (C) Pam212 keratinocytes were quantified using a Coulter counter. Values represent mean ± S.E.M. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, P≤0.05.
4.4.3 Ligand activation of PPARβ/δ and inhibition of COX2 inhibit skin tumorigenesis via independent mechanisms

Previous reports suggest that PPARβ/δ and COX2 inhibitors attenuate epithelial carcinogenesis via independent mechanisms [20,21]. Oncogenic ras transformation of primary keratinocytes results in elevated COX2 mediated prostaglandins synthesis [36]. To examine the effect of PPARβ/δ on COX2 mediated prostaglandin synthesis, prostaglandins levels were measured in wild-type and Pparb/d−null v-rasH<sup>Ha</sup>-transformed primary keratinocytes. The absence of PPARβ/δ expression did not affect PGE<sub>2</sub>, 6-Keto PGF<sub>1α</sub> and PGF<sub>2α</sub> levels in v-ras<sup>Ha</sup>-transformed primary keratinocytes (Figure 4-4). Additionally, treatment of 308 and SP1 keratinocyte cancer lines with nimesulide (100 µM) or a combination of both nimesulide (100 µM) and GW0742 (1 µM) decreased PGE<sub>2</sub>, 6-Keto PGF<sub>1α</sub> and PGF<sub>2α</sub> levels, however treatment with GW0742 (1 µM) alone did not affect prostaglandins levels (Figure 4-5).
Figure 4-4: PPARβ/δ does not affect COX2 mediated prostaglandin synthesis in v-rasH<sup>a</sup>-transformed primary keratinocytes. The effect of PPARβ/δ on prostaglandin synthesis in neoplastic keratinocytes was examined by measuring prostaglandin levels in v-rasH<sup>a</sup>-transformed wild-type or Pparb/d−null primary keratinocytes. Values represent mean ± S.E.M. Values with different letters are significantly different as determined by ANOVA and post-hoc testing, P≤0.05.
Figure 4-5: Prostaglandin concentration is lowered by COX2 inhibitors, but not influenced by PPARβ/δ agonists in neoplastic keratinocytes. The effect of ligand activation of PPARβ/δ and COX2 inhibition on prostaglandin synthesis in neoplastic keratinocytes was examined by measuring prostaglandin concentration in culture medium from keratinocyte cancer cell lines following treatment with GW0742 (1 µM), nimesulide (100 µM) or the combination of both GW0742 and nimesulide. Values represent mean ± S.E.M. Values with different letters are significantly different, $P \leq 0.05$. 
To further examine the effect of PPARβ/δ on COX2 mediated prostaglandin signaling and verse visa, PPARβ/δ, PPARγ and COX2 protein levels were examined in 308 and SP1 keratinocyte cancer lines following treatment with GW0742 (1 μM), nimesulide (100 μM) or the combination of GW0742 (1 μM) and nimesulide (100 μM). Treatment with GW0742 (1 μM), nimesulide (100 μM) or combination of GW0742 (1 μM) and nimesulide (100 μM) did not affect PPARβ/δ, PPARγ and COX2 protein levels in both keratinocyte cancer lines (Figure 4-6).

**Figure 4-6:** The effect of ligand activation of PPARβ/δ and inhibition of COX2 activity on COX2, PPARβ/δ and PPARγ expression in neoplastic keratinocytes. A representative western blot showing the protein levels of COX2, PPARβ/δ and PPARγ quantified in 308 and SP1 keratinocyte cancer lines following treatment with GW0742 (1 μM), nimesulide (100 μM) or the combination of both GW0742 and nimesulide. Values represent the fold change from control and are the mean ± S.E.M. based on analysis of 4 independent samples. Significantly (*) different than controls, \( P \leq 0.05 \).
4.5 Discussion

The role of PPARβ/δ activation in skin carcinogenesis requires clarification and elucidation of specific mechanisms due to discrepancies in the literature. A number of recent reports in the literature suggest that PPARβ/δ is a target of the COX2-regulated prostaglandin signaling pathway [28-30]. Furthermore, these reports suggested that PPARβ/δ promotes colorectal cancer growth and invasion [28-30]. However, these reports are inconsistent with data showing that PPARβ/δ inhibits inflammation and cell proliferation and induces terminal differentiation, which ultimately results in attenuation of epithelial carcinogenesis [3,9,13,20,31-34]. Furthermore, recent reports showed that PPARβ/δ and COX2 inhibitors attenuate skin and colon tumorigenesis via independent mechanisms [20, 21]. However, the combination of ligand activation of PPARβ/δ and COX2 inhibition did not caused an enhancement in the attenuation of colon tumorigenesis [21]. The combination of chemotherapeutic drugs such as 5-Fluorouracil and difluoromethyl-ornithine, with COX inhibitors have been shown to enhanced the efficacy of skin tumorigenesis attenuation than either compound alone, and could potentially serve as a strategy to treat chemotherapy resistant tumors [19].

Results from previous studies showing ligand activation of PPARβ/δ attenuates skin tumorigenesis, suggest that ligand activation of PPARβ/δ or COX2 inhibition could regress skin tumors and that the combination of both therapies could enhance regression of skin tumors than either therapy alone.
Consistent with previous studies, results from the present study showed that PPARβ/δ attenuates tumor incidence and multiplicity in a chemically-induced skin carcinogenesis model. Results from the present study also showed that COX2 inhibition is chemotherapeutic in a chemically-induced skin carcinogenesis model, as treatment with the COX2 specific inhibitor nimesulide briefly reduced tumor multiplicity in wild-type mice, but not in Pparb/d−null mice. Furthermore, combining ligand activation of PPARβ/δ with COX2 inhibition enhanced this reduction of tumor multiplicity in wild-type mice but not in Pparb/d−null mice over the same brief time span. Since none of the tumors examined in the wild-type mice were squamous cell carcinomas, it cannot be determined whether ligand activation of PPARβ/δ or COX2 inhibition or the combination of both therapies can inhibit malignant conversion. However, consistent with previous studies, the incidence of keratocanthomas was higher in Pparb/d−null mice when compared to wild-type mice, and ligand activation of PPARβ/δ attenuated this incidence. While keratocanthomas are benign lesions in humans, they can progress to malignant carcinomas in mice [35]. This result confirmed that ligand activation of PPARβ/δ could potentially inhibit malignant conversion. Consistent with this idea, squamous cell carcinomas were only found in Pparb/d−null mice, further research is needed to determine whether malignant conversion can be inhibited by ligand activation of PPARβ/δ. Additionally, further research is also needed to examine the chemopreventive efficacy of the combination of ligand activation of PPARβ/δ and COX2 inhibition in skin carcinogenesis.
As an alternative to examining malignant conversion, the effect of ligand activation of PPARβ/δ or COX2 inhibition or the combination of both therapies in keratinocyte cell lines representing different stages of neoplasia was also examined. Consistent with previous studies, ligand activation of PPARβ/δ or COX2 inhibition attenuated neoplastic cell proliferation and these effects were enhanced by the combination of both therapies. Collectively, these findings suggest that ligand activation of PPARβ/δ or COX2 inhibition can target specific cell lineages critical for the progression of chemically-induced skin cancer and inhibit cell growth; and that the combination of both therapies can enhance this inhibition.

Previous reports showed that introduction of ras oncogene into normal epithelial cells induces COX2 mediated prostaglandin synthesis, which promotes neoplastic and malignant transformation [36]. Quantification of prostaglandins levels in v-ras\(^{Ha}\)-infected keratinocytes, showed similar levels for various prostaglandins in both wild-type and Ppar\(b/d\)–null keratinocytes, suggesting that PPARβ/δ does not influence ras-induced COX2 mediated prostaglandins synthesis. Additionally, treatment of neoplastic keratinocytes (308 and SP1 cells), harboring activating ras mutation with the PPARβ/δ ligand GW0742 demonstrated that ligand activation of PPARβ/δ does not affect prostaglandin synthesis. Consistent with previous studies, treatment of neoplastic keratinocytes with the COX2 inhibitor nimesulide or a combination of GW0742 and nimesulide inhibited prostaglandins synthesis with similar efficiency.
Furthermore, this present study also demonstrated that treatment of neoplastic keratinocytes with GW0742 or nimesulide or a combination of GW0742 and nimesulide does not affect PPARβ/δ, PPARγ, or COX2 expression.

Based on results from the present studies and findings from previous work, the combination of ligand activation of PPARβ/δ and COX2 inhibition most likely regress chemically-induced skin tumors via distinct and independent signaling pathways, because the effect of COX2 inhibitor was similar in both wild-type and Pparb/d−null mice [20,21]. This study showed that COX2 and PPARβ/δ signaling pathways as distinct and independent of each other, because PPARβ/δ does not affect COX2 mediated prostaglandin synthesis and inhibition of COX2 did not affect PPARβ/δ expression. Results from Bility et al., 2008 showed that induction of terminal differentiation and concomitant inhibition of proliferation by ligand activated PPARβ/δ is a possible mechanism by which PPARβ/δ attenuates skin carcinogenesis, and might be the mechanism by which activated PPARβ/δ contributes to COX2 inhibition-mediated attenuation of skin carcinogenesis [7]. Additionally, previous studies showed that activation of PPARβ/δ inhibits of cell growth and promotes terminal differentiation in mouse and human keratinocytes [7,8,13,37]. COX2 inhibitor-mediated attenuation of carcinogenesis is mediated through the inhibition of prostaglandin mediated tumor-specific angiogenesis, cell proliferation and inhibition of apoptosis [38].
Though results from the present studies support the hypothesis that PPARβ/δ and COX2 signaling pathways are distinct and independent of each other, these signaling mechanisms might influence similar biological processes such as proliferation and differentiation and contribute additively or synergistically to the attenuation of skin carcinogenesis. Further examination is required in order to elucidate the mechanism(s) for the possible additive or synergistic effects of PPARβ/δ agonists and COX inhibitors, but the outcome is clear, both chemicals attenuate skin carcinogenesis. Further characterization of the chemotherapeutic/chemopreventive efficacy of combining ligand activation of PPARβ/δ with COX2 inhibition could lead to more effective approaches for the treatment of skin cancer. Additionally, future studies combining PPARβ/δ ligands with other chemo-therapeutic/chemopreventive agents could potentially increase the chemotherapeutic/chemopreventive efficacy of these agents additively or synergistically.
4.6 Bibliography


5.1 Discussion

The genesis of PPARβ/δ can be traced back to the cloning of the receptor in 1992 [1-3]. The role of PPARβ/δ in normal physiology and pathology remained a mystery for more than a decade. It has only been in recent years, since the development of the Pparb/d-null mouse models, coupled with development of highly specific PPARβ/δ ligands that the biological role of PPARβ/δ has begun to emerge [4-7]. Since the development of these tools, PPARβ/δ has been implicated in a number of biological processes, including glucose and lipid homeostasis, inflammation, as well as carcinogenesis [8-20]. Additionally, PPARβ/δ agonists were shown to have therapeutic effects on several metabolic diseases including diabetes and obesity [15,18,21]. Ligand activation of PPARβ/δ enhances fatty acid catabolism and energy uncoupling, and modulate lipoprotein metabolism to raise HDL cholesterol and lower triglycerides, resulting in decreased triglyceride stores in adipose tissue, improved endurance performance in skeletal muscle, and enhanced cardiac contractility, all of which contribute to improving metabolic profile. Recent studies also showed that PPARβ/δ activation in the liver suppresses hepatic glucose output, thus improving glucose homeostasis [15].
Furthermore, ligand activation of PPARβ/δ is also anti-inflammatory in macrophages, which could contribute to the stabilization of metabolic profile, as inflammation has been implicated in several metabolic syndromes [22,23]. PPARβ/δ agonists are been evaluated in clinical trials for their therapeutic efficacy in attenuating metabolic diseases. Lastly, ligand activation of PPARβ/δ has been shown to induce terminal differentiation, resulting in the inhibition of cell proliferation and induction of apoptosis, thus implicating this receptor in carcinogenesis [19,24-26]. However, a relatively small number of reports show contrary roles, which suggest that ligand activation of PPARβ/δ inhibits apoptosis, promotes cell proliferation and ultimately results in the promotion of carcinogenesis [27-32].

Due to these controversies and the use of PPARβ/δ in clinical trials, it has become critical to determine the safety of PPARβ/δ ligands as therapeutic agents in the treatment and/or prevention of metabolic diseases. The purpose of these studies aimed to definitively determine the role of PPARβ/δ in skin carcinogenesis, with the intention of clarifying current controversies in the literature. Early studies reported that PPARβ/δ is a downstream target of the oncogenic Wnt/adenomatous polyposis coli (APC)/β-catenin pathway, suggesting that it has a role in potentiating colon carcinogenesis [27]. However, more recent reports have disputed this role, suggesting that the receptor has no effect on colon polyp formation, with a large number of reports suggesting that ligand activation of PPARβ/δ attenuate colon carcinogenesis [6,25,33-35].
Furthermore, recent reports showed that Pparb/d-null mice exhibit enhanced epidermal hyperplasia in response to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), thus suggesting functional roles for PPARβ/δ in skin homeostasis and carcinogenesis [24]. Indeed, consistent with the initial observation of enhanced epithelial hyperplasia in Pparb/d-null mouse skin in response to tumor promotion, a two-stage (DMBA/TPA) carcinogenesis bioassay study showed an exacerbated onset of tumor formation, tumor size, and tumor multiplicity in the absence of functional PPARβ/δ expression, providing strong in vivo evidence that PPARβ/δ can attenuate epithelial cell proliferation and skin carcinogenesis [24].

The studies performed in chapter 2 were designed to definitively determine the role of ligand activation of PPARβ/δ in skin carcinogenesis using in vivo and ex vivo models of skin carcinogenesis. Results from the in vivo skin carcinogenesis model confirmed that PPARβ/δ protects against skin tumorigenesis, as Pparb/d-null mice exhibited enhanced skin tumor incidence and multiplicity. Furthermore, administration of GW0742 resulted in attenuation of skin tumor incidence and multiplicity in wild-type mice, but not in Pparb/d-null mice, suggesting that PPARβ/δ agonists could serve as chemopreventive agents. Malignant conversion of papillomas to carcinomas was not detected in wild-type mice in this study; therefore it cannot be determined whether ligand activation of PPARβ/δ inhibits malignant conversion.
However it must be noted that a relatively few tumors in Pparb/d-null mice converted from papillomas to carcinomas, suggesting that PPARβ/δ could inhibit malignant conversion. Additionally, keratocanthoma incidence and multiplicity was reduced in wild-type mice as compared to Pparb/d-null mice and ligand activation of PPARβ/δ enhanced this reduction. Keratocanthomas are benign lesions in humans, however they can progress to malignant carcinomas in mice, thus providing further evidence that ligand activation of PPARβ/δ could potentially inhibit malignant conversion. Further research is needed to determine whether malignant conversion can be inhibited by ligand activation of PPARβ/δ.

Tumor promotion is characterized by uncontrolled cell growth of initiated cells [36]. Pparb/d-null mice exhibit enhance epidermal hyperplasia in response to tumor promoters, suggesting that ligand activation of PPARβ/δ could inhibit epidermal hyperplasia [24]. Results from chapter 2 demonstrated that ligand activation of PPARβ/δ inhibits epidermal hyperplasia in response to a tumor promotor. Additionally, treatment of neoplastic keratinocytes in various stages of neoplasia with PPARβ/δ agonists inhibited cell proliferation, suggesting that ligand activation of PPARβ/δ can target a broad spectrum of neoplastic cells in the inhibition of skin carcinogenesis. Attenuation of skin carcinogenesis usually involves the inhibition of proliferation, induction of differentiation and apoptosis. Indeed, several reports suggest that activation of PPARβ/δ induces terminal differentiation and apoptosis in epithelial cells including keratinocytes [5,26,37-42].
Examination of the effect of ligand activation of PPARβ/δ on differentiation in chapter 2 showed that ligand activation of PPARβ/δ promotes differentiation. Keratins are intermediate filament proteins that are tough and insoluble and form an extensive cytoskeletal network that form hard but non-mineralized structures found in many animals [43-45]. Expression of specific keratins in epidermal keratinocyte is tightly regulated and serves as differentiation markers for keratinocyte differentiation. The type II keratin K5 and type I keratin K14 are expressed and constitute about 30% of the protein expressed in keratinocytes in the basal layer of the epidermis. The type II keratin K1 and type I keratin K10 are highly expressed in keratinocytes in the suprabasal layers, and are critical markers for the induction of differentiation in epidermal keratinocytes [43-45]. Ligand activation of PPARβ/δ in a skin-hyperplasia disease model promoted the expression of differentiation associated keratins. Additionally, treatment of neoplastic keratinocytes characterized by their resistance to calcium-induced terminal differentiation, with PPARβ/δ agonists resulted in the induction of differentiation associated keratins and other late differentiation markers. Furthermore, ligand activation of PPARβ/δ induced cornified envelope formation in neoplastic keratinocytes independent of the classical calcium-induced differentiation pathway.

In addition to examining the effect of ligand activation of PPARβ/δ on differentiation, chapters 2 also examined the effect of ligand activation of PPARβ/δ on apoptosis.
Results from the bulk of the literature suggest that ligand activation of PPARβ/δ does not affect apoptotic signaling; however, some reports suggest that PPARβ/δ promotes epithelial cell apoptosis while others suggest that PPARβ/δ inhibits epithelial cell apoptosis [25,41,42,46-49]. Previous reports showed that ligand activation does not affect the AKT/PDK1 cell survival pathway in epithelial cells including colon cells or human keratinocytes [25,41,42,46]. In contrast to the above reports, few studies suggest that ligand activation PPARβ/δ stimulate the AKT/PDK1 cell survival pathway, which ultimately results in the inhibition of apoptosis in various epithelial cells [47,50-52]. In particular, results from Tan et al., 2001 showed that ligand activation of PPARβ/δ increases the level of PDK1 and decreases the level of PTEN, ultimately resulting in the inhibition of apoptosis [52]. Additionally, Di-Poi et al., 2002 suggested that PPARβ/δ inhibits stress-induced apoptosis via the upregulation of anti-apoptotic signaling proteins (PDK1 and ILK) and the down-regulation of the pro-apoptotic signaling protein (PTEN) [20,49]. A subsequent study suggested that the anti-apoptotic signaling mediated by PPARβ/δ promotes efficient wound healing in a disease model [20,49]. Though the reports by Di-Poi et al., 2002 and 2003 seems to contradict the findings that PPARβ/δ promotes apoptosis, it is possible that PPARβ/δ exhibits differential response in the regulation of apoptosis under different physiological conditions [20,49]. Examination of the effect of ligand activation of PPARβ/δ on apoptosis in chapter 2 showed that PPARβ/δ does not affect the AKT/PDK1 cell survival pathway in both keratinocytes and whole skin.
Consistent with recent reports, ligand activation of PPARβ/δ selectively inhibited keratinocyte proliferation but did not affect serum deprivation-induced growth arrest or apoptosis-associated caspase activity in primary keratinocytes [53]. Furthermore, examination of the effect of ligand activation of PPARβ/δ on apoptosis-associated caspase activity in neoplastic keratinocytes showed that PPARβ/δ agonists do not affect apoptosis in neoplastic cells. These results are consistent with several reports showing that ligand activation of PPARβ/δ does not affect apoptosis, but rather inhibit proliferation and induce terminal differentiation to inhibit epithelial carcinogenesis. Overall, results from chapter 2 demonstrated that ligand activation of PPARβ/δ inhibits skin carcinogenesis via the inhibition of proliferation and the induction of terminal differentiation in neoplastic epidermal cells, but there are a number of future experiments that could be performed to more definitively demonstrate that activation of PPARβ/δ attenuates skin carcinogenesis.

Development of an epidermal-specific Pparb/d-null mouse will greatly aid in the delineation of specific mechanisms by which PPARβ/δ functions in the epidermis. Targeted deletion of PPARβ/δ in epidermal cells using the keratin 5-regulated Cre/loxP system will exclude the deleterious effects or contributing effects from other tissues and will not bias the results of epidermal-specific experiments, making it a powerful model in the study of skin carcinogenesis. This epidermal-specific Pparb/d-null mouse will also make it more feasible to mechanistically determine the function of PPARβ/δ in the epidermis.
Specifically, this model could be used to identify and characterize target genes that are being modulated by activation of PPARβ/δ and how these changes in gene expression lead to functional changes in skin carcinogenesis. Future experiments should also examine the effect of PPARβ/δ activation on skin carcinogenesis using epidermal-specific deletion of PPARβ/δ in a Cre/loxP system that is under conditional regulation (e.g. tetracycline controlled transcriptional activation). This model should be used to examine the effect of PPARβ/δ activation on the various stages of skin carcinogenesis in the two-stage skin carcinogenesis model. Results from this model could provide evidence as to which stage of skin carcinogenesis is most appropriate for the chemopreventive and/or chemotherapeutic administration of PPARβ/δ ligands.

Result from the two-stage bioassay in chapter 2 demonstrated that ligand activation of PPARβ/δ attenuates skin tumorigenesis; however this chemically-induced skin carcinogenesis model is not the best model in terms of human skin cancer relevance. Epidemiological and molecular data strongly suggest that non-melanoma human skin cancers (AK, BCC and SCC) are associated with chronic exposure to high doses of ultraviolet (UV) radiation in sunlight (Reviewed in [54]).
Although stratospheric ozone blocks UVC (below 280 nm) radiation as well as part of UVB radiation (280–290 nm) from reaching the surface of the earth, UVB (290–315 nm) and UVA (315–400 nm) reach the surface of the earth, and cause DNA damage, inflammation and erythema, sunburn, gene mutations, immunosuppression and eventually results in the development of skin cancer (Reviewed in [54]). UVB radiation-induced mutations involving the inactivation of the $p53$ tumor suppressor gene and clonal expansion of the initiated cells are believed to play a critical role in the initiation, promotion, and progression of non-melanoma skin cancers in rodents and humans (Reviewed in [54-57]). However, it must be noted that $ras$ mutations also plays a role in the development of UVB-induced skin cancer depending on the rodent strain and has also been implicated in the development of UV-induced human skin cancers [54,58]. Additionally, long-term exposure to solar UV has also been shown to promote the loss of Fas and Fas ligand interaction and accumulation of $p53$ mutations in the skin (Reviewed in [54-57]). The Fas receptor is a target for transcriptional activation by $p53$ and its activation is essential for the induction of apoptosis in aberrant cells following skin exposure to UV radiation (Reviewed in [54-57]). Future experiments should examine the effect of ligand activation of PPAR$\beta/\delta$ in the UVB-induced skin carcinogenesis bioassay model [59,60]. Tumor formation in this bioassay is initiated and promoted by repeated UVB irradiation, which induces initiation via cyclobutane-type pyrimidine dimers and other photoproducts in the animal genome, with high frequency of mutation occurring in the $p53$ tumor suppressor gene [56,57].
Additionally, UVB irradiation also induces other tumor promoting epigenetic events such as inflammation [55]. Future studies should examine the effect of ligand activation of PPARβ/δ in ex vivo human skin carcinogenesis models involving human epidermal cells harboring p53 inactivating mutation(s) such as HaCaT cell line [61]. This study should utilize RNA interference to determine the effect of aberrant PPARβ/δ expression in ex vivo human skin carcinogenesis models. Results from these future studies will provide evidence on the effect of PPARβ/δ activation on UVB-induced skin carcinogenesis, which is the most relevant rodent model for human skin cancer.

Overall, results from chapter 2 demonstrated that ligand activation of PPARβ/δ inhibits skin carcinogenesis via the inhibition of proliferation and the induction of terminal differentiation in neoplastic epidermal cells; however, results from chapter 2 do not provide evidence as to which oncogenic signaling mechanism(s) is inhibited by activation of PPARβ/δ. Studies in chapter 3 aimed to provide evidence that activation of PPARβ/δ inhibits the oncogenic ras signaling pathway and could be the mechanism or one of the mechanisms by which activation of PPARβ/δ inhibits skin carcinogenesis. Activating mutation(s) of the ras proto-oncogene is a rate-limiting factor for tumor initiation in the chemically-induced skin carcinogenesis model, which was attenuated by ligand activation of PPARβ/δ (chapter 2).
Examination of tumors produced by the, two-stage (DMBA/TPA) skin chemical carcinogenesis model reveals that nearly all tumors contained an activated c-ras\(^{Ha}\) oncogene [62,63]. In addition, malignant progression of benign tumors has been associated with over-expression, amplification, and homozygosity of oncogenic ras [62,63]. Targeted introduction of oncogenic ras into the epidermis of animals can replace the initiation step in the two-stage chemical carcinogenesis bioassay model [64,65]. Furthermore, introduction of ras oncogene into normal keratinocytes can produce tumors when grafted onto nude mice [62,63]. The present study examined the effects of PPAR\(\beta/\delta\) activation on v-ras\(^{Ha}\)-induced neoplastic and malignant transformation of primary keratinocytes. Results from chapter 3 demonstrated that PPAR\(\beta/\delta\) promotes cellular senescence in v-ras\(^{Ha}\)-infected keratinocytes, as cellular senescence was higher in v-ras\(^{Ha}\)-infected wild-type keratinocytes as compared to v-ras\(^{Ha}\)-infected \(Pparb/d\)-null keratinocytes. Introduction of ras oncogene into primary keratinocytes triggers the induction of cellular senescence; however under \textit{in vivo} conditions abrogation of neoplastic transformation is inhibited and v-ras\(^{Ha}\)-infected keratinocytes develop into tumors [62,63]. This result suggests that PPAR\(\beta/\delta\) mediated promotion of senescence might result in the inhibition of neoplastic and malignant transformation in skin carcinogenesis.
Additionally, results from chapter 3 also showed that PPARβ/δ inhibits cell proliferation in v-ras^{Ha}-infected keratinocytes, as cell proliferation was lower in v-ras^{Ha}-infected wild-type keratinocytes as compared to v-ras^{Ha}-infected Pparb/d-null keratinocytes. These results are consistent with results from chapter 2 showing that ligand activation of PPARβ/δ inhibits neoplastic keratinocytes proliferation, and suggest that inhibition of ras signaling might be a mechanism by which PPARβ/δ inhibits skin carcinogenesis. Despite PPARβ/δ dependent inhibition of proliferation and induction of senescence in v-ras^{Ha}-infected keratinocytes, PPARβ/δ did not significantly affect cell cycle and the expression cell cycle genes, suggesting that PPARβ/δ does not play a critical role in cell cycle arrest in the presence of potent cell cycle arrest factors such as the ras oncogene. Overall, these findings are consistent with previous studies showing PPARβ/δ dependent inhibition of cell growth in mouse and human keratinocytes, however these finding suggest that signaling pathways (e.g. terminal differentiation, MAPK signaling) other than cell cycle arrest might be responsible for PPARβ/δ dependent inhibition of skin carcinogenesis.

To examine other possible mechanism(s) underlying the inhibition of cell growth by PPARβ/δ in v-ras^{Ha}-infected keratinocytes, the expression and phosphorylation pattern of proteins of the MAPK signaling pathways, which plays critical roles in neoplastic and malignant transformation, were examined.
Interestingly, v-ras\textsuperscript{Ha}-infected Ppar/b-d-null keratinocytes exhibited increased levels of phosphorylated MAPK proteins when compared to similarly treated v-ras\textsuperscript{Ha}-infected wild-type keratinocytes, suggesting that PPARβ/δ inhibition of ras-mediated MAPK signaling might be a possible mechanism by which PPARβ/δ inhibits skin carcinogenesis. These findings are consistent with previous studies showing activation of PPARβ/δ inhibits MAPK signaling in both mice and human keratinocytes [38,41]. Results from chapter 3 also showed that ligand activation of PPARβ/δ induces terminal differentiation in v-ras\textsuperscript{Ha}-infected keratinocytes via a mechanism independent of calcium-induced differentiation. These results are consistent with results from chapter 2 showing induction of terminal differentiation in neoplastic keratinocytes. Since it is known that induction of terminal differentiation in keratinocytes is associated with a concomitant decrease in cell growth, this suggests that induction of terminal differentiation is one mechanism by which PPARβ/δ promotes inhibition of cell growth in oncogenic ras-infected keratinocytes. Finally, the findings demonstrating that PPARβ/δ promotes senescence and differentiation and inhibits proliferation suggest that the absence of PPARβ/δ expression could promote malignant conversion in v-ras\textsuperscript{Ha}-infected keratinocytes, since it is known that the induction of senescence in ras oncogene-infected keratinocytes is associated with a concomitant abrogation of malignant conversion in cell culture.
Indeed, results from chapter 3 showed that PPARβ/δ inhibits malignant conversion in cell culture, suggesting a role for PPARβ/δ in the abrogation of malignant conversion in skin carcinogenesis. Malignant conversion of benign tumors in the two-stage skin carcinogenesis model is associated with overexpression, amplification, and homozygosity of oncogenic ras and other oncogenes such as fos; however, the C57BL/6 mice strain used in these studies is extremely resistant to malignant conversion of papillomas [62,63]. Introduction of ras oncogene into primary keratinocytes produced tumors when grafted onto mice, and these tumors undergo malignant conversion at a higher rate as compared to the two-stage bioassay model [62,63]. Furthermore, co-infection of v-ras with v-fos oncogene rapidly converts normal keratinocytes into squamous cell carcinoma and significantly enhanced malignant conversion of tumors in xenograft models [62,63]. Results from chapter 3 showed that the absence of PPARβ/δ expression in v-ras\textsuperscript{Ha}-infected keratinocytes inhibited senescence and growth arrest and promoted proliferation and ultimately resulted in the promotion of malignant conversion in an ex vivo model. These results suggest that PPARβ/δ attenuates v-ras\textsuperscript{Ha}-induced skin carcinogenesis; however, these studies do not provide evidence about the role of PPARβ/δ in the inhibition of tumor development and malignant conversion in vivo.
Future studies should examine the effect of ligand activation of PPAR\(\beta/\delta\) on \(v\)-\(ras^{Ha}\)-induced tumor development and malignant conversion \textit{in vivo} in a keratinocyte-graft model. In this model, wild-type and \(Pparb/d\)-null keratinocytes infected with \(v\)-\(ras^{Ha}\) oncogene should be used to examine the role of PPAR\(\beta/\delta\) in tumor development and malignant conversion. In addition to examining the effect of PPAR\(\beta/\delta\) on skin carcinogenesis from an initiated/tumor cell perspective, the effect of stroma-PPAR\(\beta/\delta\) on skin carcinogenesis should also be examine. \(v\)-\(ras^{Ha}\)-infected wild-type and \(Pparb/d\)-null keratinocytes should be grafted onto both wild-type and \(Pparb/d\)-null mice inorder to the examine the effect of stroma-PPAR\(\beta/\delta\) expression on \(v\)-\(ras^{Ha}\)-induced skin carcinogenesis. Future experiments should also examine the differences in gene expression profile between wild-type and \(Pparb/d\)-null keratinocytes prior to and following \(v\)-\(ras^{Ha}\)-induced neoplastic/malignant transformation in the presence and absence of synthetic PPAR\(\beta/\delta\) ligands using microarray gene expression analysis. These studies will provide critical evidence about the various signaling pathways regulated at the transcriptional level by PPAR\(\beta/\delta\) during neoplastic/malignant transformation. Results from chapter 3 showed that PPAR\(\beta/\delta\) inhibits activation of the MAPK signaling cascade, which is downstream of Ras. Future studies should determine the molecular mechanism(s) by which PPAR\(\beta/\delta\) inhibits the MAPK signaling cascade, and also determine if inhibition of this pathway is critical for PPAR\(\beta/\delta\) mediated inhibition of skin carcinogenesis.
Results from these studies should provide definitive evidence about the mechanism(s) by which PPARβ/δ inhibit oncogenic ras signaling. Furthermore, these future studies will provide definitive evidence of the role of PPARβ/δ in oncogenic ras-induced tumor development and malignant conversion in vivo, both from a tumor cell and tumor stroma perspective.

COX2 mediated synthesis of prostaglandins is a major effector in ras signaling mediated neoplastic and malignant transformation of epithelial cells [31,66]. Furthermore, COX2-mediated prostaglandin signaling is critical in the development of various epithelial cancers [67-70]. A number of recent reports suggest that PPARβ/δ is an effector in COX2 signaling mediated promotion of colorectal cancer growth and invasion [28,29,50,51]. In contrast to the above reports, several studies, including results from chapter 2 and 3 showed that PPARβ/δ induces differentiation in a number of cell types including colon and skin, and in general inhibit cell proliferation and promote growth arrest, which ultimately results in attenuation of epithelial carcinogenesis [19,24-26]. Furthermore, recent reports showed that PPARβ/δ and COX2 inhibitors attenuate skin and colon tumorigenesis via independent mechanisms [71,72]. However, the combination of ligand activation of PPARβ/δ and COX2 inhibition did not cause an enhancement in the attenuation of colon tumorigenesis [72].
The combination of chemotherapeutic drugs such as 5-fluorouracil and difluoromethyl-ornithine, with COX2 inhibitors have been shown to enhance attenuation of skin tumorigenesis than either therapy alone, and could potentially serve as a strategy to treat chemotherapy resistant tumors [59,70,73].

Previous reports and results from chapter 2 and 3 showing that ligand activation PPARβ/δ attenuates skin tumorigenesis suggest that ligand activation of PPARβ/δ or COX2 inhibition could regress skin tumors and that the combination of both therapies could enhance regression of skin tumors [26,59]. Consistent with previous studies, results from chapter 4 showed that PPARβ/δ attenuates tumor incidence and multiplicity in a chemically-induced skin carcinogenesis model [26,40]. Results from chapter 4 also confirmed that COX2 inhibition is chemotherapeutic in a chemically-induced skin carcinogenesis model, as treatment with the COX2 specific inhibitor nimesulide briefly reduced tumor multiplicity in wild-type animals, but not in Pparβ/d-null animals. Furthermore, combination of ligand activation of PPARβ/δ and COX2 inhibition enhanced this reduction of tumor multiplicity in wild-type mice over the same brief time span. Additionally, the combination of ligand activation of PPARβ/δ and COX2 inhibition also enhanced inhibition of neoplastic keratinocyte proliferation over either therapy alone, suggesting that combining PPARβ/δ agonists with chemotherapeutic agents such as COX2 inhibitors could provide highly efficient chemopreventive and/or chemotherapeutic therapies.
Overall, results from chapter 4 demonstrated that the combination of ligand activation of PPARβ/δ and COX2 inhibition attenuates skin carcinogenesis, but there are a number of future experiments that could be performed to more definitively demonstrate that the combination of PPARβ/δ agonists and other chemotherapeutic agents could efficiently attenuate skin carcinogenesis than either therapy alone.

Future experiments should examine the chemopreventive effect of the combination of ligand activation of PPARβ/δ and COX2 inhibition on skin carcinogenesis by administering both therapies immediately after initiation of animals and not after tumor development as was done in chapter 4. Additionally, future studies should utilize other COX2 inhibitors, such as celecoxib to examine the chemotherapeutic efficacy of the combination of PPARβ/δ agonists and COX2 inhibitors. Future experiments should also utilize currently approved human skin cancer chemotherapeutic agents such as 5-flurouracil (5-FU) to examine the therapeutic potential of combining approved cancer chemotherapies with PPARβ/δ agonists [59]. The chemotherapeutic agent 5-FU has been used against various cancers for about 40 years and is the most popular and effective chemotherapeutic agent against human skin cancer [59]. 5-FU acts via several mechanisms, but principally as a thymidylate synthase inhibitor [59]. Interrupting the action of this enzyme blocks synthesis of pyrimidine thymidine, which is essential for DNA replication [59].
Future studies should also examine the effect of the combination of PPARβ/δ ligands and anti-neoplastic dietary compounds such as (-)-epigallocatechin-3-gallate, vitamin D, carotenoids, flavanoid polyphenols, isoflavones, catechins, sulforaphane and indole-3-carbinol in the prevention and treatment of skin cancer [74-80].

Overall, results from these studies showed that ligand activation of PPARβ/δ attenuates skin carcinogenesis via the induction of terminal differentiation and the inhibition of cell proliferation. Additionally, these studies suggest that inhibition of oncogenic ras signaling by PPARβ/δ could be the mechanism or one of the major mechanisms by which activation of PPARβ/δ attenuates skin carcinogenesis. Furthermore, results from these studies showed that combining PPARβ/δ agonists with chemotherapeutic agents such as COX2 inhibitors could provide highly efficient chemopreventive and/or chemotherapeutic therapies. Despite showing that ligand activation of PPARβ/δ inhibits skin carcinogenesis, possibly through the induction of terminal differentiation and inhibition of proliferation, these studies do not provide definitive evidence as to the molecular mechanism(s) by which PPARβ/δ inhibits skin carcinogenesis. Further research is needed to elucidate the exact molecular mechanism(s) by which PPARβ/δ activation attenuate skin carcinogenesis.
5.2 Bibliography


VITA

Moses Turkle Bility

EDUCATION
  •  Grade Point Average – 3.15/4.0
  •  Grade Point Average – 3.78/4.0
  •  Thesis advisor: Jeffrey M. Peters
  •  Dissertation: Modulation of skin cancer by PPARβ/δ

PUBLICATIONS


Honors
2004          Huck Institutes Fellow and Bunton-Waller Graduate Award
2003          Huck Institutes Summer Undergraduate Scholar
2000          Engineering design class award, The Pennsylvania State University