The Pennsylvania State University

The Graduate School

Department of Biochemistry and Molecular Biology

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR BETA/DELTA MODULATES ARYL HYDROCARBON RECEPTOR-DEPENDENT SIGNALING AND SKIN CARCINOGENESIS

A Dissertation in

Biochemistry, Microbiology, and Molecular Biology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2010

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ABSTRACT

Since its identification in the early 1990's, the physiological roles of the nuclear hormone receptor peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) have become better understood. This ligand-activated transcription factor is known to be a key regulator of glucose and lipid homeostasis. PPARβ/δ exhibits markedly higher expression in epithelial tissues such as the skin; therefore, this nuclear receptor been investigated in skin tumorigenesis. Most evidence has suggested that ligand activation of PPARβ/δ inhibits skin tumorigenesis, and the results of mechanistic studies have established that these effects are due to receptor-dependent alterations in tumor promotion. The studies within this dissertation examined if PPARβ/δ modulates another critical component of carcinogenesis, namely tumor initiation, and sought to delineate if PPARβ/δ modulates the balance between carcinogen bioactivation and detoxification. Utilizing *in vivo* and *in vitro* mouse and human skin models, it was observed that *Pparβ/δ*-null mice exhibited reduced cytochrome P450 (*Cyp*) mRNA induction in response to polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (B[a]P) and 7,12-dimethylbenz[a]anthracene (DMBA). *Cyp* mRNA induction was also found to be attenuated in *Pparβ/δ*-null keratinocytes at multiple times and with eleven different PAHs. Furthermore, results generated from a PPARβ/δ shRNA model in human keratinocytes revealed a similar attenuation of PAH-mediated *Cyp* mRNA induction. Additionally, experimental results with mouse keratinocytes indicated that PAH-mediated phase II enzyme mRNA induction is reduced in *Pparβ/δ*-null keratinocytes. The aryl hydrocarbon receptor (AHR) is the known transcriptional regulator of PAH-dependent phase I and II enzyme mRNA induction. Therefore, subsequent studies were designed to delineate how PPARβ/δ modulates AHR-dependent signaling in mouse and human

keratinocytes. Data generated from mechanistic studies indicated that the expression of AHR and several accessory proteins were not altered by PPARβ/δ expression. The ability of AHR to bind a ligand and translocate to the nucleus were also not modulated in a PPARβ/δ-dependent manner. Additionally, PPARβ/δ did not appear to physically interact with the AHR. Surprisingly, *Pparβ/δ*-null keratinocytes exhibited lower AHR occupancy and histone acetylation at the *Cyp1a1* promoter in response to B[a]P, as assessed by chromatin immunoprecipitation (ChIP). These findings suggested that the chromatin structure of xenobiotic metabolism promoters may be modulated by PPARβ/δ. Since DNA methylation is a common method of altering chromatin structure, this mechanism of gene regulation was examined using DNA methylation inhibitors and bisulfite sequencing. Interestingly, methylation within the *Cyp1a1* promoter was enhanced in *Pparβ/δ*-null keratinocytes. Additionally, inhibition of DNA methylation resulted in a PPARβ/δ-dependent modulation of basal *Cyp* mRNA expression. These results suggest that epigenetic regulation by PPARβ/δ contributed to the modulation of AHR signaling. To determine if modulated AHR signaling in keratinocytes results in functional changes in skin tumorigenesis, a complete skin carcinogen bioassay was performed in wild-type and *Pparβ/δ*-null mice. The multiplicity of B[a]P- or DMBA-induced skin lesions was found to be lower in *Pparβ/δ*-null mice. These observations appear to be mediated by decreased DNA adduct formation in *Pparβ/δ*-null keratinocytes as compared to wild-type cells. However, PAH exposure in keratinocytes did not change mRNA expression of the DNA damage markers activating transcription factor 3 (*Atf3)* or *p53* in either genotype. Tumorigenesis by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), a carcinogen that does not require AHR signaling or P450 bioactivation, resulted in enhanced tumorigenesis in *Pparβ/δ*-null mice. The enhanced

tumorigenesis in MNNG-treated *Pparβ/δ*-null mice supports previous evidence that PPARβ/δ modulates tumor promotion. The absence of PPARβ/δ also caused increased lesion size, more malignant tumor conversion, and increased inflammation upon MNNG or B[a]P treatment, which further indicates that PPARβ/δ functionally reduces tumor promotion. Collectively, the experiments within this dissertation identified PPARβ/δ as a critical modulator of PAH-mediated skin tumorigenesis by altering both tumor initiation and promotion. Mechanistically, the effects on tumor initiation were mediated by PPARβ/δ-dependent modulation of AHR signaling in keratinocytes. This dissertation thus established a novel molecular mechanism by which PPARβ/δ can alter skin tumorigenesis. Collectively, the results of these studies help to clarify the biological functions of PPARβ/δ in skin homeostasis and carcinogenesis.

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ABBREVIATIONS

3-MC	3-methylchloranthracene
5-Aza-dC	5-Aza-2'-deoxycytidine
[^{125}I]Br_2DpD	2-[^{125}I]iodo-7,8-dibromodibenzo-p-dioxin
[^{125}I]N_3Br_2DpD	2-Azido-3-[^{125}I]iodo-7,8-dibromodibenzo-p-dioxin
β-NF	β-naphthoflavone
ABCA1	ATP-binding cassette transporter A1
ACO	Acyl CoA oxidase
ADRP	Adipose differentiation-related protein
AF	Activator function
AH	Aryl hydrocarbon
AHR	AH receptor
AHRR	AHR repressor
ALBP	Adipocyte lipid binding protein
ANGTPL4	Angiopoietin-like protein 4
ANOVA	Analysis of variance
AOM	Azoxymethane
AP-1	Activator protein-1
aP2	Adipocyte fatty acid binding protein
APC	Adenomatous polyposis coli
AR	Androgen receptor
ARNT	AH receptor nuclear translocator
ATF3	Activating transcription factor 3
ATP	Adenosine triphosphate
azPC	Hexadecyl azelaoyl phosphatidylcholine
B[a]P	Benzo[a]pyrene
BCC	Basal cell carcinoma
BCL	B-cell lymphoma
BCT	Basal cell tumor
BER	Base excision repair
bHLH	Basic helix-loop-helix
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor
CBP	CREB binding protein
CCl_4	Carbon tetrachloride
CDK-1	Cyclin-dependent kinase-1
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CoRNR	Corepressor nuclear receptor
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
COX	Cyclooxygenase
CpG	Cytosine-phosphate-guanine
CPTI	Carnitine palmitoyl transferase I
CRABP-II	Cellular retinoic acid binding protein-II

CREB	cAMP-response element binding protein
CRP	C-reactive protein
CTE	C-terminal extension
CYP	Cytochrome P450
DAG	Diacylglycerol
DBD	DNA binding domain
DEHP	Di(2-ethylhexyl)phthalate
DiB[a,l]P	Dibenzo[a,l]pyrene
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dubecco's minimal essential medium
DMH	1,2-dimethylhydrazine dichloride
DMSO	Dimethylsulfoxide
DNMT	DNA methyltransferase
DPBS	Dubecco's phosphate buffered saline
DR1	Direct repeat 1
DRE	Dioxin response element
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGR-1	Early growth factor-1
ER	Estrogen receptor
EREG	Epiregulin
ERR	Estrogen-related receptor
EtOH	Ethanol
FAAR	Fatty acid activated receptor
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
FKBP5	FK506 binding protein 5
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GILZ	Glucocorticoid-induced leucine zipper
GLUT4	Glucose transporter type 4
GMC-SF	Granulocyte macrophage-colony stimulating factor
GR	Glucocorticoid receptor
GROα	Growth related oncogene α
GSTA1	Glutathione S-transferase Ya subunit
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HETE	Hydroxyeicosatetraenoic acid
HNF4	Hepatocyte nuclear factor 4 receptor
HODE	Hydroxyoctadienoic acid
HOX1	Heme oxygenase 1
HPV	Human papillomas virus

HRE	Hormone response element
HSP90	Heat shock protein 90
HTLV-1	Human T-cell leukemia virus type-1
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α
ICZ	Indolo[3,2-b]carbazole
ICAM	Intracellular cell adhesion molecule
IFNγ	Interferon γ
IL	Interleukin
IMCD-K2	Inner medulliary collecting duct
iNOS	Inducible nitric oxide synthase
IP-10	Interferon induced protein 10
K	Keratin
KA	Keratoacanthoma
KS	Kaposi's sarcoma
KSHV	KS-associated herpesvirus
LBD	Ligand binding domain
LC-MS	Liquid chromatography-mass spectrometry
LCoR	Ligand-dependent corepressor
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LXR	Liver X receptor
MAPK	MAP kinase
MCD	Methionine- and choline-deficient diet
MCP	Monocyte chemotactic protein
mEH	Microsomal epoxide hydrolase
miRNA	MicroRNA
MMP	Metalloproteinase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MR	Mineralocorticoid receptor
MS	Multiple sclerosis
NASH	Non-alcoholic steatohepatitis
NCoR	Nuclear receptor corepressor
NER	Nucleotide excision
NFκB	Nuclear factor κB
NHR	Nuclear hormone receptor
NMR	Nuclear magnetic resonance
NPC1L1	Niemann-Pick C1-like 1
NQO1	NAD(P)H:quinone oxidoreductase 1
NRF2	NR-E2-related factor 2
NSAID	Nonsteroidal anti-inflammatory drug
ODC	Ornithine decarboxylase
oxLDL	Oxidized LDL
PAH	Polycyclic aromatic hydrocarbon
Pap	Papilloma

PARP	Poly(ADP-ribose) polymerase
PAS	PER/ARNT/SIM
PCNA	Proliferating cell nuclear antigen
PDK4	Pyruvate dehydrogenase kinase 4
PeCDF	2,3,4,7,8-pentachlorodibenzofuran
PEPCK	Phosphoenolpyruvate carboxykinase
PG	Prostaglandin
PIMT	PRIP-interacting methyltransferase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
PR	Progesterone receptor
PRIP	PPAR-interacting protein
PTM	Post-translational modification
PUFA	Polyunsaturated fatty acid
PXR	Pregnane X receptor
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time PCR
RA	Retinoic acid
RAR	Retinoic acid receptor
RIP140	Receptor interacting protein 140
ROR	Retinoid-related orphan receptor
RXR	Retinoid X receptor
SCC	Squamous cell carcinoma
S.E.M.	Standard error of mean
shRNA	Short hairpin RNA
SRC	Steroid receptor coactivator
SMRT	Silencing mediator for retinoid and thyroid receptor
SNuRM	Selective nuclear receptor modulator
SOT	Society of Toxicology
SPR	Small proline rich protein
STAT	Signal transducer and activator of transcription
SWI/SNF	SWItch/Sugar nonfermentable
TBST	Tris buffered saline/Tween-20
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TFII	Transcription factor II family
Tg	Transglutaminase
TGF	Transforming growth factor
TNFα	Tumor necrosis factor α
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	Testicular receptor
TR	Thyroid receptor
TWEAKr	TNF-like weak inducer of apoptosis receptor
TZD	Thiazolidinedione
UCP	Uncoupling protein
UGT	UDP-glucronosyltransferase
UV	Ultraviolet
VDR	Vitamin D receptor

VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell
XAP2	Hepatitis B virus associated protein 2

ACKNOWLEDGEMENTS

The work from this dissertation has been accomplished with great help from many supportive family members, peers, and mentors. I attribute much of my success in graduate school to the wonderful guidance I have received, and I am deeply indebted to those who have contributed to my research and professional development.

I would first like to thank my dissertation advisor, Dr. Jeffrey Peters, for his mentoring as an undergraduate and graduate student. I have learned many invaluable life and scientific lessons that will always stay with me. I am grateful for his patience, enthusiasm, and support over the years. I could have never imagined that Dr. Peters would bring a young and scientifically naïve student this far when we first met while playing basketball. I attribute much of my success to the scientific ideals and drive which he has instilled within me. Additionally, this dissertation could have been completed without his large time commitment in reading and helping to improve my work. I know this took significant time away from his family and work, and I am deeply thankful for the investments he has made toward my professional and scientific development.

I would also like to thank my committee members for their support during my graduate career. I am eternally grateful for the insight and advice I received throughout the years. Dr. Wang, Dr. Mastro, and Dr. Baumrucker have been very supportive and readily available. If they did not have the answer to a question, great effort was taken to find someone that could help me. I would be sadly remised if I did not thank Dr. Perdew individually for his collaboration on this dissertation. He has been a close second mentor who has offered constant and enthusiastic scientific and professional advice over the

years. I am truly grateful for the contributions from each of my committee members, and I hope you each understand the impact you have had on my development as a scientist.

I am truly thankful to members of Dr. Peters' lab for their assistance and support throughout the years. My graduate career can be broken into two phases. During my early years, I receive invaluable training, support, and advice from many lab members, including Dr. Drew Burdick, Dr. Marjorie Peraza, Dr. Holly Hollingshead, Dr. Weiwei Shan, Amanda Burns, and Liz Girroir. Each provided inexhaustible support and mentorship to a young graduate student. Their support helped me to develop the skills necessary to be a successful graduate student. During the later years, I am thankful for the support I received from peers and mentors in the lab, including Dr. Moses Bility, Dr. Jen Foreman, Dr. Prajakta Palkar, Dr. Combiz Khozoie, Dr. J. Luis Morales, Bokai Zhu, Cherie Anderson, and Nicole Wiggins. These wonderful people offered advice and support during my experiments and the preparation of this dissertation. I am truly indebted to Dr. Prajakta Palkar for encouraging my involvement in the Society of Toxicology (SOT) and helping in the preparation of my grant and award applications. I would also like to thank members of Dr. Gary Perdew's lab for support and discussions throughout the years. Dr. Brett Hollingshead, Dr. Tim Beischlag, and Dr. J. Luis Morales were instrumental in the early design of my experimental models, and I could not have included the diverse data within this dissertation without their support. I would also like to acknowledge Dr. Iain Murray for his mentoring and scientific discussions throughout my graduate studies. He has truly had an impact on the design of many experiments contained in this dissertation, and I am thankful for his contributions. Collectively, I would not be where I am today without the support and advice from these members of the

Peters and Perdew labs, and I hope they all know how much I appreciate their help and friendship.

I am grateful to my family for their support and guidance. My parents, Kim and Greg, have always taught me to work hard and dream big, and I am eternally thankful for their love and support as I embarked on this journey. I am also grateful for the encouragement I have received from my sisters Leanne and Allison; I hope to continue to be a role model as they begin their professional careers. I would like to acknowledge my in-laws, Randy and Lori Hollis and Carl and Lori Kahle for their support and encouragement throughout the years. I would also like to recognize those members of my family who sadly could not witness my scientific development. The love and support of my grandparents was pivotal as they also encouraged me to dream big. I am deeply indebted to my great aunt Alice Patterson for her financial support that allowed me to attend Penn State and begin my scientific journey. I don't know if I will ever be able to thank my family enough for all their love, support, and encouragement, but I want everyone to know that I am thankful everyday to be a part of this family.

I would like to thank my wife Andrea Borland for her unwavering support throughout my graduate studies. I am especially thankful for her perspective and patience in listening to my research trials and tribulations. She has been a constant source of emotional strength at home as I have worked on completing this dissertation. She has also been a welcome and needed source of diversion during the frustrations of scientific research. I would not have gotten this far in life without her by my side. Thank you and I love you very much.

Chapter 1

INTRODUCTION

1.1 Nuclear receptor identification and characterization

1.1.1 Introduction to nuclear receptors

Responding to external and internal stimuli is a principal component to the maintenance of cellular, organ, and organism homeostasis. Many central biological processes are controlled at the transcriptional level to facilitate rapid and stimuli-specific biological responses. Some examples include development, cell differentiation, and the detoxification of toxicants. Modulated gene transcription is implicated in a plethora of human diseases, with a particularly strong linkage to cancer. Hormones were the first transcriptional regulatory candidates due to the inherent lipophilic nature of these compounds that would facilitate diffusion across cell membranes. Some examples include steroids, thyroid hormones, vitamin D3, retinoids, and estrogens. The development and use of radiolabeled hormones in the 1960's led to the identification of hormone binding proteins that were capable of nuclear translocation [1, 2]. In the 1970's, Ashburner linked steroid exposure to chromosome "puffing" in Drosophila [3, 4]. This discovery was the first direct observation of altered chromatin structure in response to steroids [3, 4]. The cumulative findings of Jensen and Ashburner provided a critical link between physiological responses to hormones and gene transcription [3, 4]. Future studies sought to identify and characterize the tissue- and hormone-specific receptors that regulate gene expression by DNA binding. Today these receptors are known as nuclear hormone receptors (NHRs) [5].

The existence of NHRs remained an enigma until the mid-1980's, when the discovery and characterization of several receptors ushered in a new era of

endocrinology. The first NHRs identified were the human glucocorticoid receptor (GR) [6, 7] and the human estrogen receptor (ER) [8, 9]. The reported homology of these receptors to the v-*erbA* oncogene facilitated the discovery of the thyroid receptor (TR) [10, 11]. Due to the high similarities in the cDNA coding sequence between these receptors, it was hypothesized that novel receptors could be identified through sequence homology before endogenous ligands or biological function were elucidated [12]. This method of receptor identification, termed reverse endocrinology, was used to identify the retinoic acid receptor (RAR) that mediates vitamin A-derived retinol responsiveness [13, 14]. The method of reverse endocrinology directly facilitated the discovery of a multitude of NHRs, and most of these receptors were classified as orphan receptors due to the lack of identified ligands [12]. Further application of this technique led to the identification of many nuclear hormone receptors. Furthermore, the discovery of the retinoid X receptor (RXR) [15], and its function as a nuclear receptor heterodimerization partner, signified a critical advance in our understanding of NHR function [16-22].

NHRs are generally activated by small, lipophilic molecules, such as steroids, and regulate receptor- and tissue-specific target gene transcription. The classic model of nuclear receptor transcriptional regulation (Figure **1.1**) begins with ligand binding and activation of the receptor. A ligand-dependent conformation change in the receptor leads to dissociation of receptor-bound corepressors and the recruitment of specific coactivators involved in chromatin remodeling. NHRs can then homodimerize or heterodimerize (through RXR) and bind to receptor-specific DNA sequences in target gene promoter and enhancer regions, termed “hormone response elements” (HREs). The recruitment of receptors and coactivators to the promoter leads to chromatin modifications, such as histone acetylation, that “open” the promoter region and allows

subsequent recruitment of RNA polymerase II and the transcription initiation complex (Reviewed in [23]). This causes the accumulation of processed target gene mRNA (Reviewed in [24-27]) that can be translated to functional protein and a biological effect (Reviewed in [28-33]). Collectively, these described observations are a brief synopsis of our current understanding of NHR signaling.

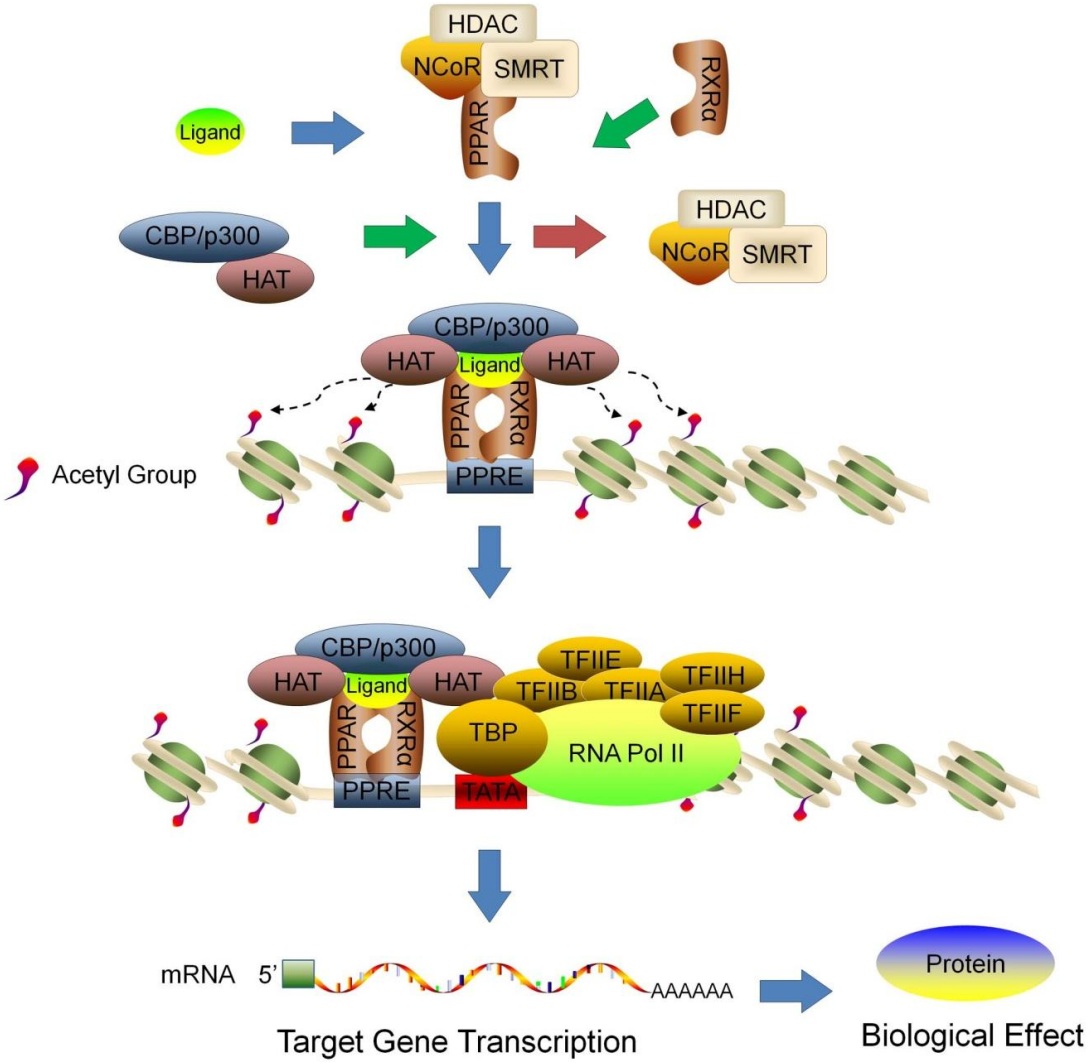

Figure **1.1**: Ligand-dependent nuclear receptor signaling. In the absence of ligand, nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs), are included in a repression complex. Associated proteins include histone deacetylases (HDACs) and corepressor such as nuclear co-repressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT). The enzymatic function of this complex maintains chromatin closure and transcriptional repression. Upon ligand binding, nuclear receptor releases corepressors and recruits coactivators, such as histone acetyltransferases (HATs) and CREB binding protein (CBP), to target gene promoters. The coactivator complex leads to histone acetylation and a relaxation of the chromatin structure. This open structure is more accessible to the general transcriptional machinery that includes TATA binding protein (TBP), transcription factor II family (TFII) of transcription factors, and RNA polymerase II (RNA Pol II). These proteins facilitate target gene mRNA production, mRNA translation, and biological effects.

1.1.2 Nuclear receptor structure

NHRs possess a modular structure with distinct domains that correspond to different receptor functions. The prototypical nuclear receptor contains five functional domains, including the NH2-terminal A/B domain, the highly conserved DNA binding domain (DBD) within domain C, the hinge region (D domain), the ligand binding domain (LBD) (E), and the COOH-terminal (F) domain. The evolutionary conservation of these domains highlights the functionality of the nuclear receptor protein structure. Furthermore, NHRs possess modular ligand-dependent and -independent functions [34] (Figure **1.2**).

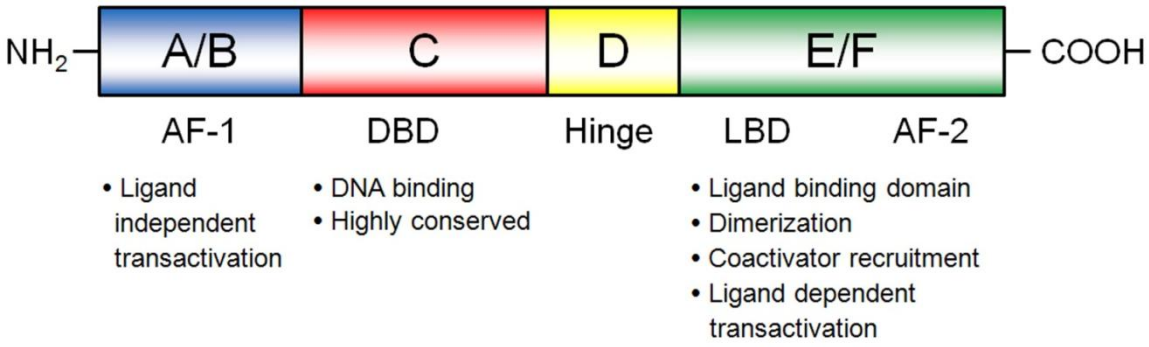

Figure **1.2**: Nuclear receptor structural domains. The N-terminal region, known as the A/B domain, contains the ligand-independent AF-1 transactivation domain. The C domain contains the highly conserved DBD. The D domain comprises the hinge region of nuclear receptors. The E/F domain contains the LBD and the ligand dependent AF-2 transactivation domain, and this domain is involved in receptor dimerization, coactivator recruitment, and transactivation.

The NH_2-terminal A/B region is the most variable domain in size and sequence, and receptor isoforms are typically generated by alternative A/B domain mRNA splicing. This region also contains the ligand-independent transactivation domain, known as AF-1, which mediates promoter- and cell-specific activities [34]. Additionally, this domain is a target of post-translation modifications (PTMs), such as phosphorylation and

sumoylation, that modulate ligand-dependent and -independent functions [35-41]. The DBD, which recognizes and binds specific DNA sequences, is the most conserved domain among the NHRs. This domain contains dual zinc finger domains and the C-terminal extension (CTE) that is primarily responsible for DNA binding [34, 42]. The results of nuclear magnetic resonance (NMR) and x-ray crystallography studies have indicated that each zinc finger contains 4 invariable cysteine residues that tetrahedrically coordinate a zinc ion [43, 44]. Located at the base of the first zinc finger is a region termed the “P box” that is responsible for discrimination of DNA recognition motifs [34]. Additionally, the “D box” within the second zinc finger mediates dimerization with other receptors [34]. The DBD also consists of two α-helical structures. The first helix in the P box facilitates binding within the DNA major groove, while the second helix in the CTE forms a right angle with the “recognition helix’ [34, 45]. The D region of nuclear receptors, also known as the hinge region, serves as a rotational axis between the DBD and the LBD. This poorly conserved region also contains the nuclear localization signal for receptors, as well as corepressor interaction domains [34]. The most COOH-terminal region (E/F) within nuclear receptors is the LBD. This multi-functional region is known to facilitate ligand binding, homo- or heterodimerization, interaction with heat shock proteins, nuclear localization, ligand-dependent transactivation, and transcriptional repression [34, 46-48]. The results of sequence alignments of nuclear receptor LBDs identified two highly conserved regions: a “signature motif” and the conserved ligand-dependent transactivation domain, termed AF-2, located near the COOH terminus [34, 49]. Structural analyses have determined that the ligand binding pocket, a three layered anti-parallel sandwich, is formed by 12 conserved α-helices numbered H1 through H12 and a conserved β-turn between H5 and H6 [34, 50-52]. The size of pocket can also vary [53]. Within this structure is a hydrophobic central core cavity, the ligand binding

pocket, created by three helices packed between the two additional layers [34, 51]. Ligand-bound receptors are known to be more compact than an unliganded counterpart, which suggests that a conformational change occurs upon ligand binding. The “mouse trap” model of receptor action suggests that the ligand bound AF-2 domain folds to close the binding pocket and exposes a functional interface for coactivator recruitment [51, 54]. Extending beyond the LDB is the COOH-terminal F domain. Notably, not all nuclear receptors possess this domain, which does not have a known function [34].

1.1.3 Nuclear receptor classifications

The application of reverse endocrinology methods largely expanded the number of identified nuclear receptors, and these receptors were found to be metazoan-specific [55]. These receptors were defined as members of the NHR superfamily, and more than one hundred distinct proteins fall within this family [56]. These include the 47, 48, and 49 receptors characterized in rats, humans, and mice, respectively [56, 57]. The results of phylogenetic analyses have indicated that nuclear receptors diverged to six distinct subfamilies. These classifications are based upon the ability of NHRs to bind DNA as homo- or heterodimers [58]. The described subfamilies became the basis for the nuclear receptor unified nomenclature system that was devised in 1999 [58]. The nomenclature was based upon the cytochrome P450 naming system (Table **1.1**) [59]. The first and largest subfamily (NR1) is the thyroid hormone receptor-like family. Receptors within this subfamily include the TRs, the RARs, the peroxisome proliferator-activated receptors (PPARs), the Rev-erbs, the retinoid-related orphan receptors (RORs), the liver X receptors (LXRs), the farnesoid X receptors (FXRs), the vitamin D receptor (VDR), the pregnane X receptor (PXR), and the constitutive androstane receptor (CAR). The

second subfamily, known as the retinoid X receptor-like subfamily, includes the hepatocyte nuclear factor 4 receptors (HNF4s), the RXRs, the testicular receptors (TRs), and the chicken ovalbumin upstream promoter transcription factors (COUP-TFs). The third subfamily is the ER-like subfamily, and its constituents include the ERs, the estrogen-related receptors (ERRs), the GRs, the mineralocorticoid receptor (MR), the progesterone receptor (PR), and the androgen receptors (AR). The fourth subfamily is known as the nerve growth factor IB-like subfamily, and the steroidogenic factor-like receptors comprise the fifth subfamily. The sixth subfamily contains the germ cell nuclear factor-like receptors. There is group of nuclear receptors that contain only one of the two conserved domains, and these receptors were assigned to the miscellaneous subfamily 0. To date, several isoforms and endogenous ligands have been identified for many NHRs (Table **1.1**).

Table **1.1**: Classification and nomenclature of mammalian nuclear hormone receptors

Class	Common Name	Receptor	Isoforms	Ligands	Reference
NR1A	Thyroid hormone receptor	TR	α, β	Thyroid hormone	[60]
NR1B	Retinoic acid receptor	RAR	α, β, γ	Retinoic acid	[14]
NR1C	Peroxisome proliferator-activated receptor	PPAR	α, β, γ	Fatty acids, fibrates, TZDs	[61]
NR1D	Reverse ErbA	RevErb	α, β	Unknown	[34]
NR1F	Retinoid-related orphan receptor	ROR	α, β, γ	Unknown	[62]
NR1H3	Liver X receptor	LXR	α, β	Oxysterols	[63]
NR1H4	Farnesoid X receptor	FXR		Bile acids	[64]
NR1I1	Vitamin D receptor	VDR		Vitamin D_3	[65]
NR1I2	Pregnane X receptor	PXR		Pregnanes, C21 steroids	[66]
NR1I3,4	Constitutive androstane receptor	CAR/MB67	α, β	Androstanes	[67]
NR2A	Hepatocyte nuclear factor 4	HNF4	α, β, γ, δ	Unknown	[68]
NR2B	Retinoid X receptor	RXR	α, β, γ	Retinoids	[19]
NR2C	Testicular receptor	TR	1-11	Unknown	[69]
NR2F	Chicken ovalbumin upstream promoter transcription factor	COUP-TF	α, β, γ	Unknown	[70]
NR3A	Estrogen receptor	ER	α, β	Estrodiol	[71]
NR3B	Estrogen-related receptor	ERR	α, β, γ	Unknown	[34]
NR3C1	Glucocorticoid receptor	GR		Glucocorticoids	[72]
NR3C2	Mineralocorticoid receptor	MR		Mineralocorticoids	[34]
NR3C3	Progesterone receptor	PR		Progestins	[73]
NR3C4	Androgen receptor	AR		Androgens	[74]
NR4A	NGF-induced clone B	NGFI-B	α, β, γ	Unknown	[34]
NR5	Steroidogenic factor 1/Fushi Tarazu factor 1	SF-1/FTZ-F1	α, β	Unknown	[75]
NR6	Germ cell nuclear factor	GCNF		Unknown	[76]
NR0B	Dosage-sensitive sex reversal/ small heterodimeric partner	DAX/SHP		Unknown	[34]

1.1.4 Nuclear receptor ligands

The scope of hormones serving as NHR transcriptional activators has expanded as novel endogenous ligands have been identified. NHR ligands are generally small, hydrophobic, and lipid soluble, and these include metabolic products, endocrine molecules, and modified lipids (Reviewed in [77]). The specificity of ligands for receptor is determined by the shape, volume, and residue interactions of the ligand-binding pocket. Endogenous ligands are still unknown for many nuclear receptors (Table **1.1**). There is strong pharmaceutical interest in identifying and characterizing the therapeutic potential of ligand activated nuclear receptors due to the ability of these receptors to modulate lipid and glucose homeostasis. The term “ligand” generally defines any molecule that binds to a receptor. Several classes of ligands exist, including pure agonists, pure antagonists, inverse agonists, and partial agonists-antagonists. Generally, pure agonists lock the receptor in an active conformation and facilitate coactivator recruitment via the “mouse trap” (Reviewed in [77]). In contrast, pure antagonists prevent the formation of active receptor structure (Reviewed in [77]). Inverse agonists were postulated as ligands that could inhibit basal transcription through increased corepressor recruitment [78, 79]. Synthetic retinoids for RARs were the first identified molecules possessing this function [78, 79]. Inverse agonists act similarly to antagonists by blocking the structural changes that facilitate corepressor release and coactivator recruitment. While ER has low corepressor affinity in the absence of exogenous ligands, some ER-specific antagonists have been shown to enhance corepressor interactions [80-83]. The PPARα antagonist GW6471 also causes a unique receptor conformation resulting in high corepressor affinity [48]. Partial agonists-antagonists are another poorly understood classification of nuclear receptor ligands. The function of these ligands is

often context-specific, as is the case with the PPARγ ligand GW0072. This molecule binds a novel region within the ligand pocket and serves as an agonist or antagonist [84-86]. Selective nuclear receptor modulators (SNuRMs), a type of partial agonist-antagonist, exert mixed ligand properties associated with tissue-specific differential recruitment of coactivators and corepressor [87-89]. Tamoxifen was the first identified SNuRM because of its estrogen-like activity in the uterus and an anti-estrogen effect in the breast (Reviewed in [90]). SNuRMs have been identified for several nuclear receptors systems (Reviewed in [90]), and the future application of these ligands as therapeutic agents highlights the importance in delineating the context-specific cues that mediate agonistic or antagonistic ligand effects.

1.1.5 Nuclear receptor coregulators

NHR signaling predicates on a multi-step process that includes ligand binding, the dissociation of corepressors, the recruitment of coactivators to modulate chromatin structure, and the formation of the pre-initiation transcriptional complex. A large number of proteins contribute to coregulation of NHR signaling, and these factors were shown to biochemically modify histones, remodel chromatin structure, or serve as bridging proteins. There is also documented evidence that ligand-activated NHRs directly interact with the transcription initiation complex [38, 91-97]. However, these interactions typically require further coactivator recruitment or enzymatic activity to exert full regulatory effects. The prevailing model is that, in the absence of exogenous ligands, receptors are DNA-bound at response elements in association with the corepressors nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) [98-102]. This interaction occurs within a region of the LBD termed the

corepressor nuclear receptor (CoRNR) box [34, 103, 104]. Although NCoR and SMRT do not possess intrinsic enzymatic activity, these proteins recruit HDACs to modify histones and condense chromatin structure [77]. Upon ligand binding, nuclear receptors undergo a conformational change that alters the corepressor interface, thus causing corepressor dissociation [54]. The closure of the “mouse trap” caused by ligand binding creates a new interface for coregulator interactions that includes ligand-dependent coactivators and corepressors (Reviewed in [105]). Coactivators represent the majority of identified nuclear receptor coregulators (Reviewed in [106]). However, the identification and characterization of receptor interacting protein 140 (RIP140) and ligand-dependent corepressor (LCoR) as ligand-dependent corepressors have sparked interested in repressive nuclear receptor transcriptional regulation [107-112]. The discovery of steroid receptor-coactivator-1 (SRC-1) as the first NHR coactivator ushered in a new era to delineate receptor transcriptional regulation and to identify novel regulatory proteins [113, 114]. This coactivator possesses intrinsic enzymatic activities to biochemically modify histones and subsequently modulate higher order chromatin structure [114]. In addition to the originally identified acetyltransferase activity of SRC-1 [114], coactivators can also directly modulate transcription through histone methylation, phosphorylation, and ubiquitination (Reviewed in [115]). The proposed “histone code” model was devised to predict the cumulative regulatory effects of histone modifications (Reviewed in [116]). While studies are beginning to dissect the step-wise progression of histone modifications in transcriptional regulation, the overall knowledge of the histone code has remained limited. In addition to coactivators and corepressors, gene regulation is also modulated through adenosine triphosphate (ATP)-dependent reorganization of chromatin structure. The reorganization occurs via the SWItch/Sugar Nonfermentable (SWI/SNF) family. This coregulator family was originally identified in *S. cerevisiae* [117],

and highly conserved eukaryotic homologues were subsequently identified and characterized [117-121]. Presumably, ligand-activated NHRs utilize SWI/SNF factors to "loosen" the promoter chromatin structure and promote recruitment of the transcription initiation complex. Therefore, it is not surprising that several NHRs employ SWI/SNF factors in ligand-dependent transcriptional regulation [122-127]. A third class of coregulators, termed "cointegrators", aid in ligand-dependent NHR transcriptional regulation. These cointegrators include cAMP-response element-binding protein (CREB)-binding protein (CBP) and p300. These factors can serve as protein "bridges" between nuclear receptors [128-130], coactivators [128, 131, 132], and the transcription initiation complex [133-135].

The orchestrated recruitment of coactivators, corepressors, cointegrators, and chromatin remodeling complexes by nuclear receptors facilitates ordered responses to biological stimuli at the transcriptional level. Most coregulators interact with multiple NHR signaling systems, and cross-talk between receptors may also play an important role in responding to endogenous and synthetic ligands. Additionally, coregulators can undergo PTMs, and this adds another level of complexity to the function of NHR coregulators (Reviewed in [106]).

1.2 Peroxisome proliferator-activated receptors

1.2.1 Overview of PPARs

The historical basis of PPAR biological function began with the description of the peroxisome, a single membrane-bound organelle originally identified in the liver and

kidney [136]. Crucial lipid metabolic enzymes reside within the peroxisome, and these enzymes are involved in fatty acid oxidation, β-oxidation, and cholesterol synthesis [137]. Throughout the 1960's and 1970's, several research groups observed that the hypolipidemic drug clofibrate caused hepatomegaly with a significant increase in the size and number of hepatic peroxisomes in rodents [138-141]. Compounds exhibiting similar hepatic biological effects became known as "peroxisome proliferators", and this diverse class of chemicals includes hypolipidemic drugs, herbicides, and industrial plasticizers [142-147]. Peroxisome proliferators, in addition to increasing rodent peroxisome number, cause enhanced hydrogen peroxide (H_2O_2) production via increased peroxisomal fatty acid β-oxidation. The resulting increase in H_2O_2 can produce oxidative stress, DNA damage, and initiation of tumorigenesis. This mechanism of indirect DNA damage, via oxidative stress, typifies peroxisome proliferators as non-genotoxic carcinogens. Throughout the 1970's and 1980's, the biochemical mechanism by which peroxisome proliferators exerted biological effects remained elusive. However, the results from several studies suggested the existence of a ligand-binding protein [148, 149]. The knowledge that peroxisome proliferators modulate gene transcription led to the hypothesis that these compounds worked in a similar fashion as steroid hormones and the steroid hormone receptors [150-152]. A breakthrough in peroxisome proliferator signaling occurred in 1990 when Isabelle Issemann and Stephen Green cloned and characterized PPAR as the receptor mediating these ligand-dependent biological effects [61].

Knowledge of PPARs leapt forward in 1992 with the cloning of three *Xenopus* PPARs [153]. PPAR isoforms have been identified in several organisms [153-157], and all PPAR isoforms, termed α, β/δ, and γ, are now known as members of the NHR

superfamily of ligand-activated transcription factors [153-157]. The three distinct PPARs are classified as PPARα, PPARβ (also known as PPARδ, NUC1, FAAR, and further referred to as PPARβ/δ), and PPARγ. The distinguishing features separating the PPARs are the ligands, tissue distribution, and biological effects of ligand activation (Table **1.2**). To date, the knowledge of PPARα and PPARγ biological functions far outweighs the knowledge of PPARβ/δ function. The following sections will provide in-depth information of the distinct functions of the PPAR isoforms.

Table **1.2**: A summary of the ligands, tissue distribution, and physiological roles of the PPAR isoforms

	PPARα	**PPARγ**	**PPARβ/δ**
Ligands	Fatty acids, Hypolipidemic drugs (fibrates), Plasticizers (Reviewed in [147])	Polyunsaturated fatty acids, Thiazolidinediones, Eicosanoids (Reviewed in [147])	Fatty acids, L-165041 [158], GW0742 [159], GW501516 [160]
Tissue distribution	Liver, Heart, Kidney, Adipose tissue [161, 162]	Adipose tissue, Heart, Colon, Intestines, Immune system [161, 162]	Ubiquitous, Highly expressed in keratinocytes, liver, brain, intestines [161-163]
Physiological role	Fatty acid catabolism, Lipid homeostasis, Rodent hepatocarcinogenesis	Adipose differentiation, Glucose homeostasis, Macrophage/immune response	Fatty acid catabolism, Epidermal differentiation, Colon/skin carcinogenesis (Reviewed in [164])

Ligand binding of PPARs induces a conformational change that allows the release of corepressors, the binding to the obligate heterodimerization partner RXRα, and the recruitment of coactivators [18] (Figure **1.3**). DNA-binding is mediated through a direct repeat 1 (DR1) sequence of AGGTCAnAGGTCA, termed a peroxisome proliferator response element (PPRE), located within the promoter region or introns of target genes [165]. Cis elements surrounding the PPRE can also influence the selectivity and relative strength of the response element [166]. The recruitment of coactivators by PPARs leads to histone modifications that “loosen” the DNA/chromatin interaction and facilitates the assembly of transcription initiation complex (Figure **1.3**). Several different classes of coactivators are known to interact with PPARs, including p300/CBP [167], SRC-1 [168, 169], and RIP140 [170]. In the absence of exogenous ligands, PPARs bind

copresssors, such as NCoR and SMRT, to cause histone deacetylation and closure of the chromatin structure. These protein associations repress basal transcriptional activity. Notably, PPARβ/δ possesses a strong interaction with HDACs and repression of transcriptional activity [171-173] (Figure **1.3**). In addition to DNA-dependent transcriptional regulation, PPARs have also been shown to antagonize other transcription factors through a mechanism termed “transrepression” (Figure **1.3**). One such transrepressive mechanism is physical interaction with transcription factors. PPARs have been shown to competitively bind with nuclear factor κB (NFκB) and activator protein-1 (AP-1) subunits to modulate inflammatory responses [174-179]. Additionally, PPAR transrepression results in altered basal and activated kinase signaling [180-182].

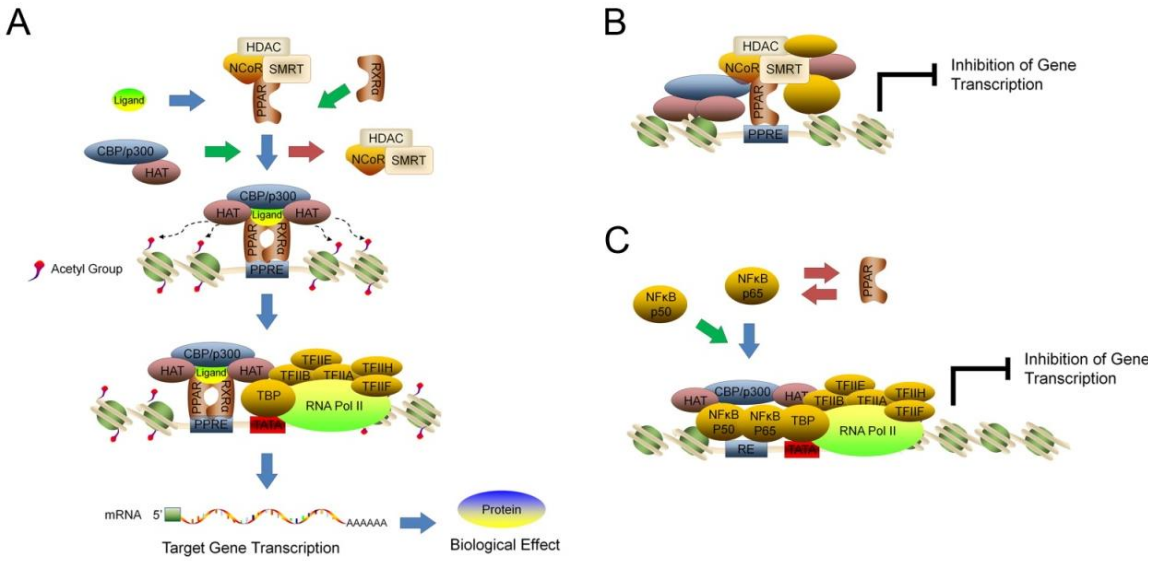

Figure **1.3**: Mechanisms of PPAR transcriptional regulation. (A) The classic mechanism of PPAR transcriptional regulation is initiated by ligand binding. A conformational change in the receptor facilitates release of copressors, heterodimerization with RXRα, DNA binding, and recruitment of coactivators. The heterodimer complex binds to PPREs in target gene promoters. This leads to chromatin remodeling and recruitment of the transcriptional initiation complex. The increased production of target gene mRNA directly leads to increased target gene protein and a biological effect. (B) The PPAR/corepressor complex has been shown to bind target gene promoters in the absence of exogenous ligands and inhibits target gene mRNA production. Ligand activation of PPARs can reverse transcriptional repression. (C) PPARs can modulate transcription by DNA-independent mechanisms through directly interacting and competitively binding with other transcription factors.

1.2.2 General properties of PPARα

The initial breakthrough in understanding the molecular events of peroxisome proliferation occurred when *Pparα* was cloned from murine liver by Issemann and Green [61]. This isoform has since been cloned from multiple organisms, including *Xenopus* [153], rat [183], rabbit [184], fish [185, 186], and human [187]. Mouse and human PPARα have an 85% sequence similarity and 91% amino acid similarity [188]. Comparisons of the human PPAR isoforms have indicated a high conservation of the

DBD (83-86%) and moderate conservation within the LBD (68-70%) [188]. PPARα is highly expressed in tissues of high metabolic and energy capacity, including the liver, kidney, heart, brown adipose tissue, and skeletal muscle [162, 189, 190]. Additionally, PPARα has functional effects in monocytes [191], vascular endothelial cells [192], and smooth muscle cells [178].

The ligands for PPARα encompass classes of structurally diverse compounds, including the originally classified peroxisome proliferators (Figure **1.4**). Natural activators of PPARα include fatty acids, such as linoleic, palmitic, and arachidonic acids. These natural ligands activate PPARα with relatively lower affinity (micromolar range) [183, 193]. Derivatives of fatty acids have also been proposed to activate PPARα, and the hydroxyeicosatetraenoic acid (HETE) lipoxygenase metabolite 8(S)-HETE has been identified as a high affinity PPARα ligand [194]. Arachidonic acid metabolites, including leukotrienes and prostaglandins (PGs), have been shown to modulate inflammatory responses by serving as PPARα ligands [174, 195]. Phosphocholine [196] and ethanolamide [197] derivatives have also been described as physiologically relevant endogenous ligands. Synthetic PPARα ligands are also widely studied and include phthalate monoesters, herbicides, and the fibrate class of hypolipidemic drugs. Some pertinent examples of fibrates include clofibrate, fenofibrate, bezafibrate, and Wy-14,643 (Reviewed in [187]).

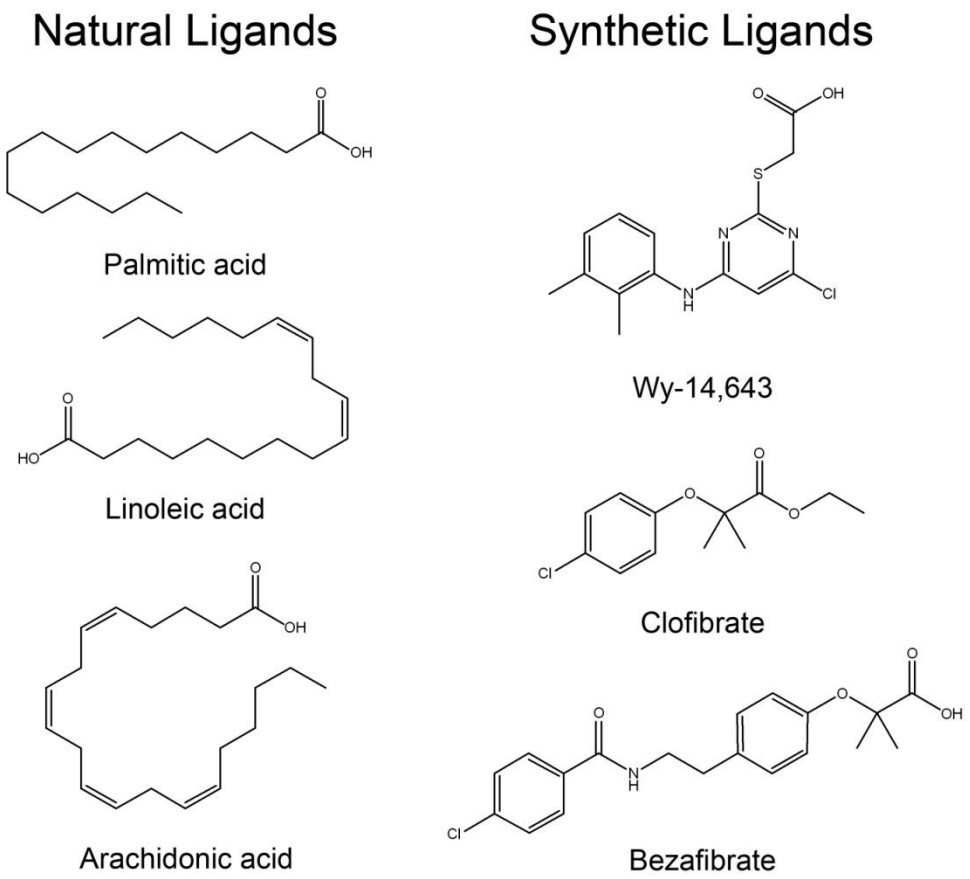

Figure **1.4**: Structures of representative PPARα ligands. PPARα ligands encompass a variety of structurally similar chemicals. Natural ligands include fatty acids and fatty acid derivatives. Synthetic ligands include phthalate monoesters, herbicides, and the fibrate class of hypolipidemic drugs.

The promise of PPARα ligands to treat dyslipidemia was challenged when hypolipidemic drugs were shown to induce hepatic peroxisome proliferation, hepatomegaly, and liver cancer in rodent models [198, 199]. Surprisingly, the carcinogenic effect of PPARα ligands have not been observed in primates or humans (Reviewed in [200, 201]). Additionally, long-term administration of two peroxisome proliferators did not cause hepatocarcinogenesis in Syrian hamsters [202]. Several hypotheses have been brought forth to explain why PPARα ligands cause cross-species receptor activation, but rodent-specific hepatocarcinogenesis. First, species differences

in receptor expression were proposed to mediate rodent carcinogenesis. Rodent hepatic PPARα expression was found to be 10-fold higher than humans [203]. However, a primary hepatocyte cell culture system has cast uncertainty onto species-specific differences in receptor expression [204]. The results of RNA analyses found that human samples expressed a truncated PPARα protein lacking exon 6 [203]. A larger study further reported that 20-50% of human *Pparα* mRNA encodes this truncated protein that is unable to bind a target gene response element [203]. The ligand affinity of human and mouse PPARα was also examined in an *in vitro* transactivation assay. The ligand affinity for mouse PPARα was found to be greater than the affinity for human receptor [200]. Therefore, the expression and functionality of PPARα may cause the species differences in hepatocarcinogenesis. The genomic sequence of PPARα target gene promoters was assessed for species differences. The human acyl CoA oxidase (ACO) promoter PPRE was found to have several mutations that rendered ACO mRNA unresponsive to PPARα ligand activation [205]. Thus, species differences in PPARα target gene nucleotide sequence may also contribute to peroxisome proliferator-dependent enhancement of rodent hepatocarcinogenesis. To address the species-specific differences in liver tumorigenesis, the *Pparα*-null mouse [206] and the humanized PPARα mouse [207] have been developed to delineate species differences in carcinogenesis. The results of comprehensive studies indicated that PPARα ligands alter lipid metabolizing enzymes in a species-independent manner. However, peroxisome proliferation, mitogenic activity, and hepatocarcinogenesis are a rodent-specific effect of PPARα ligand activation [207, 208]. Recently, rodent-specific regulation of the microRNA (miRNA) let-7C was found to alter the mRNA degradation of c-myc, a known proto-oncogene [209, 210]. While further mechanistic studies are warranted to delineate species-specific differences, the use of

the null and humanized PPARα mouse models are the best opportunity to clarify the role of this nuclear receptor in hepatocarcinogenesis.

1.2.3 Physiological roles of PPARα

PPARα is a critical regulator of lipid metabolism by altering the transcription of genes involved in fatty acid transport and metabolism. Indeed, fatty acid transport protein (FATP) and fatty acid translocase (FAT) have been shown to be PPARα target genes involved in the transportation of fatty acids across the cell membrane [211, 212]. Additionally, ligand activation of PPARα has been shown to directly regulate the transcription of acetyl-CoA [213], ACO [153, 214], enoyl-CoA hydratase/dehydrogenase multifunctional enzyme [215], liver fatty acid binding protein (L-FABP) [216, 217], and keto-acyl-CoA thiolase [218]. Collectively, these enzymes all contribute to β-oxidation of fatty acids. The mitochondrion is the primary site of fatty acid metabolism, and PPARα directly regulates the transcription of carnitine palmitoyl transferase I (CPTI). This gene is involved in the translocation of activated fatty acids to the inner membrane of the mitochondrion [219]. PPARα has also been shown to regulate the cytochrome P450 (CYP) 4A subclass (CYP4A) of enzymes involved in hepatic fatty acid metabolism [220, 221]. The specificity of PPARα in regulating these processes has been confirmed through use of the *Pparα*-null mice [206]. The absence of PPARα expression renders hypolipidemic drug incapable of inducing mRNA transcription of β-oxidation genes [206]. The results generated from transcriptional studies and null-mouse models conclusively indicate that PPARα regulates a battery of genes involved in fatty acid metabolism and utilization.

PPARα has also been implicated as a lipid sensor and modulator of cholesterol and triglyceride profiles. Dysregulation of cholesterol and free lipid levels are known risk factors for obesity, type 2 diabetes, and cardiovascular disease (Reviewed in [222]). The lipid lowering effect of PPARα ligands is thought to be through increased high-density lipoprotein (HDL) levels by direct regulation of apolipoprotein synthesis and secretion. Ligand activation of PPARα has been shown to increase HDL levels [223, 224], decrease very low-density lipoprotein (VLDL) levels [225], and decrease serum triglycerides [223, 226-230]. ApoC-III is a major component of VLDL, and several studies have indicated that PPARα ligands reduce apoC-III expression [225-227, 230-235]. Conversely, apoA-I, a component of HDL, has been shown to be upregulated by PPARα ligands [223, 224, 230, 236]. Hydrolysis of lipoproteins by lipoprotein lipase (LPL) has also been implicated in disease states, and several studies have observed a PPARα-dependent regulation of LPL expression [225, 234, 235]. Fatty acid β-oxidation and lipid sensoring also converge at several enzymes, including FABP and pyruvate dehydrogenase kinase 4 (PDK4), and both these genes have been shown to be regulated in a PPARα-dependent manner [227, 228, 237-239]. In addition to ligand-dependent regulation of lipid sensoring, PPARα polymorphisms have also been shown to alter basal apolipoprotein and serum triglyceride levels. A valine to alanine polymorphisms at amino acid 227 (V227A) in the PPARα protein sequence indicated positive health prospects by lowered cholesterol and serum triglyceride levels [240]. Conversely, the PPARα L162V polymorphism caused negative health consequences by increasing low-density lipoprotein (LDL), apoB, apoC-III, and triglyceride levels [241-244]. HDL levels were also found to be decreased in populations possessing this polymorphism [241, 245], thus L126V increases the risk for obesity, diabetes, and

cardiovascular disease. The cumulative polymorphic and ligand activation studies indicated that PPARα plays an important role in several disease states.

The expression of PPARα in vascular-associated cell types, including macrophages [191], monocytes [191], endothelial cells [246], and vascular smooth muscle cells (VSMC) [178], suggests that PPARα may alter the development of atherosclerosis. Indeed, administration of PPARα ligands has been shown to reduce atherosclerotic lesions in a PPARα-dependent manner [246-250]. Ligand activation of PPARα reduced inflammation by decreasing monocyte-macrophage migration and adhesion to endothelial cells [251, 252]. A primary mechanism of these anti-inflammatory effects is PPARα-dependent reduction of vascular cell adhesion molecular-1 (VCAM-1). PPARα has also been shown to cross-talk with several inflammatory signaling pathways, including NFκB [247, 248, 251, 253-256] , AP-1 [254, 256, 257], thioredoxin-1 [258], nitric oxide synthase [247], endothelin-1 [254, 259], angiotensin II [260]. PPARα ligands also reduced interleukin-6 (IL-6) [178, 254] , monocyte chemoattractant protein-1 (MCP-1) [256], cylcooxygenase-2 (COX2) [178, 256], and pro-inflammatory cytokine [261] mRNA induction in smooth muscle cells, T cells, and macrophage. Macrophage lipid accumulation also contributes to atherosclerosis, and PPARα directly regulated expression of the cholesterol efflux pump ATP binding cassette transporter A1 (ABCA1) [191, 248, 262]. Cumulatively, these studies indicate several possible PPARα-dependent mechanisms exist to modulate vascular cell inflammation and plaque formation in the treatment and prevention of atherosclerosis.

As well as the anti-inflammatory effects in the vasculature system, PPARα also modulates inflammation in other organ systems. This effect was first observed in

comparative inflammation studies using wild-type and *Pparα*-null mice. Treatment with arachidonic acid or its primary metabolite, leukotriene B4 (LTB4), manifested prolonged inflammation in *Pparα*-null mice as compared to wild-type controls. The modulated inflammatory response was shown to be specific, as phorbol ester-dependent inflammation did not cause a genotype-specific differential inflammation [174]. Further investigations observed that LTB4 is a PPARα ligand and mediates anti-inflammatory effects by LTB4 catabolism [174]. The neutrophil chemoattractant 8S-HETE [194], the immunomodulator DHEA-S [263], 17-beta-estradiol (E2) [264], and modified epoxyeicosatrienoic acids [265, 266] can also been described as anti-inflammatory PPARα ligands. Additionally, ligand activation of PPARα inhibits NFκB signaling [247, 248, 251, 253-256], inhibits AP-1 signaling [254, 256, 257], reduced IL-6 mRNA production and plasma section [178, 254], and induced nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α (IκBα) transcription [255]. These facts all demonstrate that ligand activation of PPARα can reduce inflammation by multiple mechanisms.

PPARα has also been implicated in the progression of several cancers. In addition to causing rodent hepatocarcinogenesis [267], PPARα has also been implicated in the development of pancreatic carcinoma [267] and Leydig adenomas [268]. The dependence of PPARα in carcinogenesis has been investigated with *Pparα*-null mice [206]. Peroxisome proliferators have been shown to cause hepatocarcinogenesis [198] that is not seen in similarly treated *Pparα*-null mice [199, 269]. Several hypotheses have been have been proposed to explain the carcinogenic effect of PPARα ligands. One proposed mechanism has basis in PPARα-dependent lipid metabolism. A byproduct of β-oxidation is reactive oxygen species, including H_2O_2, which can cause oxidative stress

and DNA damage [270-273]. Ligand activation of PPARα has also been shown to promote hepatocyte proliferation [199, 274-276] and decrease apoptosis [277-279] by several mechanisms, and the regulation of these functions directly alters carcinogenesis. Ligand activation of PPARα has been shown to modulate the expression of growth factors, oncogenes, and cell cycle control proteins. This list includes cyclin-dependent kinase 1 (CDK-1) [276, 280, 281], CDK-2 [276, 281], CDK-4 [276, 281], c-myc [276, 282-284], c-fos [282, 285], c-jun [285], c-Ha-Ras [282], proliferating cell nuclear antigen (PCNA) [276, 280, 281], early growth response factor-1 (EGR-1) [286], p53 [280], and p21 [280]. The PPARα-dependent regulation of many proliferative genes has been validated using the *Pparα*-null mouse model [199, 276]. PPARα has also been shown to regulate B-cell lymphoma-2 (Bcl-2) and Bak expression to suppress liver apoptosis [277-279]. The modulation of apoptosis by peroxisome proliferators and PPARα has been attributed to tumor necrosis factor α (TNFα) expression and oxidant production in liver kupffer cells that caused activation of hepatocytes [287-289]. Conflicting evidence suggests that kupffer cells express PPARα and activate hepatocytes [290]. Clearly more studies are needed to delineate what, if any, role PPARα plays in kupffer cells and peroxisome proliferation in the liver.

It is widely accepted that ligand activation of PPARα caused rodent-specific hepatocarcinogenesis [199, 200, 291]. Humanized PPARα mouse models have provided another method to examine why hepatocarcinogenesis by peroxisome proliferators is specific to rodents. Initial characterization of the humanized model indicated that PPARα-dependent regulation of β-oxidation is intact in both human and rodent models. However, the regulation of cell cycle regulatory genes is only observed in rodents [207, 208]. Rodent-specific regulation of miRNAs may contribute to PPARα-dependent

regulation of c-myc regulation and liver tumorigenesis [209, 210]. The lack of defined mechanisms highlights the need for further species-specific studies to delineate the safety of PPARα ligands.

In summary, there is clear evidence that PPARα plays critical roles in many physiological relevant pathways and disease states. The role of PPARα in β-oxidation and lipid sensoring has implications in atherosclerosis and dyslipidemia, which are early markers for diabetes and coronary artery disease. The utilization of PPARα ligands for the treatment and prevention of metabolic disorders and atherosclerosis has strong potential. However, PPARα-dependent rodent hepatocarcinogenesis has hindered the advancement of PPARα ligand therapies. Recent results from human and humanized mouse model systems have revealed the absence of human tumorigenic effects. Further investigations using PPARα-humanized models and ligands are necessary to delineate the species-specific differences in tumorigenesis. The role of PPARα in β-oxidation, lipid sensoring, and inflammation demonstrates how PPARα ligands could be used to prevent or treat many diseases (Figure **1.5**).

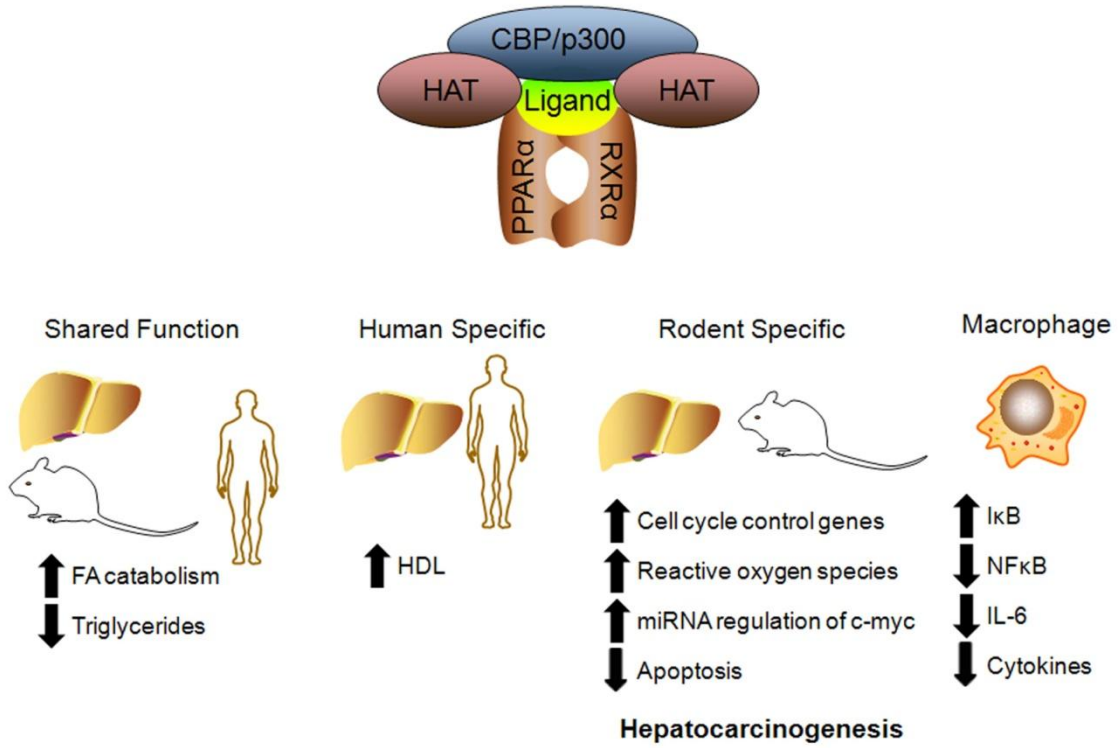

Figure **1.5**: PPARα-dependent physiological functions. Ligand activation of PPARα enacts numerous downstream effects. Ligand activation of PPARα leads to species-independent transcriptional regulation of genes involved in fatty acid catabolism in a variety of tissues. PPARα acts as a lipid sensor by responding to fatty acid ligands and directly lowering triglyceride levels while increasing HDL levels. These effects clearly implicate PPARα in metabolic syndromes and atherosclerosis. In macrophage, PPARα negatively regulates the transcription of specific cytokines through DNA-dependent and -independent mechanisms, which serves to inhibit inflammation. Activation of PPARα has been shown to cause rodent hepatocarcinogenesis that is not observed in human models. The rodent-specific carcinogenic effect of PPARα is caused by increased expression of cell cycle control genes and non-genotoxic DNA damage by reactive oxygen species. Furthermore, decreases in apoptosis and regulation of c-myc by miRNA have been demonstrated to be rodent-specific mechanisms of hepatocarcinogenesis that are not observed in human models.

1.2.4 General properties of PPARγ

The basis and cloning of PPARγ originally focused on a novel regulator of the adipocyte fatty acid binding protein (aP2) gene. The protein that bound to the aP2 promoter region was termed ARF6 [292]. This protein was later shown to be a member of the PPAR nuclear receptor superfamily and termed PPARγ [293]. This PPAR isoform has since been cloned in *Xenopus*, human, and mouse [153-155, 294, 295]. A 99% homology was found between human and mouse PPARγ, and this high conservation suggests that critical biological functions are shared in both species [188]. The human PPARγ coding sequence also contains three distinct splice variants, termed PPARγ1, PPARγ2, and PPARγ3 [296]. While PPARγ1 and PPARγ3 encode the same eight exon protein, the PPARγ1 transcript is transcribed from a further downstream promoter than PPARγ3 [296]. The PPARγ2 protein, containing only 7 exons, is transcribed from a third promoter that adds twenty-eight amino acids to the N-terminus [297]. In addition to PPARγ having distinct tissue distribution, the PPARγ subtypes are also expressed in a tissue-specific manner. PPARγ1 is known to be broadly expressed, while PPARγ2 is expressed mostly in adipose tissue [296]. PPARγ3 is mostly expressed in adipose tissue, macrophage, and colon epithelium [298, 299].

Much like PPARα, PPARγ is activated by a vast number of synthetic and natural ligands (Figure **1.6**). Polyunsaturated fatty acids (PUFAs), including linoleic, arachidonic, and eicosapentanoic acid, display preferential binding to PPARγ [300, 301]. 9- and 13-hydroxyoctadienoic acid (9-HODE and 13-HODE) and hexadecyl azelaoyl phosphatidylcholine (azPC), components of oxidized low-density lipoproteins (oxLDL), have also been shown to activate PPARγ [302, 303]. Arachidonic acid and PG

derivatives have also been shown to act as weak PPARγ ligands, including 15-deoxy PGJ2 and 12-HETE [302]. PG-H1 and PG-H2 have also been shown to be less potent PPARγ ligands [304]. The anti-diabetic thiazolidinediones (TZDs) were long used for therapeutic purposes before they were identified as PPARγ agonists. TZDs had been shown to induce adipocyte differentiation through activation of aP2 [305, 306], and the identification of PPARγ binding sites in the aP2 promoter validated TZDs as potent PPARγ ligands [307]. To date, several TZDs, including troglitazone, rosiglitazone, and ciglitazone, have been described as PPARγ ligands [308-310]. The therapeutic effects of TZDs include lowered plasma glucose levels and insulin sensitization in animal and human models of type 2 diabetes [311, 312]. Several nonsteriodal anti-inflammatory drugs (NSAIDs) have also been reported to be synthetic PPARγ ligands [313].

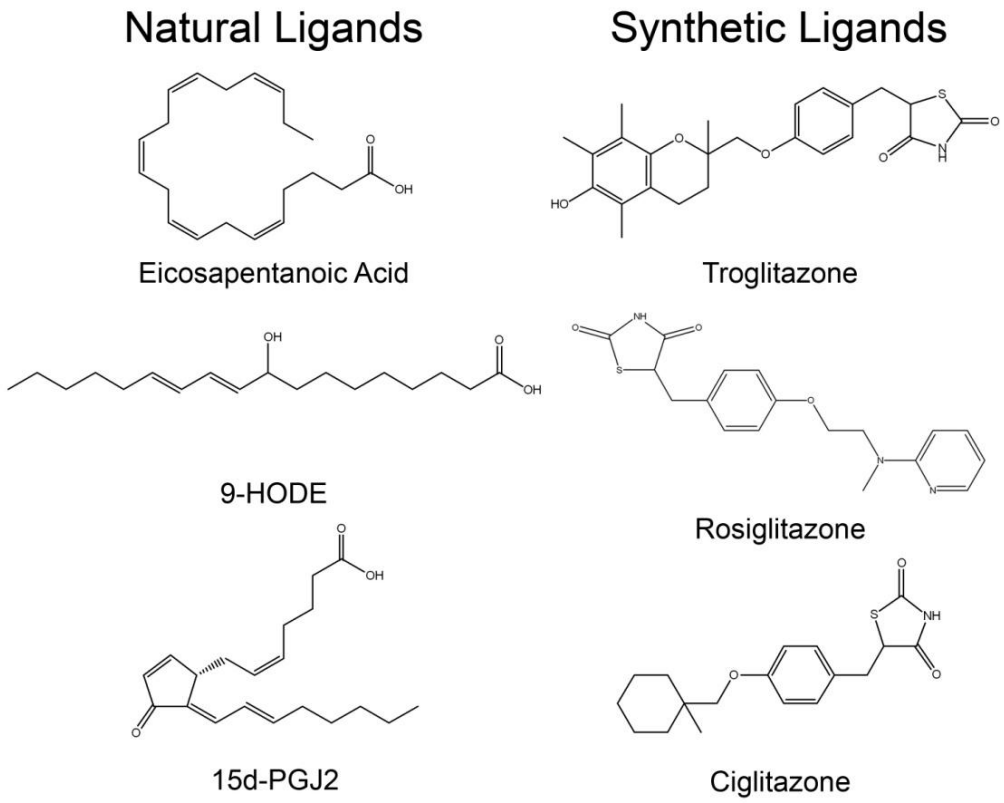

Figure **1.6**: Structures of representative PPARγ ligands. PPARγ ligands encompass a variety of structurally similar chemicals. Natural ligands include fatty acids and fatty acid derivatives. Synthetic ligands include the TZD class of anti-diabetic drugs.

Since the discovery of TZDs as PPARγ ligands, considerable interest has been placed on elucidating the physiological effects of PPARγ ligands. In the following sections, there is a discussion of how synthetic and natural PPARγ ligands modulate physiological functions in humans and rodents.

1.2.5 Physiological roles of PPARγ

The most widely studied PPAR isoforms is PPARγ, and this is mostly attributed to the physiological functions of documented ligands. In general, PPARγ is highly

expressed in the adipose, macrophages, and colon [296-299]. Lower, but detectable, expression of PPARγ has also been reported in the heart, small intestines, kidney, pancreas, spleen, and skeletal muscle [307, 314, 315]. The results of preliminary functional studies in adipocytes indicated that ligand activation of PPARγ is sufficient to initiate adipocyte differentiation via expression of aP2 [307, 315]. PPARγ has since been shown to regulate the expression of lipid metabolism and lipid uptake genes in adipocytes, including phosphoenolpyruvate carboxykinase (PEPCK) [316], acyl-CoA synthase [213], LPL [317], FATP [211], and CD36 [318]. Surprisingly, PPARγ-null mice are lethal at two developmental stages, which highlights the importance of PPARγ biological functions [319]. Heterozygous PPARγ mice were found to be viable, and these mice had reduced adipose stores. [319-321]. PPARγ-dependent adipogenesis was further recognized when a dominant negative PPARγ mutant inhibited adipocyte differentiation in the 3T3-L1 cell line [322, 323]. The results of these collective studies indicate a critical role of PPARγ in adipocyte biology.

The pharmacological interest in the TZDs stemmed from the utilization of these drugs to treat type 2 diabetes. It has been well characterized that TZDs, through activation of PPARγ, increased insulin sensitivity, reduced plasma glucose, and reduced insulin levels in animal and human models of hyperglycemia [311, 312, 324, 325]. Glucose metabolism is known to occur predominantly in the skeletal muscle. Therefore, how does low expression of PPARγ in the muscle cause striking differences in plasma glucose levels? Additionally, how does increased adipocyte differentiation by TZDs cause beneficial health effects via PPARγ? To address these apparent conflicting biological phenomena, several hypotheses have been put forth. First, PPARγ expression is several orders of magnitude higher in adipocytes, but PPARγ protein is still likely

functional in skeletal muscle and liver. The adipocyte-independent functions of TZDs have been examined in adipose-deficient mice. Surprisingly, TZD altered insulin resistance and glucose levels [326]. The results from mechanistic studies have indicated that ligand activation of PPARγ increased mitochondrial uncoupling proteins 1-3 (UCP1-3) [327] and repressed leptin mRNA production [328, 329]. These effects resulted in increased lipid metabolism and energy expenditure. Additionally, PPARγ has been reported to regulate glucose transporter type 4 (GLUT4) in insulin-responsive tissues, including muscle and fat, to regulate glucose homeostasis [330, 331]. Tissue-specific transcriptional regulation of LPL and FATP-1 by PPARγ has also been shown to contribute to blood glucose levels and diabetes [332]. LPL and FATP-1 were found to be regulated by PPARγ in adipocytes, but this regulation was not observed in muscle tissue [332]. Moreover, increased fatty acid hydrolysis and decreased muscle fatty acid uptake have been shown to improve insulin sensitivity [333]. The relative size of the adipocyte can also modulate insulin sensitivity, glucose uptake, and lipid metabolism [334]. Ligand activation of PPARγ has been observed to increase the number of small adipocytes produced [335, 336]. TZDs have also been shown to reduce muscle triglyceride levels in rodent diabetic models, and several mechanisms may contribute to these effects [337]. The modulation of muscle triglyceride levels is likely mediated through scavenging by PPARγ-dependent regulation of CD36, but TNFα levels have also been shown to be reduced in a PPARγ ligand-dependent manner [335, 336, 338]. Insulin resistance has been positively correlated to TNFα levels [338]. Therefore, PPARγ ligands may also contribute to glucose and lipid homeostasis by modulation of inflammatory signaling. Collectively, these results from a number of studies demonstrate that PPARγ plays a multi-faceted role in preventing diabetes.

PPARγ may also play a role in atherosclerosis. Initially, PPARγ was suggested to be pro-atherosclerotic because of the high receptor expression in macrophage and foam cells. Surprisingly, rosiglitazone reduced the formation of atherosclerotic lesions in mice [339]. The contributing mechanism was shown to be through regulation of the nuclear oxysterol receptor LXRα and cholesterol efflux in macrophage. LXRα has been reported to regulate ABC family members and initiate cholesterol efflux in macrophage [340]. Combined, the increased cholesterol efflux and anti-inflammatory effects caused by TZDs indicate that PPARγ ligands could be used in the prevention of atherosclerosis.

The reduction in TNFα signaling in response to PPARγ ligands strongly suggests that this receptor modulates other inflammatory signaling pathways [338]. Subsequent molecular studies have shown that PPARγ interacts with several factors in several tissues to reduce inflammatory signaling. In addition to reduced TNFα signaling in macrophages, PPARγ has additionally been shown to reduce inducible nitric oxide synthase (iNOS), metalloproteinase 9 (MMP9), and scavenger receptor A expression. [299]. PPARγ ligands reduced IL-6 and IL-1β expression in monocytes, a direct precursor to macrophages [341]. Endothelial cells also exhibited PPARγ-dependent anti-inflammatory effects by reducing interferon-induced protein 10 (IP-10), interferon-inducible T-cell α chemoattractant, and endothelin-1 [254, 342]. PPARγ also been shown to interfere with AP-1 signaling [343], as well as interfere with NFκB signaling by competitive binding to the p50 and p65 subunits [344]. Collectively, PPARγ can reduce inflammatory responses by cross-talk with several signaling pathways.

The coupled anti-inflammatory effects of TZDs and ligand-dependent adipocyte differentiation suggest that ligand activation of PPARγ can modulate proliferation and

tumorigenesis. A wide variety of evidence has shown that PPARγ inhibited human and mouse cancer cell line proliferation. These anti-proliferative effects have been observed in several tissue and cell types, including lung [345], prostate [346, 347], colon [348-352], endothelial [353], liver [354], myeloma [355], bronchial epithelium [356], stomach [357], pancreas [358], breast [359], melanoma [360-363], smooth muscle [364], ovarian [365], neuroblastoma [366], leukemia [367], and thyroid [368]. Additionally, ligand activation of PPARγ was shown to trigger cell cycle arrest in malignantly transformed adipogenic HIB-1B cells [369] and primary liposarcoma cells [370]. PPARγ ligands also reduced tumor growth or differentiation state in several rodent models of mammary carcinogenesis [371-373]. While most cell line data suggests that PPARγ is an anti-proliferative transcriptional regulator, the role of this nuclear receptor in colon carcinogenesis has remained controversial. Several studies have shown that PPARγ expression is elevated in human colon tumors and colon cancer cell lines [351, 374, 375]. The increased expression of PPARγ in colon cells has been attributed to decreases in cell proliferation and morphological changes indicative of colon differentiation [348-352]. PPARγ ligands also reduced tumor growth in colon xenografts in nude mouse models [375]. However, two independent studies have shown that TZDs increased colon polyp multiplicity in the adenomatous polyposis coli (APC) mouse colon cancer model, termed *Apc*$^{+/min}$ [376-378]. Higher levels of β-catenin and increased colon tumorigenesis were observed in *Pparγ*$^{+/-}$ mice as compared to wild-type mice in the azoxymethane (AOM) chemically-induced model of colon carcinogenesis [379]. Ligand activation of PPARγ also reduced the initiation and progression of colon carcinogenesis in the AOM model [380, 381]. The 1,2-dimethylhydrazine dihydrochloride (DMH) model of chemically-induced colon carcinogenesis similarly increased tumorigenesis in *Pparγ*$^{+/-}$ mice [352]. Surprisingly, colon carcinogenesis was found to be independent of PPARγ

heterozygosity in APC models [379]. Collectively, these data do not clearly delineate if PPARγ is pro- or anti-tumorigenic. The fact that PPARγ is an embryonic lethal phenotype does not allow clear assessment of this receptor in tumorigenesis [319]. However, tissue-specific deletion of PPARγ did not alter cancer incidence in several tissues [382]. The lack of a clear consensus suggests that further investigations and novel model systems are needed to delineate the role of PPARγ in cancer initiation and progression.

PPARγ polymorphisms have also been shown to correlate with biological consequences. The interest of PPARγ polymorphisms in the alteration of biological function has led to numerous investigations to find polymorphism-dependent phenotypes. To date, eighteen PPARγ polymorphisms have been identified (Reviewed in [383]), although there is not a clear consensus on how each polymorphism alters receptor function. The two most common identified polymorphisms are the proline to alanine (Pro12Ala) polymorphism and the silent histidine (His477His) polymorphism. The Pro12Ala allele was shown to encode a receptor that is less responsive to ligands, thus influencing type 2 diabetes and obesity. The consensus appears that the Ala-12 allele is protective or non-effective against type 2 diabetes in normal populations. Surprisingly, the Ala-12 allele was found to be deleterious in obese subjects (Reviewed in [383, 384]). Insulin sensitivity was improved with the Ala-12 allele, although the effects of this polymorphism on body mass index (BMI) remains inconclusive (Reviewed in [383-385]). The silent His477His polymorphism, a cytosine to thymidine transition at nucleotide 1431 (C1431T) was observed to lower BMI levels for any given leptin level in obese subjects (Reviewed in [384]). Additionally, the T-1431 allele reduced the risk of cardiovascular disease; however, this polymorphism is considered a risk factor for polycystic ovarian

syndrome (Reviewed in [384]). Collectively, the functions of PPARγ polymorphisms remain largely undefined, but several studies have indicated that polymorphisms may alter receptor function.

While PPARγ modulates several normal physiological functions, mutations in the PPARγ gene may also alter tumorigenesis. Several research groups have examined what contribution PPARγ mutations play in human tumors. Collectively, less than 10% of cancers were found to possess PPARγ mutations (Reviewed in [386]). Thus, PPARγ mutations did not appear to cause tumorigenesis. While the observed mutations may contribute to carcinogenesis, it is unclear whether the mutation is causal or a secondary effect of tumorigenesis. Thus, polymorphisms appear to be more relevant to physiological function than mutations are to tumorigenesis.

In summary, a wealth of evidence suggests that PPARγ plays a critical role in normal physiology (Figure **1.7**). Ligand activation of PPARγ has been targeted in the treatment of type 2 diabetes, atherosclerosis, and inflammatory disorders. Although a clear role in tumorigenesis has not been established, the general consensus is that PPARγ is protective against tumorigenesis. These facts support the use of PPARγ ligands in clinical trials (Reviewed in [387-391]). The lack of a null-mouse model, due to embryonic lethality, has made clarifying the role of this receptor difficult. However, recent advances in transgenic and tissue-specific expression mouse models may soon allow thorough dissection of PPARγ function in normal physiological function, disease states, and tumorigenesis.

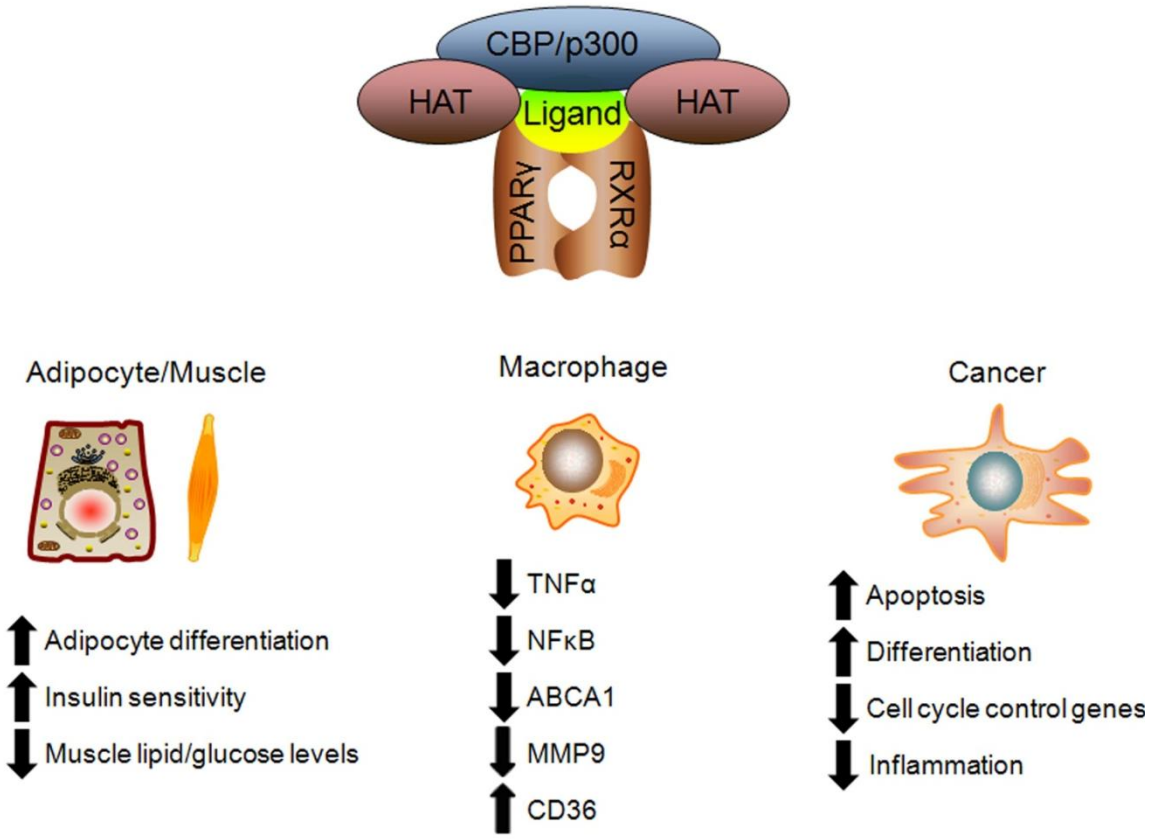

Figure **1.7**: PPARγ-dependent physiological functions. PPARγ has been shown to regulate the gene transcription involved in various cellular processes. Ligands for PPARγ have been utilized to prevent or treat various human diseases, including type 2 diabetes and atherosclerosis. PPARγ induced adipocyte differentiation and enhanced global insulin sensitivity. Ligand activation of PPARγ dampened numerous inflammatory pathways and activated lipid scavenging in macrophage. PPARγ has also been examined in numerous cancer models, and the consensus is that PPARγ is anti-tumorigenic by decreased cell proliferation, increased differentiation, and increased apoptosis.

1.2.6 General properties of PPARβ/δ

The identification of PPARβ/δ proved to be more difficult than other PPAR isoforms, and this confusion resulted in a variety of names given to this receptor. This isoform was named PPARβ when it was cloned from *Xenopus* in 1992 [153]. In

subsequent cloning from a human osteosarcoma cDNA library, it was identified as NUCI [157]. The murine isoform was then cloned from three independent laboratories and given the names PPARδ, NUCI, and fatty acid activated receptor (FAAR) [155, 392, 393]. The current convention to be used when referring to this receptor is PPARβ/δ. The human and mouse LBDs are 90% homologous and 72% homologous to the *Xenopus* receptor [188, 394, 395]. PPARβ/δ is ubiquitously expressed in rat tissues [161, 162]. Human tissues have shown similar ubiquitous expression, with notably higher expression in tissues involved in lipid metabolism, including the liver, intestine, kidney, adipose, and skeletal muscle [189]. Other analyses of PPARβ/δ distribution observed similar patterns of expression in tissues of high metabolism [155, 163, 394, 396]. Murine PPARβ/δ expression is predominantly nuclear and co-immunoprecipitates with RXRα in the absence of exogenous ligands [163]. Thus, PPARβ/δ may possess a constitutive nuclear function in the presence of yet unidentified endogenous ligands.

PPARβ/δ, like the other PPAR isoforms, has been shown to be activated by a diverse group of endogenous and synthetic ligands (Figure **1.8**). Saturated and unsaturated fatty acids are well known activators of PPARβ/δ, although the affinity for these ligands is intermediate compared to PPARα and PPARγ [300]. PUFAs, including linoleic acid, arachidonic acid, dihomo-γ-linoleic acid, and eicosapentanoic acid, have also been shown to activate PPARβ/δ in the low micromolar range [155, 300, 397]. Arachidonic acid metabolites, including 15-HETE, have also recently been shown to bind PPARβ/δ [398]. Additionally, palmitic acid and the metabolite 2-bromopalmitic acid have also been demonstrated to serve as natural PPARβ/δ ligands [392]. Eicosanoids have also been described as functional PPARβ/δ ligands, as PG A_2, D_1, and D_2 have also been observed to activate PPARβ/δ [194]. Carbaprostacyclin, a semi-synthetic PG

analogue has been suggested to be a potent PPARβ/δ activator [397], although recent investigations have challenged this observation [399]. Retinoic acid (RA) has recently been described to bind PPARβ/δ and modulate tumorigenesis [400, 401]. In contrast, two recent investigations have excluded RA as a PPARβ/δ activator [402, 403]. While the search for potent endogenous ligands for PPARβ/δ has yielded intriguing candidates, synthetic compounds have shown great promise for the pharmacological activation of PPARβ/δ. The first synthetic ligand was designed by Merck in 1997, and this compound, known as L-631033, demonstrated high selectivity for PPARβ/δ as compared to the other PPARs [404]. Another ligand, L-165041, was subsequently described and exhibited a 10-fold higher selectivity for human PPARβ/δ than other PPARs [158]. A three component library screen of ureidofibrates also categorized GW2433 as a dual activator of human PPARα and PPARβ/δ [405]. Recently, two highly potent PPARβ/δ ligands, GW0742 and GW501516, have shown 1000-fold selectivity for PPARβ/δ, with EC50s of 1.1 nM (human) and 20 nM (mouse) [159, 160]. Synthetic propenoic acid derivatives have also been recently described as PPARβ/δ activators [406]. Recently, three PPARβ/δ antagonists have been identified, although thorough examination of these compounds is needed to define the specific and off-target effects of receptor antagonism [407-410].

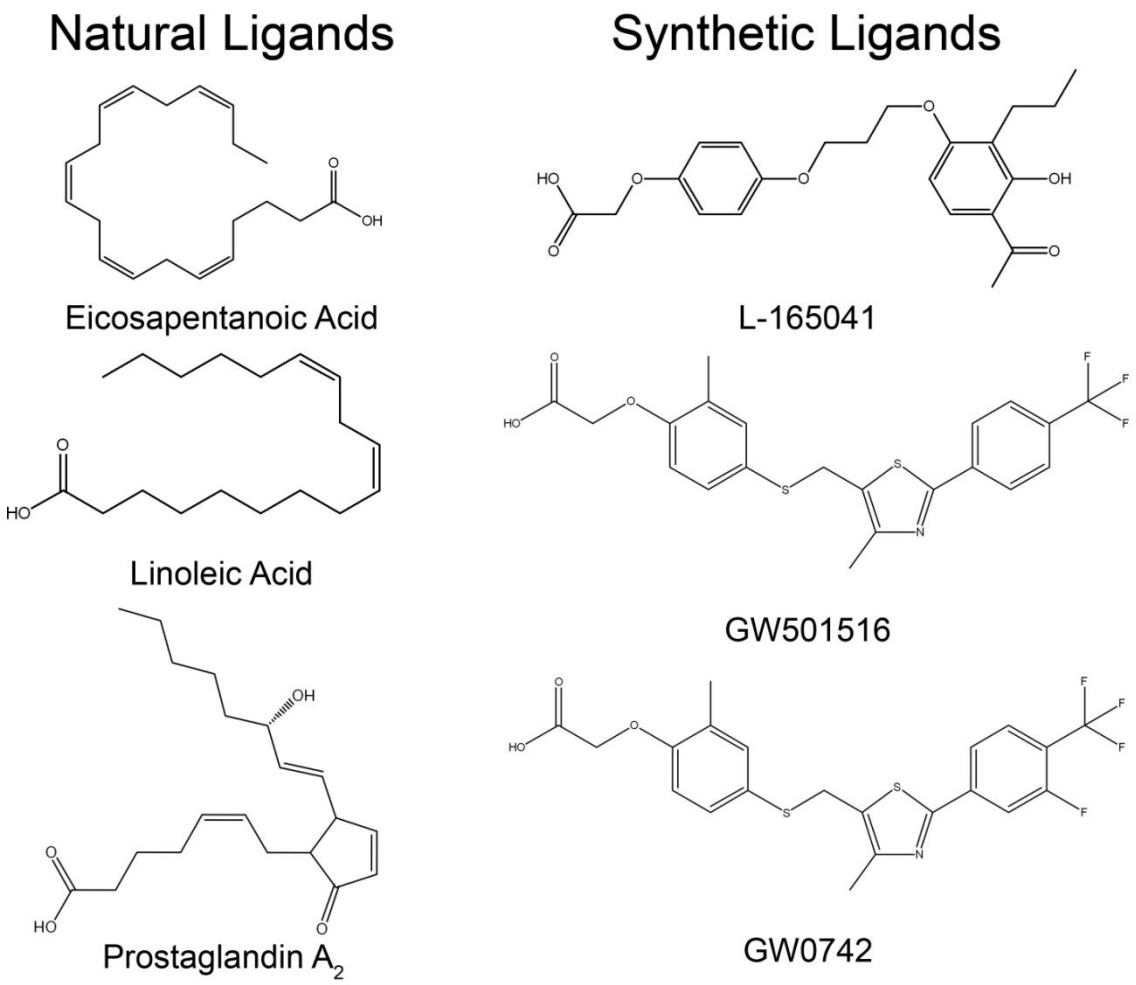

Figure **1.8**: Structures of representative PPARβ/δ ligands. PPARβ/δ ligands encompass a variety of structurally similar chemicals. Natural ligands include fatty acids, fatty acid derivatives, and eicosanoids. A number of high affinity synthetic ligands for PPARβ/δ have recently been developed, including L-165041, GW501516, and GW0742.

1.2.7 Physiological role of PPARβ/δ

Among the PPAR isoforms, the functional role of PPARβ/δ is considerably less well understood. The recent advent of high affinity synthetic agonists and null-mouse models has propelled investigations into the biological functions of PPARβ/δ in multiple

tissue types. Expression of PPARβ/δ has been detected in early embryogenesis [411] and is ubiquitously distributed [162, 163]. Notably high, and predominantly nuclear, expression of this receptor has also been observed in the liver, keratinocytes, and intestine [162, 163]. Three independent *Pparβ/δ*-null mouse models have been characterized (Table **1.3**). The first *Pparβ/δ*-null mouse, termed the Peters model, was created in 2000 by targeting a neomycin-resistance cassette to the LBD in C57Bl/6 mice [412]. The absence of PPARβ/δ resulted in reduced litter size, reduced body weight and fat deposits, increased epidermal hyperplasia in response to phorbol esters, and decreased sensitivity to NSAIDs [412]. In 2002, a *Pparβ/δ*-null mouse, termed the Barak model, was created by targeted deletion of the DBD using the Cre recombinase/loxP system. Using this model, full and conditional null mouse lines were created [413]. *Pparβ/δ*-null mice were mostly embryonic lethal due to placento-decidual contacting loosening, but the surviving pups were found to have reduced adipose stores [413]. A third *Pparβ/δ*-null mouse, termed the Nadra model, was created by neomycin-resistance targeted replacement of the PPARβ/δ DBD [414]. These mice had high embryonic lethality due to alterations in trophoblast giant cells [414]. The Nadra PPARβ/δ-null model also exhibited a hyperplastic epidermal response to phorbol esters [414], much like the Peters model [412]. Collectively, observations from all three mouse models indicate a role for PPARβ/δ in lipid metabolism by reduced adipose stores or lipid droplet formation [412-414]. The discrepancy between the models appears to be in the embryonic lethality of *Pparβ/δ*-null mice. Two of the three null models resulted in embryonic lethality [413, 414], while the Peters model had normal Mendelian distributions [412]. Several possibilities may explain the differences in lethality. First, the Peters mouse model was created by LBD disruption, as compared to the targeted deletion models. It is thus possible that the Peters model expresses a ligand-binding

mutant receptor that still possesses DNA-binding ability. However, protein expression of PPARβ/δ was not detected in this mouse model [412]. Furthermore, the Peters model was created in the C57Bl/6 mouse background and is a congenic mouse strain. However, the other models were created on mixed genetic backgrounds. It has been well established that mixed genetic backgrounds cause significantly different phenotypes [415-417], and this observation seems to be a likely explanation for the embryonic lethality observed in other *Pparβ/δ*-null mouse models [413, 414]. Collectively, results from null mouse models indicate that PPARβ/δ may be key regulator of physiological responses, disease states, and tumorigenesis (Table **1.3**).

Table **1.3**: *Pparβ/δ*-null Mouse Models

	Peters Model (2000) [412]	Barak Model (2002) [413]	Nadra Model (2006) [414]
Targeted Disruption	Ligand-binding domain	DNA-binding domain	DNA-binding domain
Method of Disruption	Neomycin-resistance cassette inserted into the last exon	Cre-loxP system	Neomycin-resistance cassette inserted into exons 4 and 5
Phenotype	Viable on C57BL/6 background	Frequent embryonic lethality on mixed genetic background	Frequent embryonic lethality on mixed genetic background
Observations	Smaller than wild-type littermates in early life Decreased gonadal adipose stores Altered myelination in corpus collossum Enhanced epidermal hyperplasia in response to phorbol esters.	Placental defects Stunted growth before puberty Decreased adipose mass	Placental defects Trophoblast giant cell differentiation defect Smaller than wild-type littermates in early life Enhanced epidermal hyperplasia in response to phorbol esters.

The decreased size and fat stores caused by PPARβ/δ disruption suggested that lipid homeostasis, fatty acid metabolism, and adipose biology are regulated by PPARβ/δ-dependent mechanisms. Evidence that PPARβ/δ regulates fatty acid metabolism has been observed in several skeletal muscle model systems. Ligand activation of PPARβ/δ has been shown to increase myogenesis and an oxidative phenotype [418-421]. Researchers have also examined the role of PPARβ/δ in lipid and cholesterol homeostasis, and L-165041 increased HDL cholesterol levels [422]. Similarly, GW501516 improved lipid profiles in insulin-resistant obese rhesus monkeys, including decreased fasting glucose, insulin, and LDL levels and increased HDL levels [160]. GW501516 also reduced adiposity and improved insulin resistance in several mouse models of obesity [418, 423]. Furthermore, *Pparβ/δ*-null mice placed on a high fat Westernized diet had increased serum triglycerides due to elevation of VLDL and reduction of LPL as compared to wild-type mice [424]. Ligand activation of PPARβ/δ also improved insulin sensitivity in a high fat diet model that was not observed in similarly treated *Pparβ/δ*-null mice [425]. Regulation of lipid homeostasis occurs in multiple tissues, and the known role of PPARβ/δ in lipid signaling suggests that this receptor can elicit biological effects in adipose tissue. Indeed, when an adipose-specific constitutively active PPARβ/δ transgenic mouse model was investigated, the presence of activated receptor correlated to a lean phenotype with reduced serum triglycerides. Further dissection of this phenotype found high fatty acid oxidation in brown adipose tissue [423]. Administration of GW501516 further promoted fat burning and was found to be protective against obesity [423]. Ligand activation of PPARβ/δ therefore reduced adipose stores by increased fatty acid catabolism and consumption of the free fatty acid pool. Mechanistic studies in adipocyte models have shown that PPARβ/δ ligands and receptor over-expression induced expression of early markers of adipose differentiation,

including adipocyte lipid binding protein (ALBP) [392, 426]. PPARβ/δ also increased expression of PPARγ and increased adipocyte differentiation, which suggests a synergism between these PPAR isoforms in regulating adipose biology [426]. Furthermore, studies using wild-type and *Pparβ/δ*-null pre-adipocytes revealed that lipid accumulation and expression of differentiation markers were markedly lower in *Pparβ/δ*-null pre-adipocytes [427]. These effects appeared to involve cyclic adenosine monophosphate (cAMP) [428]. Collectively, these results indicate that PPARβ/δ plays a key role in adipose biology and functions to improve insulin sensitivity and increase fatty acid oxidation.

Energy expenditure, lipid metabolism, and glucose homeostasis are all regulated by complex interactions between multiple organs, including adipose tissue and skeletal muscle. These observations suggest that PPARβ/δ regulates fatty acid metabolism in the muscle. PPARβ/δ is the predominant PPAR in skeletal muscle [429], and ligand activation of receptor increased fatty acid oxidation in L6 myotubes [429, 430]. Treatment of a myoblast cell line, C2C12, with a synthetic PPARβ/δ ligand or long chain fatty acids increased mRNA production of several lipid metabolism genes, including heart-FABP, FAT, LPL, and CPTI [431]. PPARβ/δ over-expression also enhanced receptor signaling while a dominant-negative receptor eliminated upregulation of lipid metabolism target genes [431]. Microarray and transcript profiling studies from two independent laboratories have shown a clear linkage between ligand activation of PPARβ/δ and lipid metabolism [418, 432]. Ligand activation of PPARβ/δ also improved insulin resistance in C2C12 muscle cells by preventing NFκB activation and diacylglycerol (DAG) accumulation [433]. Results from another study linked PPARβ/δ-dependent expression of adipophilin to increased insulin sensitivity in muscle cells, thus

presenting a novel target gene that regulates insulin sensitivity [434]. A role of PPARβ/δ in muscle energy expenditure and metabolic disorders was also established when muscle-specific expression of PPARβ/δ converted type II muscle to type I muscle [435]. This observation was pivotal because type I muscle possess higher mitochondrial and oxidative enzyme levels than type II muscle [435]. These collective results indicated that PPARβ/δ is a critical regulator in lipid metabolism and energy expenditure. These facts also suggest that PPARβ/δ ligands can be utilized in the prevention or treatment of disease states including dyslipidemia, obesity, and atherosclerosis.

Altered lipid homeostasis correlates with diseases such as atherosclerosis, and macrophages primarily regulate this disease state. PPARβ/δ has also been shown to play a critical role in macrophage function. Ligand activation of PPARβ/δ was shown to increase triglyceride accumulation by causing oxLDL-dependent lipid redistribution in macrophages. These effects were attributed to the expression of adipose differentiation-related protein (ADRP) and the loading of macrophages with VLDL [436]. Furthermore, PPARβ/δ ligands increased reverse cholesterol transport and increased HDL by upregulation of ABC-A1, increased lipid scavenging by scavenger receptor A and B expression, and by decreasing apoE expression [437, 438]. The results from these studies indicated that PPARβ/δ ligands reduced atherosclerotic lesions by increased lipid accumulation in peripheral tissues and increased HDL [439]. A seminal investigation utilizing wild-type and *Pparβ/δ*-null macrophages revealed that PPARβ/δ reduced atherosclerosis. Ligand activation of PPARβ/δ in wild-type macrophages was shown to cause dissociation between PPARβ/δ and the transcriptional repressor BCL-6 in LDL receptor-deficient mice fed a high fat diet [179]. This resulted in reduced inflammation by decreased production of multiple cytokines and chemokines [179]. Mechanistic

investigations have also shown that MCP-1 and IL-1β expression was repressed by PPARβ/δ ligands. Additionally, MCP-1 and IL-1β expression was increased by a mutation in PPARβ/δ that prevents corepressor dissociation [179]. Studies from several independent laboratories have also observed that ligand activation of PPARβ/δ attenuated inflammatory responses in macrophages, including interferon γ (IFNγ)- and lipopolysaccharide (LPS)-induced inflammation [440-443]. A further correlation between PPARβ/δ and atherosclerosis was uncovered when PPARβ/δ was shown to increase the expression of genes involved in carnitine biosynthesis and lipid mobilization [444, 445]. There is also evidence that PPARβ/δ regulated intestinal absorption and secretion of cholesterol in macrophage. Ligand activation of PPARβ/δ in mice devoid of HDL expression was found to increase fecal cholesterol excretion and decrease absorption by repression of Niemann-Pick C1-like 1 (NPC1L1) [446]. Subsequent investigations linked ligand activation of PPARβ/δ to NPC1L1 repression by enhanced excretion of macrophage or HDL-derived cholesterol in feces [438]. Recent evidence has also suggested that macrophage can be alternatively activated, termed M2 macrophage, and modulate inflammation and insulin resistance (Reviewed in [447]). While one report suggested that PPARβ/δ plays no role in M2 activation [448], two other reports observed that ligand activation of PPARβ/δ improved insulin sensitivity by shuttling macrophage to the M2 state [449, 450]. Many disease states also have foundations in macrophage immune responses. PPARβ/δ expression has been shown to be elevated in engulfing macrophage, and this indicates that PPARβ/δ modulates macrophage self tolerance and inflammation [451]. Collectively, these studies have shown that PPARβ/δ plays an important role in glucose and cholesterol homeostasis and may be used in the treatment of metabolic disorders (Figure **1.9**).

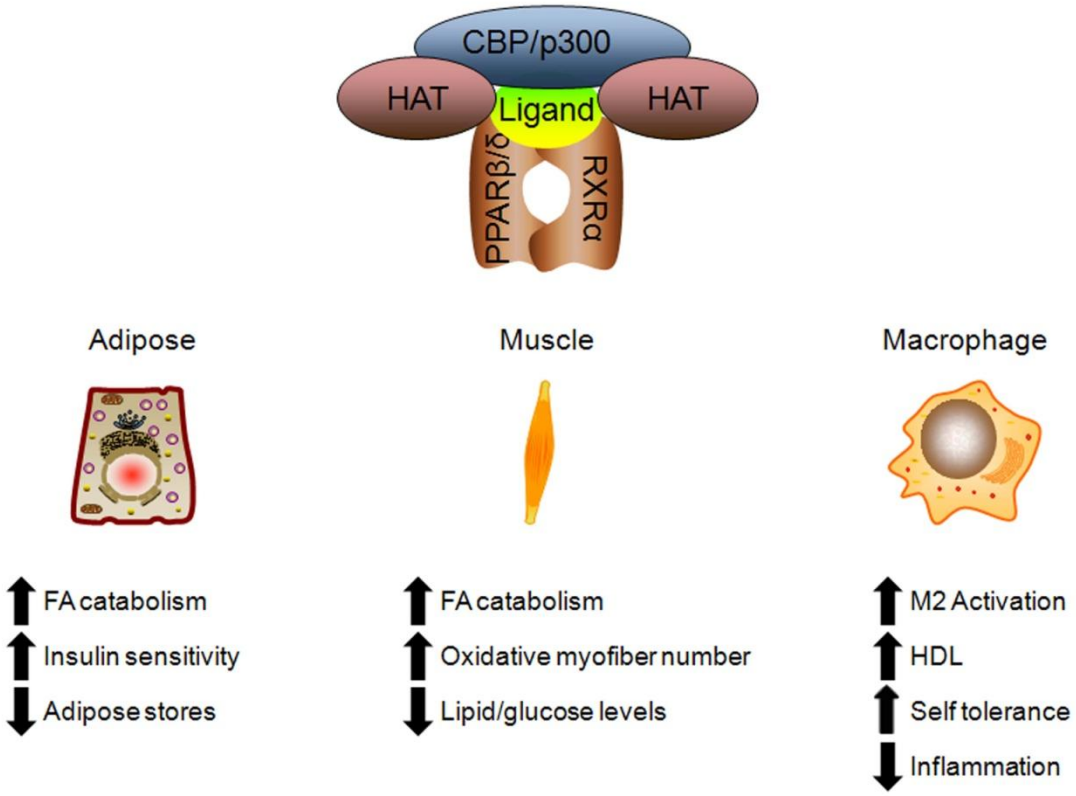

Figure **1.9**: Effect of PPARβ/δ on metabolic disorders. Ligand activation of PPARβ/δ enhanced fatty acid catabolism in adipose and muscle tissues, lowered serum triglycerides by reduced VLDL, increased HDL production in macrophage, increased alternative M2 macrophage activation. These collective effects suggest that PPARβ/δ ligands can serve as chemotherapeutic agents for the treatment and prevention of diseases associated with metabolic disorders, including dyslipidemia, type 2 diabetes, and atherosclerosis.

The expression of PPARβ/δ in tissues associated with the central nervous system (CNS) has lead to several investigations to define the function of PPARβ/δ in the brain. PPARβ/δ is expressed in fetal [411] and adult [162] brain. High receptor expression has also been observed in the hypothalamus, cerebellum, and cerebral cortex [452-455]. In contrast, PPARβ/δ expression is lower in the medulla oblongata and spinal cord [452-455]. The brain is a highly metabolic organ; therefore it is not surprising

that high PPARβ/δ expression correlates to high lipid metabolism. Analysis of female brains revealed that PPARβ/δ-dependent modulation of phospholipid and esterified fatty acid production could significantly alter lipid metabolism [456]. Ligand activation of PPARβ/δ by L-165041 has also been shown to induce expression of acyl-CoA synthetase 2 in rat brain culture [457]. Lipids serve an important function by coating and insulting neurons (Reviewed in [458]). PPARβ/δ has been shown to play a role in the myelination of neurons, as the absence of PPARβ/δ caused neuron demyelination [412]. Ligand activation of PPARβ/δ has also been shown to cause differentiation and myelination in oligodendrocytes, the primary lipid-producing cell-type in the brain [459]. Ligand activation of PPARβ/δ also increased neuroblastoma differentiation and regulated oligodendrocyte proliferation [460, 461]. The role of PPARβ/δ in inflammatory brain diseases, including multiple sclerosis (MS) and Parkinson's disease, has also been investigated. The PPARβ/δ ligands GW0742 and GW501516 were observed to reduce clinical MS symptoms [462, 463]. CNS cytotoxicity and cell death are also clinical factors in Parkinson's disease and other neurological diseases. In models of CNS cell injury and death, ligand activation of PPARβ/δ protected against toxicity and cell death [464-466]. The results of these studies suggest that PPARβ/δ ligands can be neuroprotective or utilized to treat acute neurotoxicity. More recently, PPARβ/δ has been examined in experimental autoimmune encephalomyelitis (EAE), an autoimmune inflammatory disease of the CNS. *Pparβ/δ*-null mice exhibited severe inflammation by increased CD4+ T helper cell proliferation and cytokine production [467]. Additionally, ligand activation of PPARβ/δ decreased cytokine production, including IFNγ and the IL-12 family members, in human and mouse immune cells [467]. Collectively, the high expression of PPARβ/δ in the CNS suggests that ligand activation caused strong biological effects in these tissues.

Advances in genomic analyses have recently established that PPARβ/δ polymorphisms may affect metabolic disorders and other disease states. The most common polymorphisms exist in exon 4, termed +294 T/C. This polymorphism has been associated with metabolic trait phenotypes, including increased LDL [468, 469], high baseline plasma triglyceride levels [470], and coronary lesions [470]. Surprisingly, this polymorphism was associated with decreased BMI in a Caucasian population [471]. Another common polymorphism, rs2016520, has also been linked to metabolic traits. This polymorphism was associated with reduced height, a risk factor for metabolic trait [472]. In contrast, another study linked this polymorphism to decreased BMI and increased HDL [473]. Three additional PPARβ/δ polymorphisms have been shown to increase whole body insulin sensitivity [474]. Furthermore, several other studies have associated many polymorphisms with increased diabetes [475], plasma glucose [476, 477], insulin sensitivity [476], BMI [477, 478], and interactions with calpains [478]. For all these studies suggesting a linkage between PPARβ/δ polymorphisms and metabolic disorders, three studies found no association between PPARβ/δ and metabolic diseases [477, 479, 480]. Alzheimer's disease has also been linked to dysregulated cholesterol levels, but no association was found between PPARβ/δ and Alzheimer's disease [481, 482]. Polymorphisms in PPARβ/δ have also been examined in NSAID protection of colorectal cancer. Only two studies have investigated this linkage, with one study finding no PPARβ/δ polymorphism linkage [483] and the other study describing a polymorphic enhancement of NSAID-dependent protection of colorectal cancer [484]. Apart from a role in disease states, PPARβ/δ polymorphisms have also been examined in exercise physiology and endurance. The exon 4 294 T/C was not found to be associated with exercise endurance in athletes [485]. However, a combination of this polymorphism and

a peroxisome proliferator-activated receptor gamma coactivator-1 polymorphism was associated with elite level endurance athletes [485]. Another polymorphism in exon 4 of PPARβ/δ resulted in smaller increases in training-induced oxygen consumption and increased HDL expression [486]. Although these studies revealed that PPARβ/δ polymorphisms are associated with changes in disease states and susceptibility, more studies are necessary in varying populations and ethnicities to link these polymorphisms to disease states. Additionally, these polymorphisms need to be examined with other disease state polymorphisms to assess the complex interactions between signaling pathways.

PPARβ/δ ligands have also been shown to reduce inflammation in multiple models. A common cell type that modulates inflammation is the endothelial cells. These cells line the vasculature and form the interface between the circulatory system and all other tissue systems. The predominant inflammatory molecules in these cells are TNFα or C-reactive protein (CRP). Several PPARβ/δ ligands, including GW0742, GW501516, and L-165041, reduced TNFα- or CRP-dependent induction of VCAM-1 [487-490], intracellular cell adhesion molecule-1 (ICAM-1) [487, 489] , E-selectin [487, 489], MCP-1 [488, 490, 491], and growth regulated oncogene α (GROα) [489]. Another component of the endothelial system is VSMCs and the pulmonary system. PPARβ/δ ligands reduced LPS-dependent induction of granulocyte macrophage-colony stimulating factor (GMC-SF) [492], TNFα [492], IL-6 [492], IL-1β [491, 492], MCP-1 [491], and transforming growth factor β (TGFβ) [491]. As discussed earlier, PPARβ/δ modulated inflammatory responses in disease states such as atherosclerosis. Early mechanistic studies in macrophages revealed that PPARβ/δ reduced inflammation through interaction with BCL-6 [179, 442, 493]. Furthermore, PPARβ/δ inhibited expression of MCP-5 [493], IL-

1β [179], TNFα [442], IL-6 [442], and VCAM-1 [442]. Ligand activation of PPARβ/δ has also been examined in atherosclerosis, and activation of the receptor reduced or prevented atherosclerosis [179, 440-442, 493]. Additionally, the absence PPARβ/δ in monocytes prevented the transition to M2 activated macrophages, which may also reduce atherosclerosis [449]. PPARβ/δ ligands have also been shown to redirect dendritic cells to be less stimulatory to monocyte-derived inflammation [494]. In several models of cardiac inflammation, ligand activation of PPARβ/δ decreased inflammation caused by ischemia/reperfusion or LPS. Inflammatory markers known to be blunted by PPARβ/δ ligands include TNFα [495, 496], NFκB [495], ICAM-1 [497], IL-6 [497], and MCP-1 [497]. Furthermore, the absence of PPARβ/δ expression led to exaggerated TNFα expression in LPS-mediated inflammation [496]. Ligand activation of PPARβ/δ has also been examined in liver toxicity caused by diet or chemicals. The methionine- and choline-deficient (MCD) diet is a mouse model of non-alcoholic steatohepatitis (NASH) in the liver, and ligand activation of PPARβ/δ inhibited the inflammatory markers IL-6, IL-1β, MCP-1, TNFα, and NFκB [498]. Carbon tetrachloride (CCl_4) is a chemical model of liver hepatotoxicity and inflammation that revealed PPARβ/δ-dependent reduction of inflammation. *Pparβ/δ*-null mice displayed enhanced NFκB signaling by increased expression of TNFα, TNF-like weak inducer of apoptosis receptor (TWEAKr), and S100 calcium-binding protein A6 in response to CCl_4 [499]. Ligand activation of PPARβ/δ reduced the expression of these inflammatory markers in wild-type mice, and this effect was not observed in similarly treated *Pparβ/δ*-null mice [499]. IL-6 is another inducer of liver inflammation. Hepatocytes and a human cancer cell line treated with GW501516 inhibited IL-6 inflammation through suppression of signal transducer and activator of transcription 3 (STAT3) [500]. Additionally, genome-wide analyses of mouse liver indicated that PPARβ/δ prevented the expression of inflammatory genes [501]. A role for

PPARβ/δ in skin inflammation and wound healing has also been examined using mouse genetic models and ligand treatments. PPARβ/δ protected against phorbol ester-induced hyperplasia and inflammation in two independent *Pparβ/δ*-null mouse models [412, 502]. Furthermore, ligand activation of PPARβ/δ attenuated these inflammatory responses [412, 502, 503]. It was established that inflammatory signaling, such as that caused by phorbol esters and TNFα, enhanced the expression of PPARβ/δ in skin and keratinocytes [412, 502, 504]. It was then proposed that increased PPARβ/δ expression upon skin injury enacted survival, differentiation, and remodeling signals [504, 505]. Consistent with these previous observations, a deficiency in PPARβ/δ expression reduced skin wound healing [502, 504, 506]. Furthermore, ligand activation of PPARβ/δ increased permeability barrier repair [503]. These receptor-dependent effects have been attributed to upregulation of AKT [504] and the Rho-GTPase pathways [505]. Coordinated autocrine signaling between the keratinocytes and underlying fibroblasts has also been proposed to aid in tissue repair by modulating IL-1β inflammatory responses in the fibroblasts [507]. This model has yet to be fully investigated, but it does represent an eloquent model of PPARβ/δ-dependent functionality in epithelial wound repair. Consistent with previous observations, ligand activation of PPARβ/δ prevented inflammation in the colon [508], adipocytes [509], microglia/astrocytes [463], and aggregating brain cultures [462]. Collectively, the results of these studies suggest that PPARβ/δ reduced inflammation in multiple tissues types and disease states that could be used in the prevention or treatment of multiple inflammatory diseases.

The high expression of PPARβ/δ in epithelial tissues suggests that this PPAR isoforms serves a pivotal role in epithelial proliferation and tumorigenesis. The maintenance of epithelial tissues, including skin and colon tissues, relies on a delicate

balance between cell proliferation and terminal differentiation. The function of PPARβ/δ in epithelial differentiation was first examined in keratinocytes and mouse skin. In both models, exposure to phorbol esters increased *Pparβ/δ* mRNA levels and increased keratinocyte differentiation marker expression [510]. These observations suggest that phorbol esters may produce an endogenous PPARβ/δ ligand to induce terminal differentiation. Treatment of human keratinocytes with the PPARβ/δ ligands L-165041 or tetradecylthioacetic acid also increased the mRNA production of several differentiation markers, such as transglutaminase (Tg) and involucrin [511]. Additionally, wild-type neonatal skin possessed a higher number of cornified envelopes, a morphological marker of differentiation, than *Pparβ/δ*-null skin [512] without overt changes in epidermal morphology [412]. The PPARβ/δ ligand GW0742 also increased differentiation marker expression and cornified envelope formation in wild-type primary keratinocytes, an observation that was absent in similarly treated *Pparβ/δ*-null primary keratinocytes [512]. Surprisingly, no differences in phorbol ester- or calcium-mediated differentiation of keratinocytes were observed between wild-type and *Pparβ/δ*-null keratinocytes [412, 512]. This observation suggests that PPARβ/δ is dispensable for differentiation in the presence of more potent differentiation signals. These observations have also been reported by several independent laboratories using different *Pparβ/δ*-null mouse models [502-504, 511]. In addition to the role in skin differentiation, PPARβ/δ caused differentiation in a growing list of tissues and cells. This list includes oligodendrocytes [459], macrophage [437], intestinal epithelium [513, 514], human keratinocytes [515], breast cancer cell lines [516], colon cancer cell lines [516, 517], trophoblasts [414], osteoblasts [518], hepatic embryonic stem cells [519], C6 glioma cells [520], primary keratinocytes [502-504, 511, 512], neonatal stratum corneum [521], and undifferentiated F9 cells [522]. Collectively, these observations indicate that ligand activation of PPARβ/δ

induces terminal differentiation by increased expression of proteins required for this process.

It has also been previously established that terminal differentiation is associated with concomitant decreases in cell proliferation. Thus, PPARβ/δ likely modulates cell proliferation. While PPARβ/δ promoted differentiation, the role of PPARβ/δ in cell proliferation has remained controversial (Reviewed in [164, 523]). In three independent *Pparβ/δ*-null mouse models, the absence of PPARβ/δ led to enhanced skin hyperplasia and proliferating cell nuclear antigen (PCNA) positive cells as compared to wild-type controls [412, 506, 524, 525]. Furthermore, *Pparβ/δ*-null primary keratinocytes were observed to proliferate faster than wild-type cells [402, 406, 526], and ligand activation of PPARβ/δ further decreased cell proliferation in a PPARβ/δ-dependent manner [402, 406, 512]. These effects were attributed to cyclin A expression [504] or altered protein turnover of the MAP kinase (MAPK) signaling pathway [526]. Additionally, treatment with the PPARβ/δ agonist GW1514 increased differentiation with no effect on epidermal cell proliferation [503]. Human keratinocytes and keratinocyte cell lines have also been examined for PPARβ/δ-responsiveness and cell proliferative effects. Several ligands have been examined, including L-165041, GW0742, and di(2-ethylhexyl)phthalate (DEHP). Ligand activation of PPARβ/δ inhibited cell proliferation in normal human keratinocytes [511], the N/TERT-1 human keratinocyte cell line [515], the NCTC 2544 keratinocyte cell line [527], and the HaCaT keratinocyte cell line [402]. In contrast, treatment with PPARβ/δ ligands L-165041 and GW501516 increased cell proliferation in keratinocytes isolated from psoriasis patients [528]. Retinoic acid- and epidermal growth factor-stimulated cell proliferation has also been cooperatively linked to PPARβ/δ ligand activation [401, 529]. However, these observations have been disputed by two

independent research groups [402, 403]. Ligand activation of PPARβ/δ has also been examined in somatic cells types. In the absence of PPARβ/δ, primary mouse endothelial cells [530], mouse primary hepatocytes [499], and primary mouse stellate cells [499] were observed to proliferate faster than wild-type cells. In contrast, over-expression of PPARβ/δ in mouse embryonic fibroblasts increased cell proliferation [531]. Prostacyclin analogs have been reported to modulate cell proliferation by activation of PPARβ/δ, and treatment with prostacyclins or over-expression of prostacyclin synthase inhibited proliferation in bovine endothelial cells [532], rat vascular smooth muscle cells [533-535], bovine vascular smooth muscle cells [532], human vascular smooth muscle cells [536], human and mouse lung fibroblasts [537], and the human embryonic kidney epithelial cell line HEK-293 [538]. The PPARβ/δ ligand GW501516 has shown conflicting effects on cell proliferation. In several human endothelial types, GW501516 caused increased cell proliferation [539-541]. However, GW501516 inhibited cell proliferation in rat cardiac fibroblasts [542, 543] and mouse inner medulliary collecting duct (IMCD-K2) cell line [544]. The use of cancer cell lines confounds the understanding of PPARβ/δ function in cell proliferation. Surprisingly, most reports of proliferative PPARβ/δ ligands focused on GW501516, and this ligand increased cell proliferation in the ER-positive breast cancer cell lines [540], lung cancer cell lines [545-547], liver cancer cell lines [548, 549], cholangiocarcinoma cell lines [550], and prostate cancer cell lines [540]. Additionally, L-165041 has been shown to increase proliferation in a rat glioma cell line [551]. In contrast to these observations, several PPARβ/δ ligands, including GW501516, exhibited no, or inhibitory, effects on cell proliferation. These examples include colon cancer cells lines [540, 552, 553], ER-positive and -negative breast cancer cells lines [540, 554], lung cancer cell lines [555, 556], liver cancer cell lines [552], neoplastic skin cancer cell lines [524, 557], melanoma cell lines [554, 558, 559], a skin carcinoma cell

line [406], a mammary gland cancer cell line [560], a rat glioma cell line [551], myeloma cell lines [561], a neuroblastoma cell line [460], a renal carcinoma cell line [562], and prostate cancer cell lines [540]. Collectively, the described observations indicate that ligand activation of PPARβ/δ exerted mixed effects on cell proliferation in tissues and cancer cell lines. The lack of consensus suggests that standardized methods of analysis should be used to clarify the role of PPARβ/δ in cell proliferation.

Another underlying cellular process modulating cell number and tumorigenesis is apoptosis, a highly programmed method of cell death. Similar to the conflicted role in differentiation and cell proliferation, the effect of PPARβ/δ expression and ligand activation on apoptosis has remained controversial. In terms of intrinsic apoptotic signaling, ligand activation of PPARβ/δ in human HaCaT keratinocytes increased annexin-V staining, a marker of apoptosis, in a dose-dependent manner [402]. However, these effects were not caused by poly (ADP-ribose) polymerase (PARP)-dependent pathways [402]. The increased annexin-V staining was also observed in response to PPARβ/δ ligands in a mouse mammary gland cancer cell line [560]. Similarly, GW0742 increased apoptosis in HIT-T15 hamster pancreatic β cells [563]. Other studies in normal bladder cells [564], human liver cancer cell lines [552], human colon cancer cell lines [552], medullary collecting duct cells [544], and vascular smooth muscle cells [565] have shown negligible effects of PPARβ/δ ligands on apoptosis. The majority of studies examining PPARβ/δ as an apoptosis modulator have focused on serum withdrawal-dependent or chemically-induced apoptosis. The first studies examining serum starvation-induced apoptosis found that ligand activation of PPARβ/δ by GW501516 inhibited apoptosis in human colon cancer cell lines [566-568]. The protective effect of PPARβ/δ ligands to serum starvation was similarly observed in the Sp1 keratinocyte cell

line [569]. While the results of these studies suggest that ligand activation of PPARβ/δ reduced apoptosis in the absence of serum, examination of other human colon cancer cells with this approach resulted in contrasting findings. Treatment with two PPARβ/δ ligands did not alter the proliferation of HCT116, HT29, and LS174T colon cancer cell lines in the absence of culture medium serum [552]. These effects were similarly observed in human liver cancer cell lines [552], a human breast cancer cell line [554], a mouse mammary gland cancer cell line [560], a human melanoma cell line [554], and a human keratinocyte cell line [402]. Ligand activation of PPARβ/δ also been examined in multiple models of induced apoptosis, such as ischemia and hypoxia. PPARβ/δ ligands prevented hypoxia-induced apoptosis in endothelial progenitor cells [541]. Apoptosis was also examined in ischemia-dependent kidney dysfunction, apoptosis, and organ injury. Surprisingly, ischemic wild-type mice had less kidney dysfunction and injury as compared to *Pparβ/δ*-null or heterozygous mice. Treatment with L-165041 further protected wild-type mice from injury through activation of the AKT signaling pathway [570]. H_2O_2 is a common chemical inducer of apoptosis in endothelial cells, and PPARβ/δ ligands prevented H_2O_2 apoptosis in these cells by direct regulation of the anti-apoptotic protein 14-3-3ε and prevention of Bad-dependent signaling [571]. Researchers have also examined how PPARβ/δ modulated apoptosis during short- and long-term administration of H_2O_2 in endothelial cells. Surprisingly, short-term administration of H_2O_2 decreased expression of PPARβ/δ [572], while repeated low-dose exposure enhanced PPARβ/δ expression [573]. Although the length of exposure caused large differences in PPARβ/δ expression, ligand activation of PPARβ/δ protected against H_2O_2 apoptosis in both models [572, 573]. Furthermore, reduced expression of PPARβ/δ by anti-sense oligonucleotides increased apoptosis during the short-term H_2O_2 exposure model [573]. Additional chemical mediators of apoptosis have also been examined for PPARβ/δ-

dependent modulation, and ligand activation of PPARβ/δ inhibited apoptosis caused by palmitate [563], oxLDL [565], cisplatin [547], and TNFα [574]. Cumulatively, the function of the PPARβ/δ in apoptosis remains controversial due to inconsistencies in the model systems, tissue types, and ligands examined. These conflicted observations demonstrate the need for consensus model systems and ligands to delineate the role of PPARβ/δ in modulating apoptosis.

Consistent with the controversial role of PPARβ/δ in cell proliferation and apoptosis, the role of this nuclear receptor in tumorigenesis is equally controversial. The colon is the most controversial tissue examined for PPARβ/δ-dependent modulation of carcinogenesis, and two contrasting hypotheses have emerged to explain the function of PPARβ/δ in colon tumorigenesis. The first hypothesis suggests that PPARβ/δ is a target of the APC/β-catenin pathway. Dysregulation of APC leads to increased PPARβ/δ expression and modulation of apoptosis. This hypothesis proposed that NSAIDs prevent carcinogenesis through reduced COX-dependent production of PPARβ/δ ligands. The second hypothesis postulates that ligand activation of PPARβ/δ decreases colon carcinogenesis by induction of terminal differentiation and inhibition of cell proliferation. The first hypothesis was bound on evidence that PPARβ/δ expression increased in colorectal cancers [575, 576]. Furthermore, NSAIDs decreased PPARβ/δ expression and APC signaling [575]. The expression of PPARβ/δ in colon cancers was subsequently shown to be correlated to wide-spread expression of the receptor in transformed cells, much like COX2 expression [577]. The *Apc*$^{+/min}$ mouse is a commonly used to examine predisposition to colon carcinogenesis, and these mice form spontaneous tumors. First, PPARβ/δ expression was reported to be higher in *Apc*$^{+/min}$ mice [578, 579], and this was also observed in human familial colon cancers [578].

Ligand activation of PPARβ/δ increased polyp number in a PPARβ/δ-dependent manner in the *Apc*$^{+/min}$ model [566, 568]. Results from a APC/PPARβ/δ dual genetic model indicated that that *Apc*$^{+/min}$/*Pparβ/δ*-null mice were protected against colon carcinogenesis and the purported tumor promoting effects of GW501516 [580]. Genetic disruption of PPARβ/δ in the colon epithelium reduced AOM-dependent colon carcinogenesis [581], although these observations contrasted with other *Pparβ/δ*-null mouse observations [513, 582]. Common treatments for colon cancer are NSAIDs and APC/β-catenin inhibitors. Several reports revealed that these compounds reduce APC-mediated carcinogenesis through down-regulation of PPARβ/δ and receptor-dependent modulation of vascular endothelial growth factor (VEGF) expression [579, 583, 584]. The hypothesis that PPARβ/δ enhanced colon carcinogenesis follows a similar model that has been proposed for the oncogene cyclin D1. In this model, mutations in APC result in dysregulated PPARβ/δ expression and increased tumorigenesis.

In direct contrast to this first hypothesis, a second colon cancer model suggests that PPARβ/δ inhibits colon carcinogenesis through regulation of colonic differentiation. The first evidence in support of this hypothesis arose when another *Pparβ/δ*-null genetic model was bred to the *Apc*$^{+/min}$ strain. In this model, enhanced colon tumorigenesis was observed in *Apc*$^{+/min}$/*Pparβ/δ*-null [582]. Additionally, *Pparβ/δ*-null mice had enhanced AOM-dependent colon carcinogenesis [582]. These observations confirmed the previous report that PPARβ/δ is dispensable for APC-mediated colon carcinogenesis [413]. Ligand activation of PPARβ/δ also increased colonic differentiation and apoptosis, which likely resulted in reduced AOM-dependent colon carcinogenesis in wild-type mice [513]. The combination of NSAIDs and PPARβ/δ ligands was also examined as a therapeutic treatment in colon carcinogenesis. Ligand activation of PPARβ/δ inhibited colon polyp

formation in a PPARβ/δ-dependent manner [582, 585]. Nimesulide, a prototypical NSAID, reduced polyp formation independent of PPARβ/δ expression, and the combined treatment did not exert additive benefits or effects on polyp formation [585]. These results indicated that PPARβ/δ and NSAID anti-tumorigenic effects are mediated by independent mechanisms [585]. Recent evidence in *Apc*$^{+/min}$ mice has shown that PPARβ/δ expression is unchanged in tumors and administration of nitrogen-donating NSAIDs increased PPARβ/δ expression [586]. These results challenge the observation that NO-donating NSAIDs inhibit colon carcinogenesis by reducing PPARβ/δ expression [579]. However, this examination solely relied on immunohistochemistry to examine protein expression [579]. Collectively, the role of PPARβ/δ in colon carcinogenesis remains controversial, and a clear consensus in colon carcinogenesis must be sought before PPARβ/δ ligands can be used as preventative and therapeutic agents.

In contrast to the highly disputed role of PPARβ/δ in colon carcinogenesis, PPARβ/δ has a more defined role in skin carcinogenesis. In the two-stage (initiation/promotion) skin chemical carcinogenesis model, *Pparβ/δ*-null mice were found to have enhanced tumor incidence, multiplicity, and size as compared to wild-type mice [525]. Subsequent studies revealed that PPARβ/δ protected against carcinogenesis by directly modulating the ubiquitin pool and the degradation of protein kinase C α (PKCα) signaling components [525, 526]. GW0742 inhibited chemically-induced skin carcinogenesis in a PPARβ/δ-dependent manner [524]. The results from other studies have indicated that PPARβ/δ ligands and NSAID co-administration prior to, during, or after tumor formation alters two-stage skin carcinogenesis. The presence of the NSAID sulindac, a pan COX inhibitor, prior to and throughout the bioassay inhibited tumor formation in a PPARβ/δ-independent manner through decreased PG synthesis [587]. A

second study found that the administration of the COX2-selective NSAID nimesulide with PPARβ/δ ligands after tumor formation reduced skin carcinogenesis [557]. While ligand activation of PPARβ/δ had no effect on tumor progression, nimesulide reduced tumor multiplicity in both genotypes [557]. The combined treatment of PPARβ/δ ligand and COX inhibition synergistically reduced tumor multiplicity in wild-type mice for the first twenty-five percent of the treatment protocol, and this effect was not observed in similarly treated *Pparβ/δ*-null mice [557]. These effects were attributed to decreased PG production and increased differentiation in wild-type mice. These observations suggest that PPARβ/δ ligands could be used as a chemopreventive or chemotherapeutic agent in combination with current treatments [557]. In contrast to the disputed roles of PPARβ/δ in colon carcinogenesis, PPARβ/δ reduced skin carcinogenesis and may be utilized as a therapeutic target in skin cancer.

The ability of PPARβ/δ to alter tumorigenesis in other tissue types has also been examined. PPARβ/δ expression was examined in two models of lung cancer [547, 588]. In an examination of two lung cancer tissue arrays, PPARβ/δ protein expression was reported to be elevated in tumor tissues as compared to adjacent non-tumorous tissues [547]. The results from immunostaining further revealed that cancerous cells were more immunoreactive to a PPARβ/δ antibody [547]. The ability of PPARβ/δ to alter RAF-induced lung tumorigenesis was also examined, and the absence of PPARβ/δ expression increased the incidence and score of lung adenomas [588]. Furthermore, PPARβ/δ expression was not altered as compared to non-transgenic controls in the RAF transgenic mouse model [588]. Ligand activation of PPARβ/δ also enhanced adenosquamous and squamous cell carcinoma in the mammary gland [589]. Additionally, the enhancement of mammary tumorigenesis by retinoic acid is mediated

by ligand activation of PPARβ/δ and modulation of the ratio of cellular retinoic acid binding protein-II (CRABP-II) to FABP5 [590]. In contrast, ligand activation of PPARβ/δ decreased cell proliferation, increased apoptosis, and decreased clonogenecity in the C20 mouse mammary cancer cell line [560]. PPARβ/δ expression, as observed by in situ hybridization, was also found to be enhanced in endometrial adenocarcinomas [591], although quantitative protein expression was not examined. PPARβ/δ expression was also examined in gastric tumors and found to be unchanged between tumors and adjacent normal tissues [592]. Reduced expression of PPARβ/δ in melanomas was also observed to cause superficial spreading [559], which may suggest a role for PPARβ/δ in tumor invasion and metastasis. Preliminary investigations in liver tissues using PPARβ/δ genetic models indicated that PPARβ/δ reduced chemically-induced liver hepatotoxicity by modulating the expression of inflammatory markers [499, 593]. Collectively, preliminary investigations have not conclusively revealed whether the expression of PPARβ/δ or ligand activation of receptor alters tumorigenesis, and further studies are needed to specifically delineate the role in this nuclear receptor in tumorigenesis.

The collective literature suggests that PPARβ/δ is multi-functional nuclear receptor that can modulate numerous biological responses (Figure **1.10**). Understanding these functions is critical because PPARβ/δ may be utilized as a pharmacological target to prevent or treat diseases. Although several receptor functions are strongly disputed, PPARβ/δ clearly modulates lipid homeostasis, metabolic diseases, inflammation, cell proliferation, and differentiation. Future investigations must therefore focus on delineating the mechanisms by which PPARβ/δ is reported to function within these controversial topics.

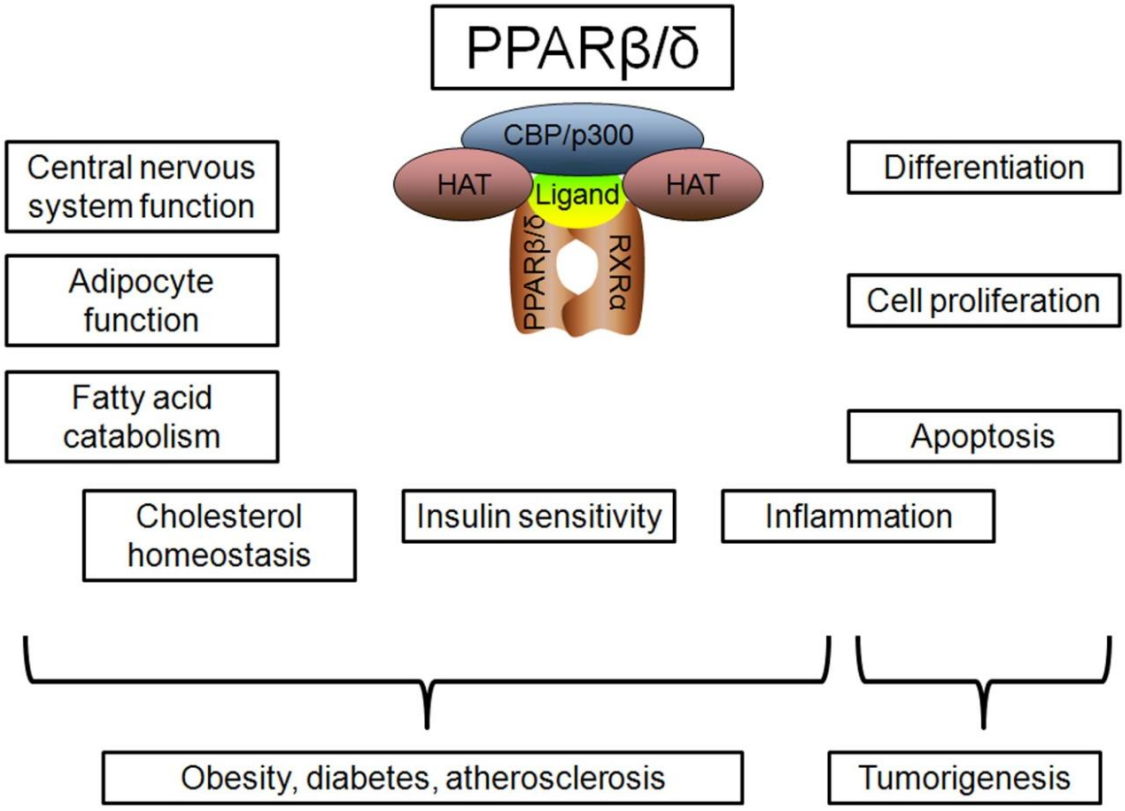

Figure **1.10**: Summary of PPARβ/δ functions. PPARβ/δ is a multi-functional nuclear receptor modulates many signaling pathways, including central nervous system function, adipocyte function, fatty acid catabolism, cholesterol homeostasis, insulin sensitivity, and inflammation. These functions play a key role in the development of disease states that include obesity, diabetes, and atherosclerosis. PPARβ/δ may also modulate tumorigenesis through regulation of cell differentiation, cell proliferation, and apoptosis.

1.3 Skin carcinogenesis

1.3.1 Structure and physiology of the skin

The skin is the largest organ in the body in terms of surface area and accounts for approximately fifteen percent of the total body weight [594]. The skin serves as the first line of defense between the host and the environment [595]. This organ also protects against many insults, including pathogenic agents, toxic chemicals, microorganisms, environmental threats, radiation, and loss of body fluids [595]. The skin can be divided into three distinct layers, termed the hypodermis, dermis, and epidermis [594]. The hypodermis is the deepest layer of the skin and primarily functions to regulate temperature and protects against mechanical injury (Reviewed in [594]). The dermis is the middle layer of the skin and is composed of fibroblasts, dendrocytes, and mast cells [594]. This layer is tightly connected to the epidermis by the basement membrane and contains connective tissue, hair follicles, blood vessels, nerve endings, and multiple glands [594]. The epidermis is the outermost layer of the skin protecting against the external environment. Continual cell proliferation is needed to maintain the epidermis, and the constant exposure of this tissue to environmental toxicants can cause cancer [594, 596, 597].

The epidermis is composed of five defined layers [594]. These layers (Figure **1.11**) are termed the stratum germinativum (basal), stratum spinosum (spinous), stratum granulosum (granular), stratum licidum, and stratum corneum (cornified) [594, 596, 597]. The epidermis is primarily composed of keratinocytes (95%), but melanocytes, Langerhans cells, and Merkel cells also populate the epidermis [594]. The

basal layer is the innermost layer of the epidermis and contains a population of keratinocyte stem cells that facilitate self-renewal and re-population of the upper layers (Reviewed in [597-599]. The progression of cells away from the basal layer begins a process termed terminal differentiation. This orchestrated and apoptotic-like process directly creates the protective and impermeable layers of the skin (Reviewed in [597-599]). The next layer of the epidermis is the spinous layer. Cells within this layer have started the process of terminal differentiation and are cuboidal in shape. These cells are physically joined by an intercellular adhesion complex termed desmosomes and begin to synthesize differentiation proteins that include keratins and cross-linking enzymes (Reviewed in [597-599]). The spinous layer also contains the dendritic-like Langerhans cells that serve as the anti-presenting cells of the epidermis [594]. The granular layer is composed of several layers of squamous and partially differentiated cells that contain assembled granular structures. These granules are composed of differentiation-related proteins and enzymes that will execute terminal differentiation. The stratum lucidum is a few thin layers of flattened, dead keratinocytes that predominantly exists in epidermal areas of high friction [594]. The cornified layer is the outermost epidermal layer and contains dead keratinocytes that are devoid of nuclei and cellular organelles. Cells in this layer have formed an intracellular matrix of lipids from the lamellar bodies (Reviewed in [597-599]). The dead keratinocytes are also surrounded by an insoluble intercellular matrix of proteins that are known as the cornified envelop (Reviewed in [597-599]). The insoluble and lipophilic nature of the cornified envelop and the intracellular lipid matrix protects the body from the outside environment (Reviewed in [597-599]). As cornified cells are sloughed off, new basal cells enter into terminal differentiation to maintain the dynamic integrity of the skin.

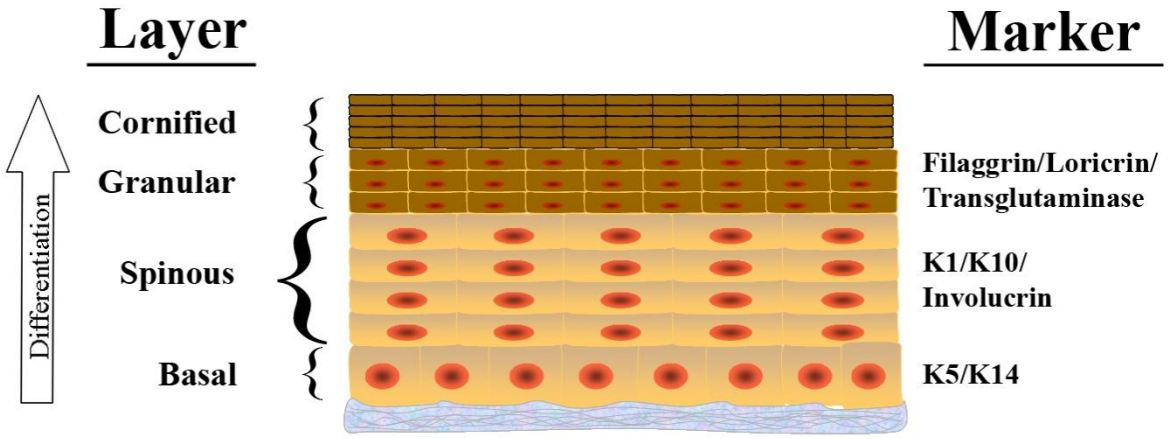

Figure **1.11**: Schematic structure of the epidermis. The epidermis is composed of four primary layers that expression layer-specific protein markers. The basal layer is the sole proliferative layer of the epidermis and is known to expression of keratin 5 (K5) and K14. Induction of terminal differentiation progresses cells into the spinous layer and expression of K1, K10, and involucrin begin to be expressed. Further progression of cells away from basement membrane leads to further differentiation marker expression, followed by intra- and inter-cellular crosslinking. The final layer of the epidermis is the cornified layer, which contains enucleated, dead, cross-linked cells that protect the body from external insults.

The structure and stability of the skin, mediated through epidermal keratinocytes, is maintained by a diverse group of proteins. The stabilizing component in the skin is a class of proteins termed keratins. These proteins are intermediate filament proteins that form the strong and insoluble cytoskeletal network of the skin. There are two classes of keratins, termed acidic and basic, that are based upon the protein amino acid sequence. Acidic keratins, also termed Type I, have molecular masses of 40-60 kiloDaltons (kDa) (Reviewed in [598-600]). In contrast, basic keratins, also known as Type II, have molecular masses of 50-70 kDa (Reviewed in [598-600]). During differentiation, acidic and basic keratins heterodimerize to form a strong filamentous structure (Reviewed in [598-600]). The expression of specific keratins is tightly regulated and serves as biochemical markers of keratinocyte differentiation. Keratin 5 (K5), K14, and K15 are

expressed in the basal layer and compose approximately thirty percent of the proteins in this layer (Reviewed in [598-601]). As cells release from the basement membrane and begin to differentiate, expression of K5, K14, and K15 is downregulated and replaced by expression of K1 and K10 (Reviewed in [598-601]). In addition to the exchanged keratin expression in the spinous layer, other proteins are expressed to commit cells to terminal differentiation. These proteins include involucrin and Tg (Reviewed in [602]). As cells progress into the granular layer, loricrin, filagrin, and small proline rich proteins (SPRs) are expressed and accumulate at the plasma membrane of cells (Reviewed in [602]). Tg is the executioner of terminal differentiation by cross-linking involucrin, loricrin, filagrin, and SPRs into a complex protein network by catalyzing ε-(γ-glutamyl)lysine isopeptide bonds between the proteins [603]. The granular layer cells exhibit greater plasma membrane permeability that allows an influx of calcium into the cell. Calcium is a crucial activator of Tg, and this initiates the cross-linking process (Reviewed in [599]). The cross-linking of insoluble proteins, in addition to the deposition of lipid at the plasma membrane, forms the insoluble and water impenetrable protective complex known as the cornified envelope (Reviewed in [598-602]). The cornified cells compose the outermost layer of the epidermis and function as the protective barrier of the skin (Reviewed in [598-602]). Collectively, the expression of many proteins determines the formation of the layers of the epidermis (Figure **1.11**).

A critical balance between proliferation and differentiation is necessary to maintain the integrity of the skin while allowing responses to physical and chemical stimuli. A prominent mediator of differentiation is calcium. It is widely known that primary keratinocyte proliferate *in vitro* under calcium concentrations below 0.05 mM. However, concentrations of calcium between 0.12 and 1.2 mM rapidly modulate the expression of

proteins involved in terminal differentiation [604, 605]. Increased calcium concentrations initiates terminal differentiation by altering the expression of differentiation related proteins, such as keratins, and by modulating the enzymatic activity of Tg (Reviewed in [599, 606]). A key regulator in this process is the PKC family of kinases, with PKCδ being a crucial initiator of terminal differentiation (Reviewed in [606]). Phorbol esters, such 12-O-tetradecanoylphorbol-13-acetate (TPA), have also been shown to modulate differentiation by causing growth arrest and induction of differentiation-related proteins [607]. Additionally, hydrocortisone [608, 609] and vitamin D [610] expression have also been shown to cause terminal differentiation in keratinocytes. Several other signaling molecules and chemicals have also been shown to promote keratinocyte proliferation, thus preventing terminal differentiation. Epidermal growth factor (EGF) and TGFα are potent keratinocyte mitogens necessary for wound repair (Reviewed in [599]). Retinoic acids have also been shown to inhibit differentiation by direct transcriptional regulation of keratins and differentiation-related proteins (Reviewed in [611]). TGFβ is another signaling molecule known to regulate differentiation. Interestingly, keratinocyte proliferation is inhibited at low concentrations of TGFβ and enhanced by high concentrations of TGFβ [612]. Proliferation and differentiation must be balanced during wound healing, also termed activation [613]. This process is critical to re-epithelialization of the wound site and the initiation of the healing process. The hallmark of activated keratinocytes is the expression of K6, K16, and K17, as well as increased proliferation and increased cellular migration [613]. Cytokines and chemokines are also released to produce paracrine and autocrine signaling [613]. These events cause the recruitment of additional cell types to the wound area [613]. Proliferation must then be reduced as the wound heals, which illustrates the importance of balancing keratinocyte proliferation and differentiation.

1.3.2 Mechanisms of skin carcinogenesis

Skin cancer is an emerging public health concern with world-wide increases in incidence. Cancer is a clinical disease in which uncontrolled cell growth leads to the formation of a tumor. The relative long latency of cancer has made identifying the initiating agents of skin carcinogenesis difficult. However, mouse models of skin carcinogenesis have been invaluable in ascertaining initiators and promoters of cancer. Several extra-cellular factors contribute to skin carcinogenesis, including viruses, ultraviolet (UV) radiation, and chemical toxicants (Reviewed in [614-617]). These factors have been shown to act independently or cooperatively, termed co-carcinogenesis, to promote tumorigenesis [616]. Tumors can be divided into two broad categories, benign or malignant, characterized by the ability of the tumor to invade the surrounding tissues [618]. Benign tumors are unable to invade the surrounding tissue and are typically encapsulated within a tissue. Examples of benign tumors in humans include papillomas and keratoacanthomas (KAs) [618]. However, KAs can progress to a malignant tumor type in murine models [619]. Malignant tumors possess the inherent ability to invade surrounding tissues and metastasize to other organ systems. Examples of malignant tumors include basal cell carcinomas (BCCs), squamous cell carcinomas (SCCs), and melanomas [618]. In general, cancers arise through a three-step process that includes initiation, promotion, and progression (Reviewed in [617, 620, 621]). Initiation is an irreversible step which causes inherent cellular damage (Reviewed in [617, 620, 621]). Promotion, on the other hand, is considered a non-mutagenic and non-carcinogenic reversible change that cannot solely cause tumorigenesis (Reviewed in [617, 620, 621]). Progression is the transformation of a benign tumor into a malignant and invasive tumor [622]. Clearly, understanding the various causes and mechanisms of skin

carcinogenesis will provide critical insights into the treatment and prevention of this increasingly deadly disease.

Viral skin carcinogenesis is the least understood of the three mediators of skin carcinogenesis. This is mostly attributed to the latent onset of carcinogenesis and poor identification of the initiators [615]. Viruses can also serve as co-carcinogens in concert with other tumorigenic factors, including immunity and inflammation [615, 616]. To date, three viruses conclusively cause skin neoplasms, and these include human papilloma virus (HPV), Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), and human T-cell leukemia virus type 1 (HTLV-1) [615]. HPV, in addition to being a causative agent in cervical cancer, has been shown to cause SCCs [615, 623, 624]. KSHV is causative in skin lesions of immuno-compromised individuals by altering host expression of cell cycle, angiogenesis, and inflammatory genes [615]. HTLV-1 skin carcinogenesis is attributed to the oncoprotein Tax and direct alteration of NFκB signaling [615]. Clearly, further understanding of the causative mechanisms of viruses in skin carcinogenesis is an important issue that must be addressed.

UV radiation is a predominant cause of skin cancer in humans, and constant exposure to UV, particularly in developing countries, has led to the increasing incidence of skin tumorigenesis. The UV spectrum encompasses the 10-400 nanometer (nm) spectrum and is composed three distinct bands: UVC (below 280 nm), UVB (280-315 nm) and UVA (315-400 nm) (Reviewed in [614]). While stratospheric ozone blocks UVC and some UVB (280-290 nm) radiation, UVB and UVA radiation does reach the earth's surface (Reviewed in [614]). The primary mechanisms of UV-induced skin carcinogenesis are DNA mutagenesis, DNA strand breaks, lipid peroxidation, and

protein cross-linking (Reviewed in [614]). While there is not a specific targeting of UV-induced DNA damage, there is a strong propensity for cyclobutane-type pyrimidine dimers and pyrimidine (6-4) pyrimidine photoproducts [625-627]. These damaging effects predominantly cause cytosine to thymidine transitions at mono- and di-pyrimidine sequences. The damaged nucleotide bases are typically replaced by adenosine nucleotides at unreadable lesion sites by semi-conservative DNA replication (Reviewed in [614]). Further studies have identified DNA transitions as the hallmark of UV-induced DNA damage [628]. UV radiation is also known to cause cytosine photohydrates, purine photoproducts, and singe-strand DNA breaks [629-631]. Preliminary studies in UV skin carcinogenesis surprisingly revealed that greater than eighty percent of pre-cancerous skin lesions have specific DNA mutations in the p53 tumor suppressor gene loci. Further investigations also found a strong correlation between p53 mutations and malignant skin cancers (Reviewed in [632, 633 , 634]). Decades of investigations have revealed that UV radiation is a causative agent in skin carcinogenesis, and persistent human exposure necessitates further investigations into the prevention and treatment of skin cancer.

In addition to UV-induced skin carcinogenesis, epidermal exposure to pollutants and chemical agents are similarly known to be causative mediators of tumorigenesis. Studies throughout the past century have described many chemicals that function as tumor initiators or promoters (Reviewed in [635]). In general, three broad chemical classes have been identified that cause irreversible DNA damage, and these classes include polycyclic aromatic hydrocarbons (PAHs), nitrosamines, and alkylating agents [621]. PAHs are metabolized by the CYP family and microsomal epoxide hydrolase (mEH) to form highly reactive diol epoxides [636-638]. The epoxide moiety is highly electrophilic and forms stable DNA adducts by covalent binding to exocyclic amino

groups of guanine and adenosine [636-638]. Alkylating agents and nitrosamines cause irreversible damage by the addition of an alkyl or methyl groups to DNA (Reviewed in [639]). N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Reviewed in [639]) and β-propiolactone [621] are prototypical alklyating carcinogens. The electrostatic nature of the nitrogen 7 position (N7) of guanine residues makes this position highly susceptible to methylation. O6 of guanine and N3 of adenine are nucleotide sites susceptible to alkylation (Reviewed in [639]). The damage caused by nitrosamines and alkylating agents causes carcinogenesis by nucleotide mutations or DNA strand breaks (Reviewed in [621, 639]). Several mechanisms exist to repair the diol-epoxide, methylated, or alkylated DNA damage, but absent or reduced DNA repair results in permanent DNA mutations [640]. Although DNA damage should occur randomly, several cancer studies using PAHs and MNNG have reported a propensity for DNA mutations in the H-ras and p53 genes [641-643]. As stated previously, tumor promoters do not possess the inherent ability to cause tumorigenesis, but these compounds enhance cancer through clonal selection and expansion of initiated cells. Prototypical promoters include TPA, anthralin, and benzoyl peroxide. These chemicals promote tumorigenesis through alteration of proliferative and inflammatory signaling pathways (Reviewed in [621]). TPA, which is functionally analogous to DAG, is able to activate the PKC pathway and enhance ornithine decarboxylase (ODC) activity, both of which are important roles in tumor promotion [617]. As chemical carcinogenesis is the more well-studied models to study mechanisms of skin carcinogenesis, subsequent discussions will focus on chemical mediators of skin tumorigenesis.

Throughout several decades of detailed skin tumorigenesis studies, two predominating models have emerged to examine the effects of chemical carcinogens as

initiators or promoters. The two models are the two-stage carcinogenesis model and the complete carcinogen model. The purpose of these different models is to discern the ability of a chemical to serve as an initiator, promoter, or both (complete) [644]. The two-stage model involves a single, subthreshold, treatment with an initiator that this followed by repeated exposure to a promoter (Reviewed in [617, 621, 644-646]). The complete carcinogen model utilizes a single or repeated dose of a carcinogen (Reviewed in [617, 621, 644-646]). The key difference between these models is that the two-stage model allows mechanistic and operational separation of initiation and promoter that cannot be examined in the complete carcinogen model [644]. A clear difference in endpoint tumor types has also been observed with these two models. Papillomas are the predominant tumor in the two-stage model, while carcinomas are the predominant tumor in the complete carcinogen model (Reviewed in [617, 620, 621]). As stated previously, mutations in the H-ras oncogene have been observed in a great majority of PAH-initiated skin tumors [642]. Additionally, initiation with MNNG has also been shown to cause mutations in H-ras [647]. Future studies utilizing these models of skin carcinogenesis must focus on identifying new carcinogens and examination of putative chemoprevention agents (Reviewed in [648-650]).

1.3.3 Mechanisms of PAH-dependent skin carcinogenesis

PAHs cause tumorigenesis through direct DNA mutation of tumor suppressors and oncogenes such as H-ras and p53. As discussed earlier, PAHs are metabolized by the CYPs and mEH to form diol epoxides [636-638]. The creation of reactive carcinogens, term bioactivation, by the P450 system is a by-product of PAH detoxification. A two-step cellular mechanism exists to hydroxylate (phase I) and

subsequently conjugate large hydrophilic groups (phase II) to the PAH (Figure **1.12**). These modifications increase the solubility and excretion of the PAH. Upon PAH insult, cellular responses lead to increased protein expression of CYPs and subsequent hydroxylation of the PAH [636-638]. A reactive, yet highly unstable, epoxide moiety can be formed. This moiety can be acted upon by mEH to form a diol that can be metabolized by phase II enzymes [636-638]. However, further hydroxylation of PAHs by CYPs causes the formation of a diol-epoxide that can adduct to and mutagenize DNA [636-638]. The main CYP contributors to bioactivation of PAHs are CYP1A1, CYP1A2, and CYP1B1, and tissue-specific expression and induction of these isoforms is tightly regulated [636-638]. The direct transcriptional responsiveness of phase I and II enzymes to PAHs suggested that these carcinogens could be ligands for a transcription factor.

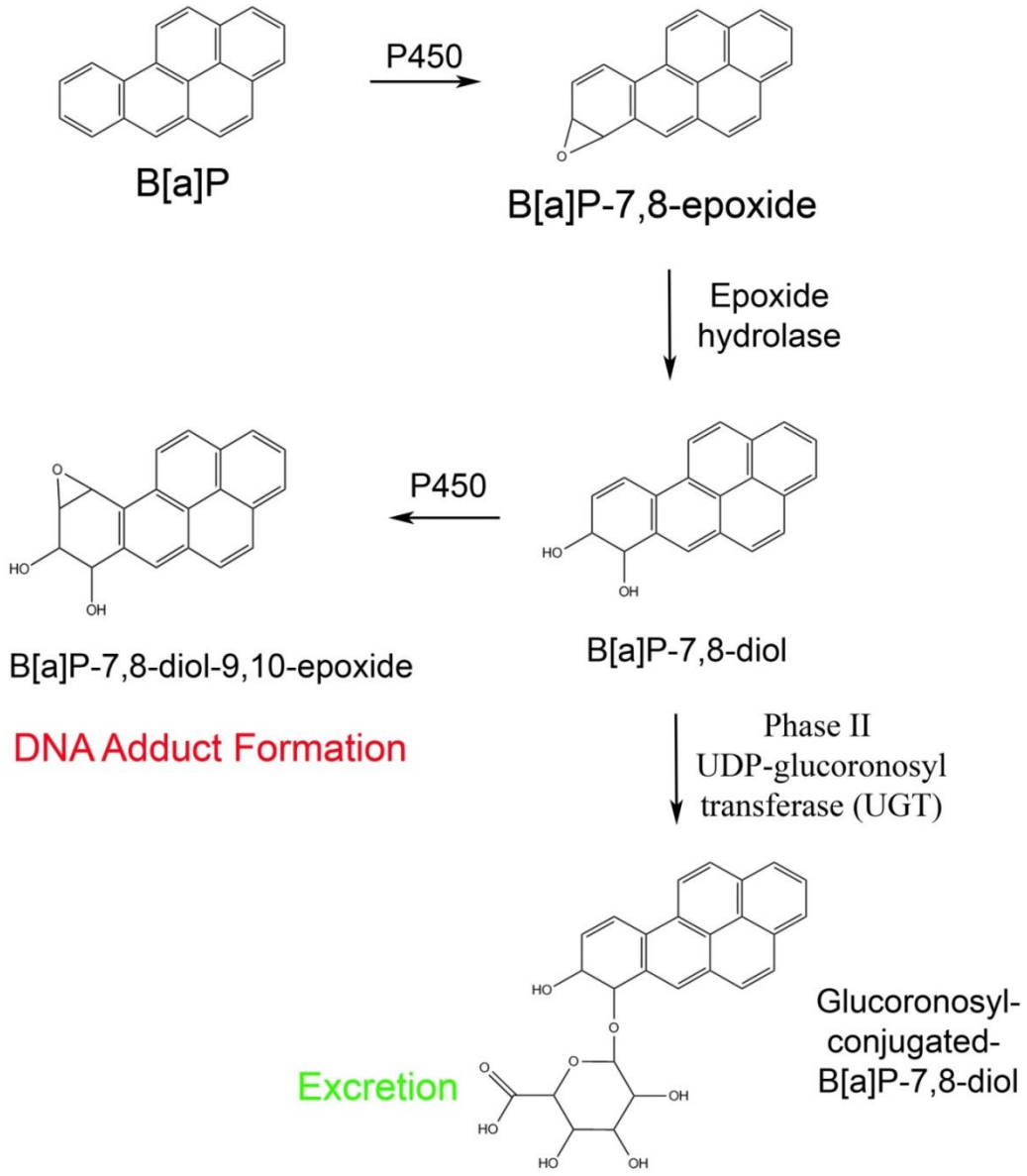

Figure **1.12**: Schematic of prototypical metabolism of PAHs by phase I and II enzymes. B[a]P is enacted upon by the cytochrome P450 enzymes to create B[a]P-7,8-epoxide. The epoxide functional group is hydrolyzed by epoxide hydrolase to form B[a]P-7,8 diol. Further chemical modification by P450s creates the highly reactive diol-epoxide group that can form DNA adducts. B[a]P-7,8-diol can also be chemically modified by phase II enzymes, such as UDP-glucoronosyltransferase (UGT) to generate a hydrophilic molecule capable of being excreted.

Researchers in the 1970's examined the ability of PAHs to induce liver CYP activity in the various strains of mice. Surprisingly, strains were found to be "responsive" or "non-responsive" to PAHs [651, 652]. Selective inter-breeding of the "responsive" and "non-responsive" strains identified an autosomal dominant trait, subsequently termed the aryl hydrocarbon (AH) locus [653]. This locus was hypothesized to be a ligand-binding transcriptional activator termed the AH receptor (AHR). Subsequent studies using a reversible, tritiated form of the prototypical PAH 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) determined that the ligand-bound AHR could bind DNA [654] and undergo nuclear localization [655, 656]. The AHR coding sequence was subsequently cloned and characterized, and sequence analysis identified this receptor as a member of the basic-helix-loop-helix (bHLH) PER/ARNT/SIM (PAS) family of transcription factors [657]. Further detailed studies throughout the past few decades have shown that the inactive cytosolic AHR exists as a heterotetrameric complex with a dimer of the heat shock protein 90 (HSP90) and hepatitis B virus X-associated protein 2 (XAP2) [658-662]. Upon nuclear translocation, the AHR was found to heterodimerize with the AH receptor nuclear transporter (ARNT) [663] and bind a DNA at dioxin response elements (DREs) of the consensus sequence 5'-TNNGCGTG-3' [664, 665].

The classic AHR signaling pathway (Figure **1.13**) has the unliganded AhR in the cytsol as part of a heterotetrameric complex that includes two molecules of HSP90 and one molecule of XAP2 [659-662]. Upon ligand binding, AHR undergoes a conformational change that results in shedding of heterotetrameric complex and nuclear translocation [666]. The ligand-bound AHR heterodimerizes with ARNT and binds to DREs located within target gene promoters. The DNA-bound heterodimer recruits histone modifying enzymes and other coactivators to loosen the DNA/histone interactions. These actions

cause the recruitment of the transcription initiation complex and production of target gene mRNAs. The group of phase I and II enzymes that are known to be regulated by the AHR has generally been termed the “AH battery”. Members of this battery include CYP1A1 [667], CYP1A2 [668], CYP1B1 [669], UDP-glucoronosyltransferase (UGT) 1a1 and 1a6, [670], NAD(P)H:quinone oxidoreductase (NQO1) [671, 672], and the glutathione S-transferase Ya subunit (GSTA1) [673]. Interestingly, AHR has also been shown to induce the expression of the oxidative stress-induced transcription factor NR-E2-related-factor 2 (NRF2) [674], which is also known to regulate the expression of phase II enzymes (Reviewed in [675]).

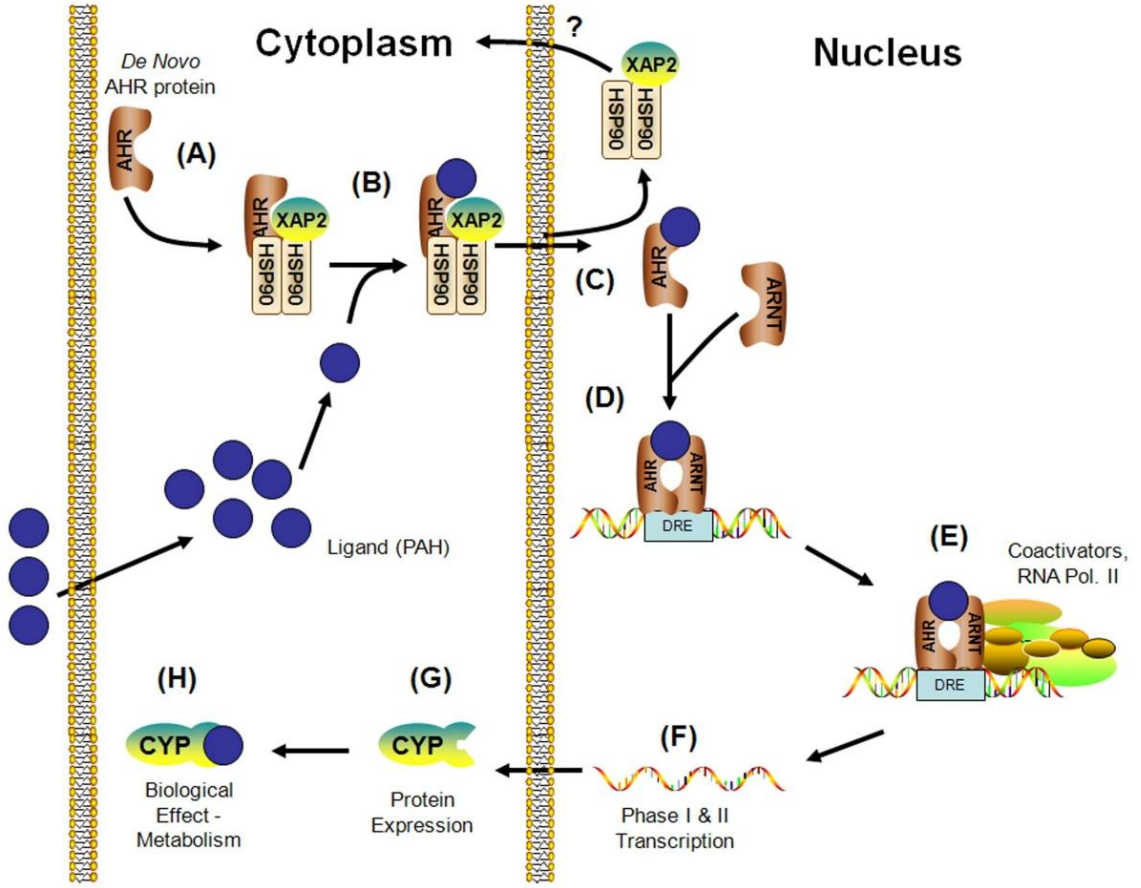

Figure **1.13**: AHR signaling pathway in response to PAHs. (A) In the absence of ligand, AHR is located in a cytosolic complex with a HSP90 dimer and XAP2. (B) Ligand binding to PAHs results in a conformational change in AHR that causes shedding of the HSP90/XAP complex and nuclear translocation. (C) AHR heterodimerizes with ARNT. (D) The heterodimer binds to receptor-specific DNA sequences, termed DREs, located within target gene promoters. (E) The heterodimer recruits histone modifying enzymes, coactivators, and the transcription initiation complex to target gene promoters. This results in increased mRNA (F) and protein (G) production of the target gene. (H) CYP expression then causes metabolism of the PAH.

1.4 Hypothesis and objectives

Previous evidence supports a clear role for PPARβ/δ in skin carcinogenesis. *Pparβ/δ*-null mice exhibit enhanced tumorigenesis as compared to similarly treated wild-type mice in two-stage models of skin carcinogenesis [525, 557]. This model utilizes a single dose of a mutagen followed by continued tumor promotion over the course of the study. While this model has been used to demonstrate a PPARβ/δ-dependent reduction in skin tumorigenesis, whether PPARβ/δ alters tumor initiation and/or promotion has not been examined to date. PPARβ/δ was first suggested to alter tumor promotion when *Pparβ/δ*-null mouse skin exhibited a hyperplastic response to the tumor promoter TPA. Furthermore, *Pparβ/δ*-null primary keratinocytes have been shown to proliferative faster than wild-type cells [402, 406, 512]. Notably, PPARβ/δ-dependent regulation of tumor initiation has not been specifically examined. The standard model for studying skin carcinogenesis is PAH exposure. AHR-dependent regulation of phase I and II enzymes maintains a critical balance between carcinogen bioactivation and detoxification. Considering these facts, the overall hypothesis of this dissertation is that PPARβ/δ modulates AHR signaling in the skin, resulting in altered tumor initiation and PAH-dependent skin carcinogenesis.

The following chapters seek to clarify the role PPARβ/δ in modulating the AHR signaling pathway and skin carcinogenesis. The objective of chapter 2 is to determine if PPARβ/δ modulates AHR signaling in human and mouse keratinocyte models. Keratinocytes are the proliferative component of the skin and the known target of PAH-dependent DNA mutagenesis. Therefore, the data generated within this chapter will be used to examine the functional components of the AHR signaling pathway in wild-type

and *Pparβ/δ*-null keratinocytes. These studies will include examination of AHR and accessory protein expression, ligand binding, nuclear translocation, heterodimerization, target gene promoter occupancy, coactivator recruitment, and the induction of target gene mRNA. Additional studies will also determine the inherent DNA structure of AHR target genes using epigenetic inhibitors and physical examination of chromatin structure. To assess whether this modulation is relevant to human skin cancer, a human keratinocyte PPARβ/δ shRNA cell line will be created and characterized. Experiments within this investigation will specifically address the *in vitro* mechanism(s) by which PPARβ/δ modulates AHR-dependent signaling in human and mouse keratinocytes and establish preliminary evidence for altered PAH-dependent skin carcinogenesis. Chapter 3 will focus on whether PPARβ/δ modulation of AHR-dependent signaling alters tumor initiation and skin tumorigenesis. The objective of chapter 3 is to determine if decreased AHR signaling in PPARβ/δ-null mice causes functional changes in PAH-dependent skin carcinogenesis. Furthermore, it is predicted that inherent genotype-specific differences in tumor promotion will result in PPARβ/δ-dependent differences in malignant conversion. These hypotheses will be specifically addressed using a complete carcinogen skin carcinogenesis bioassay and PAHs B[a]P and DMBA. These two PAHs are known to be metabolized through AHR-dependent regulation of phase I and II enzymes. This bioassay will examine if reduced tumor initiation in *Pparβ/δ*-null mice translates into reduced tumor onset, multiplicity, size, or malignant conversion as compared to wild-type mice. Furthermore, MNNG, a carcinogen that is not bioactivated through AHR signaling, will be used to delineate genotype-specific differences in tumor initiation and tumor promotion. This investigation will delineate the functional role of PPARβ/δ-dependent modulation of AHR signaling in the skin and how modulated carcinogen metabolism alters tumorigenesis and malignant tumor conversion.

Combined, these chapters will address the specificity and mechanism(s) by which PPARβ/δ modulates AHR-dependent signaling *in vivo* and *in vivo* and the consequence of this modulation on skin tumorigenesis. By clearly defining the mechanistic components of this modulation, a novel mechanism by which PPARβ/δ modulates skin carcinogenesis will be present that can thus be exploited for future assessments of skin cancer risk.

Chapter 2

MODULATION OF AHR-DEPENDENT SIGNALING BY PPARβ/δ IN MOUSE AND HUMAN KERATINOCYTES

2.1 Abstract

The nuclear hormone receptor peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) is emerging as a novel target in skin tumorigenesis. While the exact mechanism(s) mediating PPARβ/δ-dependent biological effects have yet to be delineated, it has been shown that PPARβ/δ exerts anti-proliferative, pro-differentiation, and pro-apoptotic effects in several skin models. Studies with *Pparβ/δ*-null mice have indicated that the absence of PPARβ/δ increased tumorigenicity and malignant conversion in a two-stage model of skin chemical carcinogenesis. These observations could be due to PPARβ/δ-dependent modulation of tumor initiation and/or promotion. The results from previous studies revealed that PPARβ/δ alters keratinocyte proliferation and tumor promotion. However, tumor initiation has not been specifically examined. Surprisingly, *Pparβ/δ*-null mice exhibited attenuated induction of cytochrome P450 (CYP) genes in response to polycyclic aromatic hydrocarbons (PAHs). Thus, it was hypothesized that PPARβ/δ alters carcinogen metabolism and tumor initiation in the skin. As the aryl hydrocarbon receptor (AHR) is the key transcriptional regulator of xenobiotic metabolism, this pathway was examined for PPARβ/δ-dependent modulation. Using a primary keratinocyte model, *Pparβ/δ*-null keratinocytes had reduced PAH-dependent mRNA induction of phase I and II xenobiotic metabolism enzymes, including the mRNAs encoding for *Cyp1a1*, *Cyp1b1*, NAD(P)H:quinone oxidoreductase 1 (*Nqo1)*, and glutathione S-transferase alpha 1 (*Gsta1*), as compared to wild-type keratinocytes. The attenuated PAH-dependent induction of these mRNAs in *Pparβ/δ*-null keratinocytes was not found to be caused by PPARβ/δ-dependent alterations of AHR expression, ligand binding, or nuclear translocation. However, PAH-dependent AHR promoter occupancy and histone acetylation at the *Cyp1a1* promoter were found to be significantly

reduced in *Pparβ/δ*-null keratinocytes. Thus, the chromatin structure of the *Cyp1a1* promoter may be altered in a PPARβ/δ-dependent manner. Indeed, DNA methylation was found on the *Cyp1a1* promoter in the absence of PPARβ/δ expression. A similar modulation of PAH-mediated AHR signaling was observed in a human PPARβ/δ shRNA keratinocyte model, thereby demonstrating a human relevance to skin carcinogenesis. Collectively, these observations describe a novel epigenetic mechanism by which PPARβ/δ can modulate xenobiotic metabolism mRNA induction in the skin by dysregulation of AHR signaling. The results of these studies indicate that PPARβ/δ may alter the balance between carcinogen bioactivation and detoxification in the skin.

2.2 Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand activated transcription factors and are critical regulators of lipid homeostasis. Ligand-dependent biological responses are mediated by tissue-specific expression of three PPAR isoforms, termed PPARα, PPARβ/δ, and PPARγ. Detailed studies have delineated pharmacological functions for ligand activation of PPARα and PPARγ, including the use of fibrates as PPARα-dependent modulators of dyslipidemia (Reviewed in [291]) and the use of thiazolinediones as PPARγ-dependent modulators of insulin-sensitization (Reviewed in [188]). Compared to other PPAR isoforms, much less is understood about the intrinsic functions of PPARβ/δ. This isoform is ubiquitously expressed and exhibits higher expression in epithelial tissues such as the skin [163, 523]. The exact role of PPARβ/δ in carcinogenesis has not reached a clear consensus. However, a large body of evidence suggests that PPARβ/δ inhibits tumorigenesis by inhibiting proliferation, inducing terminal differentiation, and increasing apoptosis (Reviewed in [164, 523]). Several lines of evidence have described a direct role for PPARβ/δ-dependent inhibition of skin tumorigenesis. First, *Pparβ/δ*-null mice have a hyperplastic response to the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA), and this effect has been observed in two independent null-mouse models [412, 506]. It has also been observed that *Pparβ/δ*-null primary keratinocytes, the proliferative cell type in the epidermis, proliferate faster than wild-type cells [402, 406, 525]. Furthermore, ligand activation of PPARβ/δ reduced cell proliferation in several mouse and human skin models (Reviewed in [164]). Additional studies have investigated the role of PPARβ/δ in skin carcinogenesis. Ligand activation of PPARβ/δ reduced tumor incidence, size, multiplicity, and malignant conversion in several two-stage skin chemical

carcinogenesis bioassays [524, 525, 557, 587]. Although these known effects on cell proliferation and tumorigenesis have been consistently observed, detailed studies have not delineated the mechanism(s) by which PPARβ/δ attenuated skin tumorigenesis.

The reported PPARβ/δ-dependent decreases in keratinocyte cell proliferation and two-stage skin carcinogenesis suggests that PPARβ/δ reduces tumor promotion. However, PPARβ/δ-dependent modulation of tumor initiation in the skin cannot be excluded. The prerequisite DNA mutagenesis for tumor initiation is commonly mediated by polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (B[a]P) and 7,12-dimethylbenz[a]anthracene (DMBA). These compounds are chemically modified by the phase I and II xenobiotic metabolism system, which facilitates the detoxification and excretion of chemical carcinogens. Phase I enzyme expression is induced in response to PAHs and causes direct hydroxylation of xenobiotics. However, this process also creates bioactivated PAHs that can form stable DNA adducts [676, 677]. Phase II enzymes, in addition to being induced by PAHs, mediate antioxidant responses and conjugation reactions that ultimately detoxify bioactivated compounds [677]. The critical transcriptional regulator of phase I and II enzymes is the aryl hydrocarbon receptor (AHR), a member of the basic helix-loop-helix (bHLH) Per-ARNT-SIM (PAS) family of transcription factors that is activated by a wide variety of structurally diverse compounds [657, 678].

As a sensor of PAH exposure and the primary direct transcriptional effector, the AHR is considered a key modulator of carcinogenesis. In its unliganded state, AHR is relegated to the cytosol in a complex with co-chaperones, including heat shock protein 90 (HSP90), hepatitis B virus associated protein 2 (XAP2), and p23 [662, 679-681].

Upon ligand binding, AHR undergoes a conformational change that lead to chaperone dissociation, nuclear translocation, heterodimerization with AHR nuclear translocator (ARNT), and DNA binding at dioxin response elements (DREs) [682]. The AHR/ARNT complex then causes chromatin remodeling, coactivator recruitment, and transcription of target genes, which includes, but is not limited to, phase I and II enzymes involved in the metabolism of PAHs [683, 684]. Due to the classic use of PAHs as skin carcinogens, AHR expression and activation have been extensively studied in many skin models. Studies utilizing *Ahr*-null mice have indicated that the absence of AHR drastically reduced genotoxic and PAH-dependent DNA adducts [685-687]. Interestingly, *Ahr*-null mice exhibited a different PAH metabolite profile than wild-type mice. This observation suggests that secondary metabolic pathway(s) can compensate for deficient AHR signaling [685]. Additionally, *Ahr*-null mice were found to be highly resistant to PAH-dependent skin tumorigenesis, as examined by PAH injections and complete skin carcinogen bioassays [686, 688]. These observations highlight the importance of PAH-dependent AHR signaling in skin tumorigenesis and how perturbation of this pathway altered carcinogen bioactivation and tumor initiation. Therefore, identifying new regulators of AHR signaling and carcinogen bioactivation will build upon current knowledge of how modulated xenobiotic metabolism causes skin carcinogenesis.

The current study examined whether PPARβ/δ can alter tumor initiation by modulating the balance between carcinogen bioactivation and clearance. As the AHR signaling pathway is a critical regulator of most PAH metabolism, it was hypothesized that PPARβ/δ modulates AHR-dependent metabolism of PAHs and shifts the balance of carcinogen bioactivation and detoxification. Specifically, these studies examined how

PPARβ/δ modulates AHR-dependent signaling in mouse and human keratinocyte models.

2.3 Materials and Methods

2.3.1 Chemicals and materials

GW0742 [159] was synthesized by GlaxoSmithKline (Research Triangle Park, NC). B[a]P, DMBA, dexamethasone, all-trans retinoic acid (RA), 9-cis RA, and 5-Aza-2'-deoxycytidine (5-Aza-dC) were purchased from Sigma-Aldrich (St. Louis, MO). 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) was purchased from AccuStandard (New Haven, CT), and β-naphthoflavone (β-NF) was obtained from IndoFine (Hillsborough, NJ). Indolo[3,2-b]carbazole (ICZ) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were generous gifts provided by Dr. Jan Bergman (Karolinska Institute, Sweden) and Dr. Steven Safe (Texas A&M, College Station, TX), respectively. Chrysene, 5-methyl chrysene (5-me chrysene), 6-methyl chrysene (6-me chrysene), dibenzo[a,l]pyrene (DiB[a,l]P), and M50354 were synthesized by Dr. Shantu Amin (Penn State University, Hershey, PA). 2-Azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin ([^{125}I]N_3Br_2DpD) and 2-[^{125}I]iodo-7,8-dibromo-p-dioxin ([^{125}I]Br_2DpD) were graciously synthesized by Dr. Gary Perdew (Penn State University, University Park, PA) following previously described methods [689, 690]. All treatment compounds were dissolved in dimethylsulfoxide (DMSO) except all-trans RA and 9-cis RA, which were dissolved in ethanol (EtOH). Lipofectamine® and Plus Reagent® were purchased from Invitrogen (Carlsbad, CA). Protein A/G sepharose resin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and glutathione-coated magnetic beads were purchased from Thermo Scientific (Rockford, IL). The Wizard® Genomic DNA Purification kit was purchased from Promega (Madison, WI), and puromycin was obtained from EMD Biosciences (Gibbstown, NJ). Protease inhibitor cocktail was purchased from Roche Diagnostics

(Indianapolis, IN). All primers for quantitative real-time PCR (qPCR) and bisulfite sequencing were purchased from Integrated DNA Technologies (IDT, Coralville, IA).

2.3.2 Plasmids

The MISSION™ shRNA plasmid constructs for non-target (SHC002) and human PPARβ/δ (TRCN0000010647) were purchased from Sigma Aldrich. pBOS-GST-AHR, pBOS-GST-ARNT, pcDNA3.1-Flag-AHR, and pcDNA3.1-Flag-ARNT were kindly provided by Dr. Gary Perdew [691], and pSG5-mPPARβ/δ was generously provided by Dr. Curt Omiecinski (Penn State University, University Park, PA).

2.3.3 Cell culture

HEK293T and HaCaT human keratinocytes were generously provided by Dr. Yanming Wang (Penn State University, University Park, PA) and Dr. Stuart Yuspa (National Cancer Institute, Bethesda, MD), respectively. These cell lines were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. Hepa1c1c7 cells were provided by Dr. Gary Perdew and were propagated in αMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. All cells were cultured at 37°C and 5% CO_2.

2.3.4 Isolation and treatment of primary mouse keratinocytes, fibroblasts, and hepatocytes

Primary keratinocytes and fibroblasts from wild-type and *Pparβ/δ*-null mice were isolated from newborn skin and cultured as previously described [692]. Keratinocytes were cultured in low calcium (0.05 mM) Eagle's MEM supplemented with 8% chelexed FBS, 20 IU/mL penicillin, and 20 µg/mL streptomycin. Dermal fibroblasts were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Primary hepatocytes from wild-type and *Pparβ/δ*-null mice were isolated as previously described [693], and propagated in HepatoZYME SFM media (Invitrogen) supplemented with 2.5% DMSO, 10 nM dexamethasone, 1% glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. All cells were cultured at 37°C and 5% CO_2. Fibroblasts and hepatocytes were treated for 8 h with vehicle (0.02% DMSO) or the indicated treatment. Primary keratinocytes for mRNA expression analyses were treated for 8 h with vehicle (0.02% DMSO) or the indicated treatment. A temporal analysis of PAH-dependent induction of AHR target genes was completed by treatment of primary keratinocytes with vehicle (0.02% DMSO) or 1 µM B[a]P for 1, 2, 4, 6, 8, 12, and 24 h. Total RNA was then prepared using RiboZol RNA Extraction Reagent (AMRESCO, Solon, OH) and the manufacturer's recommended protocol.

2.3.5 *In vivo* studies

For skin analyses, wild-type and *Pparβ/δ*-null mice in the resting phase of hair cycle were shaved and treated with acetone (control) or 50 µg of DMBA. After 8 h, mice were euthanized and the dorsal skin region was isolated. For mRNA analyses, skin samples were immediately homogenized in RiboZol RNA Extraction Reagent, and total

RNA was prepared using the manufacturer's recommended protocol. For skin protein analysis, dorsal skin was pulverized using liquid nitrogen-cooled mortar and pestle. Skin microsomes were prepared by Dounce homogenizing the pulverized skin in homogenizing buffer (10 mM Tris/0.25 M sucrose, pH 7.4) containing protease inhibitor cocktail (Roche). Lysates were then centrifuged at 700 x g for 10 min, followed by centrifugation of the supernatant at 105,000 x g for 45 min. The pellets were then resuspended in 0.15 M Tris buffer and immediately used for western blotting.

Liver mRNA analyses were completed by oral gavage of wild-type and *Pparβ/δ*-null mice with 50 mg/kg β-NF or vehicle (corn oil) for 8 h. Mice were euthanized and livers were removed and immediately homogenized in RiboZol RNA Extraction Reagent. Total RNA was again prepared using the manufacturer's recommended protocol.

2.3.6 RNA isolation and qPCR

The mRNA encoding AHR target genes, *Pparβ/δ*, and angiopoietin protein-like 4 (*Angptl4*), a PPARβ/δ target gene, were measured by qPCR analysis. cDNA was generated from 2.5 μg of total RNA using MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). The qPCR analysis was carried out using SYBR® Green PCR Supermix for IQ (Quanta Biosciences, Gaithersburg, MD) in the iCycler and detected using the MyiQ Realtime PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The following PCR reaction was used for all genes: 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, repeated for 45 cycles. Each PCR reaction included a no-template control reaction to control for contamination, and all real-time PCR reactions had greater than 85% efficiency. The relative mRNA value for each gene was

normalized to the relative mRNA value of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The following genes were examined: AhR repressor (*Ahrr*), *Angptl4*, cyclooxygenase 2 (*Cox2*), cytochrome P450 1a1 (*Cyp1a1*), *Cyp1a2*, *Cyp1b1*, *Cyp26a1*, epigregulin (*Ereg*), FK506 binding protein 5 (*Fkbp5*), glucocorticoid-induced leucine zipper (*Gilz*), glutathione S-tranferase alpha 1 (*Gsta1*), heme oxygenase 1 (*Hox1*), NAD(P)H:quinone oxidoreductase 1 (*Nqo1*), NF-E2-related factor 2 (*Nrf2*), *Pparβ/δ*, and UDP-glucoronsyltransferase 1a2 (*Ugt1a2*) (Table **2.1**).

Table **2.1**: Primers used for quantitative real-time PCR (qPCR)

Species	Gene	Accession	Forward Primer	Reverse Primer
Mouse				
	Ahrr	NM_009644	CTGCTGCTGGAGTCTCTCAA	CTGTCTGATGAAAGCCCAGA
	Cox2	NM_011198	TTGCTGTACAAGCAGTGGCAAAGG	TGCAGCCATTTCCTTCTCTCCTGT
	Cyp1a1	NM_009992	AGGTTAACCATGACCGGGAACTGT	TTCGCTTGCCCAAACCAAAGAGAG
	Cyp1a2	NM_009993	ACATTCCCAAGGAGCGCTGTATCT	GTCGATGGCCGAGTTGTTATTGGT
	Cyp1b1	NM_009994	GCTAGCCAGCAGTGTGATGATATT	GGTTAGCCTTGAAATTGCACTGAT
	Cyp26a1	NM007811	TTCTGCAGATGAAGCGCAGG	TTTCGCTGCTTGTGCGAGGA
	Ereg	NM_007950	TGGGTCTTGACGCTGCTTTGTCTA	AAGCAGTAGCCGTCCATGTCAGAA
	Fkbp5	NM_010220	AGGCGAGGGATACTCAAACCCAAA	TGTCGATCCCAATCGGAATGTCGT
	Gapdh	NM_008084	AAATGGTGAAGGTCGGTGTGAACG	TGGCAACAATCTCCACTTTGCCAC
	Gilz	NM_010288	TGTATGAGACCCCCATGGAG	TCCATGGCCTGCTCAATCTT
	Gsta1	NM_008181	ACTAGACCGTGAACCACAGTTGCT	ACCATGGGCACTTGGTCAAACATC
	Hox1	NM_010442	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA
	Nqo1	NM_008706	AGATGGCATCCAGTCCTCCAT	TTAGTCCCTCGGCCATTGTT
	Nrf2	NM_010902	AGCACTCCGTGGAGTCTTCCATTT	TGTGTTTAGGGCCGTTCTGTTTG
	Ugt1a2	NM_013701	AAGGCTTTCTGACCACATGGA	GGCAAATGTACTTCAGGACCAGAT
Human				
	Angptl4	NM_139314	TCACAGCCTGCAGACACAACTCAA	CCAAACTGGCTTTGCAGATGCTGA
	Cyp1a1	NM_000499	AGTGGCAGATCAACCATGACCAGA	CCGCTTGCCCATGCCAAAGATAAT
	Cyp1b1	NM_000104	CATGCGCTTCTCCAGCTTTGT	GGCCACTTCACTGGGTCATGA
	Gapdh	NM_002046	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
	Pparβ/δ	NM_006238	GACAGTGACCTGGCCCTATTCA	AGGATGGTGTCCTGGATAGCCT

2.3.7 Western blot analysis

Protein samples were isolated from dorsal skin, primary keratinocytes, and HaCaT shRNA stable cell lines as described. A total of 40 µg (skin microsomes) or 50 µg (all other samples) of protein was resolved using SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes using an electroblotting method. The membranes were blocked with 5% dried milk in Tris buffered saline/Tween-20 (TBST) for rabbit and mouse antibodies and 0.5% porcine gelatin TBST for goat antibodies. Primary antibodies were incubated overnight with gentle agitation. After incubation with biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), immunoreactive proteins on the membrane were detected after incubation with ^{125}I-labeled streptavidin. Hybridization signals for specific proteins were normalized to hybridization signal for ACTIN or lactate dehydrogenase (LDH). The following antibodies were used: anti-AHR (Affinity Bioreagents, Golden, CO, RPT1), anti-ARNT (Santa Cruz sc-8076), anti-HSP90 [694], anti-XAP2 (Novus Biologicals), anti-CYP1A1 (Santa Cruz sc-20772), anti-CYP1B1 (Dr. Craig B. Marcus, Oregon State University, Corvallis, OR), anti-COX2 (Santa Cruz sc-7591), anti-microsome epoxide hydrolase (mEH, Dr. James P. Hardwick, Northeastern Ohio University College of Medicine, Rootstown, OH), anti-human PPARβ/δ (Abcam ab21209-100) anti-LDH (Rockland, Gilbertsville, PA) and anti-ACTIN (Rockland, Gilbertsville, PA, 600-401-886).

2.3.8 Photoaffinity ligand ([^{125}I]N_3Br_2DpD) binding assay

Wild-type and *Pparβ/δ*-null primary keratinocytes were isolated and cultured as previously described [692]. Cytosolic lysates were prepared from 150 mm culture plates. Cells were trypsinized, pelleted, and were homogenized in MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, 10% glycerol pH 7.4) containing 20 mM sodium molybdate and protease inhibitors (Roche). The cytosolic fraction was obtained by centrifugation at 100,000 × g for 1 h. All binding experiments were conducted in the dark until ultraviolet (UV) cross-linking of [^{125}I]N_3Br_2DpD. Briefly, one hundred fifty micrograms of cytosolic protein was incubated at room temperature with increasing amounts of [^{125}I]N_3Br_2DpD. Ligand was allowed to bind the lysate for 20 min at room temperature, then samples were photolyzed at 8 cm with 402 nm UV light. Three percent dextran-coated charcoal was added to the photolyzed samples to remove unbound ligand. Labeled samples were resolved using 8% acrylamide-SDS-PAGE, transferred to a PVDF membrane and visualized using autoradiography. Radioactive bands corresponding to AHR relative mobility (M_r) were then excised and counted using a γ-counter.

2.3.9 Reversible ligand ([^{125}I]Br_2DpD) nuclear translocation sucrose gradient ultracentrifugation

Wild-type and *Pparβ/δ*-null primary keratinocytes were isolated and cultured as previously described [692]. Cells were grown on 150 mm cell culture dishes and treated for 1 h with [^{125}I]Br_2DpD. Following treatment, cells were washed twice with Dubecco's phosphate buffered saline (DPBS), trypsinized, and pelleted. Nuclear lysates were prepared using a Dounce homogenizer and MENG buffer containing 20 mM sodium molybdate and protease inhibitors. Nuclear protein pellets were isolated by

centrifugation at 1,000 x g for 20 min. The nuclear pellet was resuspended in MENG buffer containing 20 mM sodium molybdate, 400 mM potassium chloride, and protease inhibitors. Following a 1 h incubation on ice, soluble nuclear proteins were isolated by centrifugation at 100,000 × g for 1 h. Lysates were then layered onto 5.1-ml sucrose gradients (10-30%) prepared in MENG buffer containing 400 mM potassium chloride and centrifuged at 416,000 x g for 135 min. After centrifugation, 200 µL fractions were collected with an Isco Model 640 density gradient fractionator and counted using a γ-counter. Bovine serum albumin (4.4S) and catalase (11.3S) were routinely applied to independent sucrose gradients as external markers of mobility.

2.3.10 Glutathione S-transferase (GST) pulldown

Cos-1 cells were grown to 50% confluence before transfection with PPARβ/δ and the GST- and Flag- AHR and ARNT proteins using the Lipofectamine® transfection reagent and manufacturer's recommended protocol. Forty-eight h post-transfection, soluble protein lysates were isolated in MENG buffer with 150 mM sodium chloride and protease inhibitor cocktail using a Dounce homogenizer. Five hundred µg of respective lysates were added to 100 µL of glutathione-coated magnetic beads (Pierce) and gentle agitation for 3 h. Magnetic beads were washed three times with MENG buffer with 150 mM sodium chloride and protease inhibitors before the addition of loading dye and resolution of proteins by western blotting. The direct interaction of proteins was assessed by probing for AHR or PPARβ/δ protein expression using anti-AHR (Affinity Bioreagents, Golden, CO, RPT1) and anti-PPARβ/δ [163].

2.3.11 Chromatin immunoprecipitation (ChIP)

Wild-type and *Ppar*β/δ-null primary keratinocytes were treated for 2 h with vehicle or 1 μM B[a]P. Cross-linking was performed by the addition of formaldehyde to a final concentration of 1% and sample rocking for 10 min. The cross-linking was quenched by the addition of glycine to a final concentration of 125 mM and gently rocked for an additional 10 min. Cells were washed twice with PBS before the addition of lysis buffer (50 mM Tris-HCl pH 8, 1% SDS, 10 mM EDTA, and protease inhibitor cocktail). DNA was sheared to obtain chromatin in the range of 500-1500 bp with the Diagenode Bioruptor™ (Diagenode, Sparta, NJ). One hundred micrograms of sheared chromatin was precleared by the addition of protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h that was previously blocked with bovine serum albumin (BSA)/salmon sperm DNA (Invitrogen, Carlsbad, CA). The precleared chromatin was immunoprecipitated by gentle agitation with specific antibodies for either AHR [695], anti-acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY) as a positive control, or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as a negative control. After 4 h, the immune complexes were captured by the addition of preblocked protein A agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and an agitated overnight incubation. The beads were washed three times with a low salt wash buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% sodium deoxycholate, 1% Triton-X, 150 mM sodium chloride, and protease inhibitor cocktail) and once with a high salt wash buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% sodium deoxycholate, 1% Triton-X, 500 mM sodium chloride, and protease inhibitor cocktail). The beads were washed once with TE8 (10 mM Tris-HCl pH 8, 1 mM EDTA) and the immune complexes were released by the addition of elution buffer (100 mM sodium bicarbonate and 1% SDS). The formaldehyde cross-links were reversed by

overnight incubation at 65°C. Immunoprecipitated DNA was purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction and subject to PCR analysis for occupancy at the *Cyp1a1* promoter. The primers for *Cyp1a1* enhancer region were 5'-GTCGTTGCGCTTCTCACGCGA -3' (forward) and 5'- CACTGAGGGAGGGGTTTGAGG -3' (reverse). The amplification was carried out at the following reaction: 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s, repeated for 35 cycles. Band intensity was quantified using Image J Software (version 1.37, National Institutes of Health, Bethesda, MD). The specific values were normalized to treatment-specific inputs and verified to be greater than rabbit IgG controls. One biological replicate is displayed from a pool of N = 3 neonates used to isolate primary keratinocytes.

2.3.12 Epigenetic studies

To examine whether methylation alters transcriptional regulation, the methylation inhibitor 5-Aza-dC was supplemented into the culture media of primary keratinocytes from wild-type and *Pparβ/δ*-null mice. Keratinocytes were isolated and cultured as previously described [692], and 5 μM 5-Aza-dC was supplemented during the plating and propagation of the cells. The inhibitor was in the media for 64 h. Keratinocytes were treated 8 h with vehicle (0.02% DMSO) or 1 μM B[a]P, with the addition of fresh 5-Aza-dC.

For bisulfite sequencing, primary keratinocytes from wild-type and *Pparβ/δ*-null mice were isolated and cultured as described in Materials and Methods. Bisulfite conversion was completed using the EZ DNA Methylation-Direct kit (Zymo Research, Orange, CA) and the manufacturer's recommended protocol. A total of 2×10^5 cells per

genotype were utilized for the DNA bisulfite conversion. The promoter of *Cyp1a1* was analyzed and PCR amplification primers were designed for a putative cytosine-phosphate-guanine (CpG) islands using MethylPrimer Express (Applied Biosystems). Primers for amplification were 5'-GTTTTGGTTATAGAGTAGATATTAATG (Forward) and 5'-TTAAACTAACTCTATACCCCACAAATT (Reverse). The bisulfite converted region of the *Cyp1a1* promoter was amplified using the following protocol: 95°C for 1 min, 60°C for 1 min, and 72°C for 2.5 min, repeated for 35 cycles. The amplicon of interested was cloned using the TOPO TA Cloning® kit (Invitrogen) and the manufacturer's recommended protocol. Five plasmids per genotype containing the *Cyp1a1* promoter amplicon were sequenced at the Penn State University Nucleic Acid Facility. The generated sequencing data was then compared to the unmodified *Cyp1a1* promoter sequence to identify methylated cytosine using the Cytosine Methylation Analysis Tool for Everyone (CyMATE) program [696].

2.3.13 shRNA lentivirus stable cell creation and characterization

Briefly, the viral packaging plasmids and non-target or shPPARβ/δ plasmids were cotransfected into HEK293Tcells to produce retrovirus using the Lipofectamine® transfection reagent and manufacturer's recommended protocol. Forty eight h after transfection, the cellular supernatant containing the retrovirus was filtered with a 0.22 μm filter and used to spinoculate HaCaT cells [697]. Two days post-infection, HaCaT cells were selected with culture media supplemented with 1 μg/mL puromycin and propagated below 50% confluence for 7 d to select for stable plasmid integration. Cells were maintained in selection media throughout all subsequent experiments. For protein expression analyses, triplicate 100 mm dishes of HaCaT non-target and shPPARβ/δ

soluble lysates were prepared using MENG buffer containing 20 mM sodium molybdate, 500 mM sodium chloride, and protease inhibitor cocktail. Fifty micrograms of proteins from each sample was examined by western blotting, as described in Materials and Methods, to assess the basal expression of PPARβ/δ, AHR, ARNT, HSP90, and XAP2 as compared to the loading control ACTIN. To characterize the responsiveness of the non-target and cell lines to AHR and PPARβ/δ ligands, the HaCaT shRNA cell lines were treated with vehicle (0.02% DMSO), 0.2 μM GW0742, or 1 μM B[a]P for 8 h. Total RNA was then prepared using RiboZol RNA Extraction Reagent (AMRESCO) and the manufacturer's recommended protocol. qPCR was utilized to examine the expression of *Pparβ/δ*, *Angptl4*, *Cyp1a1*, and *Cyp1b1*. A temporal analysis of PAH-dependent inducibility of *Cyp1a1* and *Cyp1b1* was completed by treatment of the non-target and shPPARβ/δ cell lines with vehicle (0.02% DMSO) or 1 μM B[a]P for 1, 2, 4, 6, 8, 12, and 24 h. Total RNA was then prepared using RiboZol RNA Extraction Reagent and the manufacturer's recommended protocol to examine the mRNA expression of *Cyp1a1* and *Cyp1b1* by qPCR. To examine whether methylation alters transcriptional regulation, the methylation inhibitor 5-Aza-dC was supplemented into the culture media of HaCaT shRNA cell lines. 5-Aza-dC (5 μM) was supplemented during the plating and propagation of the cells. The inhibitor was in the media for 72 h, and cells were treated with 1 μM B[a]P and fresh 5-Aza-dC for the last 8 h of the study. Total RNA was then prepared using RiboZol RNA Extraction Reagent and the manufacturer's recommended protocol to examine the mRNA expression of *Cyp1a1* and *Cyp1b1* by qPCR.

2.3.14 Statistical analyses

Data were analyzed for statistical significance using either the Student's t-test, one-way analysis of variance (ANOVA), or two-way ANOVA with the Bonferroni's multiple comparison test as described in the figure legends using Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

2.4 Results

2.4.1 PPARβ/δ modulates AHR-dependent signaling in the skin and keratinocytes

To determine if PPARβ/δ modulates PAH-dependent signaling in the skin, wild-type and *Pparβ/δ*-null mice were topically treated with the PAH DMBA. Consistent with reports describing PAH-dependent induction of P450 enzymes [685], the mRNA and protein expression of both CYP1A1 and CYP1B1 were increased in PAH-treated wild-type mice (Figure **2.1A, B**)**.** Surprisingly, *Pparβ/δ*-null mice had attenuated induction of CYP1A1 and CYP1B1 mRNA and protein in response to DMBA (Figure **2.1A, B**). The protein expression of COX2 and mEH, other known PAH metabolic enzymes, were not found to be altered in either genotype following PAH treatment (Figure **2.1B**). The finding that topical exposure of PAH in *Pparβ/δ*-null mice did not cause the typical P450 mRNA and protein induction suggests that PPARβ/δ can modulate skin xenobiotic metabolism. The skin is composed of multiple cell types, including dermal and epidermal constituents, which could be mediating these PPARβ/δ-dependent changes in *Cyp* mRNA induction. Since the basal keratinocytes are the proliferative component of the epidermis and are known to be a key cellular target in skin carcinogenesis, the effect of PAHs on *Cyp* mRNA expression was examined in wild-type and *Pparβ/δ*-null primary keratinocytes. Consistent with the *in vivo* observations, *Pparβ/δ*-null keratinocytes had a significant reduction in *Cyp1a1* (Figure **2.1C**) and *Cyp1b1* (Figure **2.1D**) mRNA induction upon PAH exposure as compared to wild-type cells. It should also be noted that the basal expression of *Cyp1a1* and *Cyp1b1* were found to be lower in *Pparβ/δ*-null keratinocytes (Figure **2.1C, D**). Collectively, these observations indicate that both CYP1A1 and CYP1B1 are critical components of PAH-dependent phase I metabolism in

the skin (Reviewed in [698]). As the AHR is a documented sensor and regulatory effector of PAH exposure (Reviewed in [698, 699]), the results of these studies suggest that PPARβ/δ modulates AHR signaling. Furthermore, the primary mouse keratinocyte model can be used to examine the mechanism by which PPARβ/δ modulates PAH-dependent signaling in the skin.

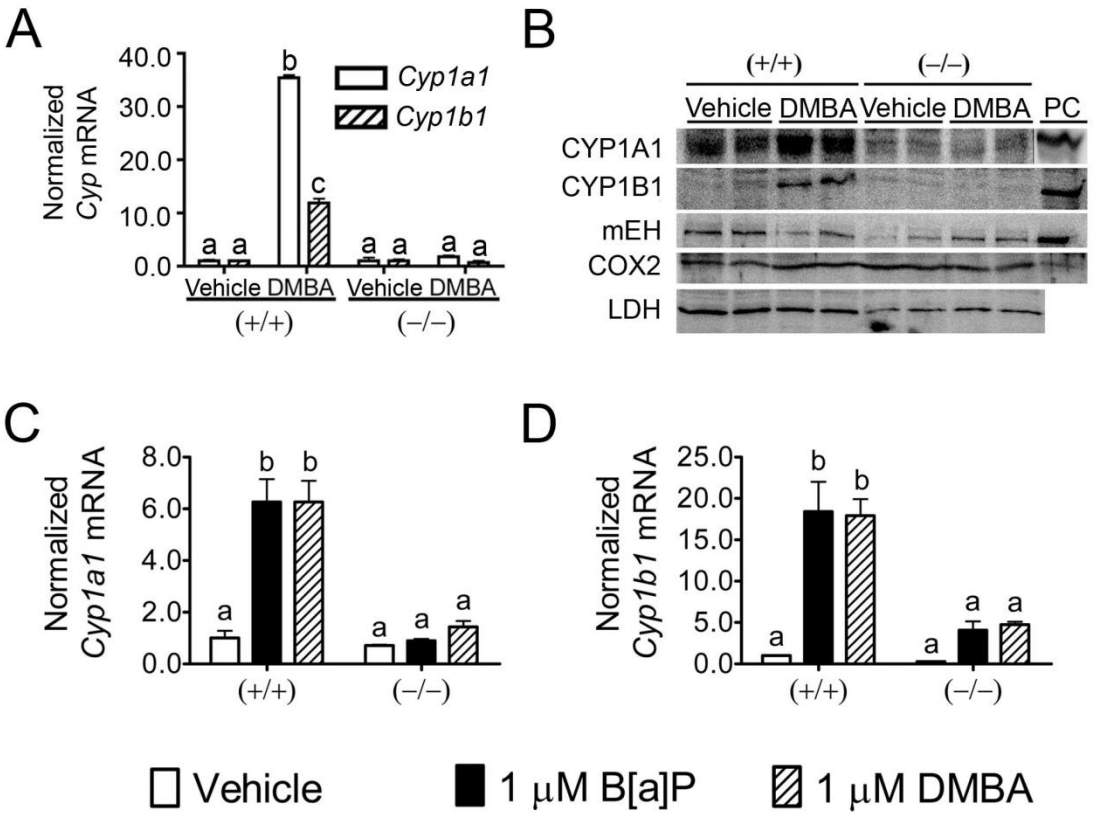

Figure **2.1**: PPARβ/δ modulates PAH-induced changes in P450 mRNA and protein expression in mouse skin and keratinocytes. Wild-type (+/+) and *Pparβ/δ* (–/–) mice (A,B) were topically treated with vehicle (acetone) or 50 μg DMBA for 8 h, and primary keratinocytes from (+/+) and (–/–) neonates (C,D) were treated 8 h with vehicle (0.02% DMSO) or 1 μM B[a]P or DMBA, as described in Materials and Methods. (A) qPCR was performed using total RNA isolated from skin to quantify the expression of *Cyp1a1* and *Cyp1b1*. Values are the average normalized fold change as compared to control and represent the mean ± S.E.M of N = 3 biological replicates. Values with different letters are significantly different, $P < 0.05$, as determined by Bonferroni's multiple comparison test. (B) Protein expression in skin microsomes of phase I enzymes CYP1A1, CYP1B1, mEH, and COX2 compared to the LDH loading control. PC = positive control (TCDD treated liver microsome protein). (C, D) Quantitative real-time PCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of *Cyp1a1* (C) and *Cyp1b1* (D). Values are normalized to (+/+) vehicle control and present mean ± S.E.M. of N = 4 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Bonferroni's multiple comparison test.

The tissue specificity of AHR modulation was further scrutinized in the liver, primary hepatocytes, and primary dermal fibroblasts to determine if PPARβ/δ globally regulates AHR signaling. Liver cells and hepatocytes were chosen because the liver and

hepatocytes are a primary target of PAH metabolism and toxicity. Dermal fibroblasts were chosen because this cell type is directly adjacent to epidermal keratinocytes. Wild-type and *Pparβ/δ*-null mice were orally gavaged with the AHR ligand β-NF to examine *Cyp* mRNA induction. Surprisingly, both genotypes had similarly increased *Cyp1a1* (Figure **2.2A**) and *Cyp1a2* (Figure **2.2B**) mRNA expression in response to β-NF. Primary hepatocytes from wild-type and *Pparβ/δ*-null mice were then treated with B[a]P to determine whether specific liver cells exhibit PPARβ/δ-dependent modulation of AHR signaling. Consistent with the tissue observations, PAH-dependent *Cyp1a1* (Figure **2.2C**) and *Cyp1a2* (Figure **2.2D**) mRNA expression was not found to be differentially modulated between the genotypes. To examine a cell-type directly adjacent to basal keratinocytes, primary dermal fibroblasts were treated with B[a]P, and *Cyp* mRNA expression was examined. Similar to the liver cell observations, PPARβ/δ expression had no effect on PAH-dependent induction of *Cyp1a1* (Figure **2.2E**) and *Cyp1b1* (Figure **2.2F**) mRNA in primary dermal fibroblasts. These experimental results provide strong evidence that PPARβ/δ-dependent modulation of AHR signaling is specific to the skin, and more explicitly, keratinocytes.

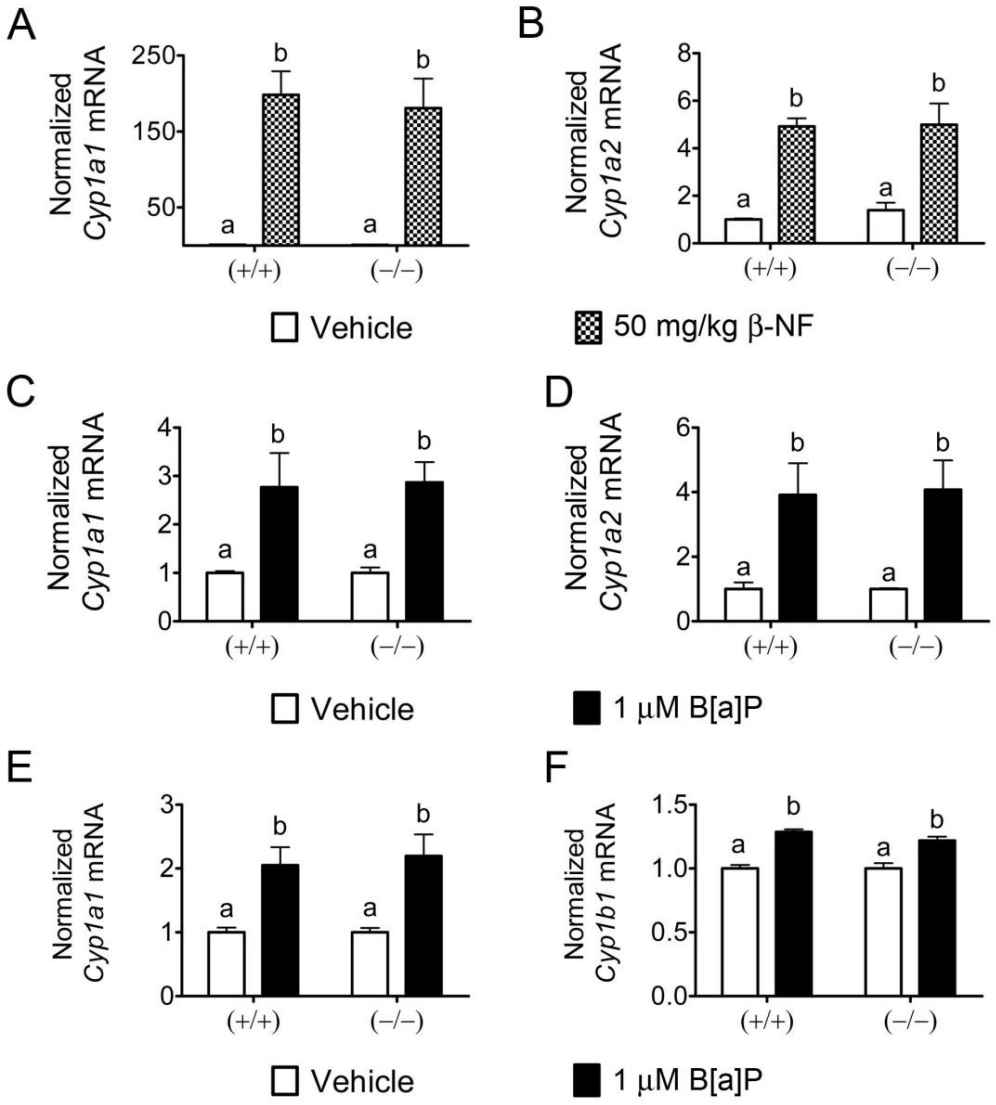

Figure **2.2**: PAH-dependent induction of *Cyp* mRNA is not modulated by PPARβ/δ expression in the liver cells or dermal fibroblasts. For liver analyses (A, B), wild-type (+/+) and *Pparβ/δ*-null (–/–) mice were isolated and treated with vehicle (corn oil) or 50 mg/kg β-NF for 8 h as described in Materials and Methods. Primary hepatocytes (C, D) and primary dermal fibroblasts (E, F) from (+/+) and (–/–) neonates were isolated and treated for 8 h with vehicle (0.02% DMSO) or 1 μM B[a]P as described in Materials and Methods. (A-F) qPCR was performed using total RNA isolated from liver (A, B), primary hepatocytes (C, D), or primary dermal fibroblasts (E, F) to quantify the expression of *Cyp1a1* (A, C, E), *Cyp1a2* (B, D) or *Cyp1b1* (F). Values are normalized to vehicle control and present mean ± S.E.M. of N = 3 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Bonferroni's multiple comparison test.

Several other nuclear receptors have also been shown to mediate ligand-dependent transcriptional effects in the skin. Thus, the ability of PPARβ/δ to modulate glucocorticoid receptor (GR)-, retinoic acid receptor (RAR)-, and retinoid X receptor (RXR) ligand-dependent signaling was examined in primary keratinocytes. Dexamethasone treatment in keratinocytes increased mRNA production of the GR target genes *Gilz* (Figure **2.3A**) and *Fkbp5* (Figure **2.3B**). However, these effects were not differentially modulated between the genotypes (Figure **2.3A**, **B**). RA isomers are also known to modulate overlapping target gene mRNAs, such as *Cyp26a1*, via RAR and RXR signaling in the skin (Reviewed in [700]). Therefore, *Cyp26a1* mRNA induction was used as a marker of RA-dependent activation of two nuclear receptor signaling pathways. *Cyp26a1* mRNA expression was increased with both retinoic acid treatments (Figure **2.3C, D**)**,** but PPARβ/δ expression had no effect on RA-dependent mRNA induction of this target gene. Collectively, PPARβ/δ did not modulate the ligand-dependent transcriptional regulation of three independent nuclear receptors, thus indicating that the observed PPARβ/δ-dependent modulation of AHR signaling in the skin is relatively specific.

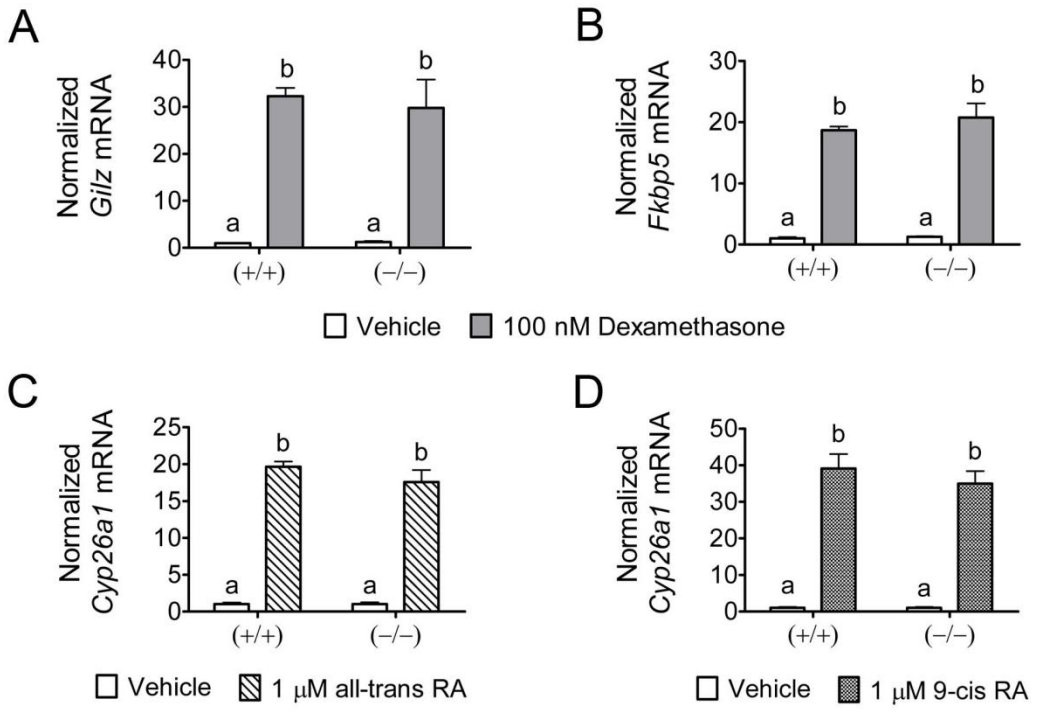

Figure **2.3**: Ligand-dependent gene regulation by GR, RAR, and RXR is not modulated by PPARβ/δ expression in primary keratinocytes. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were treated 8 h with vehicle (0.02% DMSO), 100 nM dexamethasone (A, B), 1 μM all-trans RA (C), or 1 μM 9-cis RA (D) as described in Materials and Methods. (A-D) qPCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of *Gilz* (A), *Fkbp5* (B), or *Cyp26a1* (C, D). Values are normalized to vehicle control and present mean ± S.E.M. of N = 3 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Bonferroni's multiple comparison test.

2.4.2 PPARβ/δ modulates the PAH-mediated inducibility of xenobiotic metabolism enzymes

The AHR is known to be activated by a wide range of ligands, including PAHs, arachidonic acid metabolites, flavonoids, and tryptophan oxidation products (Reviewed in [701]. Thus, the ability of PPARβ/δ to modulate AHR signaling via these structurally diverse compounds was examined in primary keratinocytes. In total, eleven AHR ligands were examined for *Cyp1a1* (Figure **2.4A**) and *Cyp1b1* (Figure **2.4B**) mRNA induction in

wild-type and *Pparβ/δ*-null primary keratinocytes. All the AHR ligands tested had significantly reduced PAH-mediated *Cyp1a1* and *Cyp1b1* mRNA induction in *Pparβ/δ*-null keratinocytes as compared to wild-type cells (Figure **2.4A, B**). This muted response appeared to be more pronounced for *Cyp1b1* induction (Figure **2.4B**) than *Cyp1a1* induction (Figure **2.4A**). Additionally, TCDD exposure caused the greatest genotype-specific modulation of *Cyp* mRNA induction. Collectively, PAH-dependent *Cyp1a1 and Cyp1b1* mRNA induction was reduced by at least 40% and 60%, respectively, in *Pparβ/δ*-null keratinocytes. *Cyp1a1* mRNA was observed to be generally more responsive to the AHR ligands, with some ligands increasing mRNA expression by greater than 100-fold (Figure **2.4A**). It is also noteworthy that the PPARβ/δ ligand GW0742 did not alter the mRNA expression of *Cyp1a1* or *Cyp1b1* in either genotype (Figure **2.4A, B**). AHR-dependent regulation of *Cyp* mRNA expression was then examined temporally. The PAH-mediated induction of *Cyp1a1* and *Cyp1b1* mRNA was found to be attenuated in *Pparβ/δ*-null keratinocytes at every time point examined (Figure **2.4C, D)**. Interestingly, a bi-phasic change in the induction of *Cyp1a1* and *Cyp1b1* mRNA was observed in both genotypes, (Figure **2.4C, D**). These collective observations revealed that PPARβ/δ modulated ligand-dependent AHR function in primary keratinocytes, and this effect was not due to ligand specificity or changes in temporal AHR signaling.

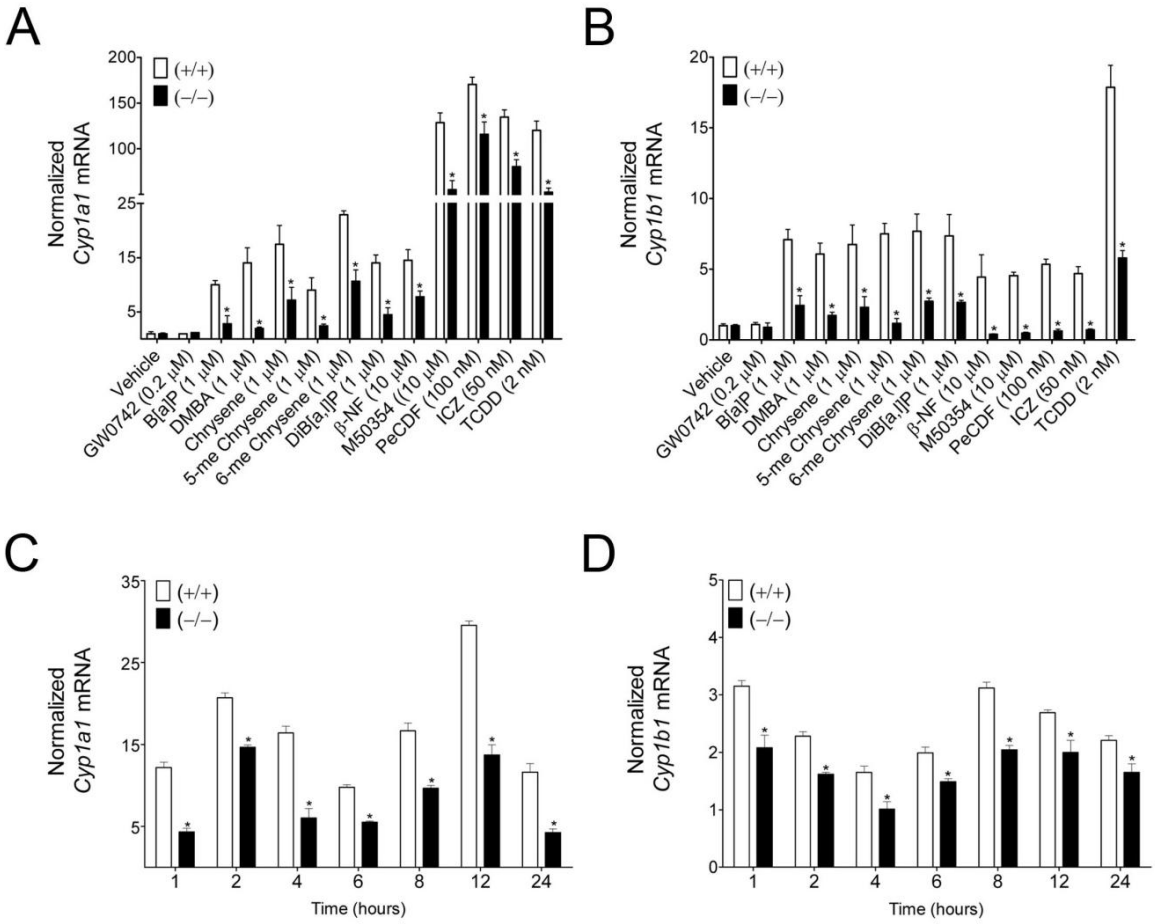

Figure **2.4**: *Cyp* ligand-dependent and temporal mRNA induction is modulated by PPARβ/δ expression in primary keratinocytes. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were treated 8 h with vehicle (0.02% DMSO), the indicated compounds (A, B), or 1 μM B[a]P for the indicated amount of time (C, D), as described in Materials and Methods. (A-D) qPCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of *Cyp1a1* (A, C) and *Cyp1b1* (B, D). Values are normalized to vehicle control and present mean ± S.E.M. of N = 3 biological replicates. Values with an asterisk, *, indicate statistically different, $P < 0.05$, groups, as determined by Student's t-test comparing genotype-specific induction by compound (A, B) or time (C, D).

In addition to directly regulating the mRNA and protein expression of phase I enzymes, the AHR has also been shown to control mRNA levels of a diverse set of target genes (Reviewed in [702]). The AHR is classically known to modulate xenobiotic metabolism, but recent studies have identified receptor-dependent regulation of cell

cycle kinetics, differentiation, inflammation, and immune responses (Reviewed in [703-705]). The collection of phase I and II enzymes that are known to be directly regulated by AHR has been termed the “AHR gene battery” [706], and the coordinated expression of these genes facilitates the metabolism and excretion of carcinogens [707]. With a PPARβ/δ-dependent modulation of phase I enzyme mRNA induction established, the ability of PPARβ/δ to modulate other classes of AHR target genes was examined in mouse keratinocytes. The PAH-dependent induction of phase II enzymes and the antioxidant sensor *Nrf2* were examined in wild-type and *Pparβ/δ*-null primary keratinocytes. The mRNA for phase II enzymes *Nqo1*, *Ugt1a2*, and *Gsta1* were all found to be significantly increased in response to PAHs in wild-type keratinocytes, and this effect was not observed in the absence of PPARβ/δ (Figure **2.5A-C**). Interestingly, *Nqo1* and *Gsta1* basal mRNA levels were significantly higher in *Pparβ/δ*-null primary keratinocytes, but PAH exposure did not alter the mRNA expression (Figure **2.5A, C**). Furthermore, PAH-mediated induction of Nrf2 mRNA was also reduced in *Pparβ/δ*-null keratinocytes as compared to similarly treated wild-type cells (Figure **2.5D**). NRF2 is a well described transcription factor that regulates antioxidant responses, and a recent hypothesis suggests that NRF2 assists in regulating the “AHR gene battery”. These observations suggest that PPARβ/δ may indirectly modulate NRF2 antioxidant signaling in response to PAHs. To delineate if all AHR target genes are regulated by PPARβ/δ, the PAH-dependent induction of two known target genes, *Ahrr* and *Ereg*, that do not contribute to phase I or II metabolism were examined. In contrast to the phase I and II mRNA modulations, PPARβ/δ expression did not alter the basal or PAH-dependent induction of *Ahrr* and *Ereg* mRNA (Figure **2.6A, B**). These observations may be caused by intrinsic differences in promoter structure or AHR coregulator occupancy in the skin that prevents PPARβ/δ-dependent modulation of some AHR target genes. The

expression of metabolism enzymes not specifically regulated by AHR, namely *Cox2* and *Hox1*, were examined in response to PAHs. COX2 has been implicated in the bioactivation of B[a]P and other genotoxic carcinogens [708, 709], and HOX1 expression is increased upon oxidative stress (Reviewed in [677]). The expression of *Cox2* and *Hox1* mRNAs were increased upon PAH exposure in wild-type keratinocytes, but this effect was not observed in similarly treated *Pparβ/δ*-null keratinocytes (Figure **2.6C, D**). Collectively, the reduced PAH-dependent induction of phase I and II enzymes in *Pparβ/δ*-null keratinocytes indicates that PPARβ/δ modulates AHR signaling and antioxidant responses to PAHs.

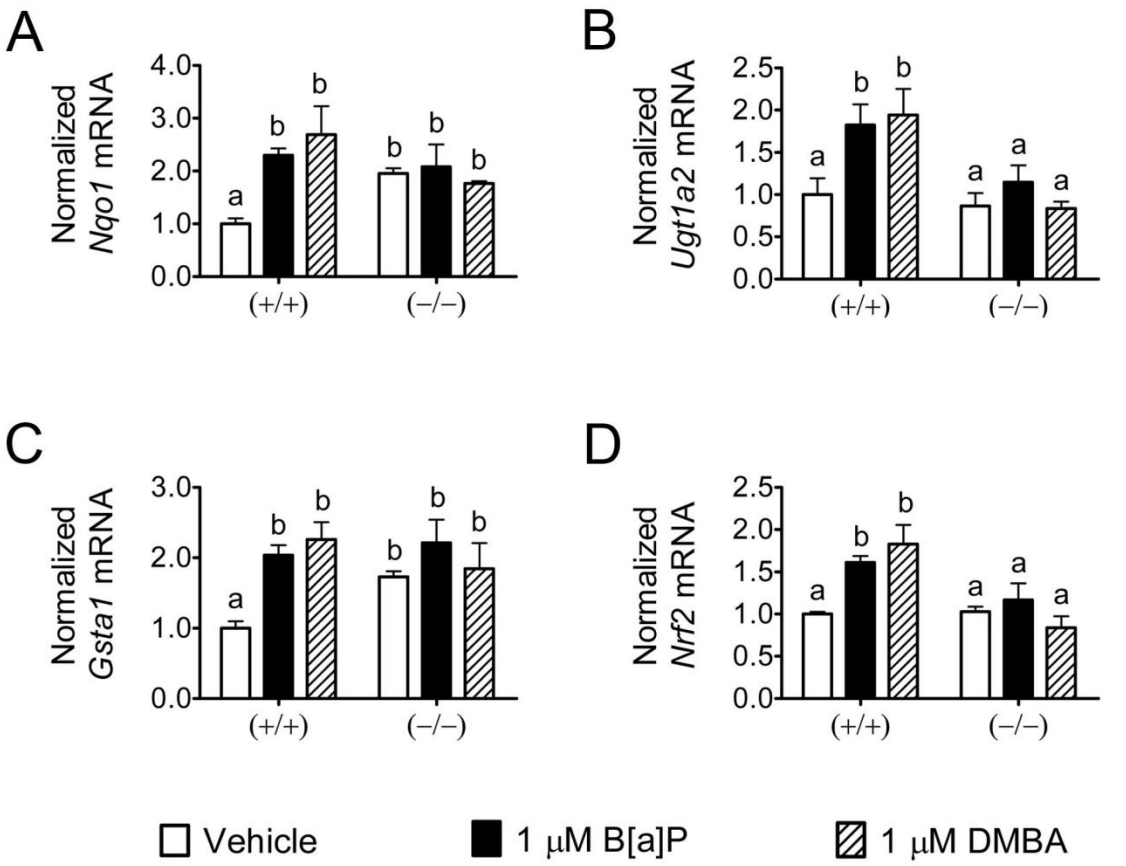

Figure **2.5**: PAH-dependent induction phase II enzymes and an antioxidant sensor are modulated by PPARβ/δ expression in primary keratinocytes. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were treated 8 h with vehicle (0.02% DMSO) or 1 μM B[a]P or DMBA as described in Materials and Methods. (A-D) qPCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of *Nqo1* (A), *Ugt1a2* (B), *Gsta1* (C), or *Nrf2* (D). Values are normalized to (+/+) vehicle control and present mean ± S.E.M. of N = 4 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Bonferroni's multiple comparison test.

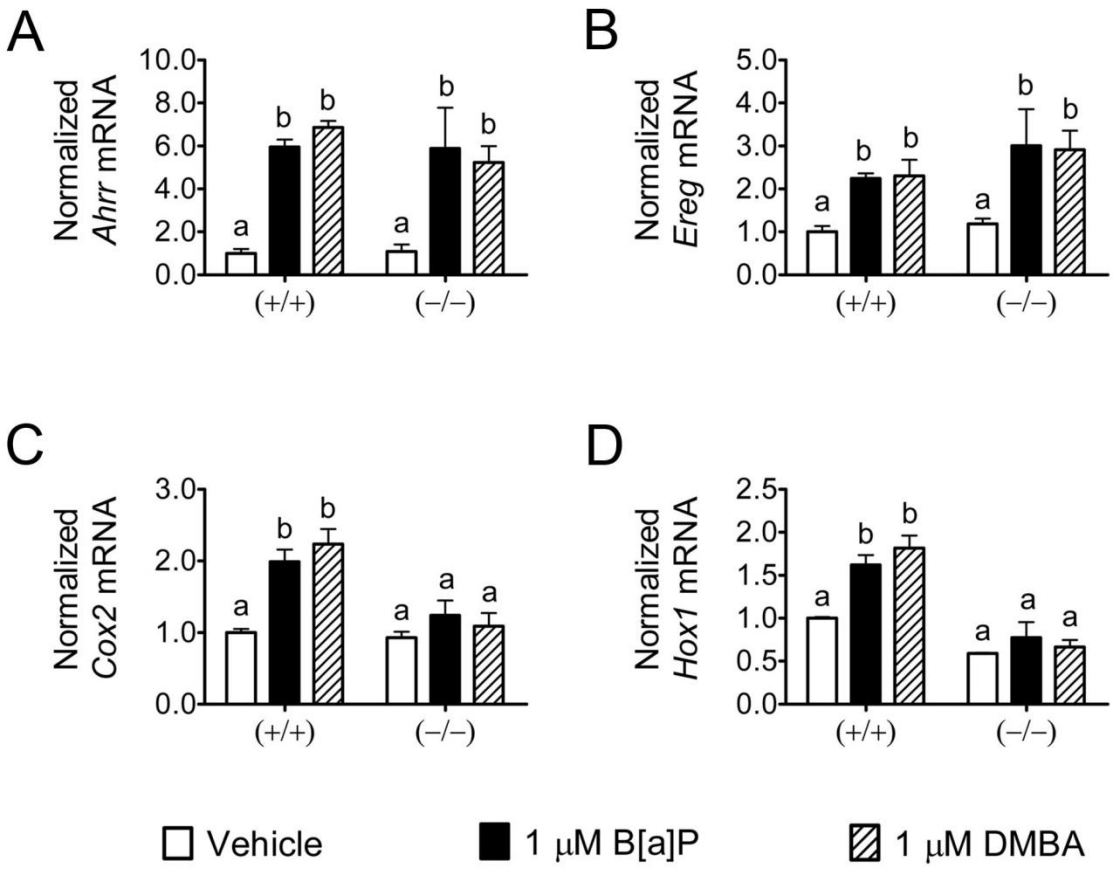

Figure **2.6**: PAH-dependent induction of AHR target genes and phase I enzymes not specifically regulated by AHR are differentially modulated by PPARβ/δ expression in primary keratinocytes. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were treated 8 h with vehicle (0.02% DMSO) or 1 μM B[a]P or DMBA as described in Materials and Methods. (A-D) qPCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of *Ahrr* (A), *Ereg* (B), *Cox2* (C), or *Hox1* (D). Values are normalized to (+/+) vehicle control and present mean ± S.E.M. of N = 4 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Bonferroni's multiple comparison test.

2.4.3 Intrinsic cytosolic functions of AHR are not modulated by PPARβ/δ

To assess the mechanism by which AHR signaling is modulated by PPARβ/δ, the intrinsic cytosolic functions of AHR, including protein expression, ligand binding, and nuclear translocation, were examined in wild-type and *Pparβ/δ*-null primary

keratinocytes. Soluble protein lysates were prepared to examine the protein expression of AHR and its accessory signaling partners, including ARNT, HSP90, and XAP2. Quantitative western blot analysis revealed that the protein expression of AHR, ARNT, HSP90, or XAP2 was similar in wild-type or *Pparβ/δ*-null keratinocytes (Figure **2.7**). Thus, PPARβ/δ does not appear to regulate the protein expression of critical AHR signaling components. The next steps in AHR signaling are the binding of ligand to the cytosolic receptor and nuclear translocation. These events were examined utilizing irreversible and reversible radioaffinity dioxin derivatives (Figure **2.8A**), known as [^{125}I]N_3Br_2DpD and [^{125}I]Br_2DpD [689, 690]. The azide derivative, [^{125}I]N_3Br_2DpD, was used to assess ligand affinity because it irreversibly cross-links to interacting proteins upon ultraviolet (UV) light exposure. Incubation of this ligand with soluble cytosolic lysates from wild-type and *Pparβ/δ*-null keratinocytes revealed a dose-dependent increase in ligand binding in both genotypes (Figure **2.8B**). Furthermore, the relative binding of cytosolic receptor was similar between wild-type and *Pparβ/δ*-null keratinocytes (Figure **2.8B**), which indicates that PPARβ/δ does not alter AHR ligand binding. It is also well established that a ligand-dependent conformational change in AHR causes dissociation from the HSP90/XAP2 complex (Reviewed in [702]). Results from biochemical studies using [^{125}I]Br_2DpD and sucrose gradient ultracentrifugation have described a distinct difference in sedimentation between the unliganded cytosolic (9S) and the liganded nuclear (6S) receptor [690]. Treatment of wild-type and *Pparβ/δ*-null keratinocytes with [^{125}I]Br_2DpD resulted in a similar pattern of 6S receptor formation (Figure **2.8D**). The sedimentation pattern of keratinocytes was identical, but at relatively lower levels, to that found in the Hepa1c1c7 cell line that expresses notably high levels of AHR (Figure **2.8D** versus **2.8C**) [710]. Collectively, the results of these investigations provide evidence that PPARβ/δ does not modulate cytosolic AHR functions.

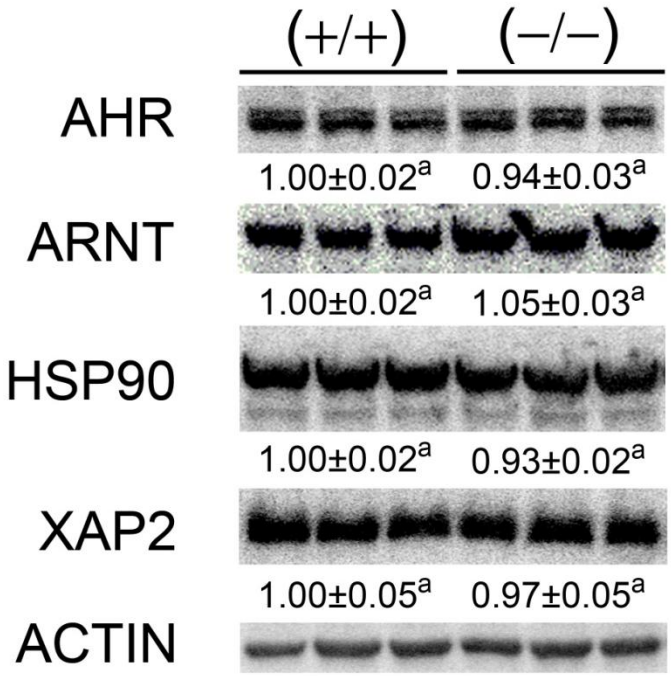

Figure **2.7**: Expression of AHR and accessory proteins is not modulated by PPARβ/δ. Quantitative protein expression of AHR and accessory proteins in wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes. Expression values are fold expression relative to (+/+) and represent mean ± S.E.M. of N = 3 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Student's t-test.

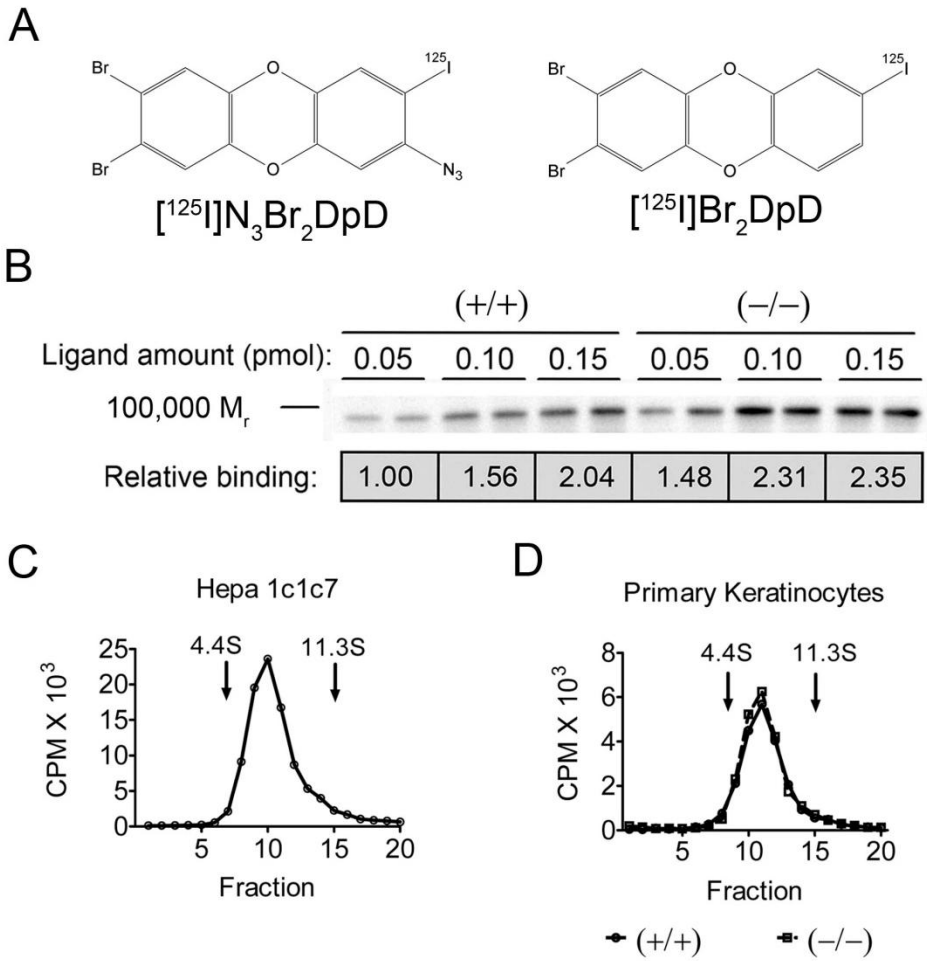

Figure **2.8**: PPARβ/δ does not alter the cytosolic functions of AHR in keratinocytes. The functionality of AHR cytosolic protein, including radioaffinity ligand binding (B), and radioaffinity ligand-induced nuclear translocation (C) was evaluated in wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes. (A) Chemical structure of the two AHR radioaffinity ligands, [^{125}I]N_3Br_2DpD and [^{125}I]Br_2DpD. (B) Affinity of cytosolic AHR for [^{125}I]N_3Br_2DpD. Cytosol from (+/+) and (–/–) keratinocytes was incubated with radioaffinity ligand as described in Materials and Methods with N = 2 technical replicates. Relative binding was determined by scintillation counting and normalized to the (+/+) 0.05 pmol average signal. (C, D) Analysis of AHR nuclear translocation using [^{125}I]Br_2DpD in Hepa1c1c7 cells (C) or primary keratinocytes (D). Cells were treated 1 h with [^{125}I]Br_2DpD and nuclear protein was subjected to sucrose gradient ultracentrifugation as described in Materials and Methods. Fractions were quantified by scintillation counting. The proteins bovine serum albumin (4.4S) and catalase (11.3) were used as external sedimentation standards.

2.4.4 The nuclear functions of AHR are modulated by PPARβ/δ

The nuclear functions of AHR, including heterodimerization and gene regulatory activities, were examined to determine whether PPARβ/δ modulated these aspects of AHR functionality. Upon nuclear translocation, AHR is known to heterodimerize with ARNT (Reviewed in [702]), and PPARβ/δ has not been described as a binding partner for the regulatory AHR complex to date. A physical interaction between AHR, ARNT, and PPARβ/δ was examined in a Cos-1 over-expression system utilizing GST- and Flag-AHR and ARNT expression constructs [691]. At wash conditions that displayed the documented interaction between AHR and ARNT (Figure **2.9A,** Lane 8, 10, and 12), PPARβ/δ was observed to non-specifically pull-down with the capture resin (Figure **2.9A**, Lane 6). The subsequent analysis considered this non-specific binding as background and was controlled for to assess the ability of PPARβ/δ to interact with the AHR complex. When GST-AHR, Flag-ARNT, and PPARβ/δ were co-expressed (Figure **2.9A**, Lane 11), an increased association of PPARβ/δ with the AHR complex was observed. However, this putative interaction did not appear to be significant when compared to the inputs and the total increase from background (Figure **2.9A**, Lane 11 compared to Lane 1, 2, and 6). Therefore, the results from these studies suggest that PPARβ/δ does not specifically interact with the AHR nuclear regulatory complex.

Upon binding with ARNT, the AHR/ARNT heterodimer binds to response elements within target gene promoters and recruits coactivators and nucleosome remodeling complexes. Collectively, these proteins alter the promoter structure of target genes, which causes the recruitment of the transcription initiation complex and increased target gene mRNA production. ChIP was performed in wild-type and *Pparβ/δ*-null

primary keratinocytes to assess whether PPARβ/δ modulated the ability of AHR to bind a target promoter and cause chromatin remodeling. Upon treatment with B[a]P, increased occupancy of AHR and increased acetylation of histone 4, a common marker of transcriptional activity, were observed at a known *Cyp1a1* promoter enhancer element in wild-type keratinocytes (Figure **2.9B**) [711-713]. These changes in AHR occupancy and histone acetylation were not observed in similarly treated *Pparβ/δ*-null primary keratinocytes (Figure **2.9B**). The results of these studies suggest that AHR is not able to access the *Cyp1a1* promoter in the absence of PPARβ/δ expression at the observed treatment time (2 h).

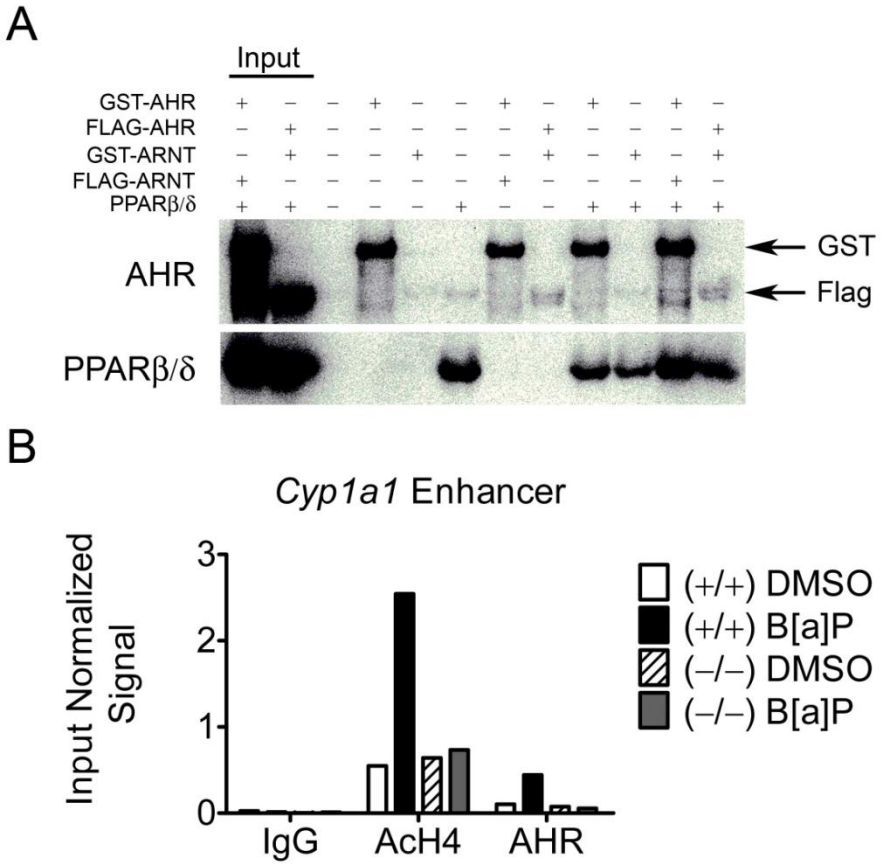

Figure **2.9**: PPARβ/δ modulates the nuclear functions of AHR. The functionality of AHR nuclear protein, including interaction with ARNT and PPARβ/δ (A) and promoter occupancy in response to PAH (B) was evaluated in Cos-1 cells (A) and wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes (B). (A) Protein expression of GST- and Flag-tagged AHR and PPARβ/δ after GST-pulldown in Cos-1 cells over-expressing AHR, ARNT, and PPARβ/δ compared to 10% input control, as described in Materials and Methods. There does not appear to be an interaction when compared to background PPARβ/δ signal. (B) ChIP to assess promoter occupancy at the *Cyp1a1* enhancer element in response to B[a]P in (+/+) and (–/–) primary keratinocytes. Keratinocytes were treated 2 h with vehicle (0.02% DMSO) or 1 μM B[a]P. ChIP and PCR quantification were carried out as described in Materials and Methods. One biological replicate is displayed as pooled from keratinocytes isolated from N = 3 neonates. IgG immunoprecipitation is used as a negative control, and AcH4 immunoprecipitation is used as a positive marker of transcriptional activation.

2.4.5 *Cyp* mRNA induction is modulated by DNA methylation in mouse keratinocytes

Thus far, PPARβ/δ appeared to modulate the occupancy of AHR at the *Cyp1a1* promoter in mouse keratinocytes. Several chromatin biochemical alterations could underlie this phenomenon. A common regulator of endogenous chromatin structure is DNA methylation and the formation of expression-silencing cytosine-phosphate-guanine (CpG) islands (Reviewed in [714, 715]). To examine whether DNA methylation causes modulated AHR signaling, 5-Aza-dC, a DNA methylation inhibitor, was supplemented into the media of mouse keratinocytes to ablate DNA methylation patterns. The basal and PAH-dependent expression of *Cyp1a1* and *Cyp1b1* was investigated by qPCR. Upon 72 h in control media, the PPARβ/δ-dependent modulation of AHR signaling, as assessed by *Cyp* mRNA expression, was observed in mouse keratinocytes (Figure **2.10A, B**). Surprisingly, a significant increase in basal *Cyp1a1* and *Cyp1b1* mRNA expression was observed upon 5-Aza-dC treatment (Figure **2.10A, B**). It is also noted that the expression of the mRNA loading control, *Gapdh*, was unchanged upon 5-Aza-dC treatment (data not shown). Maximal PAH-induced expression level of *Cyp1a1* was not affected by 5-Aza-dC in wild-type keratinocytes (Figure **2.10A**), and these results indicate that maximal *Cyp1a1* message is not altered by DNA methylation. When normalized to genotype-specific vehicle controls, 5-Aza-dC abolished PPARβ/δ-dependent modulation of *Cyp1a1* and *Cyp1b1* mRNA responses to PAH (data not shown), and this further established that DNA methylation may be causing the PPARβ/δ-dependent modulation of AHR signaling.

Bisulfite sequencing was utilized to directly examine whether methylation at the *Cyp1a1* promoter contributes to PPARβ/δ-dependent modulation of AHR signaling. MethylPrimer Express® analysis of the *Cyp1a1* promoter identified a putative 933

basepair CpG island at approximately -1481 to -548 basepairs upstream of the *Cyp1a1* transcription start site (Figure **2.10C**). Bisulfite-converted DNA encompassing this region was amplified and sequenced to assess the DNA methylation status of the *Cyp1a1* promoter. Five independent sequencing plasmid amplicons were analyzed from wild-type and *Pparβ/δ*-null keratinocyte bisulfite-converted DNA using the CyMATE program [696] (Figure **2.10C**). Examination of the resulting CyMATE methylation map revealed that *Pparβ/δ*-null keratinocytes possess higher DNA methylation at the *Cyp1a1* promoter as compared to wild-type cells (Figure **2.10C**). Enhanced promoter methylation is known to repress gene expression (Reviewed in [716]), and *Pparβ/δ*-null keratinocytes had reduced basal mRNA levels of *Cyp1a1* (Figure **2.10C**). However, the number of sites and frequency of methylated cytosines is much lower than the CyMATE predicted methylation status for the *Cyp1a1* promoter (Figure **2.10C**). These observations suggest that DNA methylation may contribute, but is not required, to altered basal *Cyp* mRNA expression and PAH-mediated induction.

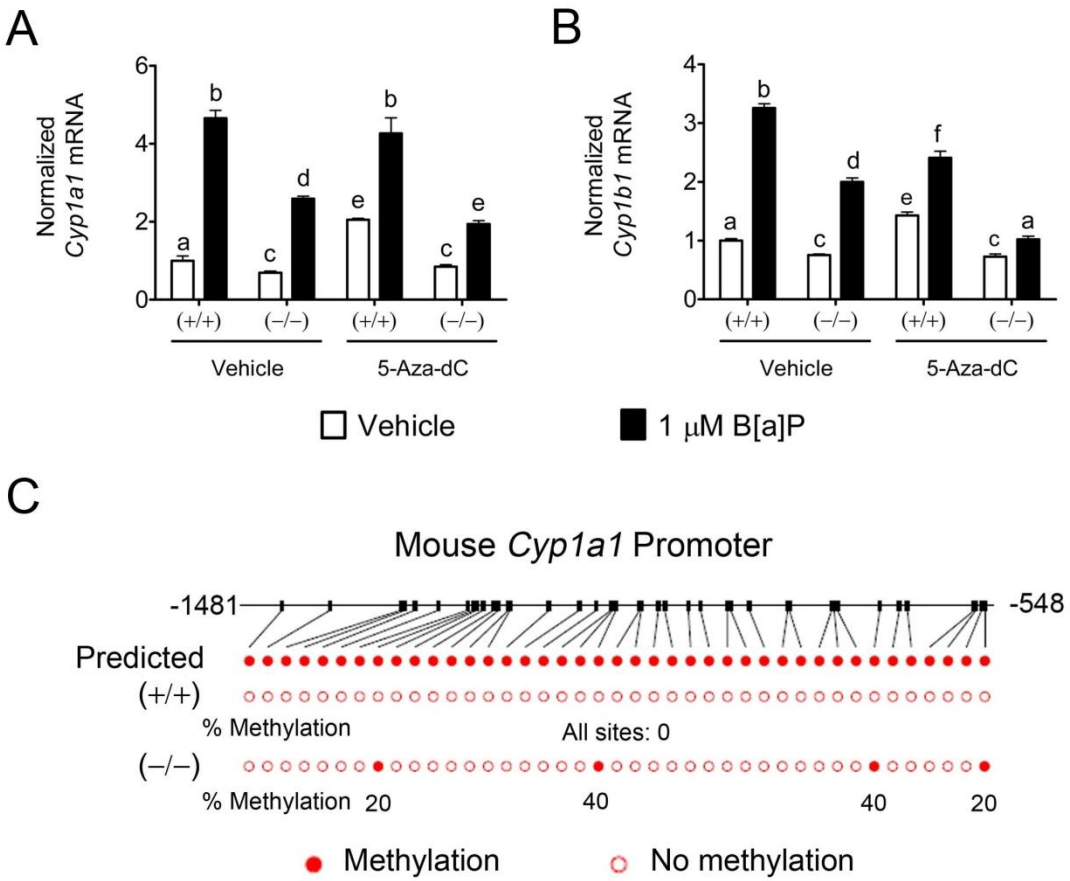

Figure **2.10**: PPARβ/δ alters *Cyp* promoter methylation in mouse keratinocytes. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were isolated as described in Materials and Methods. (A, B) qPCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of *Cyp1a1* (A) and *Cyp1b1* (B). Keratinocytes were treated with 5 μM 5-Aza-dC for 64 h prior to a 8 h B[a]P treatment, as described in Materials and Methods. Values are normalized to (+/+) vehicle control and represents mean ± S.E.M. of N = 4 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Student's t-test. (C) Bisulfite sequencing results using bisulfite converted DNA from (+/+) and (–/–) primary keratinocytes to amplify a putative methylation region within the *Cyp1a1* promoter, as described in Materials and Methods. Five sequencing plasmids were sequenced for each genotype, and CyMATE [696] was used to map and examine methylation patterns. Percent (%) methylation indicates the incidence of methylation at a particular cytosine residue.

2.4.6 Reduced PPARβ/δ expression in a human keratinocyte model modulated AHR-dependent signaling

To examine whether PPARβ/δ-dependent modulation of AHR signaling was specific to murine keratinocytes, a stable PPARβ/δ shRNA model was developed in the human HaCaT keratinocyte cell line. This cell line was chosen because it is generally used as cell line model to examine normal human keratinocyte functions [717]. Stable non-target and shPPARβ/δ heterogeneous cell populations were characterized for PPARβ/δ protein and mRNA expression. A 35% decrease in PPARβ/δ protein (Figure **2.11A**) and 60% reduction in PPARβ/δ mRNA (Figure **2.11B**) were observed in the shPPARβ/δ cell line as compared to the non-target cells. The functionality of this knockdown was assessed by examining the ligand-dependent regulation of the PPARβ/δ target gene *Angptl4*. The shPPARβ/δ cell line had significantly reduced ligand-dependent induction of *Angptl4* mRNA as compared to the similarly treated non-target cell line (Figure **2.11C)**. The results from these studies indicate that the human shRNA model system can be utilized to examine PPARβ/δ-dependent modulation of AHR signaling and predict human PAH responses.

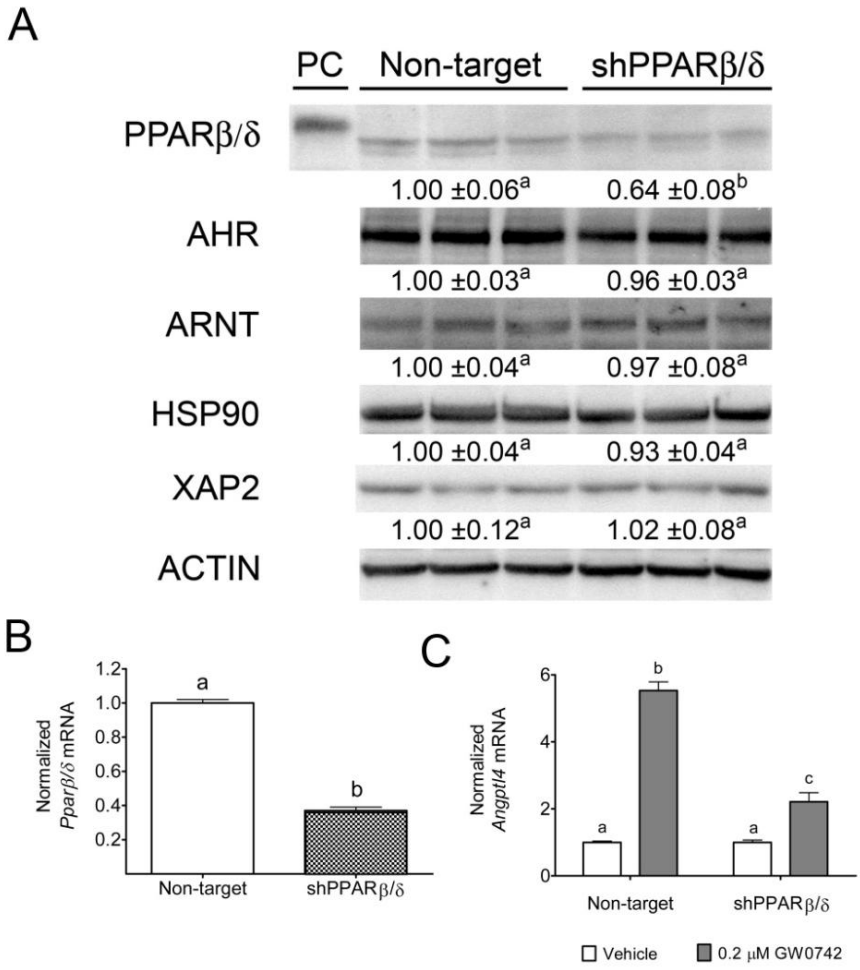

Figure **2.11**: Characterization of PPARβ/δ shRNA knockdown in human HaCaT keratinocytes. The effect of the MISSION™ shRNA plasmid system on PPARβ/δ expression and functionality in human HaCaT keratinocytes was assessed at the protein (A), mRNA (B), and ligand-dependent transcriptional regulation (C) levels. (A) Quantitative protein expression of PPARβ/δ, AHR, and accessory proteins in the HaCaT shRNA cell lines was normalized to ACTIN expression. Expression values are fold expression relative to the non-target cell line and represent mean ± S.E.M. of N = 3 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined Student's t-test. PC = positive control (lysate of Cos-1 cells transfected with hPPARβ/δ). (B, C) Quantitative real-time PCR was performed using total RNA isolated from the HaCaT shRNA cell lines to quantify the basal expression of *Pparβ/δ* (B) and ligand-dependent induction of *Angptl4* after 8 h GW0742 treatment (C). Values are the average normalized fold change as compared to the non-target cell line and represent the mean ± S.E.M of N = 3 biological replicates. Values with different letters are significantly different, $P < 0.05$, as determined by Student's t-test (B) or Bonferroni's multiple comparison test (C).

The human relevance of PPARβ/δ-dependent modulation AHR signaling was examined utilizing the characterized HaCaT shRNA model. First, the expression of AHR and the previously described accessory proteins were examined. The protein expression of AHR, ARNT, HSP90, and XAP2 were not found to be significantly altered by reduced PPARβ/δ protein expression (Figure **2.11A**). With the HaCaT model established as a relevant human model, the effect of PAH exposure was examined. Following 8 h B[a]P treatment, the shPPARβ/δ cell line had reduced *Cyp1a1* (Figure **2.12A)** and *Cyp1b1* (Figure **2.12B**) mRNA induction as compared to similarly treated non-target cells. Thus, human keratinocytes (Figure **2.12A, B**) mimicked the observed PPARβ/δ-dependent modulation of AHR signaling in mouse keratinocytes (Figure **2.12C, D**). PPARβ/δ also modulated the temporal PAH-dependent induction of *Cyp1a1* and *Cyp1b1* mRNA at exposure times beyond 1 h (Figure **2.12C, D**). These observations are strikingly similar to the modulated *Cyp* mRNA induction observed in mouse keratinocytes (Figure **2.4C, D**). Interestingly, the shPPARβ/δ cell line displayed enhanced *Cyp1a1* mRNA inducibility at 2 h as compared to the non-target cell line (Figure **2.12C)**. Although this observation cannot be discounted, the greater majority of data, including the mouse keratinocyte data, indicated that PPARβ/δ modulated AHR signaling in mouse and human keratinocytes.

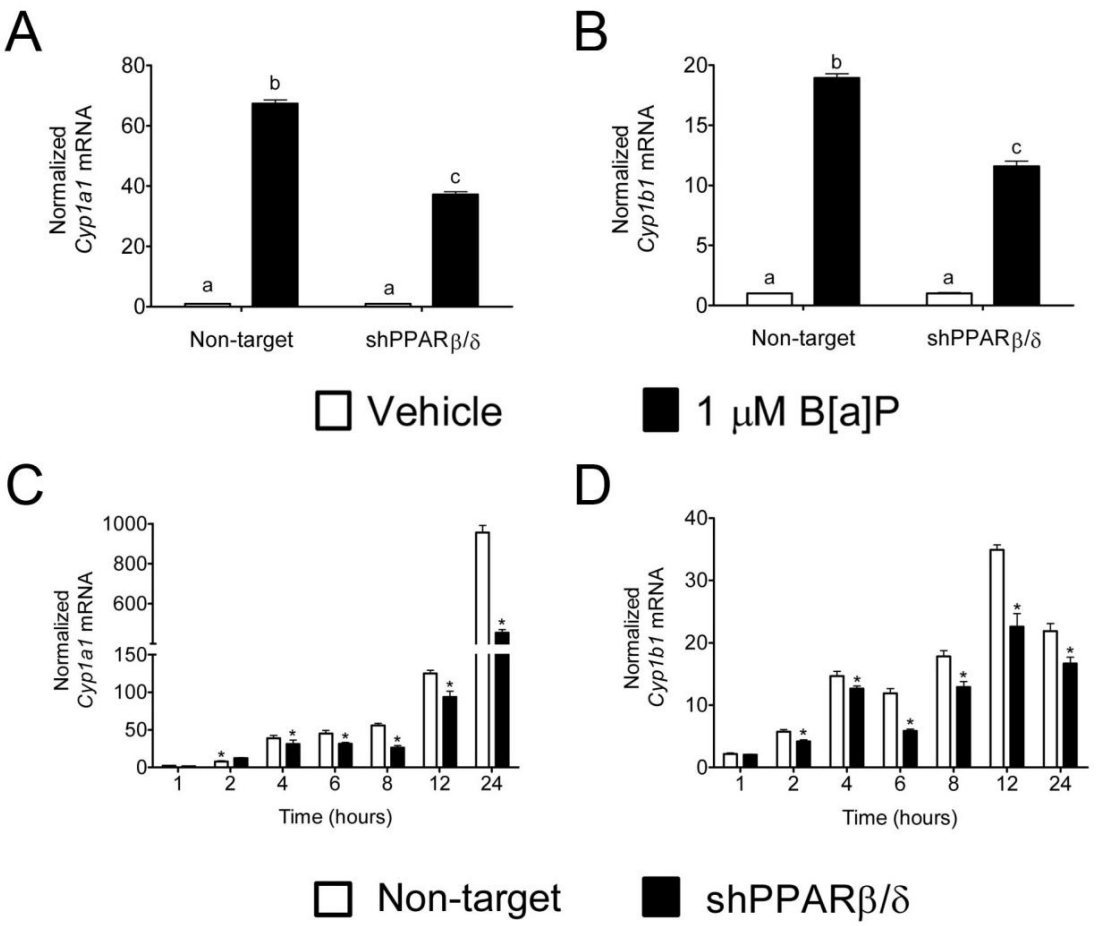

Figure **2.12**:Effect of reduced PPARβ/δ expression on PAH-dependent induction of *Cyp* mRNA in the HaCaT shRNA model system. PPARβ/δ-dependent modulation of AHR signaling was examined at 8 h (A, B) and throughout a time-course of PAH exposure (C, D). (A-D) qPCR was performed using total RNA isolated from the HaCaT shRNA cell lines to quantify the expression of *Cyp1a1* (A, C) and *Cyp1b1* (B, D). HaCaT shRNA cell lines were treated with vehicle (0.02% DMSO) or 1 μM B[a]P for 8 h (A, B) or the indicated treatment time (C, D), as described in Materials and Methods, to assess the mRNA expression of *Cyp1a1* (A, C) or *Cyp1b1* (B, D). Values are the average normalized fold change as compared to vehicle and represent the mean ± S.E.M of N = 3 biological replicates. Values with different letters are significantly different, $P < 0.05$, as determined by Bonferroni's multiple comparison test (A, B). Values with an asterisk, *, indicate a significant difference, $P < 0.05$, as determined by Student's t-test at each time point (C, D).

The ability of PPARβ/δ to modulate DNA methylation patterns in human keratinocytes was examined in the HaCaT shRNA model using the methylation inhibitor 5-Aza-dC. Under normal growth conditions, muted *Cyp* mRNA induction in response to

B[a]P was observed in the shPPARβ/δ cell line (Figure **2.13A, B**). However, a significant reduction in basal *Cyp* mRNA was not observed in the shPPARβ/δ cell line (Figure **2.13A, B**). The addition of 5-Aza-dC also did not increase basal *Cyp* mRNA expression (Figure **2.13A, B**) in accordance with the mouse keratinocyte observations (Figure **2.10A, B**). The ablation of DNA methylation did result in comparable *Cyp1a1* and *Cyp1b1* mRNA responses in the non-target and shPPARβ/δ cell lines (Figure **2.13A, B**). The results of these studies indicate that PPARβ/δ-dependent DNA methylation contributed to reduced PAH-mediated Cyp mRNA induction in human keratinocytes. Collectively, these observations establish that PPARβ/δ modulated AHR-dependent signaling in human skin.

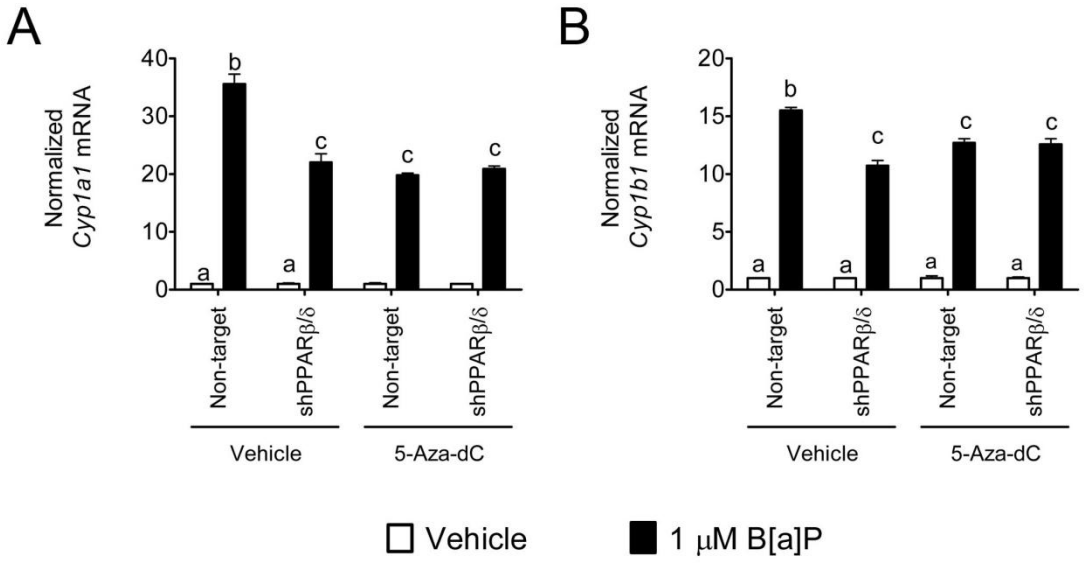

Figure **2.13**: Effect of reduced human PPARβ/δ expression on epigenetic regulation of *Cyp* mRNA induction in the HaCaT shRNA model system. (A, B) PPARβ/δ-dependent modulation of AHR signaling was examined in the presence of a DNA methylation inhibitor. qPCR was performed using total RNA isolated from the HaCaT shRNA cell lines to quantify the expression of *Cyp1a1* (A) and *Cyp1b1* (B). HaCaT shRNA cell lines were treated with 5 μM 5-Aza-dC for 72 h prior to an 8 h B[a]P treatment, as described in Materials and Methods. Values are the average normalized fold change as compared to vehicle and represent the mean ± S.E.M of N = 3 biological replicates. Values with different letters are significantly different, $P < 0.05$, as determined by Bonferroni's multiple comparison test

2.5 Discussion

The results from this study indicated that PPARβ/δ modulated AHR signaling through skin-specific alteration of endogenous DNA structure and ligand-dependent promoter occupancy. AHR protein was found to be functional in mouse and human keratinocytes by measureable assays of receptor biochemistry. Interestingly, PPARβ/δ appeared to change the endogenous chromatin structure of xenobiotic metabolism target genes. This resulted in altered ability of AHR to occupy a phase I enzyme promoter in response to PAH. The PPARβ/δ-dependent modulation of xenobiotic metabolism mRNA induction in response to PAH was observed in both mouse and human keratinocyte models. These data provided direct evidence that PPARβ/δ may alter PAH tumor initiation in mouse and human skin.

The specificity of modulated AHR signaling by PPARβ/δ in the skin is of great interest because PAHs are used as the initiating agents in skin tumorigenesis studies. It was very surprising that altered AHR signaling was not observed in the liver (Figure **2.2A, B**), a tissue that commonly exhibits high xenobiotic metabolism in response to PAHs (Reviewed in [677]). The observation that dermal fibroblasts (Figure **2.2D, E**) did not exhibit modulated AHR signaling (Figure **2.1C, D**) provided further evidence for the tissue specificity. The differences between fibroblast and keratinocyte PAH-dependent mRNA induction was fascinating because a recently proposed model suggested that PPARβ/δ initiates a paracrine signaling loop between keratinocytes and fibroblasts to alter mitogenic signaling [507]. While not specifically examined in the current study, cross-talk between keratinocytes displaying modulated

AHR signaling and the underlying fibroblasts that do not exhibit AHR modulation could contribute to PAH-dependent mitogenic or inflammatory responses. Additionally, PPARβ/δ is known to modulate inflammatory responses in several tissues, which can significantly alter tumor promotion and inflammatory signals (Reviewed in [164, 718]). While understanding the complex interactions that occur in the skin upon PAH exposure is beyond the context of these investigations, future studies should investigate PPARβ/δ-mediated paracrine signaling in skin tumorigenesis and malignant progression.

The tissue-specificity of modulated AHR signaling is hypothesized to be partly based on differences in PPARβ/δ expression. Previous studies utilizing a specific mouse antibody have shown that receptor expression is markedly higher in the skin than in the liver [163] and significantly higher in primary mouse keratinocytes than primary dermal fibroblasts [410]. These two observations indicate that relatively high PPARβ/δ expression is necessary to modulate AHR signaling. Whether this effect occurs in other tissues of high PPARβ/δ expression, such as the colon and small intestine [163], has not been specifically investigated to date. PPARβ/δ has been previously shown to reduce azoxymethane (AOM)-induced colon carcinogenesis [513, 582]. However, tumor initiation was not specifically examined, and AOM, the initiating agent, is not known to be metabolized by AHR-dependent P450s. While not previously addressed, these observations suggest that AOM colon carcinogenesis is not modulated through PPARβ/δ-dependent regulation of AHR signaling. Collectively, the described studies suggest that the relative expression level of PPARβ/δ is a critical indicator of how PAH bioactivation and detoxification is balanced, but more investigations are necessary to clarify this hypothesis.

AHR is widely considered a master regulator of PAH-dependent tumorigenesis (Reviewed in [719]). The fact that *Pparβ/δ*-null keratinocytes did not exhibit complete ablation of AHR signaling (Figure **2.1A-D**) indicates that PPARβ/δ is a secondary coregulator of xenobiotic metabolism. The use of *Ahr*-null mouse models has established that the loss of AHR expression decreased PAH-dependent skin carcinogenesis [685-688, 720]. Furthermore, PAH tumorigenesis has also been shown to be modulated in null mouse models of phase I and II xenobiotic metabolism enzymes. Decreased PAH-dependent tumorigenesis has been observed in Phase I null mice; in contrast, phase II null mice have increased PAH-dependent tumorigenesis (Reviewed in [721, 722]). The biological significance of modulated AHR signaling by PPARβ/δ in skin carcinogenesis is difficult to predict because PAH-dependent xenobiotic metabolism mRNA induction is not ablated in *Pparβ/δ*-null keratinocytes (Figure **2.1C, D)**. *In vivo* studies will be necessary to examine whether modulated AHR signaling in keratinocytes correlated to altered tumor initiation and cancer.

The novel observation of this investigation is that PPARβ/δ modulated AHR signaling in the skin by altering the chromatin structure of specific target genes. These observations are the first report of PPARβ/δ-dependent alteration in DNA methylation. Surprisingly, the promoter methylated by PPARβ/δ is not a documented target gene. Despite the decades of research examining AHR signaling, epigenetic regulation of AHR signaling has only recently been explored. The first AHR epigenetic studies examined how 5-Aza-dC and the AHR ligand 3-methylchloranthracene (3MC) altered *Cyp1a1* mRNA induction. Specific ablation of DNA methylation patterns caused enhanced 3MC-dependent induction of *Cyp1a1* mRNA [723]. Recent results in several mouse and human cell lines have also indicated that methylation of the *Cyp1a1* and *Cyp1b1*

promoters causes biological effects [724-728]. Most of these studies have utilized the DNA methylation inhibitor 5-Aza-dC [723-728] and bisulfite sequencing [724, 727]. The currently described study is the first mechanistic description of perturbed *Cyp1a1* methylation patterns by a receptor other than AHR. Results of these current studies revealed that ablation of DNA methylation patterns caused higher *Cyp1a1* basal mRNA levels in wild-type keratinocytes (Figure **2.10A**). Interestingly, the bisulfite sequencing analysis did not detect cytosine methylation in wild-type keratinocytes at the putative *Cyp1a1* CpG island (Figure **2.10C**). Therefore, alternative PPARβ/δ-dependent epigenetic regulatory mechanisms may exist to modulate AHR signaling. The bisulfite sequencing also indicated that methylation of the *Cyp1a1* promoter in *Pparβ/δ*-null keratinocytes, albeit its low incidence, altered basal *Cyp* mRNA expression. This effect manifested in reduced *Cyp1a1* mRNA levels in the absence of PPARβ/δ (Figure **2.1C**). To more thoroughly define whether PPARβ/δ establishes the putative methylation patterns in P450 promoters, the sample size of the bisulfite sequencing analysis must be increased. Several groups have reported that a sample size of twenty to thirty independent sequencing reads are necessary to establish the presence of a CpG island [696]. A second putative PPARβ/δ-dependent mechanism of epigenetic regulation emerged from the 5-Aza-dC inhibitor study, in which basal *Cyp1a1* and *Cyp1b1* mRNA levels were increased upon inhibitor treatment (Figure **2.10A, B)**. The results of these studies indicate that the *Cyp1a1* promoter was methylated, but this did not correspond to the bisulfite sequencing analysis. It is conceivable that an unidentified AHR coregulator is directly repressed by DNA methylation in a PPARβ/δ-dependent manner. In the presence of 5-Aza-dC, the mRNA expression of this co-regulator would be increased to alter basal *Cyp1a1* mRNA levels. Clearly, these newly hypothesized mechanisms of

epigenetic regulation should be tested with null mouse models and current epigenetic tools to shed light onto how PPARβ/δ caused epigenetic regulation.

The current investigations examined a novel nuclear receptor regulatory mechanism. The fact that the observed effects were ligand-independent added a level of complexity to the investigations. Identifying the epigenetic- and PPARβ/δ-dependent regulator(s) of AHR signaling is critical to elucidate the mechanism(s) causing modulated AHR signaling (Figure **2.14**). New model systems and analysis techniques are necessary to identify the PPARβ/δ target gene(s) that are regulated by receptor-dependent DNA methylation. The use of bisulfite sequencing and methylation inhibitors with null-mouse models provided a clear advantage to delineate the epigenetic regulatory mechanisms being described. Future investigations into PPARβ/δ and AHR signaling should rely on these approaches to clearly define the molecular mechanisms mediating these effects. While the current studies establish that PPARβ/δ modulates AHR signaling in human keratinocytes, this model exhibited insufficient knockdown of receptor expression to fully examine epigenetic regulation. The use of more efficient shRNAs or siRNAs directed against PPARβ/δ could provide useful alternatives to the described stable shRNA model. Another area that should be considered when elucidating human relevance is how human PPARβ/δ polymorphisms might contribute to modulation of AHR signaling. Because regulation of AHR signaling is critical in defining human toxicant exposure and tumorigenesis, creating suitable human models is necessary to clarify how these two receptors alter PAH-dependent carcinogenesis.

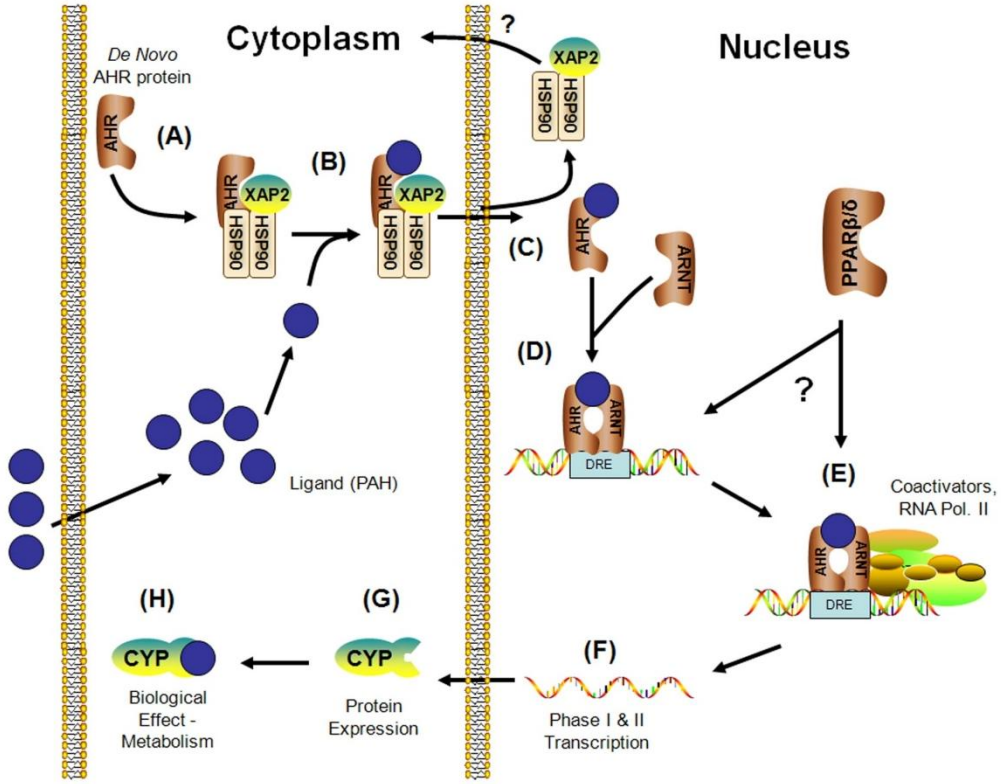

Figure **2.14**: AHR signaling pathway. (A) *De novo* protein synthesis of the AHR and accessory proteins ARNT, HSP90, and XAP2. (B) Ligand binding of AHR to PAH. (C) Nuclear translocation of AHR. (D) Heterodimerization with ARNT and DNA binding. (E) Coactivator recruitment and nucleosome restructuring. (F) Target gene transcription (Phase I and II enzymes). (G) Production of target gene protein. (H) Execution of protein enzymatic function to produce biological effect. The data presented suggests that PPARβ/δ modulates AHR signaling by altering endogenous promoter structure or AHR promoter occupancy in response to PAHs.

Collectively, these studies present a novel mechanism by which PPARβ/δ modulated AHR-dependent signaling in the skin (Figure **2.14)**. The biological effect of this modulation has yet to be established *in vivo*, but *in vitro* evidence indicated that PPARβ/δ-dependent alterations in phase I and II xenobiotic metabolism will translate into differences in skin tumorigenesis. The fact that PPARβ/δ may modulate xenobiotic metabolism mRNA induction by epigenetic regulation is exciting because the

biochemical functions of PPARβ/δ have remained largely undefined. Future studies are necessary to understand the specificity of this modulation and to identify the direct regulatory effectors. Therefore, understanding how PPARβ/δ modulated the promoter structure of xenobiotic metabolism genes could provide greater understanding of PPARβ/δ-dependent anti-tumorigenic effects in the skin (Reviewed in [164]). Since human AHR polymorphisms have not been able to explain the high variability of PAH-mediated *Cyp* mRNA induction to date (Reviewed in [729]), these described observations indicate that PPARβ/δ may be a critical effector of AHR signaling in the skin that contributes to this variability.

Chapter 3

PPARβ/δ MODULATES PAH-INDUCED SKIN CARCINOGENESIS

3.1 Abstract

Peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) is a ligand-activated member of the nuclear hormone receptor superfamily that has emerged as a putative modulator and therapeutic target of skin tumorigenesis. Ligand activation of PPARβ/δ inhibited skin tumorigenesis in two-stage chemical carcinogenesis bioassays by attenuation of cell proliferation, increased terminal differentiation, and reduced inflammatory signaling. These observations indicate that PPARβ/δ can alter tumor promotion. However, whether PPARβ/δ can also modulate tumor initiation has not been examined to date. Results from previous studies revealed that polycyclic aromatic hydrocarbon (PAH) metabolism, bioactivation, and tumor initiation may be modulated by PPARβ/δ. The hypothesis that PPARβ/δ alters PAH-dependent skin tumorigenesis was examined using a complete chemical carcinogen bioassay. Two PAHs that require AHR-dependent bioactivation to cause DNA damage were used and compared to N-methyl-N'-nitro-nitrosoguanidine (MNNG), a carcinogen that does not require AHR-dependent xenobiotic metabolism to mutate DNA. Interestingly, reduced tumorigenecity was observed in *Pparβ/δ*-null mice in response to the PAHs benzo[a]pyrene (B[a]P) and 7,12-dimethylbenz[a]anthracene (DMBA) as compared to wild-type mice. In contrast, *Pparβ/δ*-null mice had enhanced tumorigenecity in response to MNNG as compared to wild-type mice. Histopathological analysis revealed that *Pparβ/δ*-null mice had more malignant tumor types following B[a]P and MNNG carcinogenesis than wild-type mice, but this effect was not observed with DMBA-mediated skin carcinogenesis. Furthermore, *Pparβ/δ*-null mice generally exhibited enhanced infiltration of inflammatory cells within the tumors and in the tumor stroma. Further interrogation into PAH-dependent tumorigenesis revealed that *Pparβ/δ*-null primary keratinocytes possess less B[a]P-

dependent DNA adducts than similarly treated wild-type cells. However, this effect was not due to altered mRNA expression of the DNA damage markers activating transcription factor 3 (*Atf3)* and *p53*. Collectively, the results of these studies indicate that PPARβ/δ-dependent modulation of AHR signaling functionally altered PAH-mediated skin cancer. In the absence of PPARβ/δ expression, AHR-dependent PAH-mediated skin cancer is reduced, likely due to decreased bioactivation of PAHs. However, the results from these studies also demonstrate that PPARβ/δ can modulate tumor promotion based on the finding that MNNG-dependent skin cancer is enhanced in *Pparβ/δ*-null mice. Thus, PPARβ/δ has important functional roles in both tumor initiation and promotion in the skin.

3.2 Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily and function as ligand-activated transcription factors. Upon activation, PPARs cause tissue-specific biological responses via three distinct isoforms, termed PPARα, PPARβ/δ, and PPARγ. Ligand activation of PPARs results in a myriad of biological effects, including modulation of lipid and glucose homeostasis. While the pharmacological functions of PPARα and PPARγ have been extensively examined (Reviewed in [188]), knowledge of PPARβ/δ biological functions and the pharmacological utility of ligands have yet to be conclusively evaluated. PPARβ/δ expression is high in epithelial tissues, such as the skin, and is predominantly found as a nuclear protein in a constitutive complex with its obligate heterodimerization partner retinoid X receptor (RXR) [163]. These described observations indicate that PPARβ/δ is functional in the presence or absence of endogenous ligands and may exert diverse biological effects relative to the occupancy, or lack thereof, of the ligand binding domain.

PPARβ/δ expression is notably higher in many tissues that are highly proliferative and susceptible to cancer, including the liver, colon, lung, and mammary tissues. Because of this, many studies have examined the role of PPARβ/δ expression in cancer or if ligand activation of PPARβ/δ can modulate tumorigenesis. In terms of liver disease and cancer, PPARβ/δ has been predominantly examined in response carbon tetrachloride (CCl_4) and alcohol exposure. Although one report suggested that hepatocyte proliferation is enhanced by PPARβ/δ expression [730], the majority of evidence indicated that PPARβ/δ reduced liver inflammation and damage [499-501, 593, 731, 732] by modulating tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) expression [499, 500, 593] or nuclear factor κB (NFκB) activity [593]. The role of

PPARβ/δ in colon cancer has also been examined, although a consensus function for PPARβ/δ in this model has yet to be achieved. Initial investigations hypothesized that dysregulation of adenomatosis polyposis coli (APC)/β-catenin signaling and cyclooxygenase (COX)-dependent production of endogenous ligands contributed to PPARβ/δ-dependent enhancement of colon tumorigenesis [567, 568, 575, 577, 578, 581]. However, several models of tumorigenesis and ligand treatments have shown that PPARβ/δ reduced colon tumorigenesis or had no effect [413, 508, 513, 582, 585, 586, 733-735]. Similarly, a function for PPARβ/δ in lung and mammary cancer has not reached consensus. Some studies have shown that increased expression and ligand activation of PPARβ/δ enhanced tumorigenesis [547, 589, 736]. In contrast, other studies have reported decreased inflammation and tumorigenesis [588, 737]. Collectively, these findings illustrate the uncertainty of how PPARβ/δ modulates tumorigenesis. Importantly, these receptor-dependent effects must be examined in greater detail before pharmacological targeting of PPARβ/δ can be utilized for the treatment or prevention of diseases.

Non-melanoma skin tumorigenesis is emerging as a world-wide epidemic. While the exact mechanisms of skin cancer are not clearly defined, the relatively high expression PPARβ/δ in the skin suggests that a functional role for this receptor in skin homeostasis exists. Furthermore, elucidating whether PPARβ/δ causes functional effects on skin tumorigenesis may lead to novel preventative or therapeutic approaches. The first report of a PPARβ/δ-dependent function in skin tumorigenesis was that the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) caused exacerbated hyperplasia in *Pparβ/δ*-null mice [412]. This noted hyperplasia in *Pparβ/δ*-null mice has since been confirmed by another laboratory using a *Pparβ/δ* model [506]. The hyperproliferative phenotype of *Pparβ/δ*-null mouse skin has also been localized to the

keratinocyte layer [402, 502, 512, 526]. Further research found that changes in cell proliferation were mediated by PPARβ/δ-dependent regulation of kinase signaling [512, 525, 526] and keratinocyte differentiation [503, 524, 557]. Inflammation is another contributing factor to skin tumorigenesis (Reviewed in [738, 739]), and it is important to note that ligand activation of PPARβ/δ reduced inflammation in the skin [502, 512]. The functions of PPARβ/δ in skin tumorigenesis have also been investigated using two-stage chemical carcinogenesis bioassays. In these studies, ligand activation of PPARβ/δ inhibited the onset of tumor formation, tumor multiplicity, tumor size, and malignant conversion [524, 525, 557, 587]. Co-administration of PPARβ/δ ligands with other chemopreventive or chemotherapeutic approaches, such as COX inhibitors, have also indicated that PPARβ/δ can be cooperatively targeted to enhance cancer therapies [557, 587]. Collectively, these findings demonstrate that PPARβ/δ inhibited skin tumorigenesis by modulation of tumor promotion and inflammation, although the molecular mechanisms of these effects have yet to be functionally defined.

Recent evidence has suggested that PPARβ/δ modulated AHR-dependent signaling in keratinocytes to alter the balance between carcinogen bioactivation and detoxification. To assess whether these described observations result in functional differences in skin tumorigenesis, a complete chemical carcinogen bioassay was performed in wild-type and *Pparβ/δ*-null mice. The PAHs benzo[a]pyrene (B[a]P) and 7,12-dimethylbenz[a]anthracene (DMBA) were used to examine PPARβ/δ-dependent modulated of AHR signaling in the skin carcinogenesis. The carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a carcinogen that is not bioactivated through AHR-dependent signaling, was used to dissect genotype-specific differences in tumor initiation and promotion. Histopathological analyses were also performed to identify

differences in tumor types or malignant conversion between wild-type and *Pparβ/δ*-null mice. Collectively, these studies examined if PPARβ/δ-dependent modulation of AHR signaling results in functional changes in tumorigenesis and malignant conversion.

3.3 Materials and Methods

3.3.1 Chemicals and reagents

B[a]P, DMBA, and MNNG were purchased from Sigma-Aldrich (St. Louis, MO). All primers for quantitative real-time PCR (qPCR) were purchased from Integrated DNA Technologies (IDT, Coralville, IA).

3.3.2 Complete chemical carcinogenesis bioassay

Female, wild-type (+/+) and *Pparβ/δ*-null (–/–) mice [412], in the resting phase of the hair cycle (6-8 weeks of age) were shaved and topically treated weekly with 100 µg of DMBA or B[a]P or 300 µg of MNNG dissolved in 200 µL acetone. The B[a]P and DMBA dosing regimen was chosen based on previous bioassays completed in a mouse strain of similar sensitivity as the C57BL/6 strain [740-742]. The studies were carried out for 27 weeks (DMBA) or 34 (B[a]P) weeks, respectively. This time frame is known to result in one hundred percent carcinoma incidence and multiple carcinomas per mouse [742]. The MNNG dosing was chosen based on a previous study demonstrating that a high percentage of carcinoma incidence and multiplicity can be observed within 25 weeks [743] in a mouse strain of similar sensitivity as the C57BL/6 strain [740]. The onset of lesion formation, lesion number, and lesion size was assessed weekly, and mice were euthanized by overexposure to carbon dioxide at the end of the study. Lesions of greater than 2 mm in diameter were fixed in 10% neutral-buffered formalin, stored in 70% ethanol, and then paraffin embedded. Tissue sections were stained with hematoxylin and eosin (H&E) and were examined by a pathologist. The tumor

classifications defined were squamous cell carcinoma (SCC), basal cell tumor (BCT), keratoacanthoma (KA), papilloma (Pap), SC hyperplasia, and BC hyperplasia.

3.3.3 Primary keratinocyte culture and treatment

Primary keratinocytes from wild-type (+/+) and *Pparβ/δ*-null (–/–) mice were isolated from newborn skin and cultured as previously described [692]. Keratinocytes were cultured in low calcium (0.05 mM) Eagle's minimal essential medium supplemented with 8% chelexed fetal bovine serum (FBS), 20 IU/mL penicillin, and 20 µg/mL streptomycin. Keratinocytes were cultured at 37°C and 5% CO_2 until 80-90% confluence. Cells were then treated for 8 h or 24 h with vehicle (0.02% DMSO) or 1 µM B[a]P. The 8 h treatment was examined because previous studies have demonstrated that attenuated PAH-mediated inducibility of *Cyp1a1* and *Cyp1b1* mRNA occurs in *Pparβ/δ*-null keratinocytes at this time. The 24 h treatment for mRNA and DNA adduct analysis was chosen because DNA adduct formation has been observed at this time in cultured mouse keratinocytes [685, 744].

3.3.4 RNA isolation and qPCR

Total RNA from keratinocytes was prepared using RiboZol RNA Extraction Reagent (AMRESCO, Solon, OH) and the manufacturer's recommended protocol. The mRNA encoding *p53* and activating transcription factor 3 (*Atf3*) were measured by qPCR analysis. cDNA was generated from 2.5 µg of total RNA using MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). The qPCR analysis was carried out using SYBR® Green PCR Supermix for IQ (Quanta Biosciences, Gaithersburg, MD)

in the iCycler and detected using the MyiQ Realtime PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The following PCR reaction was used for all genes: 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, repeated for 45 cycles. Each PCR reaction included a no-template control reaction to control for contamination, and all real-time PCR reactions had greater than 85% efficiency. The relative mRNA value for each gene was normalized to the relative mRNA value for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The following primers were used to assess the relative mRNA expression of *Gapdh* (NM_008084), *Atf3* (NM_007498), and *p53* (NM_001127233): 5'-AAATGGTGAAGGTCGGTGTGAACG (*Gapdh* forward), 5'-TGGCAACAATCTCCACTTTGCCAC (*Gapdh* reverse), 5'-TCAAGGAAGAGCTGAGATTCGCCA (*Atf3* forward), 5' GTTTCGACACTTGGCAGCAGCAAT (*Atf3* reverse), 5'-AAAGGATGCCCATGCTACAGAGGA (*p53* forward), and 5'-GCAGTTTGGGCTTTCCTCCTTGAT (*p53* reverse).

3.3.5 B[a]P DNA adduct post-labeling

Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were isolated and propagated, as described in Materials and Methods, to examine B[a]P-dependent DNA adduct formation. Briefly, triplicate 100 mm dishes of wild-type and *Pparβ/δ*-null primary keratinocytes were treated for 24 h with 1 μM B[a]P. Genomic DNA was isolated using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI) and the manufacturer's recommended protocol. Five μg of genomic DNA was labeled with [γ-^{32}P]-ATP and polynucleotide kinase as previously described [745-747]. Ammonium sulfate thin layer chromatography was used to separate and identify [γ-^{32}P]-labeled

B[a]P-adducted nucleotides as compared to standards. The labeled signal was quantified and normalized to the total amount of nucleotides examined, being represented as adducts per 10^9 nucleotides.

3.3.6 Statistical analyses

Tumor data and DNA adduct data were analyzed for statistical significance using Fischer's exact test or Student's t-test as described in the figure legends. qPCR data was analyzed for statistical significance using two-way analysis of variance (ANOVA) with the Bonferroni's multiple comparison test as described in the figure legends. All results are reported as percentage incidence or mean ± S.E.M. using Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

3.4 Results

3.4.1 PPARβ/δ modulates PAH-dependent skin chemical carcinogenesis

To determine if modulation of AHR signaling by PPARβ/δ functionally alters skin tumorigenesis, a complete carcinogen bioassay was performed in wild-type and *Pparβ/δ*-null mice. The PAHs B[a]P and DMBA were both examined to determine if differential bioactivation of these compounds (Reviewed in [638]) caused PPARβ/δ-dependent differences in skin tumorigenesis. MNNG, a chemical carcinogen that does not bind AHR and is not metabolized by the cytochrome P450 enzymes, was also examined to identify PPARβ/δ-dependent modulation of tumor promotion during complete chemical carcinogenesis.

PAH complete chemical carcinogenesis resulted in tumor formation in both genotypes, and several genotype-dependent differences in tumorigenesis were observed between wild-type and *Pparβ/δ*-null mice. The onset of tumor formation was similar between genotypes treated with either B[a]P or DMBA (Figure **3.1A, B**). A significant increase in lesion multiplicity was observed in wild-type mice compared to *Pparβ/δ*-null mice (Figure **3.1D, E**), and this effect was observed with both B[a]P and DMBA (Figure **3.1D, E**). It is also worth noting that the differences in lesion multiplicity were not observed until the late stage of the bioassay, specifically the final 2 weeks for B[a]P treatment and the final 5 weeks for DMBA treatment. Interestingly, there was a significant increase in average lesion size in *Pparβ/δ*-null mice treated with B[a]P for the last five weeks of the bioassay (Figure **3.1G**). In contrast to the effects found with B[a]P and DMBA, the onset of lesion formation was earlier in *Pparβ/δ*-null mice treated with

MNNG (Figure **3.1C**). Lesion multiplicity and average lesion size were also found to be enhanced in *Pparβ/δ*-null mice treated with MNNG as compared to wild-type mice (Figure **3.1F, I**). The enhanced tumorigenesis in *Pparβ/δ*-null mice was not observed until the latter third of the bioassay, with a significant difference in lesion onset from week 16 to 21 (Figure **3.1C**) and a significant difference in lesion multiplicity and average lesion size from week 21 to 25 (Figure **3.1F, I**). Collectively, the described observations revealed a distinct PPARβ/δ-dependent alteration in lesion multiplicity, as wild-type mice exhibited increased PAH-mediated lesion multiplicity and *Pparβ/δ*-null mice exhibited increased MNNG tumorigenesis.

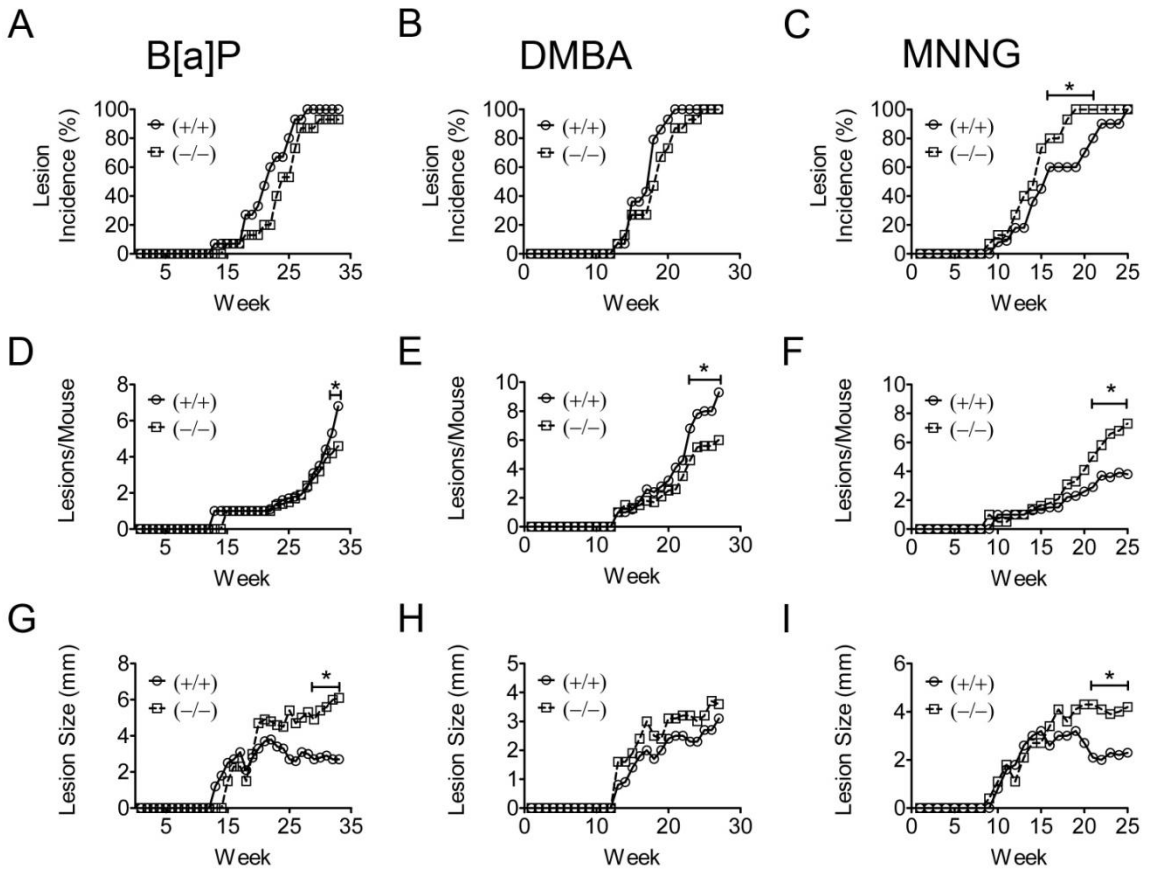

Figure **3.1**: PPARβ/δ modulates complete skin carcinogenesis. Complete chemical carcinogen testing was performed on wild-type (+/+) and *Pparβ/δ*-null (–/–) as described in the Materials and Methods. (A-C) The incidence and onset of lesion formation in mice treated with B[a]P (A), DMBA (B), or MNNG (C). (D-F) Skin lesion multiplicity in mice treated with B[a]P (D), DMBA (E), or MNNG (F). (G-I) Average skin lesion size in mice treated with B[a]P (G), DMBA (H), or MNNG (I). An asterisk, *, indicates a statistically significant, $p < 0.05$, difference between genotypes as calculated by Fischer's exact test (A-C) or one tailed Student's t-test (D-I).

3.4.2 PPARβ/δ modulates malignant conversion and inflammation

Histopathological analysis was performed to determine if differences in tumor lesions existed between the genotypes. Of particular interest with complete carcinogen bioassays was the presence of malignant tumor types [740], including squamous cell

carcinomas (SCCs), basal cell tumors (BCTs), and keratoacanthomas (KAs). Malignant tumor types are commonly generated in complete chemical carcinogen bioassays, as opposed to the propensity for non-malignant papillomas typically observed in two-stage chemical carcinogenesis bioassays [619, 740, 741]. The percentage of mice with SCCs was greater in *Pparβ/δ*-null mice treated with B[a]P as compared to similarly treated wild-type mice (Figure **3.2A**). However, the average number of SCCs per mouse was not different between wild-type and *Pparβ/δ*-null mice treated with B[a]P (Figure **3.2B**). While the percentage of mice with BCT was not different between wild-type and *Pparβ/δ*-null mice treated with either PAH (Figure **3.2C**), DMBA treatment resulted in more BCTs per mouse in *Pparβ/δ*-null mice than wild-type mice (Figure **3.2D**). The percentage of mice with KAs was also found to be greater in *Pparβ/δ*-null mice treated with both PAHs as compared to similarly treated wild-type mice (Figure **3.2E**). *Pparβ/δ*-null mice treated with both PAHs also exhibited greater KAs per mouse as compared to wild-type mice (Figure **3.2F**). PPARβ/δ-dependent alterations in tumor malignancy were also observed with MNNG treatment, with *Pparβ/δ*-null mice having a greater percentage of SCCs and KAs (Figure **3.2A, E**) than similarly treated wild-type mice. Moreover, *Pparβ/δ*-null mice treated with MNNG possessed more SCCs and KAs per mouse than similarly treated wild-type mice (Figure **3.2B, F**).

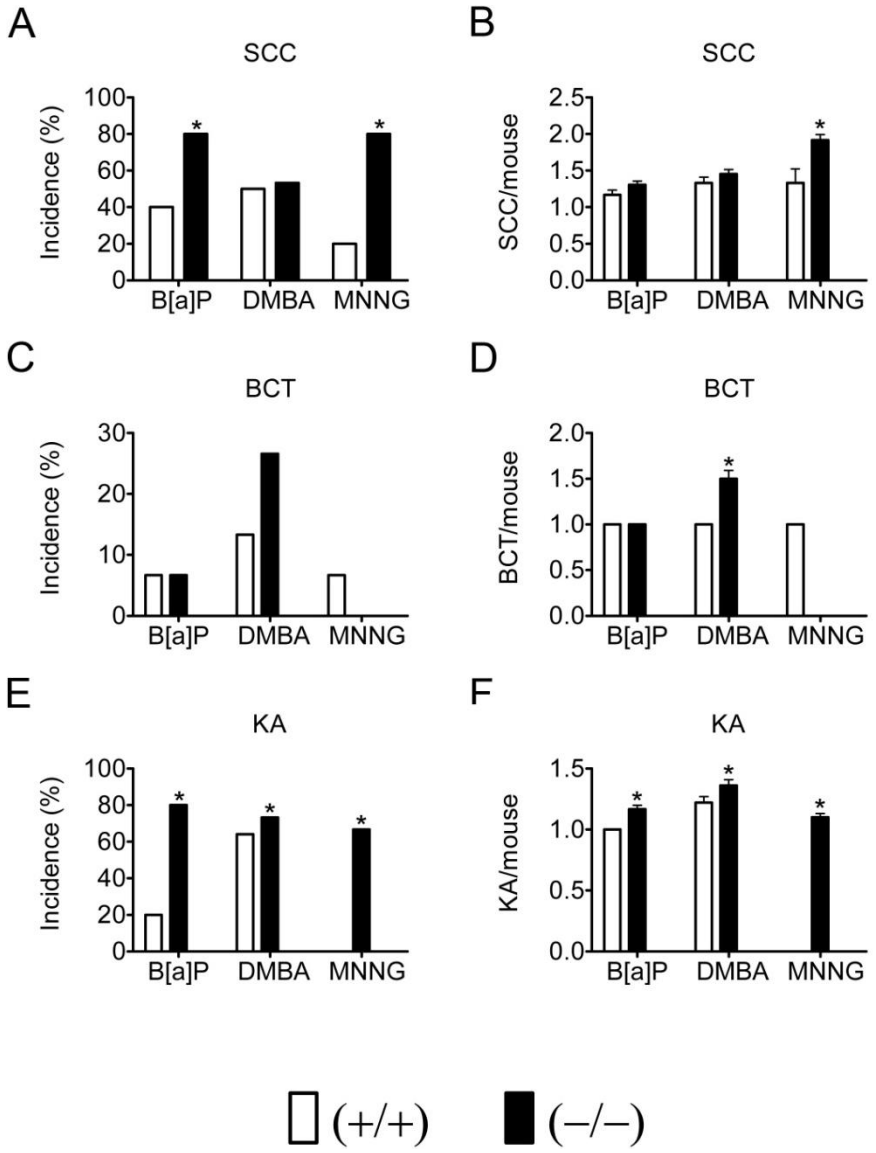

Figure **3.2**: PPARβ/δ modulates chemically-induced tumor malignancy. Complete chemical carcinogen testing was performed on wild-type (+/+) and *Pparβ/δ*-null (–/–) as described in the Materials and Methods. Lesions were examined and classified by a pathologist. (A, C, E) The incidence of SCCs (A), BCTs(C) and KAs (E) in mice treated with B[a]P, DMBA, MNNG. (B, D, F) The multiplicity of SCCs (B), BCTs (D) and KAs (F) in mice treated with B[a]P, DMBA, or MNNG. An asterisk, *, indicates a statistically significant, $p < 0.05$, difference between genotypes as calculated by Fischer's exact test (A, C, E) or one tailed Student's t-test (B, D, F).

Examination of the distribution of tumor pathologies also revealed interesting observations. The distribution of tumor pathologies observed in response to B[a]P were found to be different between wild-type and *Pparβ/δ*-null mice (Figure **3.3A**). A higher incidence of malignant tumor types was observed in *Pparβ/δ*-null mice treated with B[a]P as compared to wild-type mice (Figure **3.3B**). This was due to a higher incidence of KAs (26% versus 13%) and SCCs (32% versus 29%) in *Pparβ/δ*-null mice (Figure **3.3A**). In contrast, the distribution of tumor pathologies was not different between wild-type and *Pparβ/δ*-null mice treated with DMBA (Figure **3.4**). Striking differences in the distribution of tumor pathologies were also observed in response to MNNG (Figure **3.5A**). A higher percentage of malignant tumor types were found in MNNG-treated *Pparβ/δ*-null mice as compared to similarly treated wild-type mice (Figure **3.5B**). The malignant shift in *Pparβ/δ*-null mice was due to a greater conversion of SCC hyperplasia (orange) to SCCs (blue) and the higher percentage of KAs (green) as compared to wild-type mice (Figure **3.5A**). In general, the alterations in tumor pathologies by B[a]P and MNNG in *Pparβ/δ*-null mice were due to increased SCC conversion (Figure **3.3A** and **3.5A**). Representative tumor histopathology shows the increased epidermal invasion (E) into the muscle tissues (M) that was observed in *Pparβ/δ*-null mice treated with B[a]P and MNNG more often than similarly treated wild-type mice (Figure **3.6**).

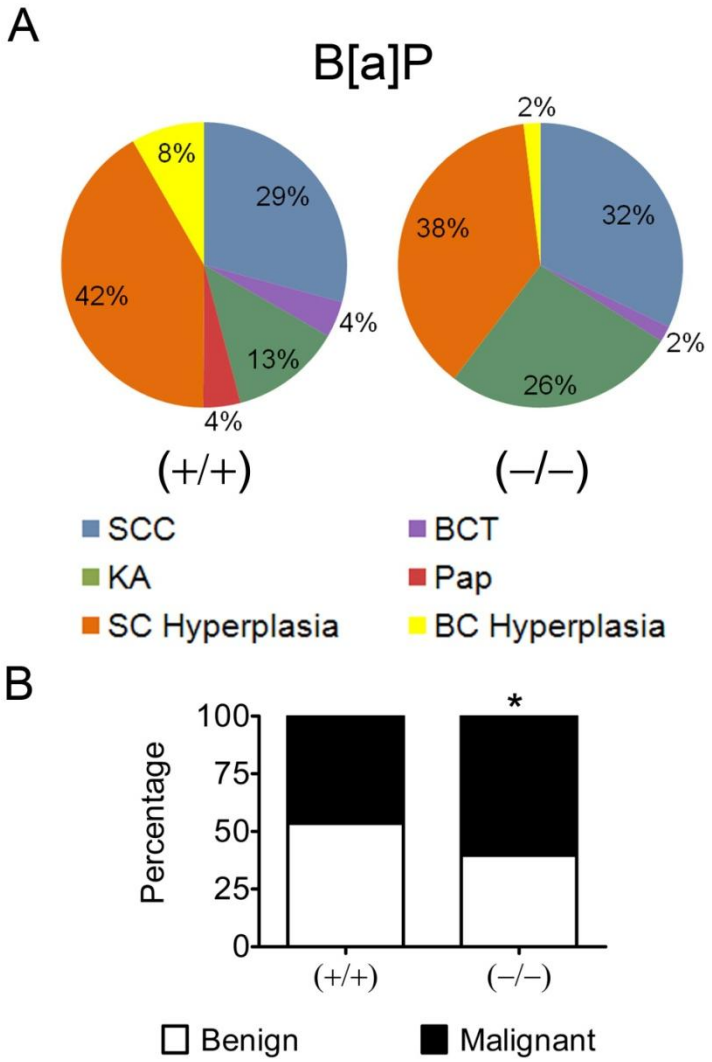

Figure **3.3**: PPARβ/δ modulates B[a]P-induced skin tumor distribution. Complete chemical carcinogen testing, utilizing B[a]P, was performed on wild-type (+/+) and *Pparβ/δ*-null (–/–) mice as described in the Materials and Methods. (A) The percentage of each pathologically classified tumor type (SCCs, BCTs, KAs, Paps, SC hyperplasias, or BC hyperplasias) in each genotype, as a percentage of total genotype tumors. (B) The percentage of pathologically examined tumors being classified as malignant (SCCs, BCTs, KAs) or benign (Paps, SC hyperplasias, and BC hyperplasias) in each genotype, as a percentage of total genotype tumors. An asterisk, *, indicates a statistically significant, $p < 0.05$, difference between genotypes as calculated by Fischer's exact test.

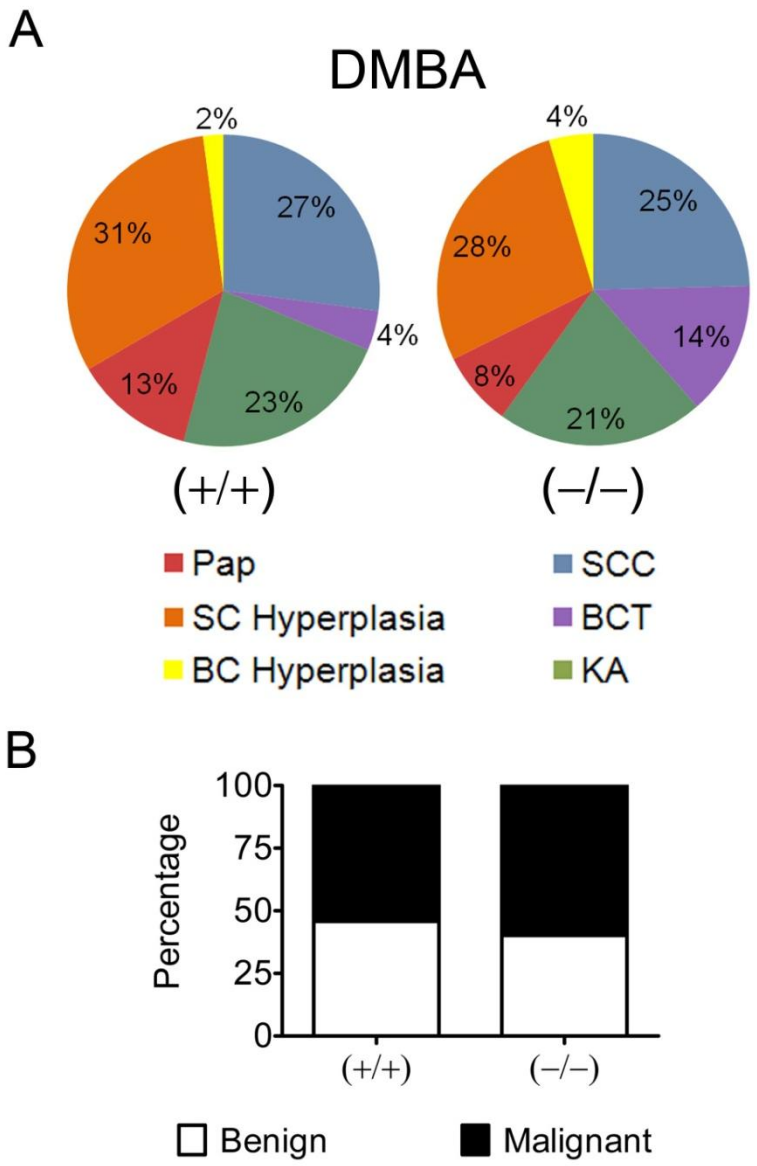

Figure **3.4**: PPARβ/δ does not modulate DMBA-induced skin tumor distribution. Complete chemical carcinogen testing, utilizing DMBA, was performed on wild-type (+/+) and *Pparβ/δ*-null (–/–) mice as described in the Materials and Methods. (A) The percentage of each pathologically classified tumor type (SCCs, BCTs, KAs, Paps, SC hyperplasias, or BC hyperplasias) in each genotype, as a percentage of total genotype tumors. (B) The percentage of pathologically examined tumors being classified as malignant (SCCs, BCTs, KAs) or benign (Paps, SC hyperplasias, and BC hyperplasias) in each genotype, as a percentage of total genotype tumors. An asterisk, *, indicates a statistically significant, $p < 0.05$, difference between genotypes as calculated by Fischer's exact test.

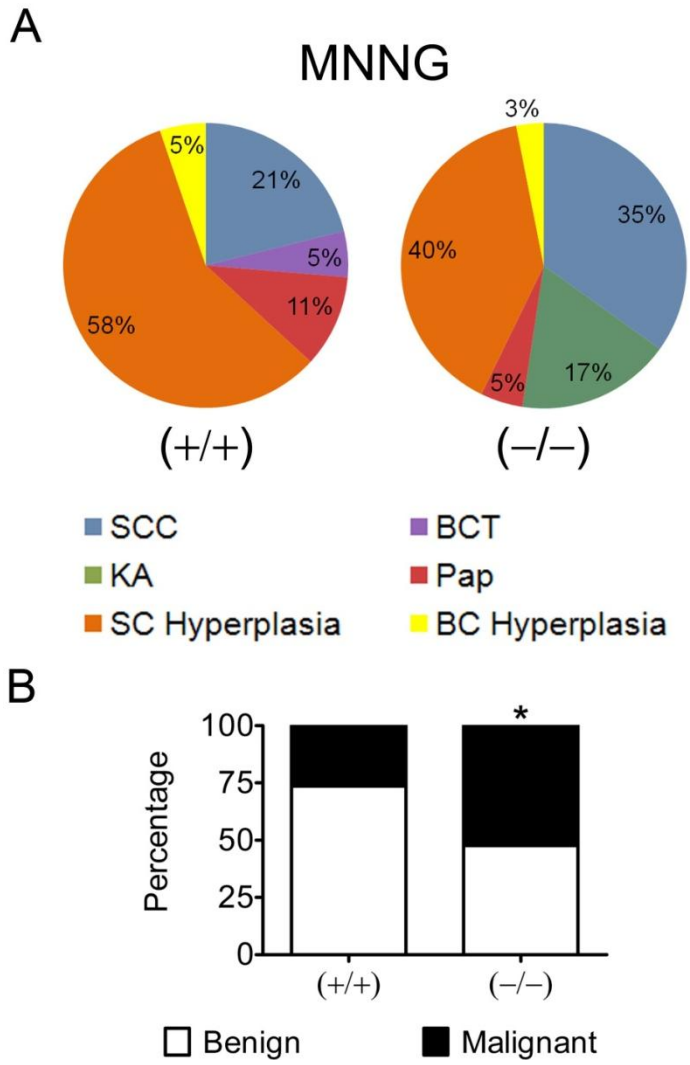

Figure **3.5**: PPARβ/δ modulates MNNG-induced skin tumor distribution. Complete chemical carcinogen testing, utilizing MNNG, was performed on wild-type (+/+) and *Pparβ/δ*-null (–/–) mice as described in the Materials and Methods. (A) The percentage of each pathologically classified tumor type (SCCs, BCTs, KAs, Paps, SC hyperplasias, or BC hyperplasias) in each genotype, as a percentage of total genotype tumors. (B) The percentage of pathologically examined tumors being classified as malignant (SCCs, BCTs, KAs) or benign (Paps, SC hyperplasia, and BC hyperplasias) in each genotype, as a percentage of total genotype tumors. An asterisk, *, indicates a statistically significant, $p < 0.05$, difference between genotypes as calculated by Fischer's exact test.

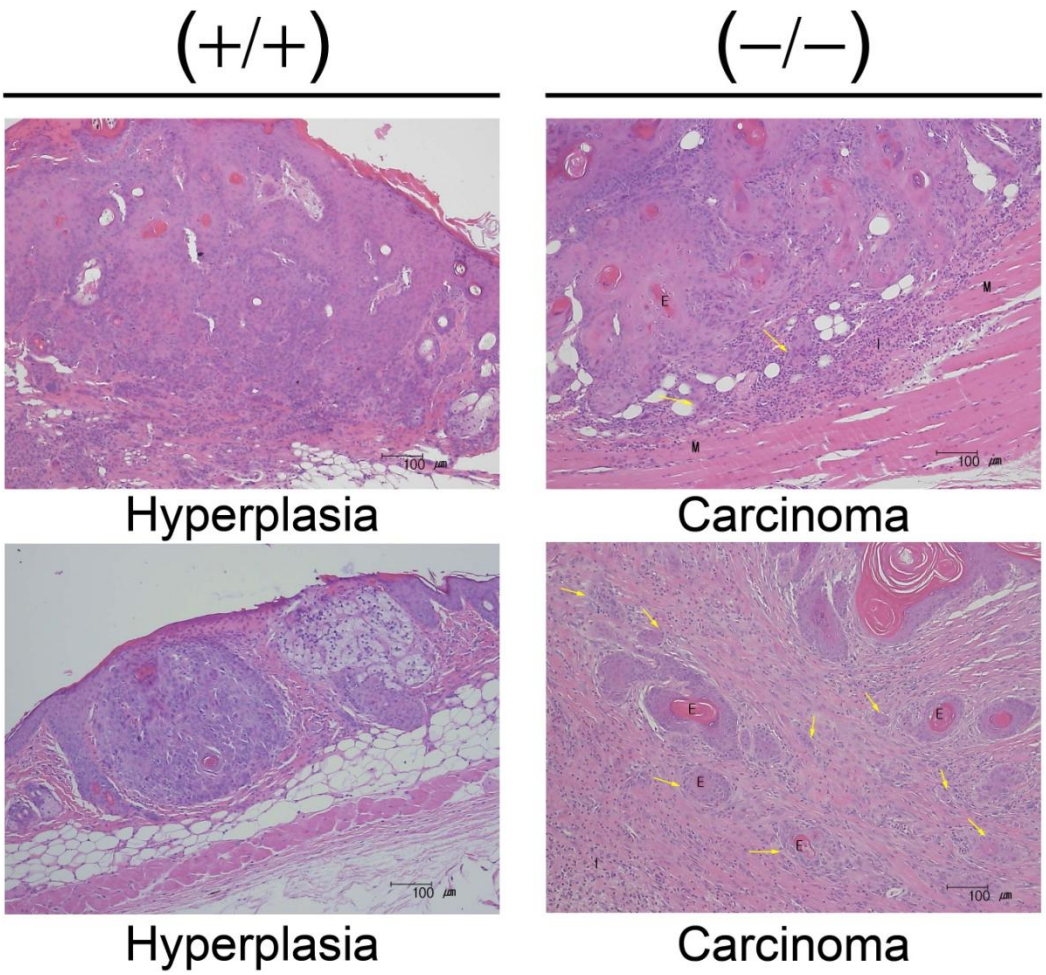

Figure **3.6**: PPARβ/δ modulates tumor invasiveness. Representative histology demonstrating enhanced tumor invasion in B[a]P-treated *Pparβ/δ*-null (–/–) mice as compared to similarly treated wild-type (+/+) mice. Arrows indicate tumor invasion from epidermis (E) to underlying muscle (M) tissue.

Inflammation has also been implicated in cancer progression and metastasis in many organ model systems (Reviewed in [738, 739]). Examination of tumor pathologies revealed that *Pparβ/δ*-null mice had increased incidence of inflammatory cell infiltrates within the tumor and in the tumor stroma (Figure **3.7**). While the severity of inflammation was not quantified, this observation supports previously described PPARβ/δ-dependent anti-inflammatory functions in the skin [502, 512].

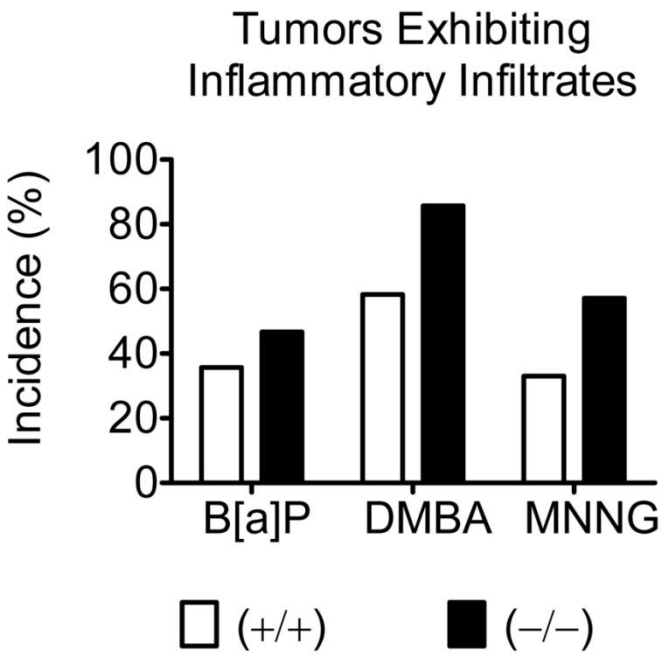

Figure **3.7**: PPARβ/δ reduces tumor inflammation. Complete chemical carcinogen testing was performed on wild-type (+/+) and *Pparβ/δ*-null (–/–), as described in the Materials and Methods. Lesions were examined microscopically, and the incidence of tumors presenting with inflammatory infiltrates was determined. There was not a statistical difference between the genotypes when analyzed by Fischer's exact test.

3.4.3 PPARβ/δ alters DNA damage

To determine whether the increased PAH-dependent lesion multiplicity in wild-type mice was due to genotoxic damage, the expression of DNA damage markers and DNA adduct formation was examined in wild-type and *Pparβ/δ*-null primary keratinocytes. The PAH-mediated mRNA induction of DNA damage markers *Atf3* and *p53* were examined at 8 and 24 h. The mRNA expression of *Atf3* and *p53* were unchanged in both genotypes following B[a]P treatment (Figure **3.8A, B**). Furthermore, mRNA changes were not observed at either the 8 or 24 h B[a]P exposures (Figure **3.8A, B**). Despite the lack of *Atf3* and *p53* mRNA induction upon PAH exposure, the ability of

PPARβ/δ to alter DNA adduct formation was examined in keratinocytes using a [γ-^{32}P]-B[a]P-postlabeling method [745-747]. There was not a significant difference in adduct formation between the genotypes (Figure **3.9**). However, a p-value of 0.07 between the DNA adduct values for wild-type and *Pparβ/δ*-null keratinocytes suggests that there was decreased DNA adduct formation in *Pparβ/δ*-null keratinocytes. Overall, PPARβ/δ did not modulate the mRNA expression of DNA damage markers *Atf3* and *p53* in response to B[a]P (Figure **3.8A, B**). However, *Pparβ/δ*-null keratinocytes appeared to have decreased B[a]P-dependent DNA adduct formation (Figure **3.9**). These observations indicated that DNA adduct formation is a contributing mechanism to the decreased PAH-dependent lesion multiplicity observed in *Pparβ/δ*-null mice (Figure **3.1D, E**).

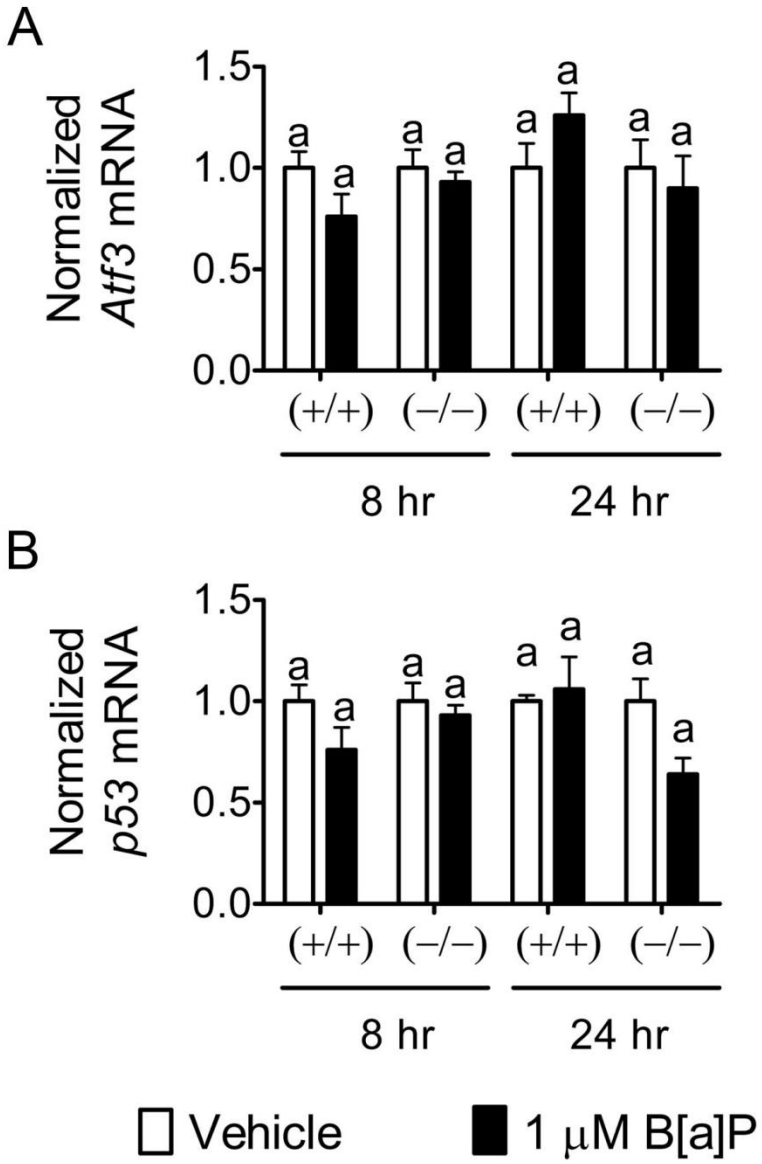

Figure **3.8**: PAH-dependent induction of DNA damage markers is not modulated by PPARβ/δ expression in primary keratinocytes. Wild-type (+/+)and *Pparβ/δ*-null (–/–) primary keratinocytes were treated 8 or 24 h with vehicle (0.02% DMSO) or 1 μM B[a]P as described in Materials and Methods. (A, B) qPCR was performed using total RNA isolated from primary keratinocytes to quantify the expression of *Atf3* (A) or *p53* (B). Values are normalized to (+/+) vehicle control and present mean ± S.E.M. of N = 3 biological replicates. Different letters indicate statistically different, $P < 0.05$, groups, as determined by Bonferroni's multiple comparison test.

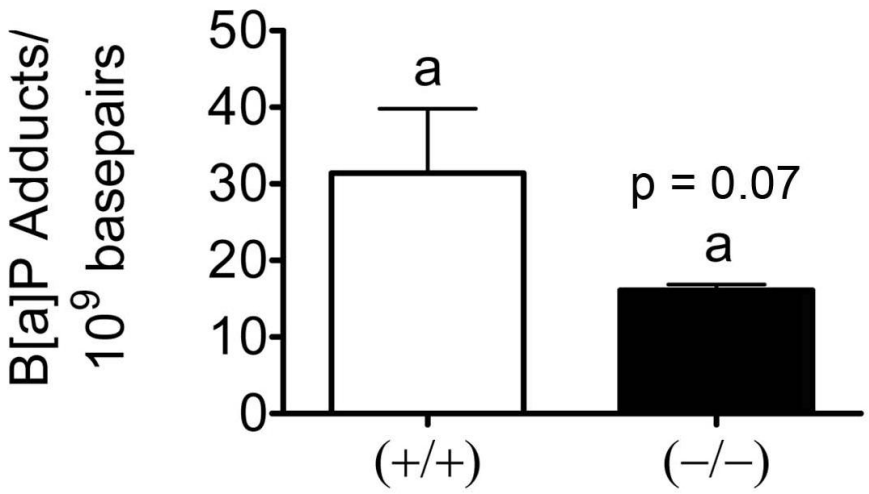

Figure **3.9**: PAH-dependent DNA adduct formation is reduced in *Pparβ/δ*-null keratinocytes. Wild-type (+/+) and *Pparβ/δ*-null (–/–) primary keratinocytes were treated 24 h with 1 μM B[a]P and subjected to the [γ-^{32}P]-post-labeling protocol, as described in Materials and Methods. Quantitative DNA adduct formation was determined by [γ-^{32}P]-post-labeling of B[a]P-adducted nucleotides. Values are normalized to (+/+) nucleotide content and represent the mean ± S.E.M. of quantified adducts per 10^9 basepairs from N = 3 biological replicates. The one-tailed Student t-test p-value of the difference was 0.07.

3.5 Discussion

These described studies mark the first time a complete chemical skin carcinogenesis bioassay has been performed in *Pparβ/δ* mouse models. Previous studies in primary keratinocytes have shown that PPARβ/δ modulated the PAH-mediated mRNA induction of *Cyp1a1* and *Cyp1b1* through alteration of AHR signaling. The purpose of these investigations was to examine if modulation of AHR signaling by PPARβ/δ in keratinocytes caused functional alterations in PAH-dependent skin tumorigenesis. Indeed, *Pparβ/δ*-null mice exhibited reduced lesion multiplicity upon B[a]P and DMBA treatment as compared to similarly treated wild-type mice (Figure **3.1D, E**). This observation was likely due to decreased PAH bioactivation, and *in vitro* DNA adduct analysis demonstrated that *Pparβ/δ*-null keratinocyte possessed less B[a]P-dependent DNA adducts than similarly treated wild-type keratinocytes (Figure **3.8C**). Previous evidence has also described that PPARβ/δ modulated AHR signaling in human keratinocytes. Because decreased PAH-dependent AHR signaling in *Pparβ/δ*-null keratinocytes resulted in a reduction of lesion multiplicity in *Pparβ/δ*-null mice, it is plausible that human PAH-dependent skin carcinogenesis would also be modulated by PPARβ/δ expression. Interestingly, the induction of *Cyp1a1* and *Cyp1b1* mRNAs in response to PAHs are greatly varied in humans, but AHR polymorphisms have not been identified that account for this variability. The fact that PPARβ/δ modulated AHR signaling in human keratinocytes suggests that PPARβ/δ contributes to the variation in human PAH-mediated *Cyp* mRNA induction. Collectively, the results of these studies indicated that PPARβ/δ-dependent modulation of AHR signaling leads to functional differences in murine tumor initiation and susceptibility to skin chemical carcinogenesis.

The use of the chemical carcinogen MNNG greatly enhanced these studies by allowing tumor initiation and promotion to be specifically dissected in *Pparβ/δ* mouse models. MNNG caused tumor initiation and mutagenesis through DNA methylation. Therefore, MNNG carcinogenesis does not require AHR-dependent induction of xenobiotic metabolism enzymes for bioactivation, and therefore caused cancer through a mechanism that is independent of the AHR. Surprisingly, *Pparβ/δ*-null mice treated with MNNG exhibited an earlier onset of lesion formation, enhanced lesion multiplicity, and enhanced average lesion size than wild-type mice. Additionally, the resulting tumor pathology revealed that MNNG-treated *Pparβ/δ*-null mice had more malignant tumor types than wild-type mice. The results from these studies indicate that PPARβ/δ modulated tumor promotion, which validated previously described PPARβ/δ-dependent reductions in two-stage skin chemical carcinogenesis and malignant conversion [524, 525, 557, 587]. Enhanced average tumor size and malignant conversion were also observed in *Pparβ/δ*-null mice treated with B[a]P as compared to wild-type mice. Surprisingly, this observation revealed that *Pparβ/δ*-null mice exhibited reduced PAH-mediated lesion multiplicity, yet possessed enhanced B[a]P-dependent average lesion size. Collectively, PPARβ/δ modulated tumor promotion and further suggests a paradoxical role of PPARβ/δ in modulating skin tumor initiation and promotion.

The complete chemical carcinogen bioassay used in these studies specifically examined two modes of DNA mutagenesis. Surprisingly, PPARβ/δ differentially modulated these modes of skin carcinogenesis. PAHs cause mutagenesis through P450 bioactivation and subsequent DNA adduct formation (Reviewed in [748]). While PAHs such as B[a]P and DMBA are structurally similar, these two carcinogens are differentially metabolized by P450s to form the ultimate carcinogens benzo[a]pyrene-7,8-diol-9,10-

epoxide (BPDE) and DMBA-3,4-diol-1,2-epoxide (DMBADE) [749-752]. The bulky DNA adducts formed by PAHs are commonly repaired by the nucleotide excision repair (NER) system [753]. MNNG mutations, on the other hand, are commonly repaired by the base excision repair (BER) system (Reviewed in [639]). The fact that PAHs and MNNG had opposing effects on lesion multiplicity between wild-type and *Pparβ/δ*-null mice indicates that NER and/or BER may be regulated by PPARβ/δ. This hypothesis has never been specifically examined, but receptor-dependent alterations of BER and/or NER could explain the documented PPARβ/δ-dependent differences in chemical carcinogenesis observed in several tissues (Reviewed in [164]).

PPARβ/δ has also been shown to reduce inflammation in several models of tumorigenesis and organ damage [499-501, 508, 513, 524, 557, 582, 585-587, 593, 731, 732]. The observed increase in inflammatory infiltrates within the tumor and in the tumor stroma of *Pparβ/δ*-null mice confirmed these previous conclusions (Figure **3.87**). Inflammation is a known contributing factor to tumorigenesis (Reviewed in [738, 739]), although the complete mechanisms have yet to be delineated. It is known that diverse inflammatory cell populations alter the tumor environment, thus affecting invasion and chemotherapeutic treatments (Reviewed in [738, 739]). The fact that wild-type mice had reduced inflammatory cells infiltrates indicated that inflammatory signaling or the populations of skin inflammatory cells may be modulated by PPARβ/δ. While immuno-phenotyping of wild-type and *Ppparβ/δ*-null mice has not thoroughly established genotype-dependent changes in immune cell populations, preliminary evidence revealed that PPARβ/δ modulated immunological inflammatory responses (Reviewed [164, 718]). Identifying differential PPARβ/δ immune responses in skin tumorigenesis, invasion, and metastasis would drastically shift the biological relevance and therapeutic targeting of

this nuclear receptor. Additionally, the complete carcinogen bioassay would be a highly useful method to assess the *in vivo* effects of PPARβ/δ-dependent alteration in tumor immunity.

Collectively, the results from these studies indicated that PPARβ/δ-dependent modulation of AHR signaling in keratinocytes leads to functional differences in skin tumorigenesis. Reduced PAH-mediated lesion multiplicity was observed in *Pparβ/δ*-null mice, and this appears to be caused by decreased DNA adduct formation and tumor initiation. The use of two classes of chemical carcinogens also revealed that PPARβ/δ modulated both tumor initiation and tumor promotion in the skin. Further studies are necessary to delineate the molecular mechanisms mediating these effects, but preliminary observations indicated that tumor inflammation or differences in inflammatory responses contributed to the enhanced tumor malignancy in *Pparβ/δ*-null mice. It should also be emphasized that these differences in tumorigenesis between wild-type and *Pparβ/δ*-null mice were observed in the absence of exogenous PPARβ/δ ligands. Future studies should focus on delineating the effects of PPARβ/δ ligands or combined chemotherapeutic regimens on skin complete chemical carcinogenesis in *Pparβ/δ* mouse models. Collectively, the results of these studies indicated that PPARβ/δ is a critical effector of murine skin tumorigenesis by modulating tumor initiation, promotion, and inflammation. Given that PPARβ/δ also modulated AHR signaling in human keratinocytes, further investigations are necessary to determine whether the described PPARβ/δ-dependent alterations in murine skin tumorigenesis will exist in humans.

Chapter 4

DISCUSSION

Since it was first cloned nearly two decades ago [153, 155, 157], the biological functions of the nuclear hormone receptor peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) have remained a mystery. The last decade has seen great advancements in the knowledge of this PPAR isoform, and this can be attributed to the development of *Pparβ/δ*-null mouse models [412-414] and high affinity synthetic ligands [159, 188, 754]. The use of these novel tools has implicated PPARβ/δ in a number of biological processes, including glucose and lipid homeostasis, inflammation, and carcinogenesis (Reviewed in [164, 188]). PPARβ/δ is now recognized as a critical metabolic regulator and has the potential to be a therapeutic target in metabolic diseases such as diabetes and obesity. Recent evidence has also suggested anti-inflammatory effects for PPARβ/δ ligands that may influence metabolic disease progression and cancer (Reviewed in [164, 718]). In keratinocytes, ligand activation of PPARβ/δ increased terminal differentiation, attenuated cell proliferation, and increased apoptosis (Reviewed in [164). In contrast, it has been suggested that PPARβ/δ ligands can reduce apoptosis and increase cell proliferation (Reviewed in [164]). Therefore, clarifying the role of PPARβ/δ in proliferation and tumorigenesis is vital so that this nuclear receptor can be targeted for disease prevention and treatments (Reviewed in [164]).

The function of PPARβ/δ in tumorigenesis has mostly been investigated in highly proliferative epithelial tissues, such as the colon and skin. The role of PPARβ/δ in colon carcinogenesis remains unclear. Through the use of multiple *in vivo* and *in vitro* models, the greater breadth of investigations have suggested that ligand activation of PPARβ/δ attenuated colon tumorigenesis (Reviewed in [164, 755]). In contrast, the biological role of PPARβ/δ in skin tumorigenesis appears to be more clearly defined. In response to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), *Pparβ/δ*-null mice had

exacerbated epidermal hyperplasia as compared to wild-type mice [412, 506]. Furthermore, *Pparβ/δ*-null primary keratinocytes have been shown to proliferate faster than wild-type cells [402, 406, 512, 526]. Mouse models of skin tumorigenesis have also consistently observed that ligand activation of PPARβ/δ reduced tumorigenesis [524, 525, 557, 587]. These anti-tumorigenic effects have been attributed to increased terminal differentiation [512, 524] and decreased keratinocyte proliferation [525, 526]. Results of these studies indicated a functional role for PPARβ/δ in altering tumor promotion, but tumor initiation, another critical component of tumorigenesis, has never been examined. The purpose of the described studies was to examine if PPARβ/δ modulates xenobiotic metabolism *in vitro* and to define if this modulation results in functional alterations in skin tumorigenesis. Collectively, mouse and human keratinocyte models were used to delineate the signaling pathway modulated by PPARβ/δ *in vitro*, and the functional significance of this modulation was examined using *Pparβ/δ* mouse models and a complete carcinogen bioassay.

The studies described in chapter 2 and 3 were specifically designed to examine if PPARβ/δ modulates skin tumor initiation. These analyses were dissected into an *in vitro* characterization and an *in vivo* analysis of skin tumorigenesis. First, the *in vitro* component determined that *Pparβ/δ*-null mice have reduced polycyclic aromatic hydrocarbon (PAH)-dependent cytochrome P450 (*Cyp*) mRNA induction by attenuation of aryl hydrocarbon receptor (AHR) signaling. These effects were found to be caused by reduced occupancy of AHR at the *Cyp1a1* promoter in response to PAHs and by epigenetic regulation of phase I mRNA expression. Secondly, the functional significance of reduced PAH-dependent AHR signaling in *Pparβ/δ*-null mice was examined using a complete carcinogen bioassay. These studies indicated that decreased AHR signaling in

Pparβ/δ-null mice resulted in decreased PAH-mediated lesion multiplicity that can be attributed to decreased DNA adduct formation. Increased tumor size was also observed in *Pparβ/δ*-null treated with B[a]P and MNNG, and this observation suggests that is also a potent modulator of tumor promotion. Thus, PPARβ/δ can modulate two critical components of skin tumorigenesis, namely tumor initiation and promotion.

Several conclusions can be drawn from the studies described in chapters 2 and 3. First, PPARβ/δ modulated AHR signaling in human and mouse keratinocytes *in vitro*. Importantly, these effects manifested into receptor-dependent alterations in skin complete chemical carcinogenesis. The results of these studies indicated that PPARβ/δ modulated tumor initiation and altered the critical balance between carcinogen bioactivation and detoxification. Secondly, regulation of DNA methylation contributed to the observed modulation of AHR signaling by PPARβ/δ *in vitro*. Finally, *Pparβ/δ*-null mice exhibited enhanced tumor promotion and inflammation, which supports previous hypotheses describing the anti-inflammatory functions of PPARβ/δ (Reviewed in [164, 718]). Collectively, these observations suggest that PPARβ/δ modulated skin tumorigenesis by specific modulation of critical components of carcinogenesis, including tumor initiation, promotion, and inflammation.

The global objective of these investigations was to ascertain the biological functions of PPARβ/δ in skin tumorigenesis. The data presented in chapter 2 indicated that PPARβ/δ can imprint DNA methylation patterns in the absence of an exogenous ligand, and this described epigenetic regulation is a novel function for PPARβ/δ. Further investigations are necessary to determine how exogenous or endogenous PPARβ/δ ligands alter DNA methylation patterns. Epigenetic regulation in toxicology is an

emerging field of interest, and future studies are needed to establish the PPARβ/δ-dependent mechanisms of epigenetic gene regulation. Methylation inhibitors, such as 5-Aza-2'-deoxycytidine (5-Aza-dC), and bisulfite sequencing should be utilized to identify the existence of PPARβ/δ-dependent DNA methylation patterns in phase I and II enzymes promoters. Genomics approaches, such as microarrays, can also be used to examine how PPARβ/δ modulates global methylation patterns. These studies should utilize *Pparβ/δ* mouse models and DNA methylation inhibitors to uncover information on PPARβ/δ-dependent epigenetic regulation. Additionally, microarray analyses could be used to identify novel target genes that are differentially expressed in wild-type and *Pparβ/δ*-null keratinocytes exposed to PAHs. Chromatin immunoprecipitation-sequencing (ChIP-Seq) (Reviewed in [756]) is another technique that should be used to examine endogenous and epigenetic gene regulation. Genomic analyses and ChIP-Seq data could be collectively used in a bioinformatics approach to identify and validate putative targets of PPARβ/δ-dependent epigenetic regulation. The ChIP-Seq technique could also be utilized to identify methylation patterns and epigenetic PPARβ/δ-dependent binding sites. A commonly examined DNA methylation marker is 5-methylcytosine, and antibodies have been developed to examine this DNA modification in ChIP or ChIP-Seq [757]. Additionally, enzymes that modulate DNA methylation, such as DNA methyltransferase 1 (DNMT1) or DNMT3B, could be examined as PPARβ/δ-dependent epigenetic effectors [758, 759]. It has recently been described that a PPAR coregulator PPAR-interacting protein (PRIP)-interacting methyltransferase (PIMT) is a nucleotide methyltransferase, and this could be the PPARβ/δ-dependent epigenetic regulator [760]. Clearly, the novelty of PPARβ/δ-dependent epigenetic regulation opens many avenues that could be investigated to elucidate how PPARβ/δ functions as an epigenetic gene regulator.

A key observation (chapter 2) that influenced future studies was the fact that PAH-dependent AHR occupancy at the *Cyp1a1* promoter was not increased in *Pparβ/δ*-null keratinocytes. This observation was supported by the observation that histone acetylation, a common marker of transcriptional activity, at the *Cyp1a1* promoter was not increased by PAH exposure in *Pparβ/δ*-null keratinocytes. These preliminary ChIP observations suggested that the occupancy, or lack thereof, of a specific AHR coregulator could mediate silencing of the *Cyp1a1* promoter. Several AHR coregulators have been examined by ChIP, including corepressors and coactivators (Reviewed in [684]). Future investigations should examine differential coregulator occupancy at AHR target genes upon PAH exposure in wild-type and *Pparβ/δ*-null keratinocytes. Some known AHR-dependent coregulators that could be examined include steroid receptor coactivator-1 (SRC-1) [712, 761], SRC-2 [712], SRC-3 [712], p300 [762], receptor interacting protein 140 (RIP140) [763], Brahma regulated gene-1 (BRG-1) [713], DNMT1 [764], and histone deacetylase 1 (HDAC1) [764]. Additionally, several histone modifications, including acetylation of histone H3 lysine 14 (H3K14) or H4K16, have also been shown to be regulated by AHR coregulators and should be examined in PPARβ/δ keratinocyte models [764]. Collectively, these ChIP studies will establish if differential coregulator occupancy at AHR target genes caused *Pparβ/δ*-null keratinocytes to have attenuated mRNA induction of phase I and II enzymes mRNA in response to PAHs.

It must be emphasized that the majority of this dissertation investigated the effect of PPARβ/δ on AHR signaling in the absence of exogenous PPARβ/δ ligands. The work described in chapter 2 established that ligand activation of PPARβ/δ did not alter *Cyp* mRNA expression, and further studies examined the ligand-independent modulation of

AHR signaling by PPARβ/δ. The novel studies described in chapter 2 revealed that PPARβ/δ regulated the PAH-dependent production of phase I and II enzyme mRNAs in the absence of exogenous PPARβ/δ ligands. Research described in chapter 3 also indicated that ligand-independent functions of PPARβ/δ modulated PAH-mediated tumor initiation and skin carcinogenesis. As this dissertation focused on the functions of PPARβ/δ in the absence of an endogenous ligand, future investigations should seek to understand how PPARβ/δ ligands alter skin complete carcinogenesis. In the two-stage model of skin carcinogenesis, ligand activation of PPARβ/δ reduced tumorigenesis [524, 525, 557, 587]. These effects are likely mediated by increased differentiation, increased apoptosis, and decreased proliferation of keratinocytes [402, 406, 502, 503, 512, 521, 524-526, 557, 587]. It can therefore be predicted that wild-type mice treated with PPARβ/δ ligands will have reduced tumorigenesis in the complete carcinogen bioassay. Furthermore, ligand-dependent increases in terminal differentiation would likely alter the PAH-mediated tumor histopathologies in wild-type mice. It is also predicted that ligand activation of PPARβ/δ would specifically reduce the incidence of malignant tumors in wild-type mice. Furthermore, the anti-inflammatory effects of PPARβ/δ ligands (Reviewed in [164, 718]) may also contribute to decreased malignancy or tumor size in wild-type mice during the complete carcinogen bioassay. Interestingly, several PPARβ/δ antagonists have recently been described and characterized [407-410]. However, antagonism of PPARβ/δ has shown contrasting effects on cell proliferation [409, 410]. The use of these antagonists in skin carcinogenesis bioassays could also help to dissect how the state of the ligand binding domain, antagonized or ligand bound, alters skin tumorigenesis. Therefore, future research of ligand activation and antagonism of PPARβ/δ within the complete carcinogen bioassay could support previous evidence for

the chemopreventive and chemotherapeutic potential of this nuclear receptor in skin carcinogenesis.

The studies within chapters 2 and 3 focused on how PPARβ/δ modulated AHR signaling to alter the induction of xenobiotic enzyme mRNA expression and skin tumorigenesis. To thoroughly address the biological significance of modulated AHR signaling by PPARβ/δ, future *in vitro* and *in vivo* studies should examine one or all three independently created *Ahr*-null mouse models [667, 688, 765]. Inclusion of these models would provide vital information regarding the specificity for PPARβ/δ modulation of PAH-dependent tumorigenesis. In particular, the use of *Ahr*-null primary keratinocytes could address whether increased production of phase II enzyme mRNA in response to PAHs was directly caused by AHR. The fact that PAH-mediated *Nrf2* mRNA induction was significantly reduced in *Pparβ/δ*-null keratinocytes suggests that other pathway(s) contributed to the observations described in chapter 2. The use of *Ahr*-null mice in a complete carcinogen bioassay would also provide a strong context for the significance of PPARβ/δ-dependent modulation of AHR signaling. *Ahr*-null mice have previously been examined in PAH complete carcinogenesis [686, 688] and were found to be completely resistant to B[a]P complete carcinogenesis [688]. This observation indicated the essential requirement of AHR-mediated bioactivation of B[a]P to cause skin tumorigenesis. This same mouse strain was also examined in dibenzo[a,l]pyrene (DiB[a,l]P) complete carcinogenesis, and *Ahr*-null mice exhibited significantly reduced tumorigenesis as compared to wild-type mice [686]. In contrast to B[a]P complete carcinogenesis, *Ahr*-null mice were not entirely resistant to DiB[a,l]P-dependent *Cyp* mRNA induction, DNA adduct formation, and carcinogenesis [686]. These findings revealed that DiB[a,l]P can be metabolized by another pathway in the absence of AHR.

Similar reductions in PAH-mediated DNA adduct formation have also been observed in another *Ahr*-null mouse model, but notable levels of DNA adducts were observed in the absence of AHR [637]. These effects on DNA adduct formation were thought to be due to relatively high basal levels of CYP1B1 expression that contributed to AHR-independent PAH metabolism, bioactivation, and DNA adduct formation [637]. Therefore, gaining further understanding of how PPARβ/δ modulates the AHR-dependent and -independent mechanisms of PAH carcinogenesis will provide useful information to understand the mechanisms of PAH-dependent skin carcinogenesis. An important mouse model within these investigations would be a double *Ahr*-null/*Pparβ/δ*-null mouse strain. This mouse model would provide valuable information about the dependency of these two receptors in PAH-mediated skin carcinogenesis. The noted reductions in litter sizes in both *Ahr*-null and *Pparβ/δ*-null mice suggest that a double null mouse model would be difficult to create [412, 766]. However, tissue-specific null strategy, such as a keratin-5-regulated Cre/LoxP system, could be employed to create this double-null mouse model in the skin. Collectively, the inclusion of new mouse models addressing PPARβ/δ-dependent modulation of AHR signaling will help elucidate the specificity of AHR functions in response to PAHs and to define the effects of PPARβ/δ on PAH-dependent skin tumorigenesis.

The combined purpose of the work described in chapters 2 and 3 was to delineate the role of PPARβ/δ in modulating tumor initiation. These results from these studies indicated that PPARβ/δ modulated tumor initiation in skin tumorigenesis. Research described in chapter 2 established that PPARβ/δ modulated the PAH-dependent mRNA induction of many phase I and II enzymes involved in carcinogen bioactivation and detoxification. Significant reductions in PAH-mediated phase I and II

enzyme mRNA induction were observed in *Pparβ/δ*-null keratinocytes. It was reported in chapter 3 that *Pparβ/δ*-null mice had reduced PAH-dependent lesion multiplicity. These results indicated that tumor initiation, and DNA adduct formation, are altered in a PPARβ/δ-dependent manner. Surprisingly, modulation of AHR signaling by PPARβ/δ did not result in a statistically significant change in DNA adduct formation between wild-type and *Pparβ/δ*-null mouse keratinocytes. The p-value of difference between adduct values was 0.07, which indicated that *Pparβ/δ*-null keratinocytes have less B[a]P-mediated DNA adducts than wild-type cells. There are several contributing factors that may account for the similar levels of B[a]P-dependent DNA adducts between the genotypes. First, the [γ-^{32}P]-B[a]P-postlabeling technique is a qualitative measure of adduct formation that does not specifically identify the types of stable adducts formed. The metabolites formed during PAH-specific exposure and metabolism have been extensively examined, including the identification of the ultimate carcinogen chemical moieties for B[a]P and DMBA [744, 767-771]. A metabolomics-based approach [772-774], utilizing liquid chromatography-mass spectrometry (LC-MS), would facilitate precise identification of different PAH-dependent metabolites formed in wild-type and *Pparβ/δ*-null keratinocytes or mice. Furthermore, the inclusion of *Ahr*-null mice or keratinocytes in these metabolomics studies could delineate AHR-dependent and -independent metabolites and to further profile the specific reactive metabolites created by PAH-mediated AHR signaling in these mouse strains.

The collective results from chapters 2 and 3 established that PPARβ/δ is a coregulator of xenobiotic metabolism that altered the balance between carcinogen bioactivation and detoxification. To date, numerous effectors of AHR signaling have been examined in ligand-dependent signaling and tumorigenesis (Reviewed in [684,

703]). Classic coregulators of AHR signaling include the heterodimerization partner AHR nuclear translocator (ARNT) and the accessory proteins hepatitis B associated protein 2 (XAP2), p23, and heat shock protein 90 (HSP90). Surprisingly, null models for these critical coregulators are embryonic lethal [775-778]. Recently, an epidermal-specific *Arnt*-null mouse model revealed that this protein is required for B[a]P-dependent tumor initiation [779], thus establishing the necessity of AHR/ARNT heterodimer in skin tumorigenesis. The studies in chapter 2 established that PPARβ/δ did not alter the protein expression of AHR, ARNT, XAP2, and HSP90 in human or mouse keratinocytes. Several phase I and II enzyme null models have also been developed to examine the effect of altered carcinogen bioactivation during tumorigenesis. Although CYPs are highly conserved and serve important functions in xenobiotic metabolism, viable null mouse models have been created for *Cyp1a1* [780], *Cyp1a2* [781, 782], and *Cyp1b1* [783, 784]. As expected, these null mouse models each exhibited marked differences in PAH-dependent xenobiotic metabolism, carcinogen bioactivation, and carcinogenesis [780-784]. Microsomal epoxide hydrolase (mEH) is another critical effector of PAH bioactivation that has been examined with a null mouse model, and this enzyme is also required for PAH-dependent carcinogenesis [785]. Several phase II enzyme null mouse models have also been examined in tumorigenesis. Specific deletion of NAD(P)H:quinone oxidoreductase 1 (*Nqo1*) [786] and glutathione s-transferase pi (*Gstp*) [787] exhibited enhanced PAH tumorigenesis. The enhanced tumorigenesis in phase II enzyme-null mice is not surprising, given the fact that the enzymes are necessary for the detoxification of bioactivated carcinogens. Collectively, the results of this dissertation indicated that PPARβ/δ should be considered a critical AHR coregulator that is involved in balancing carcinogen bioactivation and detoxification.

PPARβ/δ also modulated AHR signaling in human keratinocytes (chapter 2), but the exact mechanism(s) have yet to be conclusively defined. The human HaCaT shRNA model was used to show that reduced PPARβ/δ expression resulted in reduced PAH-mediated induction of *Cyp* mRNAs. Other models of modulated PPARβ/δ expression could be exploited in the HaCaT cell line to delineate specific receptor-dependent mechanisms of gene regulation. The Migr1 retroviral expression system has been utilized to stably expression proteins in numerous cell model systems [697]. This bicistronic enhanced green fluorescent protein (eGFP) expression system allows for the isolation of cells expressing a protein of interest cooperatively with eGFP. In particular, this model could be used to dissect whether PPARβ/δ modulated AHR signaling by DNA-dependent mechanisms or by corepressor/coactivator recruitment to AHR target gene promoters. Additionally, expression systems for several functional PPARβ/δ mutations have been previously described, including mutations in the DNA binding domain [172], a corepressor-interacting domain [172], a coactivator-interacting domain [538], and the ligand binding domain [788]. Exploitation of these mutations in a HaCaT Migr1 model system could dissect the mechanism(s) by which AHR signaling was modulated by PPARβ/δ in human keratinocytes. The DNA binding mutant model could be specifically used to examine if PPARβ/δ must bind within AHR target gene promoters to enhance PAH-mediated Cyp mRNA induction, or whether PPARβ/δ is an indirect transcriptional regulator of AHR signaling. Furthermore, other mutant models would establish if PPARβ/δ modulated AHR signaling by the dissociation or recruitment of coregulators to phase I and II enzyme promoters in response to PAHs. These HaCaT mutant models could also be used in bioinformatics approaches such as microarrays or ChIP-Seq to identify the functional effects of PPARβ/δ mutations. In addition to the utility of these models to examine modulated AHR signaling by PPARβ/δ, a methodical and

comprehensive examination of PPARβ/δ mutations in a single cell line could quell discrepancies and provide great insight into the true biological function of PPARβ/δ.

While the effects of human PPARβ/δ polymorphisms on PAH-mediated AHR signaling were not addressed within this dissertation, alterations in PPARβ/δ function due to polymorphisms may cause significant biological effects. It has been established that humans exhibit great variability in *Cyp* mRNA induction in response to PAHs, but polymorphisms in AHR have not been identified to date to explain this phenomenon (Reviewed in [729]). It has been shown within this dissertation that PPARβ/δ modulated PAH-mediated AHR signaling in human keratinocytes (chapter 2). It is thus plausible that PPARβ/δ polymorphisms may contribute to the variability of *Cyp* mRNA induction in response to PAHs. Several human PPARβ/δ polymorphisms have been described [468-486, 789], but the functions of these polymorphisms have not provided any direct evidence as to how PPARβ/δ modulates AHR signaling. The use of the Migr1 expression model might be of great use to delineate the functionality of these polymorphisms in a cell line model system, such as HaCaT cells. In addition to defining which, if any, polymorphisms alter the ability of PPARβ/δ to modulate AHR signaling, this model could establish which receptor polymorphisms are biomarkers of susceptibility for PAH exposed populations. This information could then be utilized in risk assessment analyses to establish exposure risks in populations possessing specific PPARβ/δ polymorphisms.

The development of new *Pparβ/δ* mouse models could also greatly aid in elucidating the functional roles of this nuclear receptor in skin tumorigenesis. First, an epidermal-specific *Pparβ/δ*-null mouse model would allow PAH-mediated skin

tumorigenesis to be evaluated without the confounding effects from other organs or cell types, such as inflammatory cells. This model could be created through a keratin 5-regulated Cre/LoxP system to specifically delete PPARβ/δ in the mouse epidermis. Mouse strains that have equivalent immunological responses would allow specific dissect of whether the reduced malignant conversion and inflammation in wild-type mice was due to genotype-dependent inflammatory responses (chapter 3). As discussed earlier, a PPARβ/δ DNA-binding mutant mouse model could also be used to determine if PPARβ/δ-dependent alters complete chemical carcinogenesis by DNA-dependent or -independent mechanisms. These mouse models could be created globally or be epidermal-specific, as both models would help dissect how PPARβ/δ modulated AHR signaling. Furthermore, these models could also be highly valuable in defining epigenetic regulatory mechanisms exerted by PPARβ/δ.

PPARβ/δ ligands are known to reduce inflammation and tumorigenesis in the skin (Reviewed in [164, 718]). There have also been attempts to establish the chemotherapeutic potential of PPARβ/δ ligands and the combined use of these ligands with known therapeutic agents. Specifically, ligand activation of PPARβ/δ has been investigated in cooperation with COX inhibitors in colon and skin carcinogenesis. First, co-administration of GW0742 and the COX2 inhibitor nimesulide was examined in colon tumorigenesis. Co-treatment of GW0742 did not enhance the therapeutic effects of nimesulide [585]. Similarly, nimesulide treatment was examined in combination with GW0742 in skin chemical carcinogenesis. This purpose of this study was to examine how co-administration of PPARβ/δ ligands and nimesulide affected tumorigenesis in mice possessing tumors. While GW0742 or nimesulide did not markedly alter tumorigenesis, co-administration of these agents reduced tumorigenesis in a PPARβ/δ-

dependent manner [557]. These results indicated that PPARβ/δ ligands could be used in combination with other chemotherapeutic agents to attenuate tumorigenesis. Several types of chemotherapeutic agents are currently being tested as cancer treatments, including plant polyphenols, curcumin, resveratrol, lycopene, retinoids, and NSAIDs (Reviewed in [790-798]). Future studies combing these agents with PPARβ/δ ligands in *Pparβ/δ* mouse models could establish if ligand activation of PPARβ/δ increases the efficacy of these chemotherapeutic agents. Additionally, these studies could identify novel PPARβ/δ-interacting pathways in skin tumorigenesis that could be investigated in the future.

The presence of basal cell tumors (BCTs) was a surprising observation of the complete carcinogen bioassay. This tumor type is the most common form of skin malignancy (Reviewed in [799, 800]), but BCTs are generally only observed in rat skin carcinogenesis models [741]. BCTs are predominantly formed by ultraviolet (UV) light exposure (Reviewed in [799, 800]). The presence of this tumor type, and subsequent enhancement in DMBA-treated *Pparβ/δ*-null mice, was a novel observation that must be further investigated. The C57BL/6 strain of mice, on which the *Pparβ/δ* mouse models are based [412], are known to be highly sensitive to complete chemical carcinogenesis [740]. Therefore, it is not unexpected that a highly sensitive mouse strain could develop a rare tumor type. The results of this bioassay indicate that DMBA-treated *Pparβ/δ*-null mice may possess a unique susceptibility to BCTs in a complete carcinogenesis model. Furthermore, PPARβ/δ could significantly alter BCT formation in a UV-induced skin carcinogenesis model. Interestingly, UV radiation has been proposed to create endogenous AHR ligands [801-804], and modulated AHR signaling by PPARβ/δ could contribute to the DNA damage caused by UV-induced skin tumorigenesis.

The observation that PPARβ/δ significantly reduced skin tumorigenesis suggests that this nuclear receptor may also modulate PAH-dependent tumorigenesis in other epithelial tissues, such as the breast or colon. Surprisingly, many models of breast tumorigenesis rely on DMBA initiation (Reviewed in [805]), and PPARβ/δ-dependent modulation of AHR signaling could be a contributing mechanism of breast tumorigenesis. While the function of PPARβ/δ has not been broadly examined in breast cancer, several studies have investigated the effects of PPARβ/δ ligands on breast cancer cell line growth. Ligand activation of PPARβ/δ caused contrasting effects on cell proliferation and differentiation [516, 540, 554, 560, 590]. These observations indicated that ligand activation of receptor exerted some effects on cell proliferation and differentiation, but conclusive investigations are still necessary. It would be of great interest for future studies to examine breast tumorigenesis in *Pparβ/δ* mouse models, specifically to delineate if PPARβ/δ also alters tumor initiation in this tissue. Proof of concept studies in a PPARβ/δ shRNA human breast cancer cell line could establish if altered PAH metabolism occurs in this tissue. Similarly, colon carcinogenesis could be examined to determine if PPARβ/δ modulated AHR signaling and tumor initiation in this tissue. *Pparβ/δ* mouse models have been used to investigate colon chemical carcinogenesis; however the initiating agent was azoxymethane (AOM) (Reviewed in [755]). This carcinogen is metabolized by CYP2E1 and causes DNA damage through alkylation [806]. Because CYP2E1 is not known to be regulated by AHR [806], these observations indicate that AOM initiation in colon carcinogenesis may not be modulated by PPARβ/δ. PAH-mediated colon carcinogenesis has not been vastly studied because most PAH exposure comes from soil or food contamination [807, 808]. However, *Cyp* mRNAs are induced in response to PAHs, and DNA adducts have been observed [807-

809]. These observations suggest that PPARβ/δ-dependent modulation of tumor initiation should be investigated in other models of epithelial tumorigenesis. It will be of great interest for future investigations to determine if PPARβ/δ modulates PAH-mediated tumor initiation in other epithelial tissues by modulating AHR signaling. These studies could then establish if PPARβ/δ expression can be utilized as a biomarker of modulated PAH-dependent tumor initiation and carcinogenesis.

Overall, the results from this dissertation support the hypothesis that PPARβ/δ modulates the balance between carcinogen bioactivation and detoxification in mouse skin tumorigenesis by specific modulation of AHR-dependent signaling. Reduced *Cyp* mRNA induction in response to PAHs was observed in human and mouse *Pparβ/δ* keratinocyte models. These results suggest that tumor initiation and PAH-dependent skin tumorigenesis would be modulated in both species. In support of this hypothesis, *Pparβ/δ*-null mice exhibited decreased PAH-mediated lesion multiplicity. However, the effect of modulated AHR signaling by PPARβ/δ on human skin tumorigenesis was not examined. It was also observed that PPARβ/δ potentially reduced tumor promotion, as observed by genotype-dependent changes in tumor size upon B[a]P- and MNNG-dependent skin carcinogenesis. This may be due to changes in cell proliferation or genotype-dependent inflammatory responses. Thus, PPARβ/δ can modulate many components of skin tumorigenesis, including tumor initiation, tumor promotion, and inflammation. Further research is necessary to dissect and mechanistically define how PPARβ/δ modulated AHR signaling and tumor initiation, but the conclusion of this dissertation is that PPARβ/δ is a critical modulator of mouse and human skin tumorigenesis.

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PUBLICATIONS (4 most recent)

Palkar, P.S., **Borland M.G.**, et al. Cellular and pharmacological selectivity of the PPARβ/δ antagonist GSK3787. *Molecular Pharmacology* (2010). 78(3): 419-430. PMID: 20516370.

Genovese, S., Foreman, J.E., **Borland, M.G.**, et al. A natural propenoic acid derivative activates peroxisome proliferator-activated receptor-β/δ (PPARβ/δ). *Life Sciences* (2010). 86(13-14): 493-498. PMID: 20153754.

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