EXAMINING OF THE ROLES OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS IN LIVER CANCER

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ABSTRACT

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) and PPARα have both been shown to modulate liver cancer, however the mechanisms by which these receptors influence liver cancer have not been fully elucidated. The focus of this study was to examine the role of PPARs in liver cancer using two different models. To examine the role of PPARβ/δ in liver cancer, a transgenic mouse line expressing a portion of the Hepatitis C virus genome that causes liver cancer (HCV mice) was crossed with a Pparβ/δ-null mouse line. Liver tissue from HCV and HCV X Pparβ/δ-null mice were used to determine the effect of genetically disrupting the Pparβ/δ gene on kinase-dependent signaling proteins known to influence cancer promotion. To examine the role of PPARα in liver cancer, liver samples from wild-type, Ppara-null mice and humanized PPARα mice were used to determine if lipid peroxidation influenced tumorigenesis. A phosphoprotein screen was performed using liver tissue from HCV and HCV X Pparβ/δ-null mice and candidate proteins whose phosphorylation status was altered by the absence of PPARβ/δ expression were tentatively identified. Western blot analysis revealed an increase in the relative expression of the p50 and p65 subunits of NFκB and SOCS2 in the HCV X Pparβ/δ-null mice as compared to control HCV mice. Quantitative PCR (qPCR) was performed to determine if target genes of NFκB, Tnfa and Il-6, were altered due to this difference detected in the subunits of NFκB. However, no changes in the expression of Tnfa or Il-6 mRNAs were observed between groups. To examine a relatively novel role of PPARα in liver cancer, lipid peroxidation was assessed in the liver of wild-type, Ppara-null mice, and humanized PPARα mice treated with or without the non-genotoxic carcinogen, GW7647. A TBARS assay was performed to measure the amount of malondialdehyde (MDA), a by-product of lipid peroxidation, in the three
genotypes treated with or without GW7647 for 18 months. No significant differences in the level of MDA were found between any genotype of treatment groups. Results from this study indicate that PPARβ/δ represses NFκB in a protective mechanism in a viral-induced liver cancer model. Further studies are needed to determine if PPARβ/δ-dependent modulation of other NFκB target genes contribute to this protective effect of Pparβ/δ. Results from this study also indicate that despite the known steatotic phenotype observed in older Ppara-null mice as compared to controls, the low incidence of liver tumors observed in Ppara-null mice is not due to differences in MDA formation. Further studies are needed to determine if other lipid peroxides may contribute to the low incidence of tumorigenesis found in Ppara-null mice compared to controls. Future experimentation should be done to better understand the effects of PPARβ/δ and PPARα in tumor formation and hepatocarcinogenesis so specific targets can be identified and therapeutic treatments can be developed against liver cancer.
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INTRODUCTION

Liver Cancer

Liver cancer is responsible for 9% of all cancer deaths in humans, killing approximately 745,000 people per year\(^1\). Liver cancer accounts for 6% of all cancers diagnosed worldwide and is one of the most common causes of cancer-related deaths in males\(^1\). Infections by hepatitis B virus (HBV) and hepatitis C virus (HCV) are responsible for the majority of primary liver cancer cases worldwide\(^2\). HBV and HCV begin as acute infections; however in 75-85% of cases, the virus stays in the body and progresses into chronic disease\(^3\). Chronic HCV can lead to long-term liver problems, such as cancer\(^3\). Approximately 2.7-3.9 million people are infected with chronic hepatitis C virus in the United States alone\(^3\). There is a vaccine to prevent the infection of HBV but currently there is no vaccine to prevent the infection of HCV\(^3\). HCV is a contagious virus that is spread through the blood of infected individuals\(^3\). Although there is not a vaccine to prevent HCV, there are some treatments to treat both acute and chronic infections\(^3\). However, since hepatitis C virus is the least understood of the hepatitis viruses, researchers are working diligently to better understand the virus in hopes to develop treatments and preventative measures to alleviate the total number of liver cancer cases overall\(^2\).

Along with viral induction, liver cancer can also be chemically induced. The liver is responsible for numerous vital functions of the body including carbohydrate and fat metabolism, lipid synthesis, storing chemicals, and detoxifying and removing toxic chemicals, drugs and carcinogens\(^4\). When mutations or imbalances occur in the liver, it is possible that conjugation
can occur between toxic metabolites and cellular macromolecules, such as nucleic acids and proteins, and could result in cancer\(^4\).

Numerous studies have suggested that oxidative stress that results in lipid peroxidation is involved in inflammation and cancer\(^5\). A major contributor to oxidative stress is reactive oxygen species (ROS). Cellular metabolism can potentially leave behind free radical ROS by-products\(^5\). The cells of many forms of cancer have been shown to have increased levels of ROS. ROS have the potential to promote cell proliferation and differentiation when in moderate amounts; however, when ROS are in overabundance, they can cause oxidative damage that can be harmful to the host\(^5\). Studies have also shown that tumor cells often have increased levels of oxidative damage products\(^5\). Lipid peroxidation is an indicator of oxidative stress and cellular injury. By studying chemical processes in liver cells, such as ROS and lipid peroxidation, potential treatments can be developed.

**Models to Study Liver Cancer**

Numerous models have been used to study liver cancer in mice, namely through transgenics, chemicals and transplants. The most common gene mutations in liver cancer are p53 and CTNNB1, therefore creating models that have induced mutation or deletion of p53 or overexpression of β-catenin mutants are useful in modeling human liver cancer\(^6\). It has been shown that mouse models that have either a single p53-deleted mutation or a combination with other mutations, such as CTNNB1 transgenic mice, develop liver cancer in result of these genetic alterations. When studying the mechanisms of liver cancer, it is important to incorporate signature features of the disease, namely chronic inflammation, injury, and fibrosis\(^6\). To examine
virally-induced liver cancers, HBV and HCV transgenic mice can be created. This is useful because mice are not susceptible to human HBV or HCV\(^6\).

On the other hand, chemically-induced liver cancer can be examined by administering the carcinogens or mutagens, such as diethylnitrosamine (DEN). This, along with reactive oxygen species, can result in DNA damage. When DNA damage of this sort occurs in the liver, it can lead to tumor development. This model only causes cancer in mice if it is combined with a promoting agent such as phenobarbital or the hepatotoxin CCl\(_4\)\(^6\). Chronic administration of non-genotoxic carcinogens can also cause liver cancer in mouse models. Another way to induce tumor formation in mice is to delete the MDR2 gene, which encodes for the excretion of phospholipids into bile in liver cells. The deletion of this gene can lead to damagingly high concentrations of bile salts in bile\(^6\). This model has exhibited that the NFκB pathway promotes the development of liver cancer\(^6\).

The last significant way to study liver cancer in mouse models is through the use of transplants. This occurs by placing tumor fragments of cancerous cells into mice\(^6\). Cells can either be injected directly into the liver or injected into the spleen and carried to the liver. In most instances, the recipient mouse livers lack the inflammation and fibrosis factors that can ultimately lead to liver cancer. To better study the effects of fibrosis, scientists can initiate liver fibrosis using pretreatment, followed by transplantation with cancer cells\(^6\).

**Peroxisome Proliferator-Activated Receptors**

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that are involved in numerous biological responses, such as immune function, proliferation, differentiation, glucose homeostasis and lipid homeostasis\(^5\). PPARs have three
isoforms found in all mammalian species: PPARα, PPARβ/δ and PPARγ. The role of PPARs in carcinogenesis is not clear. PPARα is expressed in many tissues that require fatty acid oxidation as an energy source and is also a big contributor to maintaining lipid homeostasis, especially in the liver during starvation. Since PPARα is known to maintain lipid homeostasis by modulating signaling molecules that control gene expression, it has been the target for lipid-lowering fibrate drugs by increasing the oxidation of lipids. PPARα has also been favorable in diabetes treatment, due to its ability to improve insulin resistance through changes in gene expression. However, it has been observed chronic administration of PPARα agonists can cause hepatocarcinogenesis in rats and mice. Since this does not occur in Ppara-null mice fed PPARα agonists, this demonstrates that PPARα is required to mediate liver cancer in rodents.

When a ligand binds to PPARα, expression of target genes is modulated, such as the peroxisomal protein, acyl CoA oxidase (ACO). ACO has been thought to contribute to liver cancer induced by PPARα agonists in rodents because it produces hydrogen peroxide as a by-product of long chain fatty acid catabolism. It has been hypothesized that induction of ACO and increased hydrogen peroxide could be a cause of hepatocarcinogenic effects of PPARα agonists, but this remains unclear to date. With the activation of PPARα, cell proliferation increases and apoptosis is inhibited in rodents. PPARα could also potentially lead to the activation of Kupffer cells, which leads to oxidative stress. Overexpression of ACO can also induce an increase in oxidative stress. It is not known for certain, but it is hypothesized that the oxidative stress increase is the cause of the DNA damage that leads to tumor formation in the liver.

PPARβ/δ regulates lipid homeostasis and glucose and has also been shown to be a key player in regulating hepatic gene expression. Ligand activation of PPARβ/δ is known to increase fatty acid metabolism, regulate the sensitivity of insulin, decrease serum triglycerides,
prevent obesity, and influence gastrointestinal function\(^7,12\). It has also been shown that activation of PPAR\(\beta/\delta\) can enhance terminal differentiation in keratinocytes, intestinal epithelium and osteoblasts, which lead scientists to further study the effects of PPAR\(\beta/\delta\) in tumor development and cancer\(^7\).

However, scientists are not yet sure of the effects of PPAR\(\beta/\delta\) in regards to carcinogenesis. Some studies have shown that expression of PPAR\(\beta/\delta\) is upregulated in cancer cells while others have shown that expression of PPAR\(\beta/\delta\) is down-regulated in cancer cells\(^7\). Further, some studies show that ligand activation of PPAR\(\beta/\delta\) agonists either increase or decrease tumorigenesis\(^7\). One indisputable effect of PPAR\(\beta/\delta\) is that PPAR\(\beta/\delta\) inhibits inflammation in the liver due to repression of nuclear factor, NF\(\kappa\)B\(^7\). Since inflammation is inhibited in these cases, certain cytokines have reduced expression, such as tumor-necrosis factor \(\alpha\) (TNF\(\alpha\)), interleukin 1\(\beta\) (IL-1\(\beta\)) and interleukin 6 (IL-6)\(^7\). Since the role of PPAR\(\beta/\delta\) is ambiguous in regards to carcinogenesis, it is a central topic in cancer research.

When a ligand binds to a PPAR, a conformational change occurs that allows the PPAR to form a heterodimer with retinoid X receptor (RXR)\(^7\). This action allows the complex to regulate gene expression while also playing a role in triglyceride metabolism, glucose homeostasis, and inflammatory responses\(^13\). PPARs interact with other transcription factors of various pathways, such as the AP-1 signaling pathway and p50 and p65 of the NF\(\kappa\)B signaling pathway\(^14\). Within the liver, PPARs regulate inflammatory immune responses by repressing the AP-1 and NF\(\kappa\)B pathways\(^14-15\). This represses inflammation by inhibiting cytokines and inflammatory protein production\(^14-15\).

SOCS2 is a member of the suppressor of cytokine signaling family. It is a negative regulator of the JAK/STAT pathway, whose role involves transcription and activation of genes\(^16\).
Studies have found that low gene expression of SOCS2 has been observed in hepatocellular, breast, ovarian and pulmonary cancers\textsuperscript{17}. A study by Cui et. al. demonstrated that increased expression of SOCS2 inhibits the metastasis of hepatocellular carcinoma and proposed that SOCS2 is a potential target for treatment of hepatocellular carcinoma\textsuperscript{18}.

SOCS2 has not been as extensively studied as SOCS1 and SOCS3 and the effects of SOCS2 on PPARβ/δ are not yet known. SOCS1 is known to regulate the activation of M1-macrophage by inhibiting the JAK2/STAT1 pathway as well as the TLR/NFκB signaling pathway\textsuperscript{19}. SOCS3 is thought to associate with M1 macrophages and illicit pro-inflammatory immune responses, which makes SOCS3 a potential therapeutic target in inflammatory diseases\textsuperscript{18}. One study noted that macrophages lacking PPARγ led to an upregulation of SOCS3\textsuperscript{19}.

The cyclin-dependent kinase (CDK) inhibitor, p27, is a tumor suppressor protein that regulates G0 to S phase transitions during the cell cycle. This occurs by binding to cyclin-dependent kinases. Therefore, p27 plays a role in cell proliferation\textsuperscript{20}. P27 has shown to have both positive and negative functions in regards to cancer\textsuperscript{20}. Sue et. al. demonstrated that PPARβ/δ enhances transcriptional activation of p27\textsuperscript{21}.

Protein kinase C-delta (PKC-δ) is a kinase that also has interactions with PPARs that affect gene expression. PKCs can regulate PPARα by phosphorylation through multiple sites\textsuperscript{22}. A study by Kim et. al. showed the role of PPARβ/δ in modulating PKC-α levels. PPARβ/δ reduces cell proliferation by regulating PKC-α activity\textsuperscript{23}. The potential interactions between PPARβ/δ and PKC-δ have not been widely studied.
**HCV-Induced Liver Cancer**

Although some patients have been cured from Hepatitis C virus, many people continue to suffer from its infection. There is still a need to study other pathways using HCV-induced liver cancer models. Recent studies are focusing on PPARs and their potential to be used as therapeutic targets against chronic HCV. Infection by chronic HCV can lead to liver inflammation, hepatocyte fat accumulation and diabetes. At least parts of all of these side effects are controlled by PPARs\(^{24}\). It is suggested that PPAR\(\alpha\) expression is impaired by HCV infection which is supported by a study that showed hepatocytes infected with HCV had low levels of PPAR\(\alpha\) compared to what is usually observed\(^{24}\). The roles of PPAR\(\alpha\) in HCV infection have been studied more extensively than the roles of PPAR\(\beta/\delta\). For this reason, it is necessary to study the effects of PPAR\(\beta/\delta\) in HCV-induced liver cancer so that drug targets can potentially be identified. This will be examined by crossing HCV transgenic mice with \(Ppar\beta/\delta\)-null mice and observing how this affects various pathways.

**Non-Genotoxic Carcinogens**

Aside from viral induction, liver cancer can also be chemically induced by non-genotoxic carcinogens. A 1997 study by Peters, Cattley and Gonzalez showed that chronic administration of \(Ppara\) to mice and rats resulted in hepatomegaly and eventually carcinogenesis and tumors of the liver\(^ {10}\). In this study, they evaluated the effects of the non-genotoxic PPAR\(\alpha\) agonist, Wy-14,643, and its potential effects on DNA synthesis and carcinogenesis in \(Ppara\)-null mice. Since Wy-14,643 is a PPAR\(\alpha\) agonist, it activates the expression of PPAR\(\alpha\) so that its effects can be studied. Over the course of the experiment, \(Ppara\) wild-type mice and \(Ppara\)-null mice were fed either a control diet or a diet containing 0.1% Wy-14,643, over various spans of time. It was
observed that after 11 months, 100% of the Ppara wild-type mice consuming the Wy-14,643 diet had developed tumors. At the same 11 month mark, it was also observed that the Ppara-null mice consuming the Wy-14,643 diet showed no signs of carcinogenesis and remained virtually unaffected\textsuperscript{10}. Since Wy-14,643 is a PPAR\alpha agonist and 100% of the wild-type control fed 0.1% Wy-14,643 developed tumors, this illustrates that PPAR\alpha mediates DNA synthesis and carcinogenesis\textsuperscript{10}.

The conclusions of this study were contradicted by studies that showed liver tumor formation in Ppara-null mice. In 2005, Hays, et. al performed a similar experiment to test the effects of PPAR\alpha in hepatocarcinogenesis with 0.5% bezafibrate used as the PPAR\alpha agonist\textsuperscript{25}. After 12 months of consuming the 0.5% bezafibrate diet, Ppara wild-type mice developed preneoplastic foci, adenomas and hepatocellular carcinoma while one Ppara-null mouse developed a single microscopic adenoma\textsuperscript{25}. This prompted further studies focusing on the mechanisms for tumor development in Ppara-null mice.

Studies have shown that Ppara-null mice have a lot of lipids in their livers. Some fatty acids, such as palmitic acid, were oxidized less in Ppara-null mice than Ppara wild-type mice; other fatty acids, such as lignoceric acid, had no significant differences in expression between Ppara wild-type and Ppara-null mice\textsuperscript{26}. This suggests that constitutive expression of enzymes involved in \(\beta\)-oxidation is independent of PPAR\alpha\textsuperscript{26}. In the same study, an abnormal amount of lipids were observed in Ppara-null mice. An overabundance of liver fat can potentially lead to cancer due to decreased levels of fatty acid oxidation\textsuperscript{26}.

Due to the defects in lipid metabolism that Ppara-null mice experience, lipids accumulate in the liver which can lead to damage. In a 2004 study by Howroyd et. al., they tested the effects of aging and tumor development in Ppara wild-type and Ppara-null mice\textsuperscript{27}. They found that
hepatocellular carcinomas and hepatocellular adenomas were present in older $Ppara$-null mice and not in $Ppara$ wild-type mice$^{27}$. This study showed that old $Ppara$-null mice develop can liver cancer, even though they do not express PPAR$\alpha$. However, the mechanisms underlying this effect could be entirely different than that observed for PPAR$\alpha$ agonists since there is increased steatosis observed in $Ppara$-null mice as compared to controls, which is a risk factor for liver cancer. Further, the number of tumors observed in aged $Ppara$-null mice is markedly different than that observed with wild-type mice fed PPAR$\alpha$ agonists$^{27}$.

Overall, mice and rats are more susceptible to peroxisome proliferators and hepatocarcinogenesis than humans and primates. The reason for this is not entirely known but it is hypothesized that it is due to humans and primates having lower hepatic levels of $Ppara$ compared to mice and rats$^{26}$. To study the effects of peroxisome proliferators in humans, humanized $PPAR\alpha$ ($hPPAR\alpha$) mice were generated by replacing the mouse gene with the homolog in humans, and treated with or without a PPAR$\alpha$ agonist$^{28}$. The study consisted of a long-term feeding study comprised of control $Ppara$ wild-type mice and $hPPAR\alpha$ mice. Each group was either fed a control diet or a diet containing the PPAR$\alpha$ agonist, Wy-14,643. After the experiment, it was observed that the incidence of liver tumors in wild-type $Ppara$ mice was 71% while the incidence of liver tumors in $hPPAR\alpha$ mice was only 5%$^{28}$. This suggested that differences in mouse and human PPAR$\alpha$ structures are the cause for the organisms’ variability in their susceptibility to tumor development in the liver. However, PPAR$\alpha$ is still believed to maintain lipid homeostasis in humans and its study remains pertinent in understanding the mechanisms of liver cancer development.
Purpose of Study

Overall, by learning more about the properties and expression of PPARs, they can be used as drug targets against various diseases and cancers. Although there is evidence suggesting that PPARs offer a role in virally induced and chemically induced liver cancer, their roles are not clearly defined. The purpose of these studies was to examine and analyze the roles of PPARβ/δ and PPARα in liver cancer. The role of PPARβ/δ was examined by identifying phosphoproteins that may be altered in expression between wild-type HCV mice and HCV X Pparβ/δ-null mice. The effects of ligand activation of PPARβ/δ were analyzed. A list of potential proteins was determined using Kinexus Antibody Microarray data and quantitative Western blots were run to verify if there were any differences in expression levels between the wild-type and knockout HCV X Pparβ/δ liver whole lysates. Using the results from the Western blot analysis, target genes of significant proteins were used for quantitative PCR (qPCR) analysis to further understand the potential role of PPARβ/δ.

To study the potential role of PPARα in liver cancer, six mouse liver treatment groups were examined: adult Ppara wild-type (control and GW7647), perinatal Ppara wild-type (control and GW7647), adult Ppara knockout (control and GW7647), perinatal Ppara knockout (control and GW7647), adult humanized PPARα (control and GW7647) and perinatal humanized PPARα (control and GW7647). A Thiobarbituric Acid Reactive Substances (TBARS) kit was used to determine lipid peroxidation of the cells. The amount of lipid peroxidation could be quantified due to the binding of malondialdehyde (MDA) with thiobarbituric acid. The MDA is a by-product of lipid peroxidation and binds to the thiobarbituric acid provided by the assay kit. The quantified result shows the level of damage done to the liver. If there is substantially more damage to the knockout mice than the wild-type, this shows that PPARα plays a role.
It was hypothesized that HCV-transgenic mice will exhibit differences in protein expression levels between $Ppar\beta/\delta$ controls and $Ppar\beta/\delta$-null models. The proteins that exhibited differences in expression levels in the Kinexus Antibody Microarray analysis were also expected to show similar differences in expression levels in Western blot analysis. If differences were shown in Western blot analysis, it was also hypothesized that target genes of the proteins would exhibit similar differences in expression levels in qPCR as seen in the Western blots.

Since PPARα is known to mediate carcinogenesis in mice and PPARα agonists are also known to cause liver cancer in mice, it was hypothesized that $Ppara$ control mice would have lower lipid peroxidation compared to the $Ppara$ GW7647-treated control group. Since $Ppara$ knockout mice are known to have accumulated lipids in the liver due to a decrease in lipid oxidation, it was hypothesized that $Ppara$-null control and GW7647-treated samples would have high lipid peroxidation compared to $Ppara$ wild-type controls. The same results were expected in humanized PPARα control and GW7647-treated samples.
MATERIALS AND METHODS

**Kinexus Antibody Microarray Analysis**

A Kinexus Antibody Microarray analysis was performed on liver lysate samples from control and HCV X *Pparβ/δ*-null samples. This technique tracks the expression levels or mutations of many genes on a single microarray slide. For this Kinexus Antibody Microarray analysis, 50 μg of lysate protein from each sample (wild-type control and HCV X *Pparβ/δ*-null) were covalently labeled with a fluorescent dye. Free dye molecules were removed by gel filtration. The samples were then incubated and unbound proteins were washed away. Signal quantification was performed with *ImaGene* 8.0 from BioDiscovery (El Segundo, CA). Z-ratios were calculated by taking the difference between the averages of the observed protein Z-scores and dividing by the standard deviation of all of the differences for that particular comparison.

**Western Blot Analysis**

18-month old mouse HCV liver whole lysates were used for Western blot analysis. Six samples were HCV wild-type control and six samples were HCV X *Pparβ/δ*-null. Each liver sample was 30 μg of protein per sample. 1 μL of liver sample, 5 μL of 3X Laemmli dye, and 9 μL of water were aliquotted into an Eppendorf tube and heated at 95°C for 5 minutes. Then, the samples were cooled on ice for 5 minutes and briefly centrifuged before being loaded into a 10% SDS-polyacrylamide gel, along with a molecular weight marker. A 1X Tris/Glycine/SDS buffer was used as the gel was run at 100V for 90 minutes.
The protein was then transferred to a PVDF membrane by first soaking the membrane in methanol and then in 1X Western transfer buffer (200 mL 10X Western transfer buffer, 400 mL methanol, 1400 mL ddH₂O) (10X Western transfer buffer: 60.6g Tris base, 288g glycine). The gel and membrane were placed in a Bio-Rad mini transfer apparatus along with 1X Western transfer buffer and run at 120V for one hour. After the protein was transferred to the membrane, the membrane was incubated with 5% milk/TBST buffer while shaking for 30 minutes. Then, the membrane was washed with TBST and the primary antibody was added and left to shake overnight. The primary antibodies used over the course of the study were PKC-δ, SOCS2, NFκB p50, NFκB p65 and p27.

After discarding the primary antibody, the membrane was washed twice with TBST for 10 minutes each time. Then, the secondary antibody was added and was incubated while shaking for one hour. The secondary antibodies used were anti-mouse and anti-rabbit. Next, the membrane was washed twice with TBST for 10 minutes each time. The membrane was then incubated with 1 μL of ¹²⁵I-streptavidin in 5 μL TBST and left to shake for 30 minutes. After discarding the radioactive solution, the membrane was washed twice with TBST buffer for 10 minutes each. Then, the membrane was placed in a cassette with a phosphorscreen for 3 days. The phosphorscreen was observed on a phosphorimager. All of the protein expressions were normalized to β-ACTIN.

**RNA Isolation**

RNA was isolated from five wild-type and five HCV x Pparβ/δ-null liver samples. RNA was isolated by Dr. Gayathri Balandaram. 1 mL of Ribozol was added to each liver tissue sample and then each sample was homogenized. The samples were incubated at room temperature for 10
minutes and then 200 μL of chloroform was added. The tubes were shook vigorously and then incubated for 3 minutes at room temperature. The samples were then centrifuged at 12,000 x g for 15 minutes at 4°C. The top, aqueous layers containing RNA were removed. The samples were transferred to RNase-free tubes and 0.5 mL of isopropanol was added to each tube to precipitate the RNA. The samples were incubated for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatants were then removed and the pellets were washed with 1 mL of 75% ethanol and then centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol was then removed and the pellets were left to air dry for 5 minutes. The pellets were re-dissolved in DEPC water. The concentrations of the RNA samples were determined using NanoDrop. The samples were then stored at -80°C.

**Northern Blot Analysis**

The quality of the isolated RNA was determined using a Northern blot to detect clear 28S and 18S bands representing the RNA subunits. Agar and water were mixed and then microwaved until boiling. Then, the mixture was cooled in a refrigerator for 5 minutes before adding 10X MOPS and 37% formaldehyde. The gel was poured into an Owl gel electrophoresis box and left to cool. 30 μL of loading buffer with ethidium bromide was added to each Eppendorf tube. Then, 2.5 μg of RNA and appropriate amounts of DEPC water were prepared for each sample and added to the Eppendorf tubes. The samples were vortexed, heated at 65°C for 5 minutes, and then placed on ice for 5 minutes. The samples were then centrifuged briefly and loaded into the gel wells. The gel was run at 150V for 35 minutes. The gel was observed using a gel imager.
cDNA Synthesis

cDNA was synthesized to further use for qPCR. The RNA samples were prepared at 100 ng/μL. A Supermix was prepared containing 5x RT buffer (10 μL), 10 mM dNTPs (10 μL), 25X random primers (2 μL), M-MLV Reverse Transcriptase (0.25 μL) and DEPC water (2.75 μL). 25 μL of each RNA sample (2.5 μg) was added to each PCR tube. 25 μL of DEPC water was added to one PCR tube to serve as a No Template Control. Then, 25 μL of Supermix was added to each PCR tube and mixed. The “RT-Real” program on the Thermal Cycler (25°C for 10 minutes, 37°C for 120 minutes, 4°C forever) was run and the cDNA was stored at -20°C.

qPCR

qPCR was used to further analyze the gene expression levels of target genes of significant proteins from Western blot analysis. The target genes analyzed were Tnfa and Il-6. Standard curve samples (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng) were prepared using cDNA (NT 104). Primer stocks for forward and reverse primers were made to a concentration of 7.5 pmol/μL. The unknown stocks were prepared using 1 μL of cDNA and 99 μL of DEPC water. SYBR primer mixes were made using SYBR green (12.5 μL), forward primer (1 μL), and reverse primer (1 μL). 10.5 μL of standard, sample and No Template Control (DEPC water) were added to a well in a 96-well plate. 14.5 μL of SYBR green mix was added to each respective well. The wells were sealed with caps and centrifuged at 1500 rpm for 1 minute. The “Finnzyme SYBR Protocol” program (Cycle 1: 95°C for 15 minutes, Cycle 2: 95°C for 30 seconds, Cycle 3 (60X): 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, Cycle 4 (60X): 60°C for 10 seconds, Cycle 5: 30°C for 10 seconds) was run on the Real Time PCR machine. The results were analyzed using the “MY IQ” software and normalized to GAPDH.
Protein Isolation

To investigate the effects of PPARα in liver cancer, protein was isolated from liver tissue samples. The samples used in this experiment were adult and perinatal liver samples. Within the adult and perinatal samples, each group contained 6 different treatment groups: Ppara wild-type (control and GW7647), Ppara knockout (control and GW7647), and humanized PPARα (control and GW7647). GW7647 is a PPARα agonist. Five liver tissue samples of each treatment group were prepared. The liver tissue samples were placed in liquid nitrogen and 0.025g of each sample were weighed. The weighed samples were placed in Eppendorf tubes and placed back in liquid nitrogen. RIPA buffer with one mini EDTA-free tablet (protease inhibitor) was prepared. The tissue samples were added to a cooled glass homogenizer followed immediately by 250 μL of RIPA buffer with protease inhibitor. The samples were homogenized with a glass pestle until the samples were homogenous (pestle moved up and down ~30 times). Then, the homogenized samples were pipetted into Eppendorf tubes and centrifuged at 4°C at 1600 x g for 10 minutes. The supernatants were stored at -80°C.

Determining Protein Concentration

The protein concentration of each sample was determined using a BCA standard curve. Standards were prepared using a BCA Protein Assay Kit. 50 μL of each standard was added to a 96-well plate. 2 μL of each unknown was mixed with 48 μL of 0.1 N NaOH in a well of the 96-well plate. Then, the BCA substrate mix was prepared and 200 μL was added to each well. The 96-well plate was incubated at 37°C for 30 minutes. The absorbances of the samples were
measured using a Spectracount plate reader at 570 nm. A standard curve was prepared using the BCA standards to calculate the protein concentration (μg/mL) of each sample.

**MDA Assay**

To measure the amount of lipid peroxidation of each liver sample, an MDA assay was performed using a TBARS Assay Kit. The adult samples and perinatal samples were run separately. First, a color reagent was prepared using 1.06g TBA, 100 mL TBA acetic acid and 100 mL TBA NaOH. 100 μL of sample or standard were added to a 15 mL conical tube. 100 μL of SDS Solution was then added to the tube and mixed. 4 mL of the color reagent was added to each tube. The tubes were then boiled for one hour. After an hour, the tubes were immediately placed on ice for 10 minutes to stop the reaction. The tubes were then centrifuged at 4°C at 1600 x g for 10 minutes. 1 mL of each sample was pipetted into a cuvette and the absorbances were read on a Spectrophotometer (Spectronic Genesys 5) at 530 nm. The standard curve was used to determine the concentration of MDA (μM) of each sample.

**Statistical Analysis of Data**

Prism 6.05 was used to determine statistical significance using an unpaired t-test. Significance was set at $p < 0.05$. 
RESULTS

HCV and PPARβ/δ

A Kinexus analysis was run with liver samples from wild-type and HCV X Pparβ/δ-null. The liver samples were taken from 18-month old mice. The proteins were labeled with a fluorescent dye before so that the signals could be quantified. The phosphoproteins and their Z-ratios were given for analysis (Table 1).

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Z-ratio (HCV Wild-type)</th>
<th>Z-ratio (HCV X Pparβ/δ-null)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-kappa-B p50 nuclear transcription factor</td>
<td>-2.36</td>
<td>2.53</td>
</tr>
<tr>
<td>NF-kappa-B p65 nuclear transcription factor</td>
<td>-2.07</td>
<td>3.63</td>
</tr>
<tr>
<td>p27 cyclin-dependent kinase inhibitor 1B</td>
<td>-2.04</td>
<td>-2.24</td>
</tr>
<tr>
<td>Protein-serine kinase C delta</td>
<td>2.67</td>
<td>-2.24</td>
</tr>
<tr>
<td>Suppressor of cytokine signaling 2</td>
<td>-2.29</td>
<td>-2.28</td>
</tr>
</tbody>
</table>
A negative Z-ratio indicates a decrease in expression or phosphorylation compared to the HCV wild-type control and a positive Z-ratio indicates an increase in expression or phosphorylation compared to the HCV wild-type control. Proteins listed here show that PPARβ/δ promotes the expression or phosphorylation of this protein.

Based on these results, proteins that were listed in Table 1 suggested that PPARβ/δ promotes expression or phosphorylation of those particular proteins. From there, a list of proteins was chosen for validation by Western blot analyses. The proteins chosen included: PKC-δ, SOCS2, NFκB p50, NFκB p65 and p27.

Western blot analysis was performed on the five chosen proteins to determine any significant differences in expression between wild-type liver samples and HCV x Pparβ/δ-null liver samples. The liver samples were taken from 18-month old mice. The samples were run through an SDS-polyacrylamide gel, transferred to a PVDF membrane and incubated with primary antibodies specific to the protein of interest. Depending on the primary antibody, either the membrane was then incubated with either anti-mouse or anti-rabbit secondary antibody. After labeling with 125I-streptavidin, the membrane was incubated in a cassette with a phosphorscreen and then observed on a phosphoimager. All of the levels of expression were normalized to β-ACTIN (Figure 1).
Figure 1. PPARβ/δ represses hepatic expression of SOCS2, NFκB p50 and NFκB p65 in HCV-transgenic mice.

Western blot analysis shows significantly higher expression of SOCS2 and the p50 and p65 subunits of NFκB in HCV X Pparβ/δ-null mice compared to HCV mice. Six HCV wild-type control liver samples and six HCV X Pparβ/δ-null liver samples were used and the average expression levels of the samples were taken. Protein expression levels were quantified using Opti-Quant software and normalized to β-ACTIN. Numerical values are presented as mean ±
Statistical significance was determined using an unpaired t-test set at $p < 0.05$. Statistical significance was indicated by an asterisk (*).

The proteins that had significant differences in expression levels were SOCS2, NFκB p50 and NFκB p65. In all of these cases, HCV X Pparβ/δ-null had higher expression levels compared to the control (Figure 2).

**Figure 2.** Quantification of hepatic expression of proteins in wild-type and HCV X Pparβ/δ-null.

HCV X Pparβ/δ-null expression significantly higher in SOCS2, NFκB p50 and NFκB p65. For each protein, HCV X Pparβ/δ is represented as the bar on the left and HCV X Pparβ/δ-null is represented as the bar on the right. Values are represented as mean ± SEM (n=6). Protein
expression levels were normalized to β-ACTIN. Statistical significance was determined using an unpaired t-test set at \( p < 0.05 \). Statistical significance was indicated by an asterisk (*).

Since SOCS2, NFκB p50 and NFκB p65 all had increases in expression in HCV X Pparβ/δ-null, this may mean enhanced signaling that promotes cancer. NFκB p50 had the most significant difference in expression (~3 times higher).

To further examine these proteins, qPCR analysis was done using target genes of NFκB: Tnfa and Il-6. Before qPCR analysis could be done, RNA had to be isolated and cDNA had to be synthesized using liver samples from the same mice the Western blots were performed with. RNA was isolated by Dr. Gayathri Balandaram using a Ribozol procedure. To determine the quality of the RNA that had been previously isolated, a Northern blot was run to detect the 28S and 18S ribosomal subunits (Figure 3). Five wild-type samples and five HCV X Pparβ/δ-null samples were used.
Figure 3. Northern blot confirms quality of RNA by showing distinct 28S and 18S ribosomal subunits.

RNA samples were run on a formaldehyde agarose gel stained with ethidium bromide to visually observe the 28S and 18S ribosomal subunits. Five HCV wild-type control liver samples (lanes 1-5) and five HCV X Pparβ/δ-null liver samples (lanes 6-10) were used.

Since the Northern blot showed distinct bands representing the 28S and 18S ribosomal subunits, the RNA was quality enough to continue to use for cDNA synthesis and qPCR. The RNA concentrations were determined using NanoDrop and the RNA samples were adjusted to 100 ng/μL in preparation for cDNA synthesis.

After cDNA was synthesized by reverse transcriptase, qPCR was performed using NFκB target genes: Tnfa and Il-6. The qPCR reactions were performed using SYBR green primer mixes. Standard curves were included to verify that technique was sufficient and would yield
accurate results. One experiment was run for \textit{Tnfa} and a separate experiment was run with \textit{Il-6}. The \(R^2\) value for both experiments was 0.998, which indicated that the technique was sufficient. \(C_T\) values were determined for each sample based on where the curve for each sample crossed the threshold (Figure 4). This is where enough amplified product accumulates to yield a fluorescent signal that is detectable. \(C_T\) values are primarily determined by the amount of template presented at the start of an amplification reaction.

![Amplification charts](image)

**Figure 4.** Amplification charts of (A) \textit{Tnfa} experiment and (B) \textit{Il-6} experiment show where \(C_T\) values were derived from.

Two major peaks are observed in both graphs: one for \textit{GAPDH} and one for the target gene. \textit{GAPDH} was used to normalize \textit{Tnfa} and \textit{Il-6}.

Due to the increase of expression in the knockout samples in the Western blot analysis of \textit{NFkB}, it was hypothesized that the knockout samples of the target genes would also show an increase in expression compared to the controls. The relative expression values were calculated by the \(C^{\Delta\Delta C_T}\) (Livak) Method, using the \(C_T\) values of the target genes and \textit{GAPDH}. The
calculation results in a ratio that expresses the relative expression of the knockout gene to the control. The ratio of wild-type to knockout in \textit{Tnfa} and \textit{Il-6} were 0.585 and 0.715, respectively. In both experiments, the relative expression was decreased in the knockout compared to the controls (Figure 5).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Relative expression of (A) \textit{Tnfa} and (B) \textit{Il-6}.}
\end{figure}

\textit{In both (A) and (B), the knockout (-/-) samples had lower expression, however, there was no statistical significance in either experiment. Values are represented as mean ± SEM (n=5). Statistical significance was determined using an unpaired t-test set at p < 0.05.}

\textbf{Lipid Peroxidation and PPAR\textgreek{a}}

The potential role of PPAR\textgreek{a} in liver cancer was examined by measuring the levels of lipid peroxidation in adult and perinatal mice. Each group contained \textit{Ppara} wild-type (control and GW7647), \textit{Ppara} knockout (control and GW7647) and humanized PPAR\textgreek{a} (control and GW7647) liver samples. Five liver samples of each condition were used to isolate protein. Once protein was isolated, it was used to run a TBARS assay to measure lipid peroxidation through
binding of MDA to TBA to form an adduct. The concentration of adduct formed was measured by a spectrophotometer to determine the relative amount of lipid peroxidation that occurred. It was hypothesized that there would be higher lipid peroxidation in the Ppara knockout and humanized PPARα liver samples compared to the Ppara wild-type control. In perinatal mice, the wild-type control, wild-type GW7647, knockout control and humanized PPARα control treatment groups had lower concentrations of MDA (μM) than knockout GW7647 and humanized PPARα treatment groups (Figure 6).

**Figure 6.** Highest levels of lipid peroxidation observed in Ppara knockout and humanized PPARα control treatment groups.

Values are represented as mean ± SEM (n=5). Statistical significance was determined using an unpaired t-test set at p < 0.05.
Concentrations of MDA were also measured in adult mice. It was also hypothesized that the \textit{Ppara} knockout and \textit{PPARa} humanized treatment groups would yield higher concentrations of MDA compared to the \textit{Ppara} control treatment groups. In this experiment, all of the MDA concentrations fell within the similar standard deviations with the exception of the wild-type \textit{Ppara GW7647} treatment group, which had a lower concentration of MDA (Figure 7).

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure7.png}
\caption{Wild-type GW7647 treatment group had a lower concentration of MDA compared to other treatment groups, suggesting it experienced less lipid peroxidation.}
\end{figure}

Values are represented as mean ± SEM (n=6). Statistical significance was determined using an unpaired t-test set at $p < 0.05$. 
PPARβ/δ and HCV-Induced Liver Cancer

The roles of PPARs in liver cancer are still unclear and controversial. Some studies have shown that expression of PPARβ/δ is up-regulated in cancer cells while others have shown that expression of PPARβ/δ is down-regulated in cancer cells. Further, some studies show that ligand activation of PPARβ/δ agonists either increase or decrease tumorigenesis. Viral induction of liver cancer through hepatitis C virus has been more studied with PPARα than PPARβ/δ. Therefore, part of this study served to examine the potential roles of PPARβ/δ in virally induced liver cancer. The Kinexus Antibody Microarray analysis served as the starting point in the study to examine potential proteins that exhibited differences in phosphorylation or expression between wild-type and Pparβ/δ-null mouse liver samples. Based on their calculated Z-ratios, the proteins that were found to have their phosphorylation status altered by the absence of PPARβ/δ expression were NFκB p50, NFκB p65, SOCS2, p27 and PKC-δ.

From there, the selected proteins were subject to Western blot analysis to further examine their expression levels between wild-type and Pparβ/δ-null liver samples. NFκB p50, NFκB p65 and SOCS2 all showed a significant increase of expression in HCV X Pparβ/δ-null samples. PKC-δ and p27 showed no significance in expression levels. NFκB p50 and NFκB p65 are both transcription factors that are inhibited by binding of PPARs in the liver. This represses inflammation by inhibiting cytokines and inflammatory protein production. Since NFκB p50 and p65 are both involved in transcription and inflammatory aspects of the immune response,
knockout of PPARβ/δ could result in overactive NFκB p50 and NFκB p65 which could lead to an over-active immune response. This enhanced signaling has the potential to cause cancer.

On the other hand, the transcriptional regulation of suppressor of cytokine signaling 2 (SOCS2) expression has not been extensively studied. SOCS1 and SOCS3 are both known to inhibit NFκB activation and therefore they are able to regulate TLR4 signaling. Since SOCS1 and SOCS3 inhibit NFκB activation, they would be expected to have a similar expression in Pparβ/δ-null compared to controls since PPARβ/δ is also an inhibitor of NFκB. However, the role of SOCS2 in relation to PPARβ/δ is unclear. Since the expression of SOCS2 was higher in HCV X Pparβ/δ-null compared to wild-type controls, it is suggested that PPARβ/δ would result in a decrease in immune response due to the suppression of cytokines.

To further study the NFκB p50 and NFκB p65 proteins, target genes for these proteins were chosen for quantitative PCR (qPCR) analysis. Tumor necrosis factor alpha (Tnfa) was the first target gene chosen for analysis. TNFα is an inducer of the transcription factor, NFκB. This pathway is responsible for a number of functions including cell proliferation, differentiation and apoptosis and is also a major regulator in inflammation. Interleukin-6 (Il-6) was the other target gene chosen for analysis. IL-6 acts as a cytokine that functions in inflammation and B-cell maturation. It is an inducer of acute phase response and is essential in the final differentiation of B-cells into antibody secreting cells. IL-6 is important in liver regeneration, metabolic functions in the liver and acute phase response in the liver. IL-6 signaling can be activated by the NFκB signaling pathway through the binding of a fragment of the IL-6 promoter to the NFκB protein.

Since Tnfa and Il-6 are target genes of NFκB, they were expected to yield similar results to the Western blot analysis by having increased gene expression in HCV X Pparβ/δ-null.
compared to wild-type control liver samples. After RNA isolation and cDNA synthesis by reverse transcriptase, gene expression of \textit{Tnfa} and \textit{Il-6} was analyzed by qPCR and normalized to GAPDH. Neither \textit{Tnfa} nor \textit{Il-6} had a significant \( p \)-value associated with it. The expression of mRNA for \textit{Tnfa} and \textit{Il-6} were not different. Since there was no significance, no definitive conclusions can be made from the qPCR experiment. \textit{Tnfa} and \textit{Il-6} did not cause functional differences in HCV X \textit{Pparβ/δ} 18-month old liver samples. Although the chosen target genes did not appear to cause functional differences, other NFκB target genes may be affected. Possible considerations for future experiments include using other target genes within NFκB pathways and using mice younger than the 18-month old mice used in this study. Looking at tumor tissue samples rather than the liver tissue samples may also yield more significant results as tumor samples can better illustrate carcinogenesis. Future experiments can also be done to study target genes of the SOCS2 protein to further understand its role in liver carcinogenesis.

There are other potential ideas to address the role of PPARβ/δ in HCV-induced liver cancer. Although there is currently no commercially available HCV vaccine, there are several vaccines that are being tested and developed\textsuperscript{3}. Therefore, there is the potential to develop combination therapies using one treatment to target HCV and another to target PPARβ/δ. However, further studies on the effects of PPARβ/δ and pathways leading to liver cancer need to be better understood before administration of this form of therapy. Another idea would be to explore various ligands that can be developed to prevent liver cancer. New nonhepatotoxic ligands can be developed to use PPARs as therapeutic targets\textsuperscript{36}. Therefore, it is necessary to understand the roles of PPARβ/δ in HCV therapy so that treatments can be developed and liver cancer can be avoided.
**PPARα and Non-Genotoxic Carcinogens**

Aside from viral induction, liver cancer can also be chemically induced. It has been shown that chronic administration of PPARα agonists to mice and rats resulted in hepatomegaly and eventually carcinogenesis and tumors of the liver\(^\text{10}\). In a 1997 study by Peters, Cattley and Gonzales, the effects of the non-genotoxic PPARα agonist, Wy-14,643, were evaluated in *Ppara* wild-type and *Ppara*-null mice\(^\text{10}\). The results of this study showed that 100% of the *Ppara* wild-type mice consuming the Wy-14,643 diet developed tumors, while the *Ppara*-null mice fed the same diet were virtually unaffected. This study showed that PPARα mediates DNA synthesis and carcinogenesis in a mouse model\(^\text{10}\).

The conclusions of this study have been contradicted in other studies that found that *Ppara*-null mice are capable of developing tumors in their livers. A study by Hays et. al study in 2005 tested the effects of PPARα in liver cancer in mice by using 0.5% bezafibrate as the PPARα agonist\(^\text{26}\). This study showed that *Ppara* wild-type mice fed the 0.5% benzafibrate diet developed tumors while one *Ppara*-null mouse developed a single microscopic adenoma\(^\text{26}\). This raised the question: by what mechanism can *Ppara*-null mice develop tumors?

One hypothesis to potentially explain the tumor formation in *Ppara*-null mice involves the property that *Ppara*-null mice have an increased amount of lipids in their livers compared to wild-type controls\(^\text{26}\). Studies have shown that certain fatty acids, such as palmitic acid, are less oxidized in *Ppara*-null mice than *Ppara* wild-type mice; however other fatty acids, such as lignoceric acid, had no significant differences in expression between the two sets of mice\(^\text{27}\). This suggests that the expression of enzymes involved in β-oxidation is independent of PPARα\(^\text{27}\). Due to these findings, it was hypothesized that increased levels of liver fat lead to hepatic tumor formation and carcinogenesis in mice.
Another factor that can lead to hepatic tumor formation is the age of the mouse. A 2004 study by Howroyd et. al. tested the effects of aging and tumor development in Ppara wild-type and Ppara-null mice. The results showed that older Ppara-null mice contained tumors in their livers while Ppara wild-type mice possessed no tumors. This suggests that old Ppara-null mice are predisposed to tumor formation in the liver compared to younger Ppara-null mice and Ppara wild-type mice. However, this difference could be due in part to steatosis since this is a risk factor for liver cancer.

Humanized PPARα samples were included in this study to examine the differences between the susceptibility of mouse and humans to peroxisome proliferators. Studies have shown that mice are more susceptible to peroxisome proliferators and hepatocarcinogenesis than humans. The reason for this is not definitively known but it is hypothesized that it is due to humans having lower hepatic levels of PPARα compared to mice. One study showed that the PPARα agonist, Wy-14,643, led to tumor formation in 71% of wild-type Ppara mice while only 5% of humanized PPARα mice developed tumors. This study further illustrated the differences in susceptibility to tumor formation by PPARα agonists in humans and mice.

Through these studies, it was theorized that the age of the mouse and the level of lipids in the liver are both factors that can influence tumor development in Ppara-null mice. Therefore, this study tested these theories by determining the levels of lipid peroxidation in various treatment groups in mice. Lipid peroxidation is an indicator of cellular injury and oxidative stress and is elevated when β-oxidation is decreased. Since malondialdehyde (MDA) is a by-product of lipid peroxidation, a TBARS assay was performed to measure the amount of MDA, and therefore the amount of lipid peroxidation, in Ppara wild-type, Ppara-null and hPPARα liver tissue samples. This PPARα model included the non-genotoxic carcinogen, GW7647. The experiment
was performed in both perinatal and adult mouse samples. In both experiments, it was expected that the \textit{Ppara}-null and \textit{hPPAR\alpha} liver samples (both control and GW7647 treatments) would yield higher concentrations of MDA compared to the PPAR\alpha controls.

In the perinatal mouse experiment, the highest levels of lipid peroxidation were observed in \textit{Ppara} knockout and humanized PPAR\alpha control treatment groups, although there was no significance with these results. Since it has been shown that older \textit{Ppara}-null mice tend to develop tumors\textsuperscript{26}, it is possible that the mice in the perinatal experiment were not old enough to exhibit the anticipated results. In the adult mouse experiment, wild-type GW7647 treatment group had a lower concentration of MDA compared to other treatment groups, suggesting it experienced less lipid peroxidation. It was expected that the \textit{Ppara} GW7647 control treatment group would have a higher concentration of MDA compared to \textit{Ppara} wild-type control due to the property of PPAR\alpha agonists to increase the rate of tumor formation in the liver. A possible explanation for this reversal of roles could be that the GW7647 was breaking down the lipids in the TBARS assay and giving the assay less substrate to work with.

Although there were no significant results by measuring the amount of lipid peroxidation by the TBARS assay, future studies can be performed to further analyze the formation of tumors and chemically induced hepatocarcinogenesis in \textit{Ppara}-null mice. One option could be to look at metabolomics, the study of a set of metabolites in a tissue or organism, to look for other lipid peroxide by-products besides MDA that can lead to the accumulation of fat in the liver\textsuperscript{37}. Gas chromatography-mass spectrometry (GC-MS) could also be used to examine other lipid peroxides\textsuperscript{38}. These methods could help to uncover the mechanisms by which fatty livers in knockout mice can lead to tumors and carcinogenesis.
Conclusions

Overall, results from this study indicate that PPARβ/δ represses NFκB in a protective mechanism in a viral-induced liver cancer model. PPARβ/δ was shown to have an influence on the expression of NFκB p50, NFκB p65 and SOCS2. The knockout of the PPARβ/δ gene causes an increase in the expression in the aforementioned proteins, which can lead to enhanced signaling that promotes cancer. Future studies can further examine the target genes of NFκB p50, NFκB p65 and SOCS2 to better understand the effects of PPARβ/δ in the respective pathways. Although, the low incidence of liver tumors observed in Ppara-null mice is not due to differences in MDA formation, future studies can identify other lipid peroxide by-products that may have an effect on tumor formation and carcinogenesis. Further studies are needed to determine if other lipid peroxides may contribute to the low incidence of tumorigenesis found in Ppara-null mice compared to controls. With further studies on the effects of PPARβ/δ and PPARα in tumor formation and hepatocarcinogenesis, specific targets can be identified and therapeutic treatments may be developed against liver cancer.
BIBLIOGRAPHY


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