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Farnesol-Mediated Regulation Of Hepatic Lipid Metabolism In Heparg Cells

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FARNESOL-MEDIATED REGULATION OF HEPATIC LIPID METABOLISM IN HepaRG CELLS

by

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DEDICATION

In dedication to my parents, Damodar and Tara Pant, for always believing in me, loving me and letting me be who I am, and my husband, Anurag Dawadi, for his unwavering support, patience and encouragement.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Hepatic steatosis and non-alcoholic fatty-liver disease (NAFLD)

Fatty liver or hepatic steatosis is a pathological condition characterized by excessive accumulation of triglycerides (TGs) in the form of small (microvascular) or large (macrovascular) lipid droplets inside hepatic cells in amounts > 5-10% of total liver weight (Weiss et al., 2014). A healthy liver can become fatty due to several factors. Some of the causative agents of steatosis are high alcohol consumption, pregnancy, toxins, virus, such as hepatitis A and B, and use of drugs like aspirin, steroids, tamoxifen and tetracycline (Tolman and Dalpiaz, 2007). Steatosis occurring in the absence of any significant alcohol consumption, drugs or viruses is known as NAFLD (Loomba and Sanyal, 2013). Due to its frequent association with metabolic disorders such as obesity, diabetes mellitus, and dyslipidemia, NAFLD is also considered as the hepatic manifestation of metabolic syndrome (Loomba and Sanyal, 2013).

According to a 2012 report, the prevalence of NAFLD in the general USA population is about 20%-30% (Bedogni et al., 2014) and 34% in the population of the 14 European countries (Blachier et al., 2013). Similarly, studies from Japan, Hong Kong, Taiwan, Indonesia, and India show the prevalence of NAFLD to be 9-14%, 16%, 11-41%, 30% and 5-28% respectively (Parkash and Hamid, 2013). Further, NAFLD has higher prevalence in obese or diabetic patients with rates reaching up to 90% in morbidly obese subjects (LaBrecque et al., 2014). Additionally, studies from diverse populations, such as Chinese, European and Taiwanese show that NAFLD is present in a disproportionately higher percentage of obese children compared to the general population (Wiegand et al., 2010; Yang and Huang, 2010; Fan, 2013). This high rate of pediatric NAFLD is extremely concerning given the sharp rise in

childhood obesity, even in underdeveloped countries where many of the children used to be underweight.

1.2 Pathogenesis of NAFLD

NAFLD is a progressive disease and incorporates a wide spectrum of liver abnormalities that range from hepatocellular steatosis, steatohepatitis, fibrosis and ultimately, cirrhosis (Dowman et al., 2010). Pathologically, it occurs as two distinctive forms. The first one is the simple hepatic steatosis, also known as non-alcoholic fatty liver (NAFL), which is a benign condition very common in obese people, and is almost non-progressive (Masarone et al., 2014). In 5 - 20% of these patients, NAFL develops into a more severe and progressive form known as non-alcoholic steatohepatitis (NASH), which features hepatic swelling, and is histologically indistinctive from alcohol-induced hepatic steatosis (Weiss et al., 2014). Development of NAFL and its progression to NASH is often described by two-hit hypothesis, where the 'first hit' is the lipid accumulation in hepatocytes resulting from an imbalance in inflow of lipids into liver and amounts being degraded or exported from liver. The 'second hit' is defined by generation of oxidative stress and inflammation caused by infiltration of cytokines that results in steatohepatitis (Dietrich and Hellerbrand, 2014). 10–20% of NASH patients also develop fibrosis due to chronic liver injury and wound healing. NASH affects around 2-3% of total world population and progresses to hepatic cirrhosis in <5% of patients, which affects around 0.05–0.3% of the general world population (Weiss et al., 2014). The endpoint of NASH prognosis is hepatocellular carcinoma, which is a fatal unless the patient receives liver transplant.

1.3 Risk factors for NAFLD

NAFLD development is a complex process that involves intricate interactions between host and environmental factors. As mentioned above, one of the major risk factors for NAFLD is the metabolic syndrome. Obesity, insulin resistance, and diabetes are independent predictors of NAFLD and are very closely associated with NAFLD prevalence (Younossi et al., 2011). For instance, studies show that prevalence of NAFLD in the general US population is between 27-34% (LaBrecque et al., 2014); however, prevalence of NAFLD is around 70% or more in patients with obesity and type-2-diabetes mellitus (T2DM) and 50% in patients with hyperlipidemia, and around 60% of obese patients undergoing gastric bypass surgery display the more severe form of NAFLD (Dietrich and Hellerbrand, 2014). Age is another important risk factor for NAFLD development. The older population has higher NAFLD prevalence with rates peaking at the age of 40s and 60s in men and in women respectively (Mishra and Younossi, 2012). Also, benign NAFL has larger likelihood of progressing into NASH in older patients leading to greater mortality in elderly compared to the younger patients (Ong et al., 2008). However, age-associated risk for NAFLD is confounded by the fact that prevalence of other independent risk factors, such as obesity and metabolic syndrome, is also higher in older patients.

Gender is another risk factor for NAFLD, and several studies across different population groups show that men are at greater risk to develop NALFD than women (Chen et al., 2008; Williams et al., 2011; Mishra and Younossi, 2012). A retrospective study of patients who underwent gastric bypass surgery found that out of 58 men and 307 women, 60.3% men had NASH compared to 30.9% of women (Arun et al., 2006), even though both genders had similar composition of other risk factors, such as age, obesity and other metabolic syndromes.

Risk for NAFLD is also associated with race and ethnicity. In the USA, the prevalence of steatosis is highest in Hispanics followed by whites and then black (Browning et al., 2004). Even though the higher prevalence of hepatic steatosis in Hispanics was associated with a higher percentage of obesity and insulin resistance, these factors were not associated with the lowest prevalence in Blacks (Browning et al., 2004). Further, NAFLD patients in Asians countries usually have lower rates of obesity compared to the NAFLD patients in western countries. According to a report in Japan, only 20-30% of NAFLD patients were obese, whereas in USA, almost 60-80% of NAFLD patients suffered from obesity (Eguchi et al., 2012).

The discovery of ethnic differences in NAFLD prevalence as well as reports of familial clusters of NAFLD patients (Willner et al., 2001) suggest that genetic susceptibility is another important risk factor for NAFLD. Several studies indicate that polymorphisms in the adiponutrin/patatin-like phospholipase-3 (PNPLA3) gene, which encodes a 481 amino acid protein related to adipose TG lipase, to be one of the most important contributors to variation in NAFLD prevalence among different populations (Anstee et al., 2011). One of the variants of the gene, rs738409, is significantly associated with increased TG content in human liver and elevated levels of serum transaminase levels, which is an indicator of hepatic injury (Romeo et al., 2008). This variant is also a strong risk predictor for NAFLD in Asian and Hispanic populations (Romeo et al., 2008; Zhang et al., 2015a). Another variant of the same gene, rs6006460, however, seems to have protective effects against NAFLD, and is abundant in African–Americans associated with low NAFLD prevalence but is rare in European–Americans and Hispanics (Romeo et al., 2008).

1.4 Hepatic TG accumulation during NAFLD

Hepatic accumulation of TGs is the hallmark of NAFLD. Under normal physiological conditions, fatty acid-flux through the liver is maintained in a steady state with only small amounts of TGs being stored in hepatocytes (Kawano and Cohen, 2013). Fats from diet are assembled as triglyceride-rich chylomicrons, which are secreted into plasma and are primarily distributed to adipose tissues. These circulating chylomicrons can also undergo lipolysis to generate non-esterified fatty acids (NEFAs) that are delivered to liver, and are taken up by the hepatic plasma membrane receptors, such as fatty acid transport proteins (FATPs) and fatty acid translocase (FAT; CD36) (Reddy and Sambasiva Rao, 2006; Kawano and Cohen, 2013). When there is abundance of carbohydrates, fatty acids are also synthesized in liver by de novo lipogenesis. Fatty acids acquired either through diet or lipogenesis are esterified and used as substrates for TG biosynthesis in liver, which are either stored within the hepatocytes or exported in the form of very low-density lipoproteins (VLDL) particles into the bloodstream. Under fasting conditions, fatty acids are degraded through oxidation, primarily in mitochondria and peroxisomes, in order to release energy in the form of ATP (Browning and Horton, 2004). Any imbalance between acquisition of fatty acid and its clearance from hepatocytes, either through export or oxidation, disturbs lipid homeostasis in liver leading to intracellular accumulation of TGs.

The major mechanisms behind a metabolic imbalance in liver include: 1) increased supply of fatty acids from plasma, either from higher intake of dietary fats or from a higher rate of chylomicron lipolysis; 2) increased rate of de novo lipogenesis; 3) reduced rate of fatty acid oxidation; 4) decreased secretion of TGs in the form of VLDL particles (Browning and Horton, 2004; Kawano and Cohen, 2013). Both extra-hepatic and hepatic events influence the

above processes in order to cause perturbations in hepatic lipid homeostasis. The major extra-hepatic factors include higher intake of dietary fat, increase in the body fat mass due to visceral and peripheral obesity that leads to development of insulin resistance and makes adipocytes less susceptible to the antilipolytic effects of insulin. This increases the plasma pool of NEFAs, and therefore more fatty acids end up being taken up by the hepatocytes. (Anderson and Borlak, 2008). Intra-hepatic factors mostly include changes in the expression or activity of genes that are responsible for fatty acid uptake, lipogenesis, fatty acid oxidation, TG storage and export from the liver. These genes and the cellular processes they regulate are controlled by both transcriptional and post-transcriptional modifications. An important group of molecules involved in transcriptional regulation of hepatic lipid metabolic processes are nuclear receptors and are described below.

1.5 Nuclear receptors: Lipid metabolism and NAFLD

Nuclear receptors (NRs) are ligand-activated transcription factors that regulate a broad range of metabolic, detoxifying, and regulatory processes through complex transcriptional networks in response to diverse ligands (natural or synthetic), including lipids, bile acids, vitamins, xenobiotics, hormones, drugs, and toxins (López-Velázquez et al., 2012). Several of these NRs, such as the peroxisome proliferator-activated receptors (PPARs), farnesoid X receptors (FXR), liver X receptor (LXR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are involved in transcriptional regulation of key hepatic lipid-metabolizing genes that regulate several critical metabolic processes, which are often dysregulated during NAFLD, such as lipolysis, fatty acid uptake, oxidation, storage, and export (Wagner et al., 2011). NRs play central roles in hepatic homeostasis, and because of their involvement in the metabolism of fatty acid, cholesterol and bile salts, hepatic inflammation and fibrosis, they are

regarded as important therapeutic targets for NAFLD or NASH treatment (George and Liddle, 2008; Machado and Cortez-Pinto, 2014; Mazzoccoli et al., 2014). Involvement of some of the NRs, such as FXR, CAR and PPARs in hepatic lipid metabolism as well as in NAFLD pathogenesis are described below.

1.5.1 Farnesoid X receptor

One of the NRs that is emerging as a major player in NAFLD pathogenesis and treatment is FXR. It is a bile acid receptor that is activated by bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA). FXR is highly expressed in tissues like liver, kidneys and intestines. In addition to regulating bile acid homeostasis, FXR plays important roles in hepatic lipid metabolism especially by regulating lipogenesis. Hepatic fatty acid synthesis is mostly controlled by the sterol regulatory element binding protein 1c (SREBP-1c), which regulates the expression of several important lipogenic genes including fatty acid synthase (FAS) and stearoyl-coenzyme A desaturase 1 (SCD1)(Xu et al., 2014). Activation of FXR increases the expression of the short heterodimer partner (SHP), which downregulates expression of the liver X receptor (LXR) and its target gene SREBP-1c. Thus, FXR activation suppresses expression of SREBP-1c target enzymes through the SHP-LXR-SREBP-1c pathway leading to reduced lipogenesis, and therefore less fatty acid is available as precursor for triglyceride biosynthesis (Watanabe et al., 2004). Also, FXR has been shown to suppress hepatic lipogenesis and TG accumulation through a SREBP-1c-independent pathway that involves FXR-mediated inhibition of the carbohydrate response element-binding protein (ChREBP), which is another crucial gene responsible for hepatic glucose and lipid metabolism (Li et al., 2013). FXR activation has also been shown to increase clearance of hepatic TGs by modulating expression of apolipoproteins (Xu et al., 2014) and enhance fatty acid oxidation

by inducing the expression of PPARα and its target gene, carnitine palmitoyltransferase 1 (CPT1) (Pineda Torra et al., 2003).

In NAFLD patients, hepatic expression of FXR is decreased while the expression of LXR, SREBP-1c and FAS are increased, which is associated with increased TG synthesis (Yang et al., 2010). Expression of FXR is also suppressed in liver of obese mice and FXR-null mice develop spontaneous hepatic steatosis (Xu et al., 2014). One of the most studied FXR agonists is INT-747 (also known as Obeticholic acid (OCA)), a synthetic bile acid analogue and a 6α-ethyl derivative of CDCA, which is reported to suppress steatohepatitis and improve glucose and insulin tolerance and lower the expression of lipogenic genes animal models of steatosis (Patel et al., 2015). In a clinical study where NAFLD patients with type 2 diabetes mellitus were given either 25 (n=20) or 50 mg (n=21) OCA for 6 weeks, increase in insulin sensitivity, and reduction fibrosis and inflammation markers were observed. OCA has also been studied in a larger clinical trial known as the farnesoid X receptor ligand obeticholic Acid in non-alcoholic steatohepatitis (FLINT) study where NASH patients were treated with 25 mg OCA for 72 weeks (Neuschwander-Tetri et al., 2015). Results showed that OCA improved liver histology in 45% of the patients compared to the observed 21% in control group. Decrease in the serum alanine aminotransferase levels, a marker for liver damage accompanied these effects. However, OCA caused pruritus in 23% of the patients and also led to 20% increase in the LDL-cholesterol levels, which required withdrawal of treatment. This increase in risk for atherosclerosis suggests that beneficial advantage of long-term OCA treatment against NAFLD/NASH needs to be further evaluated.

1.5.2 Peroxisome proliferator-activated receptors

PPARs are another group of NRs that have been shown to play an important role in steatosis and pathogenesis of NAFLD. While the three PPAR isotypes, PPARα, PPARβ/δ, and PPARγ have different tissue and ligand specificity, they all are involved in regulating the expression of genes involved in overall energy homeostasis. PPARγ is abundant in adipose tissues, PPARβ/δ has ubiquitous expression and PPARα is predominant in liver (López-Velázquez et al., 2012). In hepatocytes, PPARα acts as a lipid sensor that detects increases in free fatty acids, and then transcriptionally induces fatty acid oxidation by regulating expression of genes involved in mitochondrial, peroxisomal and microsomal oxidation (Sambasiva Rao and Reddy, 2004). PPARα also modulates expression of fatty acid binding and transport enzymes, lipases and apolipoproteins in hepatocytes (Mandard et al., 2004; López-Velázquez et al., 2012).

Involvement of PPARα in NAFLD/NASH pathogenesis is supported by studies on PPARα deficient animal models where absence of PPARα is associated with reduced hepatic fatty acid oxidation and development of severe hepatic steatohepatitis, which can be reversed by administration of PPARα agonists (Sambasiva Rao and Reddy, 2004). Expression of PPARα is also suppressed in steatotic human liver. In the liver of obese and NAFLD patients, hepatic TG accumulation is associated with increased expression of SREBP-1c and FAS, while the mRNA level of PPARα is suppressed, resulting in metabolic imbalance followed by steatosis due to a higher rate of lipogenesis than lipid oxidation (Pettinelli et al., 2009). In preclinical studies, pharmacological activation of PPARα by fibrates, such as fenofibrate suppresses hepatic TG accumulation and ameliorates steatosis in animal models (Tailleux et al., 2012). However, such strategies have not been successful in clinical studies yet, and

activation of PPARα has not produced conclusive results, which might be partially attributable to insufficient sample size and incomplete data (Tailleux et al., 2012; Machado and Cortez-Pinto, 2014). However, this discrepancy could also be the result of the species-specific differences that exist in PPARα activity between rodents and human regarding expression, ligand-responsiveness and identity of its target genes (Sambasiva Rao and Reddy, 2004). Therefore, further studies are required to determine the usefulness of targeting PPARα for pharmacological intervention against NAFLD.

1.5.3 Constitutive androstane receptor

The constitutive androstane receptor is mostly known for its role in xenobiotic metabolism; however, it is also responsive towards endogenous metabolites, such as androstane, progesterone and estrogen, and is reported to be involved in regulation of energy homeostasis, including lipid and glucose metabolism (Wada et al., 2009). CAR has been shown to inhibit lipogenesis by transcriptionally increasing levels of the Insig-1 protein, which in turn reduces the levels of active SREBP-1c and thereby, suppresses expression of lipogenic genes that are transcriptional target of SREBP-1c (Roth et al., 2008). Additionally, during fasting, CAR has been shown to compete with PPARα for the binding sites on the promoter of PPARα-target gene 3-hydroxyacyl CoA dehydrogenase, which is involved in the peroxisomal β-oxidation (Kassam et al., 2000). In rodents, pharmacological activation of CAR has been shown to produce contradictory results regarding development of steatosis. Depending on the animal model, CAR has been shown to either have no effect on hepatic lipid accumulation, induce abnormal *de novo* lipogenesis and insulin resistance, increase serum TG and hepatic lipogenesis, or suppress lipogenesis, increase β-oxidation and improve insulin sensitivity (Naik et al., 2013). Further, SREBP-1c, which is increased in steatotic liver, has been reported to

impair CAR-mediated activation of its target genes by hindering CAR's ability to interact with co-activators, suggesting that CAR activity is itself altered during NAFLD (Roth et al., 2008).

As discussed above, NRs, such as PPARs, CAR and FXR regulate various critical metabolic processes involved in the development of hepatic steatosis and pathogenesis of NAFLD. Given their roles in lipid metabolism and suggestions of cross talk between different NRs, they could be attractive candidates for pharmacological interventions against NAFLD.

1.6 Farnesol: An isoprenoid-derivative of the mevalonate pathway

The isoprenoid pathway, also known as the mevalonate pathway for cholesterol biosynthesis, is the source of several important metabolic intermediates that are involved in a variety of biological processes (Edwards and Ericsson, 1999)**.** As depicted in figure 1, the first step of the pathway involves condensation of two molecules of acetyl-CoA to acetoacyl CoA, which is then metabolized by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase to HMG-CoA (Figure 1). HMG-CoA-reductase (HMGCR) reduces this compound to mevalonate, which is then phosphorylated by mevalonate kinase to produce 5-phosphomevalonate. Additional phosphorylation and decarboxylation of 5-phosphomevalonate leads to production of isopentenyl pyrophosphate (IPP), which is the basic isoprene subunit used to build several other isoprenoid compounds. Isomerization of IPP produces dimethylallyl pyrophosphate (DMAPP), which together with IPP is condensed by the enzyme farnesyl pyrophosphate synthase (FPPS) to the 10-carbon geranyl pyrophosphate (GPP). A second molecule of IPP is then added to GPP by FPPS producing farnesyl pyrophosphate (FPP), which is the major branch-point intermediate in the mevalonate pathway.

Under basal conditions, the majority of the FPP is metabolized by the enzyme squalene synthase to produce squalene, which is the first committed step towards synthesis of cholesterol. Alternatively, FPP and IPP are combined by the enzyme geranylgeranyl pyrophosphate (GGPP) synthase to form GGPP, a twenty-carbon compound. FPP also function as the precursors to several longer chain isoprenoids including ubiquinone (coenzyme Q), dolichol, dolichyl phosphate, heme O, heme A, and isopentenyl tRNA (Goldstein and Brown, 1990). Additionally, FPP and GGPP function as substrates for protein prenylation reactions, which are important post-translational modifications that are required for cellular signaling by small GTPases (Zhang and Casey, 1996).

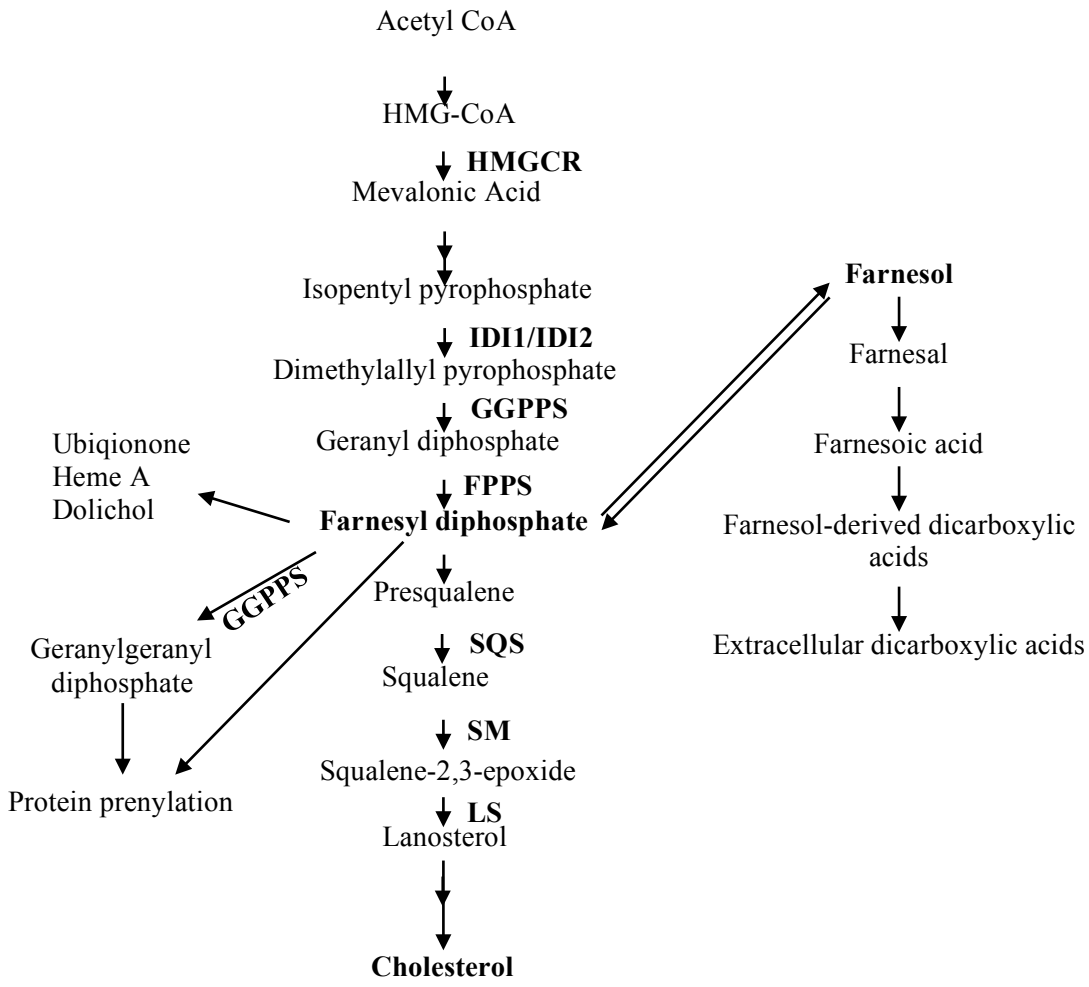


Figure 1. Schematic of mevalonate pathway for cholesterol synthesis. Two molecules of acetyl-CoA are utilized as the initial substrates to synthesize cholesterol through a series of enzymatic reactions. Farnesol is one of the isoprenoids derivatives of the pathway, which is produced by dephosphorylation of the branch point intermediate, farnesyl pyrophosphate (FPP). Farnesol can be sequentially metabolized to produce dicarboxylic acids, which are excreted through urine. 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), isopentenyl-diphosphate delta isomerase 1 (IDI1), isopentenyl-diphosphate delta isomerase 2 (IDI2), geranylgeranyl diphosphate synthase 1 (GGPPS), farnesyl pyrophosphate synthase (FPPS), squalene synthase (SQS), squalene monooxygenase (SM), lanosterol synthase (LS).

One of the non-sterol derivatives of FPP is farnesol, which produced by sequential dephosphorylation of FPP by phosphatases. Farnesol is a 15-carbon acyclic sesquiterpene alcohol ($C_{15}H_{26}O$) and is the building block of most of the acyclic sesquiterpenoids. Farnesol is colorless under standard conditions and is hydrophobic, therefore miscible with oils. Apart from its endogenous synthesis in animals, farnesol can be acquired via consumption of fruits and aromatic plants, including citrus, sage, spearmint, lemon grass, strawberries, tomatoes, peaches and plant-based essential oils. (Duncan and Archer, 2008). It has a sweet aroma and is routinely used as a fragrance in perfumes.

1.6.1 Physiological roles of farnesol

Farnesol and its metabolites (collectively known as farnesoids) are involved in several biological processes. Farnesol has been shown to mediate anti-tumor and chemotherapeutic responses in human cancer cell lines such as lung carcinoma, leukemia, oral squamous carcinoma, colon cancer and pancreatic adenocarcinoma (Rioja et al., 2000; Joo et al., 2007; Wiseman et al., 2007; Au-Yeung et al., 2008; Scheper et al., 2008). Similar results have also been observed in *in vivo* animal studies against hepatocellular carcinoma and pancreatic cancer. (Burke et al., 1997; Crowell, 1999; Burke et al., 2002; Ong et al., 2006). Many of these anti-cancer properties of farnesol are the result of its anti-proliferative effect on cancer cells caused by cell-cycle arrest and induction of apoptosis (Joo and Jetten, 2010). Farnesol-mediated apoptosis can occur through various cellular processes including regulation of HMG-CoA reductase and CTP:phosphocholine cytidylyltransferase α (CCTα), generation of reactive oxygen species, activation of nuclear receptors, induction of endoplasmic reticulum (ER) stress, and activation of the NF-κB signaling pathway (Joo and Jetten, 2010). The IC_{50} value for anti-proliferative effect of farnesol varies between 25 to 250 μM, and neoplastic cells being

are more susceptible to farnesol-induced apoptosis than are non-malignant cells (Melnykovych et al., 1992; Adany et al., 1994). Farnesol is also involved in other biological processes such as microbial quorum sensing and biofilm formation (Albuquerque and Casadevall, 2012), synthesis of isoprenoids and protein prenylation (Crick et al., 1995; Thai et al., 1999), and cellular differentiation (Hanley et al., 2000). Farnesol also modulates the expression of several Phase I and Phase II drug-metabolizing enzymes, such as cytochrome p450 (1A, 2A1-3, 2B1/2, 2C11/12, 2E1, 3A1/2, 4A1-3, 19) in rats, suggesting that farnesol can potentially affect drug metabolism and change drug efficacy or toxicity (Horn et al., 2005a). Finally, farnesol can also regulate lipid metabolic pathways, which is described in detail below.

1.6.2 Regulation of lipid metabolism by farnesol

Several *in vivo* and *in vitro* experiments in rodents and hepatoma cell lines have evaluated the effects of farnesol on lipid metabolism. Some of the first reports that implicated farnesol in regulation of lipid metabolic processes were studies where inhibition of the cholesterol biosynthesis pathway by squalene synthase inhibitors (SSIs) resulted in endogenous accumulation of farnesol. SSI inhibitors, such as zaragozic acid (squalestatin-1; SQ1), inhibit the enzyme squalene synthase **(**Figure 1**)** causing FPP accumulation and directing the pathway towards producing large amounts of farnesol-derived dicarboxylic acids (Bostedor et al., 1997; Vaidya et al., 1998). SQ1 was previously known to decrease serum cholesterol levels in marmosets, and lower cholesterol production from isolated rat hepatocytes and in rat liver (Baxter et al., 1992). Similarly, another SSI, RPR-107393, also reduced plasma cholesterol concentrations in rats (Amin et al., 1997). Taken together, these SSI-mediated effects on cholesterol levels, as well as the evidence that treatment with SQ1 causes increases in farnesol and its metabolites, suggested that farnesol or its derivatives could be involved in

SSI-mediated effects on cholesterol metabolism. Indeed, farnesol was implicated as the non-sterol derivative of the mevalonate pathway that degraded HMGCR and inhibited cholesterol synthesis through feedback regulation (Correll et al., 1994; Keller et al., 1996).

Several other studies that evaluated the effects of SSIs on lipid metabolism followed these findings. The SSI YM-53601 not only lowered plasma non high-density lipoprotein (HDL)-C levels in rhesus monkeys, but also significantly reduced plasma TG levels (Ugawa et al., 2000). Similarly, Hiyoshi et al. showed that treatment with ER-27856, a potent SSI prodrug, decreased plasma cholesterol levels in rabbits through a low-density lipoprotein (LDL) receptor-independent mechanism, and also reduced plasma TG levels and TG biosynthesis (Hiyoshi et al., 2001). Further, the SSI TAK-475 reduced nonHDL-cholesterol and TG levels in marmosets, cynomolgus monkeys, beagle dogs and Wistar fatty rats. In rats, the lipid-lowering effect of TAK-475 was associated with suppression of hepatic TG secretion and upregulation of LDL receptors (Nishimoto et al., 2003). These SSI-mediated decreases in cholesterol and TG levels were later shown to be mediated via accumulation of intracellular farnesol and its derivatives in primary cultured rat hepatocytes (Hiyoshi et al., 2003). Also, direct treatment with farnesol suppressed TG and cholesterol biosynthesis, while SSIs suppressed fatty acid biosynthesis as well (Hiyoshi et al., 2003). The isoprenols farnesol and geranylgeraniol regulated expression of several hepatic lipid metabolic genes including adipocyte fatty acid-binding protein (aP2), carnitine palmitoyltransferase 1 liver (CPT1A) and acyl-CoA synthetase (ACS) in 3T3-L1 and HepG2 cells (Takahashi et al., 2002), which provided some mechanistic insights behind farnesol-mediated regulation of lipid metabolism. Additionally, oral administration of farnesol lowered serum TGs, increased fatty acid oxidation rate and suppressed lipogenesis in rats (Duncan and Archer, 2008). In the same study, farnesol

upregulated expression of the fatty acid oxidation enzymes, CPT1A and fatty acyl-CoA oxidase (ACOX) and suppressed mRNA expression, protein levels and activity of the lipogenic enzyme FAS. Finally, in mice, co-administration of farnesol with high-fat diet lowered serum glucose and hepatic TG levels compared to high fat-only fed animals and farnesol treatment suppressed TG biosynthesis (Goto et al., 2011). Genes responsible for fatty acid oxidation, such as CPT1A and ACS were also upregulated, while expression of genes regulating lipogenesis such as FAS and SREBP-1c were suppressed in farnesol-treated animals. These data showed that in rodents, farnesol has hypotriglyceridemic properties that are mediated via suppression of TG and fatty acid synthesis and upregulation of fatty acid oxidation, and demonstrates the potential of farnesol or its derivatives to be used as a therapeutic approach against hyperlipidemia and its pathophysiological consequences.

1.6.3 Effect of farnesol on nuclear receptors

Studies have shown that farnesol modulates cellular processes through activation of nuclear receptors, such as PPARα and FXR in rodents. In the H4IIEC3 (rat hepatoma) cell line, farnesol increased activity of the PPARα target gene ACOX, and both farnesol and its derivative, farnesoic acid, increased the PPAR-response element (PPRE)-dependent ACOX-luciferase activity in CV1 cells that were co-transfected with a rat PPARα expression plasmid (O'Brien et al., 1996). In wild-type mice, topical farnesol treatment increased expression levels of the marker genes for terminal epidermal differentiation, which was absent in PPARα-/- animals (Hanley et al., 2000). These findings are further supported by data showing that farnesol treatment upregulated PPARα mRNA levels as well as expression of PPARα target genes in clone-9 cultured rat hepatocytes (Duncan and Archer, 2008). Additionally, dietary farnesol increased the expression of fatty acid oxidation genes in the livers of high fat-fed mice,

which are regulated via PPARα (Goto et al., 2011). This farnesol-mediated upregulation of fatty acid oxidation gene expression was absent in PPARα-/- mice.

Farnesol-mediated regulation of human PPARα has not been clearly elucidated; however, several studies show that farnesol can mediate PPRE-dependent transactivation as discussed below. In human keratinocytes, *trans,trans*-farnesol has been shown to activate PPRE-dependent luciferase activity and increase PPARα mRNA levels in normal human kidney epithelial (NHK) cells (Hanley et al., 2000). In CV1 cells, which is a cell line derived from kidney of African green monkey, farnesol induced both PPARα/GAL4 and PPARγ/GAL4 luciferase reporter activity (Hanley et al., 2000; Takahashi et al., 2002). Farnesol also increased PPRE luciferase activity when cells are cotransfected with full-length human PPARα and PPARγ cDNA in CV1 cells. Additionally, in human PPARα overexpressed HepG2 cells, farnesol treatment increased the expression of PPARα target genes ACS and CPT1A, suggesting that farnesol can potentially activate both PPARγ and PPARα-mediated responses in human (Takahashi et al., 2002). In HT-29 and HCT116 colon cancer cells, farnesol increased PPARγ promoter activity and farnesol-mediated anti-proliferative effects in these cells were suppressed in the presence of PPARγ antagonist (Au-Yeung et al., 2008).

Farnesol is a known FXR agonist (Forman et al., 1995a) and has been shown to regulate several physiological responses through FXR. Farnesol-mediated activation of FXR has been suggested to induce proliferation of MCF-7 breast cancer cells (Journe et al., 2008). Also, in mice, farnesol was found to suppress expression of the lipogenic genes SREBP-1c and FAS through FXR activation, and thereby decrease the intracellular pool of fatty acids that are substrates for TG biosynthesis. This effect was suggested to be the mechanism through which farnesol suppressed TG biosynthesis in mice independently of the PPARα pathway (Goto et

al., 2011). However, in rats, farnesol reduced FAS but not SREBP-1c mRNA levels, and down-regulation of FAS by farnesol was caused by a 9-cis retinoic acid-mediated mechanism that involved down-regulation of retinoid X receptor (Duncan and Archer, 2008).

Recently, Moore *et al*. [29] have shown that activation of CAR in mice reduces lipogenic genes and increases the expression of genes involved in β-oxidation and suggested that CAR activation may be a suitable therapeutic approach to treat diabetes and fatty liver disease. Since accumulation of farnesoids has been shown to activate CAR-regulated genes, such as CYP2B1 in primary rat hepatocytes (Kocarek and Mercer-Haines, 2002), it is possible that farnesoid-mediated lowering of hepatic lipid may occur through CAR activation.

1.7 HepaRG Cells: A hepatoma cell model to study human hepatic lipid metabolism

Primary human hepatocytes are regarded as the gold-standard cell culture model to study human hepatic functions. However, their unpredictable availability, batch-to-batch variability in viability and function, loss of certain differentiated properties over time in culture, failure to replicate, and relatively short lifespan makes it difficult to use them as a routine experimental model of human liver (Guillouzo, 1998). To overcome these difficulties, hepatoma cell lines, such as HepG2 cells, have been developed that retain some liver-specific properties. However, these hepatoma cell lines fail to capture the overall gene expression pattern that is found in human liver, such as for CYP, and NRs, such as PPARs and CAR, which makes them unsuitable for studying many aspects of normal human hepatic physiology (Aninat et al., 2006).

HepaRG is a recently developed bipotent hepatic cell line that was derived from a human hepatocellular carcinoma, and it expresses most of the liver-specific enzymes and high levels of CYPs (Guillouzo et al., 2007). When seeded at low density, undifferentiated HepaRG

cells grow into an elongated cell type that shows markers for hepatic progenitor cells. The undifferentiated HepaRG cells can be maintained in sub-confluent culture for many weeks and they fully retain their proliferative capacity (Aninat et al., 2006). After reaching confluency at around 10 days after plating, the cells show two distinct morphologies: one forming clusters of granular cells resembling hepatocytes surrounded by the second cell type that is more flattened and has clear cytoplasm. Upon addition of 2% DMSO to the culture medium, the hepatocyte-like cells are further differentiated into more granular cells that resemble mature hepatocytes with bile canaliculus-like structures and express hepatocyte-specific markers that can be maintained in culture for several weeks. The second group of flattened cells expresses markers for biliary epithelial cells. At confluency, the hepatocyte-like cells make up 50-55% of the total cell population. The unique feature of HepaRG cells compared to other hepatic cell lines, such as HepG2, is that HepaRG cells express most of the drug-metabolizing enzymes and other liver-specific genes, and therefore resemble primary cultured human hepatocytes more closely than do other human hepatic cell lines (Aninat et al., 2006; Hart et al., 2010; Gerets et al., 2012). Moreover, the fact that HepaRG cells can be cultured over multiple weeks allows extended studies of liver metabolism, xenobiotic toxicity, and other liver functions to be performed.

Comparative microarray analysis shows that upon DMSO stimulation, HepaRG cells express a vast number of genes similar to primary cultured human hepatocytes, including high mRNA levels of CYPs, such as CYP2B6, 3A4, 1A2, 2D6, and 4F3B (Aninat et al., 2006; Gerets et al., 2012; Samanez et al., 2012). In addition, HepaRG cells have been described as a suitable model to study hepatic lipid and glucose metabolism since they secrete TG and apo-B rich lipoprotein as well as express SREBP-1c and FAS that can be further induced by glucose

(Guillouzo et al., 2007; Samanez et al., 2012). Furthermore, HepaRG cells have been shown to have high and sustainable expression of NRs such as PPAR, CAR, FXR and pregnane X receptor (PXR) (Aninat et al., 2006; Samanez et al., 2012). PPARα agonist treatments have been shown to increase the expression of PPAR target genes, such as fatty acid binding protein (FABP4) and carnitine palmitoyltransferase 1 (CPT1), suggesting that the pathway is responsive to external stimulation (Rogue et al., 2012). Both FABP4 and CPT1 play important roles in lipid metabolism where they are regulate fatty acid transport across membranes and catalyze the rate-limiting step in β-oxidation respectively (Nguyen et al., 2008). Therefore, HepaRG's similarities to primary human hepatocytes, continual expression of hepatic enzymes for a considerable time in culture, presence of functional NRs in measurable amounts and functional lipid metabolic pathways support the use of this cell system to study farnesol-mediated effects on human hepatic lipid homeostasis.

1.8 Objective of the dissertation

The major objective of the research described below is to investigate the effects of farnesol on hepatic lipid metabolism in human liver under condition that leads to intrahepatic accumulation of triglycerides and to elucidate any underlying mechanisms behind such effects. **As an overarching hypothesis, we propose the that farnesol treatment changes the expression of genes involved in human hepatic lipid homeostasis and that these changes occur through altering the activity of one or more of the nuclear receptors PPARα, FXR, and CAR.** This hypothesis is based on the evidences described above that demonstrate farnesol's ability to suppress TG accumulation in the hepatocytes of rodents and its capability to modulate the activity of the three nuclear receptors. The hypothesis would be tested through two specific aims.

1) Specific aim 1: To identify the farnesol-mediated changes in the expression of genes involved in lipid metabolism, where HepaRG cells will be treated with farnesol, and pathway-based microarray analysis will be performed using a customized PCR Array for genes involved in hepatic lipid metabolism.

2) Specific aim 2: To determine the roles of NRs in farnesol-mediated changes in human hepatic lipid-metabolizing gene expression. For this purpose, we will determine the identity of the potential receptor and the impact of its knockdown on farnesol-mediated effects on gene expression.

CHAPTER 2: FARNESOL SUPPRESSES TRIGLYCERIDE ACCUMULATION AND REGULATES HEPATIC LIPID METABOLISM THROUGH PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARα) PATHWAY IN STEATOTIC HepaRG CELLS.

2.1 Introduction

Intrahepatic triglyceride (TG) levels are regulated through a delicate balance between: 1) the amount of free fatty acid that either enter hepatic cells from plasma or that is synthesized de novo and 2) the amount of fatty acid that is catabolized through oxidation or that is secreted into plasma as triglycerides (Koo, 2013). Any change in the balance between these complex metabolic pathways results in more fatty acid entering or produced in the hepatocytes than is eliminated, which increases intrahepatic fat content (Anderson and Borlak, 2008; Musso et al., 2009). When TG accumulates inside hepatic cells to amounts > 5% of total liver weight, the liver is classified as steatotic (Bedogni et al., 2014). Hepatic steatosis occurring in absence of any significant alcohol use, drugs or viruses is the hallmark of non-alcoholic fatty liver disease (NAFLD) (Loomba and Sanyal, 2013), which is considered to be the hepatic manifestation of the metabolic syndrome (Angulo, 2007). Even though NAFLD has become the most common liver disease today with its worldwide prevalence ranging from 11 to 40%, there are no established therapeutic regimens except for weight loss (Kawano and Cohen, 2013).

Farnesol is a non-sterol isoprenoid that is produced endogenously through the dephosphorylation of farnesyl pyrophosphate (FPP), a key branch-point intermediate of the cholesterol biosynthetic pathway (Goldstein and Brown, 1990). Farnesol is also widely present in plants such as peaches, tomatoes, strawberries, corn and lemongrass (Duncan and Archer, 2008). Physiologically, farnesol serves as the intermediate in the elimination of excess FPP and can also function as a signaling molecule in a variety of cellular processes, such as feedback regulation of sterol biosynthesis, vasoconstriction, apoptosis, cell proliferation, and

lipid metabolism (Correll et al., 1994; Edwards and Ericsson, 1999). Regarding the latter, several *in vivo* and *in vitro* studies in rodents have shown that farnesol treatment improves metabolic abnormalities in obese animals, decreases expression of fatty acid synthesis genes, increases expression of fatty acid oxidation genes, and lowers TG levels in plasma and in cultured hepatocytes (Takahashi et al., 2002; Duncan and Archer, 2008; Goto et al., 2011). Similarly, farnesol and its metabolites have also been implicated in the TG lowering effects produced by inhibitors of squalene synthase, the enzyme that catalyzes the conversion of FPP to squalene, which is the first committed step in sterol biosynthesis (Hiyoshi et al., 2003). These studies suggested that farnesol might be useful as an early therapeutic strategy to alleviate steatosis and NAFLD progression.

Most of the regulatory effects of farnesol on lipid metabolism in the above studies were shown to be mediated through nuclear receptors such as the peroxisomal proliferator receptors (PPARα; NR1C1 and PPARγ; NR1C3), farnesoid X receptor (FXR; NR1H4) and retinoid X receptor β (RXRβ) (Takahashi et al., 2002; Duncan and Archer, 2008; Goto et al., 2011). These ligand-activated transcription factors regulate expression of several lipid-metabolizing genes involved in fatty acid oxidation, synthesis, uptake, storage, transport and elimination (Chawla et al., 2001; and and Moller, 2002; Wagner et al., 2011). While the above studies demonstrate that farnesol can produce beneficial effects on rodent hepatic lipid metabolism, the relevance to human is not clear, since activity of nuclear receptors can differ substantially across species (Gonzalez and Shah, 2008; Kiyosawa et al., 2008; Ross et al., 2010). Additionally, farnesol and/or its metabolites activate constitutive androstane receptor (CAR; NR1I3) in primary cultured rat hepatocyte (Kocarek and Mercer-Haines, 2002). However, whether CAR activation has any role in farnesol's ability to regulate hepatic lipid metabolism has not yet

been studied, which is important given CAR's involvement in lipid metabolism and its crosstalk with PPARα (Xiao et al., 2013). In this study, we used oleic acid (OA)-overloaded HepaRG cells as a model for human hepatic steatosis in order to determine farnesol's effects on various lipid-metabolizing pathways as well as any involvement of PPARα, CAR or FXR signaling in the farnesol-mediated effects. We found that farnesol treatment lowered TG levels and increased fatty acid oxidation rate in the OA-overloaded HepaRG cells. Farnesol treatment also changed the expression of several lipid-metabolizing genes, including several that were not previously associated with farnesol treatment in rodent studies. Most of the farnesol-mediated changes, especially those involved in mitochondrial β-oxidation, occurred via PPARα-dependent mechanisms.

2.2 Materials and method

Materials

Trans,trans-farnesol, dimethyl sulfoxide (DMSO), and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO), 3-[2-[2-Chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid (GW4064), oleic acid (OA), hematoxylin, Oil Red O (ORO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fatty acid free bovine serum albumin (FF-BSA) were purchased from Sigma Chemical (St. Louis, MO). 2-[[4-[2-[[(Cyclohexylamino)carbonyl](4-cyclohexylbutyl)amino]ethyl]phenyl]thio]-2-methylpropanoic acid (GW7647) and N-((2S)-2-(((1Z)-1-Methyl-3-oxo-3-(4-(trifluoromethyl)phenyl)prop-1-enyl)amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4 (GW6471) were purchased from Tocris (Minneapolis, MN). Williams' medium E was purchased from Invitrogen (Carlsbad, CA), recombinant human insulin (Novolin R) from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ), and TaqMan and

SYBR Green PCR master mix were purchased from Applied Biosystems (Foster City, CA). Additional materials were obtained from the sources listed below. [9,10-^{3}H(N)]-OA was purchased from PerkinElmer (Waltham, MA).

HepaRG cell culture and differentiation

HepaRG cells were obtained from Biopredic International under a Material Transfer Agreement with INSERM-Transfert (Paris, France). The cells were plated at a density of 2.4 X 10^{4}cells/cm^{2} on culture plates (6-well plates unless otherwise indicated) in HepaRG growth medium (Williams' Medium E supplemented with 10% FBS, 5µg/ml insulin, 0.1M triamcinolone acetonide, and 100u/ml penicillin and 100ug/ml streptomycin) for 14 days followed by HepaRG differentiation medium 1 (HepaRG growth medium supplemented with 1% DMSO) for 48 hours and HepaRG differentiation medium 2 (HepaRG growth medium supplemented with 2% DMSO) for a further 14 days. Culture medium was renewed every 2-3 days. Differentiated HepaRG cells were then cultured in supplemented Williams' medium E with 2% FBS for 72 hours. Drug the treatments were begun 96 hr after removal of HepaRG differentiation media and were done in DMSO-free serum-free HepaRG treatment media. Prior to treatment, all cells were maintained for 24 hr in serum-free supplemented Williams' E medium (HepaRG treatment medium). Drugs were added to the culture medium as concentrated stock solutions (1000X) in ethanol or DMSO at the concentrations indicated in the individual figure legends.

Farnesol and oleic acid treatments

1000X Farnesol stocks (10, 30, 100, 300 and 1000 mM) were prepared in 100% ethanol. For farnesol treatments, the required volume of farnesol stock was added to 0.7% FF-BSA in HepaRG treatment medium-2 and allowed to complex for 30 min. FF-BSA+ 0.1%

ethanol was used as a vehicle control for all farnesol studies. For oleic acid (OA) treatment, OA was first complexed with to FF-BSA at a 6:1 molar ratio (OA: BSA). Briefly, an 8mM OA: FF-BSA stock was prepared by adding 4.52 mg OA into 1 ml of 8.8% FF-BSA solution in HepaRG treatment media-2, vortexed, and warmed at 37^0C until the cloudiness disappeared. Required volumes of stock were then diluted in HepaRG treatment media-2 to obtain the desired final OA concentrations.

Measurement of cell viability

The MTT assay was utilized to assess treatment effects on cell viability, as has been previously described in detail (Mosmann, 1983). Briefly, MTT was dissolved at a concentration of 1 mg/mL in 1X phosphate buffered saline (PBS) and sterile-filtered prior to use. At selected times following treatment, culture medium was replaced with 0.3 mL of MTT solution, and cells were further incubated for 30 min at 37^0C. Following incubation, cells were washed with PBS, and formazan was extracted with 0.3 ml 100% isopropanol with continuous shaking at room temperature for 30 min. Aliquots (40 µl) of isopropanol extracts were diluted in isopropanol (160 µl) and absorbance was measured at 560 nm using a SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). Each sample was measured in triplicate, and each experiment was repeated 3 independent times.

Measurement of neutral lipids by ORO staining

ORO staining was used to determine the cellular levels of neutral lipids such, as triglycerides and cholesterol esters, as previously described (Ramirez-Zacarias et al., 1992). Briefly, 0.35 g ORO was dissolved in 100 ml isopropanol and filtered. A working solution of ORO was prepared fresh by mixing 6 ml of ORO stock with 4 ml of water, and was filtered after incubating at room temperature for 20 min. Forty-eight hr after treatment, HepaRG cells

were washed in PBS and then fixed in PBS containing 10% formalaldehyde (w/v) for 1 hr. Fixed cells were washed three times with water and then incubated in isopropanol (60%) for 10 minutes at room temperature. Following incubation, isopropanol was removed and cells were allowed to dry completely, and then 1 mL of a filtered ORO solution was added to each well. The cells were then incubated for 30 min at room temperature and rinsed four times with water. Nuclei were counterstained briefly using hematoxylin. ORO-stained lipid vesicles were visualized at 200X using a phase-contrast microscope equipped with a digital camera (Olympus; Waltham, MA). To quantify differences in neutral lipid accumulation, ORO was extracted from the cells by adding 100% isopropanol to each well and incubating at room temperature for 15 min with gentle agitation. The absorbance of the extracted ORO was read at 510 nm. Experiment was repeated 3 independent times.

Total RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Following treatments, HepaRG cell cultures were harvested and total RNA was extracted and column purified using the Purelink RNA isolation kit (Ambion; Carlsbad, CA). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies; Grand Island, NY) following the manufacturer's instructions. Relative changes in gene expression were then assayed using either TaqMan or Power SYBR Green Master Mix and the StepOnePlus Real Time PCR system (Applied Biosystems; Foster City, CA). Primer pairs were purchased from Integrated DNA Technologies (IDT; Coralville, IA) and their sequences are described in **Appendix C**. Tata-binding protein (TBP) was used as the endogenous control (ID Hs.PT.58v.39858774; IDT). Relative fold-changes in mRNA levels were calculated using the comparative CT (ΔΔCT)

method (User bulletin no.2, Applied Biosystems, Foster City, CA) and all changes were normalized to the CT values for the endogenous control TBP. All assays were performed in duplicate, and each experiment was repeated three or more times.

Measurement of hepatic fatty acid oxidation rate

Differentiated HepaRG cells were cultured in 12-well plates at a density of 2.4 X 10^4cells/cm^2. Following treatment, fatty acid oxidation rates in intact cells were determined as described previously (Zhang et al., 2012), using [9,10-^{3}H(N)]-OA as substrate and tritiated water as the measured end product of oxidation. Briefly, a complex of FF-BSA:OA (8mM OA) was prepared as described above. [9,10-^{3}H(N)]-OA was included to achieve a final radiolabel concentration of 1μCi per mL in the treatment medium. The mixture was incubated for 30 min and then diluted in HepaRG treatment medium. 1 ml of medium containing the [^{3}H]-OA was added to each well and the cells were incubated at 37 ºC for 3 hr, after which the medium was collected and protein precipitated by adding 100% trichloroacetic acid to a final concentration of 9% (v/v) and centrifugation. The supernatants were alkalinized by adding 6N NaOH and then were loaded onto columns containing 0.2 g/mL Dowex 1x2-400 resin (Sigma) diluted in water. Tritiated water was eluted from the columns using 1.5 mL water into scintillation vials containing 10 mL Ultima-Flo™ M scintillation cocktail (PerkinElmer; (Waltham, MA) and radioactivity was measured using a Beckman-Coulter liquid scintillation counter (model LS3801; Indianapolis, IN). Results were corrected for nonspecific radioactivity obtained from medium containing [^{3}H]-OA that was not added to HepaRG cultures. Experiments were conducted in quadruplicate, and repeated 3 independent times.

Measurement of hepatic lipid-metabolizing genes expression using customized PCR arrays

96-well RT² Profiler™ PCR Array plates were purchased from Qiagen that were custom designed to contain primers for selected genes involved in human hepatic fatty acid synthesis, fatty acid oxidation (mitochondrial, peroxisomal), triglyceride biosynthesis, fatty acid transport, and cholesterol biosynthesis. Prototypical target genes for assessing activation of nuclear receptors CAR (CYP2B6), FXR (short heterodimer partner, SHP), and PPARα (Plin2) were included as positive controls. Four housekeeping genes were also included for normalization. A complete list of the genes can be found in **Appendix C.** Following treatment, HepaRG cells were harvested, and total RNA was isolated using the Purelink RNA isolation kit (Ambion). cDNA was then prepared using the RT^2 First Strand Kit (Qiagen) and PCR was performed using the RT² SYBR Green qPCR Master Mix according to manufacturer's (Qiagen) recommendations using StepOnePlus Real Time PCR system. Fold changes in expression were analyzed using the web-based RT^2 Profiler^{TM} PCR Array Data Analysis system (Qiagen), which is based on the $\Delta\Delta C_t$ method, and raw data were normalized to housekeeping genes. Treatment-induced changes in mRNA levels were then normalized to the mRNA of the vehicle controls and these values were visualized as a heat map that was generated using GENE-E software v. 3.0.204 (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). Each experiment was conducted 2 independent times.

Statistical analysis

All results are presented as mean ± S.E.M. Significance testing was performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software; San Diego, CA). As

appropriate, treatment groups were compared using one- or two-way analysis of variance with the Newman-Keuls correction for multiple comparisons or one-sample t-tests against the hypothetical value of 1. $P < 0.05$ was considered statistically significant.

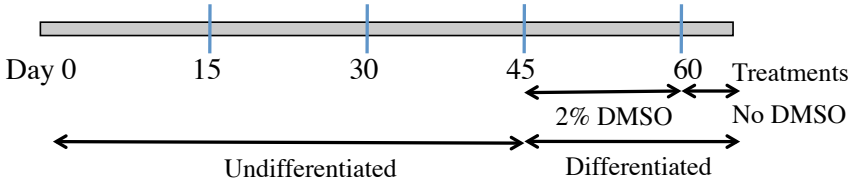
2.3 Results

2.3.1 HepaRG cell culture and differentiation.

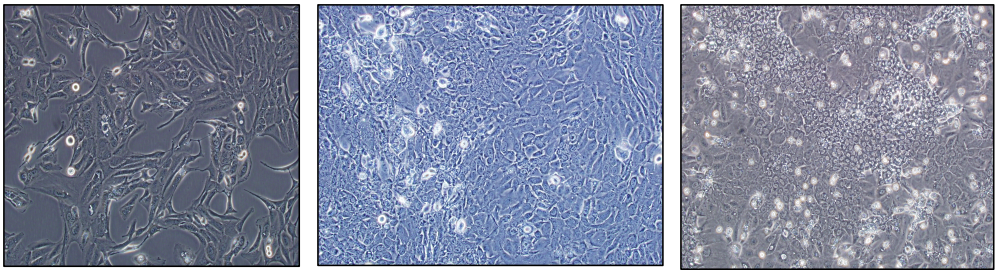
Differentiated HepaRG cells have been previously shown to be a suitable in vitro system for studying hepatic lipid metabolic processes (Samanez et al., 2012; Brown et al., 2013). We therefore used HepaRG cells as a model to evaluate the effects of farnesol on human hepatic lipid metabolism. The timeline for HepaRG culture expansion and differentiation is described in Figure 3.1A, and is detailed in the methods. HepaRG cell morphology during early (day 4) and late (day 14) growth phases, and during the differentiation phase (day 7) is shown in Figure 3.1B. As seen in the figure, differentiated HepaRG cells exist as a mixture of hepatocyte-like cells with bile canaliculus-like structures and colangiocyte-like cells in an approximate 1:1 ratio.

Figure 2.1

A



B



Undifferentiated HepaRG (Day 4) **Undifferentiated HepaRG (day 14)** **Differentiated HepaRG (day 7)**

Figure 2.1 HepaRG cell culture and differentiation (A) Timeline of HepaRG cell culture and differentiation. **(B)** Undifferentiated HepaRG cells in culture at day 4 and day 14, and differentiated HepaRG cells at day 7 after being cultured in differentiation medium containing 2% DMSO.

2.3.2 OA treatment induces accumulation of neutral lipids in differentiated HepaRG cells.

Differentiated HepaRG cells were exposed to increasing concentrations of OA (0.33, 0.66 and 1mM), a known inducer of steatosis in human hepatic cells, including HepaRG cells (Ricchi et al., 2009; Anthérieu et al., 2011), for 48 hours. Oil Red O staining revealed a concentration-dependent increase of intracytoplasmic lipid droplets (stained in red) in response to OA treatment (Figure 2.2A). Quantification of the staining showed that absorbance was significantly increased by 1.3-, 1.7- and 1.9- fold at 0.33, 0.66 and 1mM OA, respectively, compared to the vehicle control (Figure 2.2B).

Figure 2.2

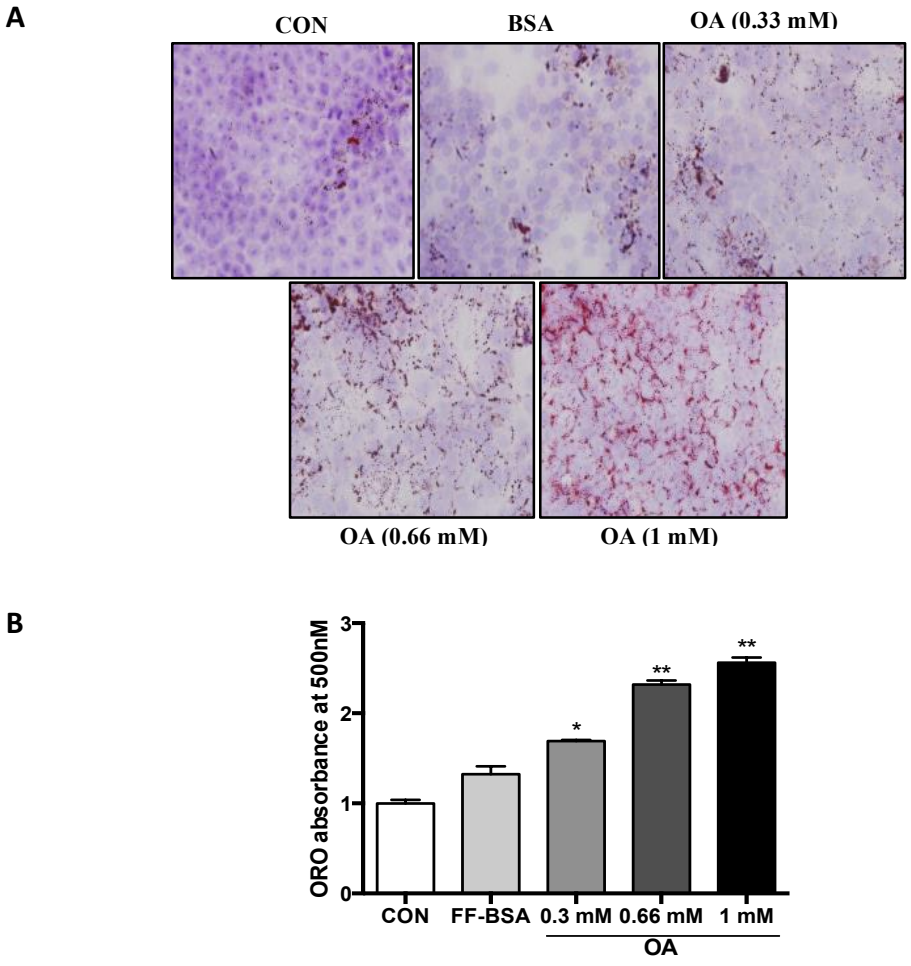


Figure 2.2 Determination of neutral lipid accumulation by Oil Red O (ORO) staining in OA-overloaded HepaRG cells. Differentiated HepaRG cells were incubated for 48 hr in HepaRG treatment medium-2, either alone (CON; untreated) or containing FF-BSA +EtOH (0.1%) (FF-BSA; vehicle control), either alone or complexed with 0.33mM, 0.66mM, or 1mM OA. Treatments were replaced once after 24 hours. **(A)** ORO was used to stain the neutral lipids (triglycerides and cholesterol esters) and cells were photographed with a phase-contrast microscope at 200X magnification. The lipids are stained in red. **(B)** Quantification of ORO staining: ORO was extracted the cells in 100% isopropanol and the absorbance was measured at 510nM. Each bar is the mean ± SEM, n= 4. *, ** Significantly different than the vehicle control (FF-BSA), *P < 0.01, **P<0.001.

2.3.3 Optimization of FF- concentration for farnesol treatment in HepaRG cells.

Given the hydrophobic nature of farnesol, FF-BSA was used as a carrier to facilitate uptake of farnesol into the HepaRG cells. For this purpose, we first determined the optimal concentration range for preparing FF-BSA and farnesol complex. Previous study in our lab has shown that farnesol can increase the expression of CYP2B1 gene in primary rat hepatocytes. We therefore measured the mRNA levels of the CYP2B6 gene, which is a human homologue of CYP2B1, as a reporter of farnesol's effects on HepaRG cells. As shown in the Figure 2.3, treatment with increasing concentration of FF-BSA (0.03-0.3%) had no effects on the expression of CYP2B6 in HepaRG cells. Further, treatment with 100μM farnesol complexed to FF-BSA increased the expression of CYP2B6 to similar levels for all concentrations of FF-BSA used. Based on these data, farnesol was complexed with FF-BSA so that the final FF-BSA concentration was 0.07% in the treatment media, which is same to that for media containing the FF-BSA: OA complex.

2.3.4 Selection of optimal dose and treatment time for farnesol treatment in HepaRG cells.

Next, we treated HepaRG cells with 100μM farnesol for 24, 48 and 72 hr in order to determine an optimal culture time. Again, using the CYP2B6 gene as a reporter for farnesol treatment, we measured the changes in its mRNA levels in response to farnesol exposure. Compared to the FF-BSA (vehicle) control, farnesol had no effect on the expression levels of CYP2B6 gene at 24 hr (Figure 2.4A). However, it increased the CYP2B6 mRNA levels at 32 and 48 hr. In order to determine an optimal dose of farnesol, we treated HepaRG cells with increasing concentrations of farnesol (50, 100, and 300μM) for 48 hr (Figure 2.4B) and measured the mRNA levels of the CYP2B6 gene. Farnesol treatment resulted in concentration-dependent increase in the CYP2B6 mRNA levels for 50 and 100μM concentrations, where as

the levels went down at 300μM. Based on our observations, we selected 100μM of farnesol as our optimal dose and 48 hr as our optimal culture time for future experiments.

Figure 2.3

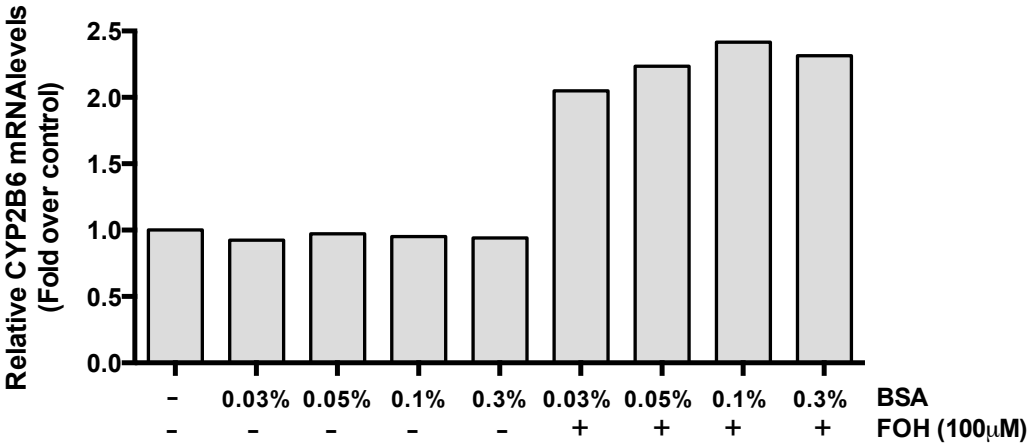


Figure 2.3 Optimization of FF-BSA treatment in HepaRG cells. Differentiated HepaRG cells were cultured for 48 hr in HepaRG treatment medium-2 alone or containing the increasing concentrations of FF-BSA (0.03, 0.05, 0.1, 0.3%) either alone or in combination with farnesol (FOH, 100μM). Treatments were replaced once after 24 hours. 48 hr after treatment, cells were harvested for RNA isolation and cDNA synthesis, and mRNA levels of CYP2B6 was quantified by *q*RT-PCR, as described in Methods. Each bar represents the normalized mRNA values from a single experiment.

Figure 2.4

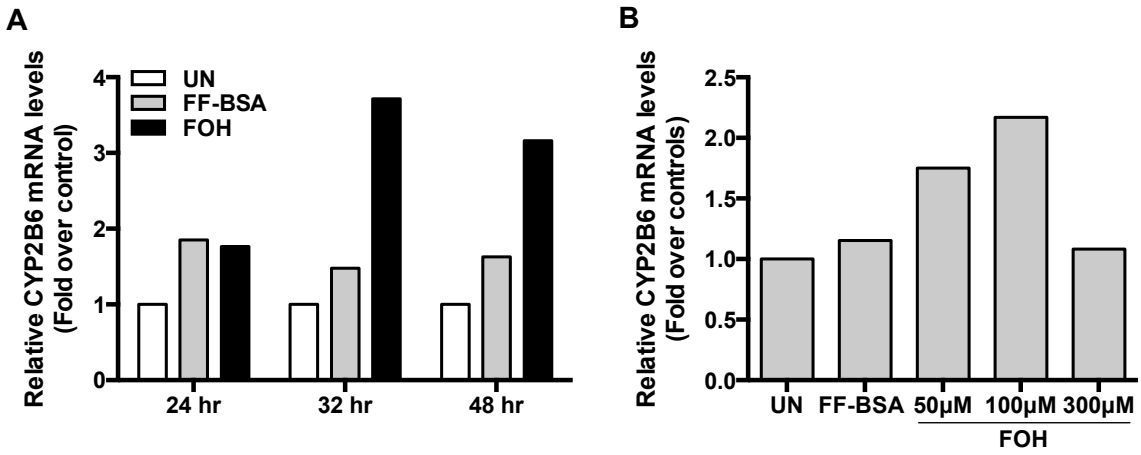


Figure 2.4 Dose- and time-dependent effect of farnesol on CYP2B6 gene expression in HepaRG cells. (A) Differentiated HepaRG cells were cultured in HepaRG treatment medium-2 alone (UN; untreated) or containing either FF-BSA+ EtOH (0.1%) (FF-BSA; vehicle control) or farnesol (FOH; 100µM) for 24, 32 or 48 hr. **(B)** Differentiated HepaRG cells were cultured in HepaRG treatment medium-2 alone (UN; untreated) or containing either FF-BSA+ EtOH (0.1%) (FF-BSA; vehicle control) or farnesol (FOH; 50, 100, or 300 µM) for 48 hr. **(A) and (B)** Following treatments treatment, cells were harvested for RNA isolation and cDNA synthesis, and mRNA levels of CYP2B6 was quantified by *q*RT-PCR, as described in Methods. Each bar represents the normalized mRNA values from a single experiment.

2.3.5 Farnesol has no toxic effects on differentiated HepaRG cell cultures.

In order to evaluate whether farnesol caused any cytotoxicity in HepaRG cultures, differentiated HepaRG cells were exposed to increasing concentrations of farnesol (10, 30, 100, 300 and 1000µM) for 24 or 48 hr, and cell viability was measured by the MTT assay. Farnesol treatment had no effect on MTT absorbance at any of the concentrations or treatment times that were tested (Figure 2.5)**.** Also, no changes in cellular morphology were noted when the cells were observed under phase-contrast microscopy (data not shown).

Figure 2.5

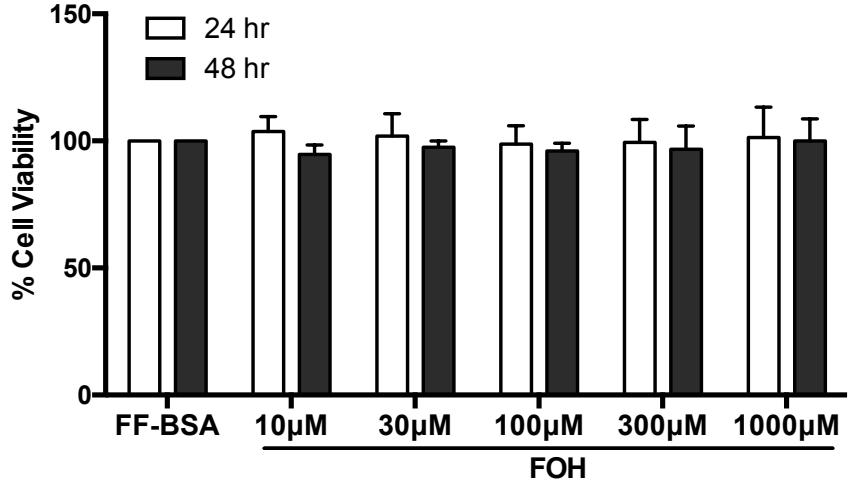


Figure 2.5 Determination cell viability in farnesol-treated HepaRG cells. Differentiated HepaRG cells were cultured for either 24 or 48 hr in HepaRG treatment medium-2 containing either FF-BSA+ EtOH (0.1%) (FF-BSA; vehicle control), alone or complexed with 10μM, 30μM, 100μM, 300μM or 1000μM farnesol (FOH). Cells viability was determined by the MTT assay, as described in Methods. Each bar represents the mean MTT absorbance ± S.E.M of three independent experiments normalized to the FF-BSA group (n=3).

2.3.6 Farnesol suppresses OA-induced lipid accumulation in HepaRG cells.

To determine the effect of farnesol on intracellular TGs accumulation in OA-overloaded HepaRG cells, differentiated HepaRG cells were pre-incubated with either FF-BSA (0.7%) or 0.66mM OA (complexed with 0.7% FF-BSA) for 24 hr followed by treatment with either vehicle control (Ethanol; 0.1%) or farnesol (100μM) in the absence or presence of OA for another 48 hr, and intracellular TG levels were measured. Farnesol treatment had no effects on the TG levels under control conditions. Exposure to OA increased the amount of intracellular TG levels by 7.7–fold in HepaRG cells when compared to FF-BSA-treated controls (Figure 2.6). This OA-induced increase in TG levels was suppressed significantly by 24.4% when HepaRG cells were co-treated with 100μM farnesol.

Figure 2.6

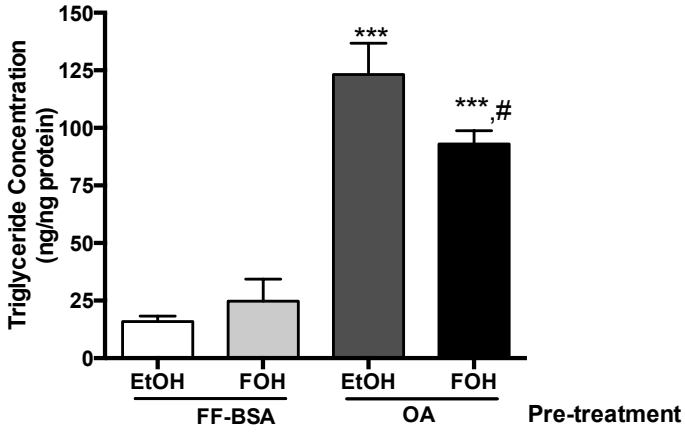


Figure 2.6 Effect of farnesol treatment on intrahepatic TG levels in steatotic HepaRG cells. Differentiated HepaRG cells were cultured for 24 hr in HepaRG treatment medium-2 containing FFA-BSA + EtOH (0.1%) or 0.66 mM oleic acid (OA) for 24 hrs. Thereafter, cells were incubated for 48 hr in pre-treatment medium (i.e., with FFA-BSA or OA) containing either EtOH (0.1%) (vehicle control) or farnesol (FOH; 100μM). Cells were then harvested for TG measurement, as described in Methods. The amount of TGs was normalized to protein for each sample. Each bar is the mean ± SEM from three independent experiments (n=3). ***, Significantly different from FF-BSA: EtOH group, $p<0.001$. #, Significantly different from OA:EtOH group, $p<0.05$.

2.3.7 *Farnesol activates lipid-sensing nuclear receptors in HepaRG cells.*

Since farnesol has been previously shown to mediate physiological responses, including lipid metabolism, through activation of PPARα, CAR and FXR, we hypothesized that one or more of these receptors could be involved in the observed farnesol-mediated reduction of TG in our hepatic steatosis model. For this, we first determined whether farnesol affected the activity of CAR, PPARα or FXR in HepaRG cells. Differentiated HepaRG cells were treated with either farnesol or known agonists for the individual nuclear receptors for 48 hr, and expression levels of their respective target genes were measured by qRT-PCR (Figure 2.7). We found that treatment with the CAR agonist CITCO (0.1μM), PPARα agonist GW7647 (10μM) and the FXR agonist GW4064 (1μM) significantly increased the expression of their target genes CYP2B6 (CAR), perilipin 2 (PLIN2, PPARα) and short heterodimer partner (SHP,

FXR) by ~4-fold, ~5-fold and ~3-fold, respectively, compared to vehicle-treated control. Treatment with farnesol (100μM) significantly increased the expression of CYP2B6 by 2-fold and PLIN2 by 1.7-fold. However, farnesol treatment had no effect on expression of SHP. These findings indicate that farnesol can modulate activity of CAR and PPARα, but not FXR, at least under the current experimental conditions in HepaRG cells.

Figure 2.7

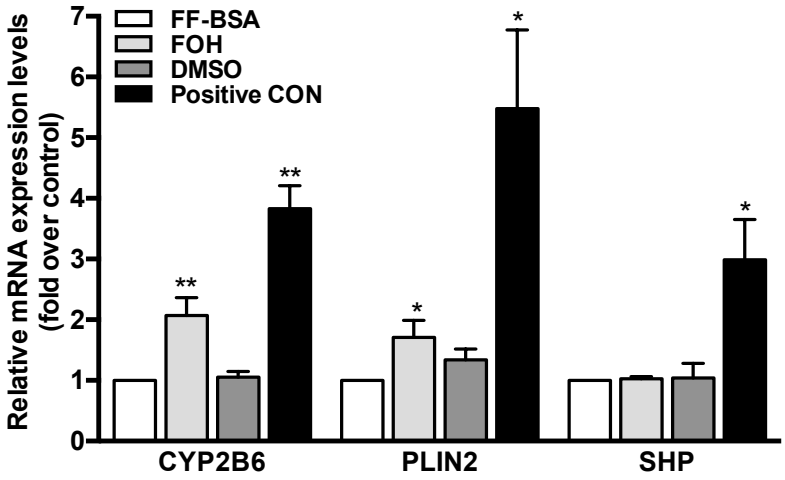


Figure 2.7 Effect of farnesol on the activity of nuclear receptors in HepaRG cells. Differentiated HepaRG cells were cultured in HepaRG treatment medium-2 containing either FF-BSA+ EtOH (0.1%) (FF-BSA; vehicle control), farnesol (FOH, 100μM), DMSO (0.1%) or the respective agonist for each nuclear receptor: CITCO (CAR; 0.1μM), GW7647 (PPARα; 10μM) or GW4064 (FXR; 1μM). Treatments were replaced once after 24 hours. 48 hr after treatment, cells were harvested for RNA isolation and cDNA synthesis, and mRNA levels of CYP2B6, PLIN2 and SHP were quantified by *q*RT-PCR, as described in Methods. Each bar represents the mean ± S.E.M. of normalized mRNA values from three (PLIN2) to four (CYP2B6, SHP) independent experiments. *, ** Significantly different from the vehicle control (BSA), * $P<0.05$; ** $P<0.01$.

2.3.8 Farnesol changes the expression of hepatic lipid metabolizing enzymes in steatotic HepaRG cells.

In order to investigate mechanisms behind farnesol-mediated suppression of lipid accumulation in OA-overloaded HepaRG cells, we designed a customized PCR array and measured changes in the expression of genes involved in various lipid-metabolizing pathways in human liver, which included genes responsible for fatty acid synthesis, fatty acid oxidation, TG biosynthesis, fatty acid uptake and transport, lipoprotein secretion and cholesterol biosynthesis. The list of genes is shown in **Appendix A**. HepaRG cells were treated either with farnesol, OA, OA and farnesol, or agonists for PPARα, CAR and FXR. mRNA levels for all genes were measured. The results are represented as a heat map in Figure 2.8 and are presented in detail in the **Appendix B**.

Our data shows that activation of CAR in HepaRG cells by CITCO resulted in upregulation in the mRNA levels of CYP2B6, ELOVL3, ACADS, ACOT12, PPARα, AGPAT2, FASN and ACOT1 and suppressed the expression of NR0B2, CD36, CYP4A11, and ACOT6. Similarly, activation of FXR by GW4064 upregulated expressions of SHP, PPARG, APOA5, ACSL5, AGPAT2, ELOVL3, ACADS, PPARA, AGPAT9, FASN, ACSL3, AGPAT6, AGPAT1, while genes, such as NR1H4, DGAT2, FABP1, SCD, ACOT6, CYP2B6, DGAT1, LIPC, CYP4A11, ACOX2, CYP2E1, and PCK1were downregulated. A large number of genes regulating hepatic fatty acid oxidation, and fatty acid uptake and transport were upregulated by PPARα activation, which included ACAA1, CD36, FABP1, PLIN2, ACADS, PCK1, HADHB, ACAA2, SLC27A2, CPT1A, APOA5, AGPAT2, HADHA, CYP4A11, ACSL5, ACOT12, ACSL4, ACADM, ACOT1, CPT2, ACADVL, ACACB, ACSL3, DHDDS,

SCD, ACOX1, AGPAT9, LIPC, EHHADH, SREBF1, PPARG, and ACOX2. Among the genes studies, the only gene downregulated by activation of PPARα was SHP.

Farnesol treatment increased the expression of CYP2B6, FABP1, ACOX2, SLC27A2, ACOT12, ELOVL4, PPARG, ELOVL3, FDFT1, PDSS1, APOA5, LIPC, ACAA2, ACAT2, ACAT1, and PLIN2, majority of which were also upregulated by PPARα activation. Also, farnesol reduced the mRNA levels of AGPAT2, ACACB, CYP2E1, PCK1, SLC27A1, and SREBF1. Incubation in OA increased the expression of APOA5, PCK1, ACOT12, HADHB, CD36, PLIN2, AGPAT2, AGPAT9, ACADS, ACAA2, ELOVL3 and CYP2E1 while expression of ACOX2, SHP, CAR, SCD5, GPAT2, SREBF1, CYP2B6, IL6, and SCD were suppressed. Co-treatment of farnesol in OA-overloaded HepaRG cells upregulated expression of APOA5, ACOT12, PCK1, ELOVL3, ACADS, CYP2B6, CYP2E1, PLIN2, ACAA2, CPT1A, PPARα, ACSL5, HADHB, ELOVL6, AGPAT6, HMGCR, ACSL4, HADHA, ACOX1 and ACADM while expression of RABGGTA, AGPAT1, SCD, GPAT2 and SHP were downregulated.

Figure 2.8

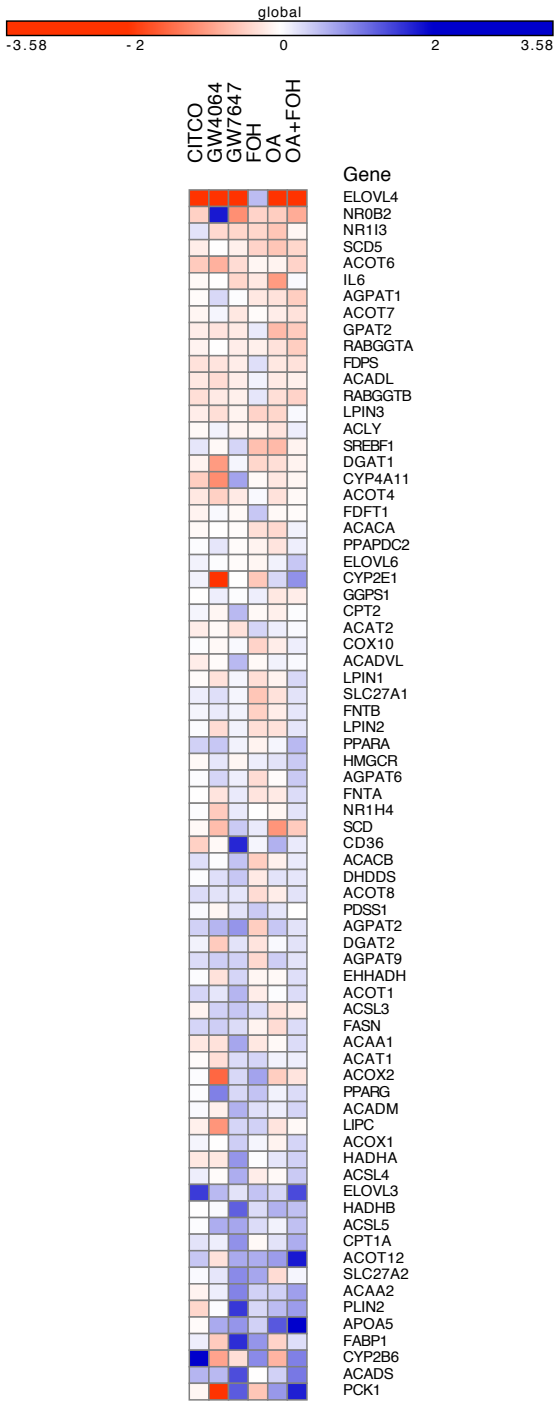


Figure 2.8 Effect of farnesol on hepatic lipid-metabolizing gene expression in HepaRG cells. Differentiated HepaRG cells were cultured in HepaRG treatment medium-2 containing either vehicle control (FF-BSA) or OA (0.66mM) for 24 hr. Thereafter, FF-BSA pre-treated group was treated with either the vehicle control (FF-BSA+EtOH (0.1%) or farnesol (100μM,

FOH) for 48 hr. The OA pre-treated group was incubated in medium containing OA in combination with one of the following: FOH (100μM), DMSO (0.1%), CITCO (0.1μM), GW7647 (10μM) or GW4064 (1μM) for 48 hr. Treatments were replaced once after 24 hours. Cells were then harvested for RNA isolation and cDNA synthesis, and mRNA levels of 85 lipid-metabolizing genes, positive controls for nuclear receptors and internal controls were measured using the custom RT^2 Profiler^{TM} PCR Array kit, as described in Methods. Fold changes in expression were analyzed using the web-based RT^2 Profiler^{TM} PCR Array Data Analysis system (Qiagen) that is based on the $\Delta\Delta C_t$ method, and raw data were normalized to housekeeping genes. Treatment-induced changes in gene expression were then normalized to the expression values of the vehicle control and presented as a heat map that was generated using GENE-E software. The microarray experiment was conducted 2 independent times.

2.3.9 Farnesol upregulates expression of fatty acid oxidation genes in steatotic HepaRG cells.

We validated the results from our microarray experiments by qRT-PCR using self-designed primers, as described in **Appendix C**. Since fatty acid oxidation was the major pathway modulated by farnesol, we measured the mRNA levels of mitochondrial and peroxisomal β-oxidation genes in HepaRG cells that were treated similarly to the array experiments. Under control conditions, farnesol had no significant effects on the expression of mitochondrial β-oxidation genes except for hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit (HADHA) **(**Figure 2.9**)**. OA treatment significantly increased expression of HADHA (~1.5-fold), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit (HADHB, ~1.5-fold), and acetyl-coenzyme A acyltransferase 2 (ACAA2, ~1.3-fold). Co-treatment with farnesol further increased the expression of HADHA (~1.2-fold), HADHB (~1.2-fold), and ACAA2 (~1.3-fold), when compared to the OA-only treated samples. In contrast, neither farnesol nor OA had any effect on the expression of peroxisomal β-oxidation genes except for ACOX2, which was suppressed by both farnesol (~1.2-fold) and OA (~1.3-fold). Additionally, expression of the cytosolic thioesterase, Acyl-CoA thioesterase 12

(ACOT12) increased by ~1.5-fold over control in response to farnesol treatment and ~1.8-fold by OA treatment. Co-treatment with farnesol further increased the OA-mediated increase in ACOT12 expression by ~1.5-fold.

Figure 2.9

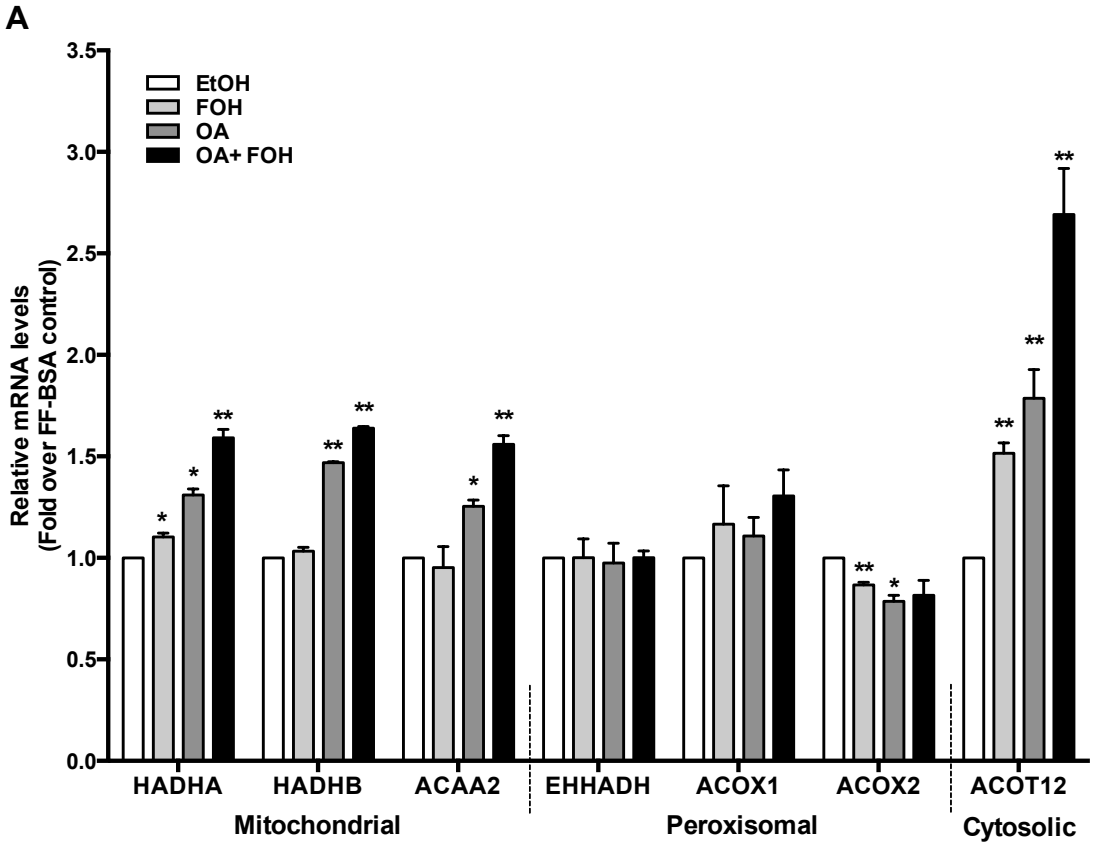


Figure 2.9 Effect of farnesol on lipid-metabolizing gene expression in OA-overloaded HepaRG cells. Differentiated HepaRG cells were cultured in HepaRG treatment medium-2 containing either vehicle control (FF-BSA) or OA (0.66mM) for 24 hr. Thereafter, the FF-BSA pretreated group was treated with either FF-BSA+EtOH (0.1%) (FF-BSA; vehicle control) or farnesol (FOH, 100μM) for 48 hr. The OA pretreated group was treated either with FF-BSA+EtOH (0.1%) (OA) or farnesol (OA+FOH) in media containing OA (0.66 mM). Treatments were replaced once after 24 hours. Cells were then harvested for RNA isolation and cDNA synthesis, and mRNA levels of genes regulating fatty acid oxidation were quantified by *q*RT-PCR, as described in Methods. Each bar represents the mean ± S.E.M. of normalized mRNA levels from three independent experiments (n=3) and are normalized to the FF-BSA treated control. *, ** Significantly different from vehicle control (FF-BSA); *, $P<0.05$; **, $P<0.01$.

2.3.10 Farnesol treatment increases fatty acid oxidation in OA-overloaded HepaRG cells.

We determined the effect of farnesol on overall fatty acid oxidation rate in OA-treated HepaRG cells using oleic acid radiolabelled with tritium (^3H) as the precursor and measured the amount of titrated water, which is the end product of lipid oxidation **(**Figure 2.10**)**. In OA-overloaded HepaRG cells, farnesol significantly increased total fatty acid oxidation rate by ~1.5-fold. The oxidation rate was also increased by treatment with 10μM GW7647 (a known PPARα agonist and positive control for fatty acid oxidation). Taken together, our findings indicate that under steatogenic conditions, farnesol increases the hepatic fatty acid oxidation rate by regulating the expression of mitochondrial and microsomal oxidation enzymes but not the peroxisomal oxidation.

Figure 2.10

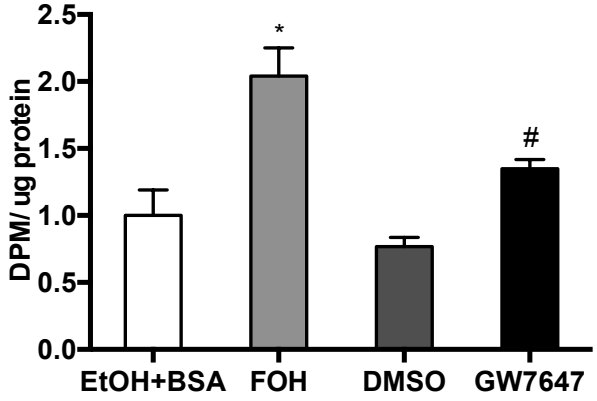


Figure 2.10 Effect of farnesol on fatty acid oxidation rate in OA-overloaded HepaRG cells. Differentiated HepaRG cells were pretreated with 0.66mM OA in HepaRG treatment medium-2 for 24 hr. Cells were then treated with either the FF-BSA+EtOH (0.1%) (FF-BSA; vehicle control) or farnesol (100µM; FOH), DMSO (0.1%), or GW7647 (10µM; positive control for fatty acid oxidation activation) for 48 hr. Treatments were replaced once after 24 hours. Following treatments, cells were prepared to measure the fatty acid oxidation rate as described in Methods. Values were normalized to protein and all samples were normalized to the EtOH+BSA treated controls. Each bar represents the mean ± S.E.M. of normalized values from three independent experiments (n=3). *, Significantly different from untreated (UT) group; P<0.01. #, Significantly different from DMSO group; *P*<0.05.

2.3.11 Farnesol modulates expression of hepatic enzymes in OA-treated HepaRG cells.

Farnesol also modulated the expression of various other metabolic enzymes as shown in Figure 2.11. Farnesol suppressed expression of SREBP1c (~ 1.2-fold), and increased expression of APOA5 (~ 1.3-fold) and ACOT12 (~ 1.5-fold) under control conditions. OA treatment suppressed expression of SREBP1c (~ 1.4-fold) and SCD (~ 2.8-fold), and increased the expression of APOA5 (~ 2.5-fold) and PCK1 (~ 2.4-fold). Co-treatment with farnesol further increased OA-mediated induction of APOA5 (~ 1.6-fold), and reversed the OA-mediated suppression of SCD (~ 1.4-fold). However, farnesol did not change the mRNA levels of PCK1 in both control and OA-treated conditions.

Figure 2.11

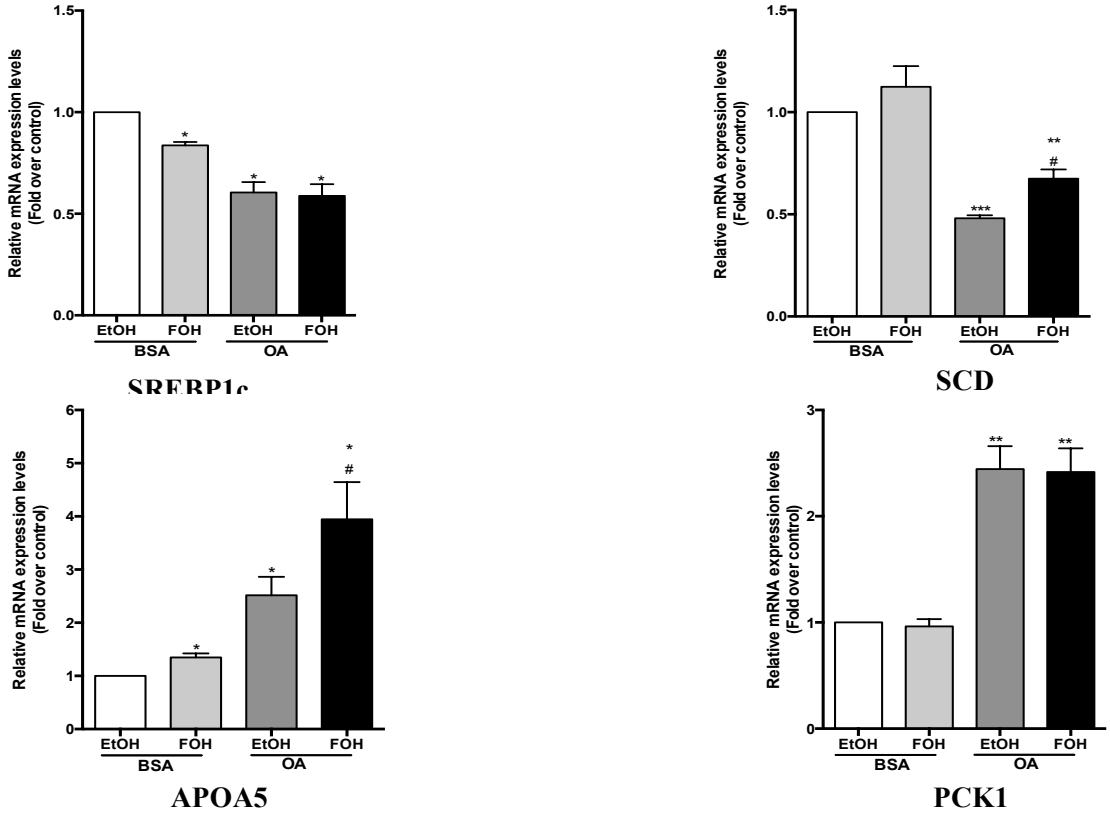


Figure 2.11 Effect of farnesol on hepatic enzymes in OA-overloaded HepaRG cells. Differentiated HepaRG cells were pretreated for 24 hr in HepaRG treatment-2 containing FF-BSA, either alone (BSA) or complexed with 0.66mM OA. The cells were then incubated for 48 hr in pre-incubation medium (i.e., BSA or OA) containing either 0.1% EtOH or 100μM farnesol (FOH). Treatments were replaced once after 24 hours. Cells were then harvested for RNA isolation and cDNA synthesis, and mRNA levels of genes were quantified by *q*RT-PCR, as described in Methods. Each bar represents the mean ± S.E.M. of normalized mRNA levels from three independent experiments (n=3) and are normalized to the EtOH-BSA treated control. *, Significantly different from EtOH:BSA group, $P<0.05$. #, Significantly different from EtOH:OA group, $P<0.05$.

2.3.12 Farnesol modulates expression hepatic drug-metabolizing enzymes in OA-overloaded HepaRG cells.

We also measured the changes in the expression of various cytochromes P450, which are primarily involved in drug metabolism and microsomal oxidation (Zanger and Schwab, 2013), response to farnesol treatment in OA-overloaded HepaRG cells. As shown in Figure 2.12, OA significantly downregulated the mRNA levels of CYP1A2 (~ 1.8-fold), CYP2B6 (~ 2.6-fold) and CYP3A4 (~1.2-fold) while it increased the levels of CYP2E1 (~1.3-fold). Farnesol significantly upregulated CYP3A4 (~1.9-fold) and CYP2B6 (~2.6-fold) mRNA levels in control HepaRG cells, and reversed the OA-mediated suppression of CYP1A2, CYP3A4 and CYP2B6 in OA-overloaded cells **(**Figure 2.12**)**. Also, farnesol suppressed mRNA levels of CYP2E1 in control cells (~ 1.5-fold) and reversed its OA-mediated upregulation in OA-overloaded HepaRG cells.

Figure 2.12

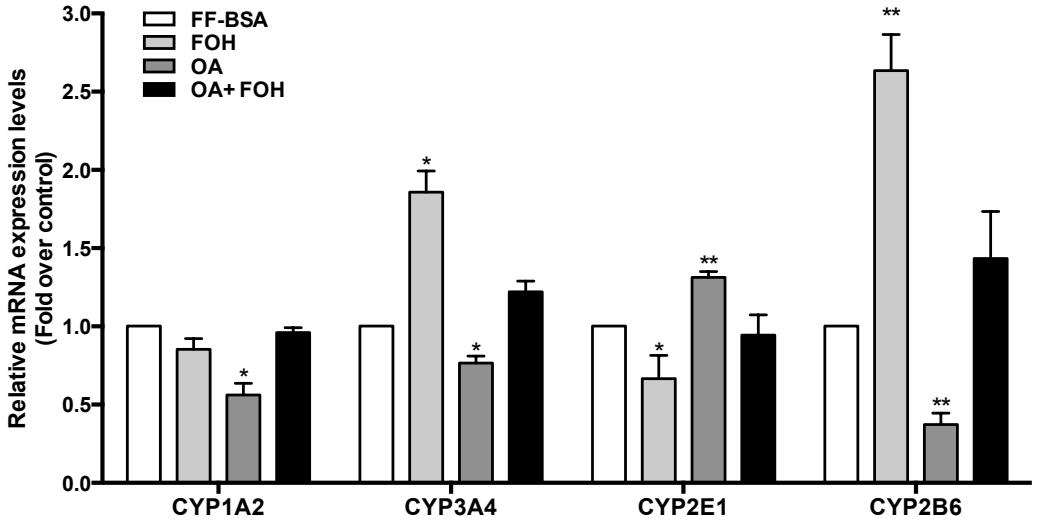


Figure 2.12 Effect of farnesol on hepatic drug-metabolizing enzymes in OA-overloaded HepaRG cells. Differentiated HepaRG cells were cultured in HepaRG treatment medium-2 containing either vehicle control (FF-BSA) or OA (0.66mM) for 24 hr. Thereafter, FF-BSA pretreated group was treated with either the vehicle control (FF-BSA+EtOH (0.1%); FF-BSA) or FOH (100 µM) for 48 hr. The OA pretreated group was treated in the same way in media containing OA (0.66 mM). Treatments were replaced once after 24 hours. Cells were then harvested for RNA isolation and cDNA synthesis, and mRNA levels of genes involved in drug metabolism were quantified by *q*RT-PCR, as described in Methods. Each bar represents the mean ± S.E.M. of normalized mRNA levels from three independent experiments (n=3) and are normalized to the FF-BSA treated control. *, ** Significantly different from vehicle control (FF-BSA); *, $P<0.05$; **, $P<0.01$.

2..3.13 Farnesol changes expression of mitochondrial β-oxidation genes through PPARα.

Next, we determined whether the observed changes in mitochondrial β-oxidation gene expression involved PPARα as suggested by our customized array data. Differentiated HepaRG cells were treated with GW7647 (10μM) in control and OA-treated HepaRG cells and changes in gene expression were measured by qRT-PCR. In control HepaRG cells, PPARα activation significantly induced expression of HADHA (~2-fold), HADHB (~1.6-fold) and ACAA2 (~1.8-fold), except for ACOT12 (Figure 2.13). Treatment with OA also upregulated HADHA (~1.4-fold), HADHB (~1.6-fold), ACOT12 (~1.9-fold) and ACAA2 (~1.4-fold) and this OA-mediated induction in HADHA, HADNB and ACAA2 was increased further by ~1.5-fold, ~1.4-fold and ~1.3-fold by GW7647 co-treatment, except for ACOT12. This suggests that except for ACOT12, all three genes are regulated by PPARα and that PPAR target genes are upregulated by OA treatment in HepaRG cells. Next, OA overloaded HepaRG cells were treated with farnesol in the presence or absence of the PPARα antagonist GW6471 (10μM) for 48 hr and expression of HADHA, HADHB and ACAA2 was measured using qRT-PCR (Figure 2.14). Validation of PPARα antagonism by GW6471 treatment is shown in Figure 2.14A where co-treatment with GW6471 dose-dependently suppressed GW7647-mediated induction of FABP1, which is a PPARα target gene. GW6471 treatment significantly antagonized the farnesol-mediated induction of HADHA, HADHB and ACAA2 expression (Figure 2.14B) suggesting that under steatogenic conditions, farnesol increases the expression of mitochondrial β-oxidation genes through activation of PPARα in HepaRG cells.

Figure 2.13

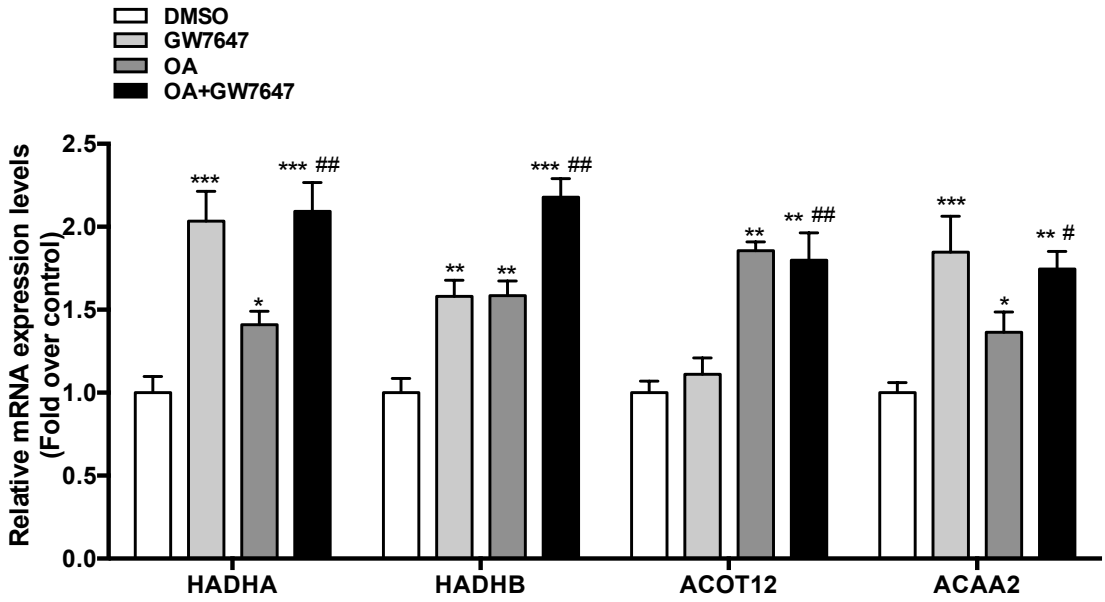


Figure 2.13 Effect of PPARα activation on mitochondrial β-oxidation genes in HepaRG cells. Differentiated HepaRG cells were cultured in HepaRG treatment medium-2 containing either the vehicle control DMSO (0.1%) or the PPARα agonist GW7647 (10μM) in control or OA (0.66mM)-treated HepaRG cells for 48 hr. Treatments were replaced once after 24 hours. Cells were then harvested for RNA isolation and cDNA synthesis, and mRNA levels of mitochondrial β-oxidation genes were quantified by *q*RT-PCR, as described in Methods. Each bar represents the mean ± S.E.M. of mRNA levels normalized to the DMSO control from three (HADHB, ACAA2; n=3) or four (HADHA, ACOT12; n=4) independent experiments. *,**,*** Significantly different from vehicle control (DMSO); *, P<0.05; **, P<0.01, ***, P<0.001. #, ## Significantly different from OA treated groups. #, P<0.05; ##, *P*<0.01.

Figure 2.14

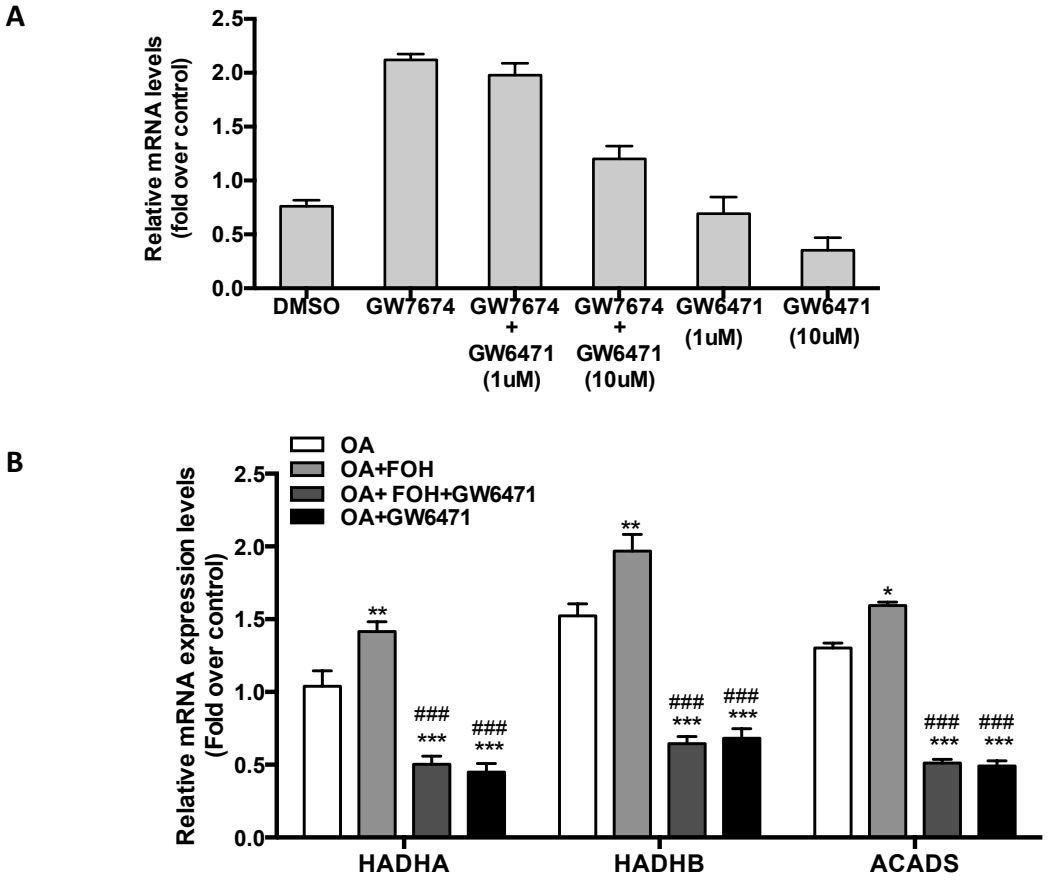


Figure 2.14 Effect of PPARα antagonist on farnesol-mediated upregulation of mitochondrial β-oxidation genes in HepaRG cells. A) Validation of PPARα antagonism by GW6471. Differentiated HepaRG cells were treated with either vehicle control (DMSO; 0.1%), the PPAR agonist GW7674 (10μM) alone or in combination with the PPARα antagonist GW6471 (1 or 10 μM), or GW6471 (1 or 10 μM) alone for 48 hrs. Treatments were replaced once after 24 hours. Cells were then harvested for RNA isolation and cDNA synthesis, and mRNA levels of FABP1 were quantified by *q*RT-PCR, as described in Methods. Each bar represents the mean mRNA levels normalized to the DMSO control from two independent experiments. B) **Effect of GW6471 on farnesol-mediated upregulation of mitochondrial β-oxidation genes.** Differentiated HepaRG cells were pre-treated with OA (0.66mM) for 24 hr in HepaRG treatment medium-2 followed by a 48 treatment with medium containing OA (0.66mM) either alone or in combination with farnesol (100μM; FOH), FOH and the PPAR antagonist GW6471 (10μM), or GW6471 alone. Treatments were replaced once after 24 hours. Cells were then harvested for RNA isolation and cDNA synthesis, and mRNA levels of mitochondrial β-oxidation genes were quantified by *q*RT-PCR, as described in Methods. Each bar represents the mean ± S.E.M. of mRNA levels normalized to the DMSO control from three (HADHA, ACAA2; n=3) or four (HADHB; n=4) independent experiments. **, *** Significantly different from OA only treated group; **, P<0.01; ***, P<0.001. #, Significantly different from OA + FOH treated groups. ###, P<0.001.

2.4 Discussion

The pathogenesis of NAFLD involves dysregulation of lipid metabolic pathways that are regulated by nuclear receptors, such as the PPARs (Sambasiva Rao and Reddy, 2004; Tailleux et al., 2012), FXR (Sinal et al., 2000), CAR (Yamazaki et al., 2007), pregnane X receptor (Sookoian et al., 2010), and liver X receptor (Ducheix et al., 2013). Therefore, pharmacological manipulations of these nuclear receptors are regarded as potential approaches for NAFLD treatment (López-Velázquez et al., 2012; Machado and Cortez-Pinto, 2014). Several synthetic nuclear receptor activators, including fibrates (PPARα), thiazolidinediones (PPARγ), GW501516 and MBX-8025 (PPARβ/δ) (Tailleux et al., 2012), and obeticholic acid (FXR) (Adorini et al., 2012) have been tested in clinical trials for treatment of NAFLD. In addition to synthetic agonists, these nuclear receptors are also targeted by several classes of endogenous compounds, including bile salts (FXR) (Wang et al., 1999) and lipoproteins, eicosanoids, fatty acids, and prostaglandins (PPARs) (Kliewer et al., 1997; Schupp and Lazar, 2010). Here, we have studied farnesol, an endogenous isoprenoid that suppresses TG accumulation in the hepatocytes of high-fat diet-fed mice (Goto et al., 2011) and the serum of Sprague–Dawley rats (Duncan and Archer, 2008) through activation of PPARα, PPARγ, and/or FXR (Forman et al., 1995; Takahashi et al., 2002; Hiyoshi et al., 2003; Goto et al., 2011). However, extrapolation of farnesol's effects on lipid metabolism from rodents to human is complicated by interspecies differences in the activities and functions of these receptors; particularly for PPARα, which in known to regulate diverse sets of lipid-metabolizing genes in mouse and human hepatocytes (Rakhshandehroo et al., 2009), possibly due to differences in ligand affinity, the PPREs in the target genes, and the transcriptional co-regulators that are recruited (Ammerschlaeger et al., 2004; Gonzalez and Shah, 2008; Yang et al., 2008). These disparities may explain why pharmacological modulation of PPARα, while preventing

steatosis in preclinical animal studies, has not proven as effective for treatment of steatosis in clinical trials (Tailleux et al., 2012). In view of the above-described differences, we evaluated whether farnesol treatment could suppress TG accumulation under steatogenic conditions in human hepatic cells, using OA-treated HepaRG cells as our model. Farnesol treatment lowered intrahepatic TGs by nearly 25% in OA-loaded HepaRG cells, suggesting that farnesol can potentially suppress steatosis in human hepatocytes.

Farnesol-mediated activation of human PPARα has not yet been clearly defined. In transfection assays conducted in CV1 cells, farnesol treatment activated a chimeric receptor containing the human PPARα ligand-binding domain and GAL4 DNA-binding domain as well as the full-length receptor (Takahashi et al., 2002). Also, in the same study, HepG2 cells engineered to overexpress human PPARα, farnesol increased expression of PPARα target genes. In our initial studies, we found that farnesol treatment increased expression of the PPARα target gene PLIN2, showing that farnesol can activate endogenous PPARα in HepaRG cells. Farnesol treatment also increased expression of CYP2B6, indicating that farnesol can also modulate human CAR-mediated responses. Farnesol has been previously shown to activate FXR in cultured rat hepatocytes (Goto et al., 2011). However, farnesol treatment did not change expression of the FXR target gene SHP in our study even though FXR was robustly activated by the positive control agonist GW4064, which might be attributable to farnesol being a relatively weak FXR agonist (Forman et al., 1995) or to species differences in FXR activation.

Farnesol upregulates genes involved in fatty acid oxidation in cultured rat hepatocytes (Duncan and Archer, 2008) and the livers of high fat-fed mice (Goto et al., 2011). The results from our customized microarray and qRT-PCR analyses indicated that farnesol treatment of

OA-loaded HepaRG cells increased the expression of several genes that are responsible for hepatic lipid oxidation (i.e., HADHA, HADHB, ACAA2, and ACOT12), which translated to increased fatty acid oxidation. Farnesol treatment has also been reported to suppress expression of the lipogenic genes SREBP-1c and FAS in obese mice (Goto et al., 2011) and FAS but not SREBP-1c in cultured rat hepatocytes (Duncan and Archer, 2008). Our microarray data indicated that farnesol treatment suppressed the expression of SREBP-1c but not FAS in HepaRG cells, suggesting that farnesol-mediated regulation of hepatic lipid-metabolizing gene expression can differ across species.

OA-treated HepaRG cells are reported to show decreased expression of lipogenic genes and increased expression of genes regulating fatty acid oxidation and lipid droplet formation. (Anthérieu et al., 2011). Also, treatment of HepaRG cells with the fatty acid palmitate, either alone or in combination with OA in1:2 ratio changes the metabolic profile to one that resembles NAFLD, including abnormal mitochondrial metabolism, altered fatty acid oxidation, and development of insulin resistance (Brown et al., 2013). When these OA-loaded HepaRG cells are treated with PPARα or dual PPARα/γ agonists, TG accumulation is suppressed, which is accompanied by increases in fatty acid oxidation, upregulation of genes regulating fatty acid oxidation, and downregulation of lipogenic genes (Rogue et al., 2014), suggesting that activation of PPARs in OA-treated HepaRG cells can reverse steatosis. In the current study, farnesol treatment of control or OA-loaded HepaRG cells increased expression of several lipid-metabolizing genes that were also upregulated by PPARα agonist (GW7647) treatment. GW7647 treatment of control and OA-loaded HepaRG cells increased expression of the mitochondrial β-oxidation proteins HAHDA, HADHB, and ACAA2, while the amount of ACOT12 mRNA was not changed. ACOT12 is a cytoplasmic enzyme that catalyzes the

hydrolysis of acetyl-CoA, and the enzyme's activity is regulated by ADP/ATP levels (Swarbrick et al., 2014). ACOT12 in rats has a peroxisome proliferator responsive element (PPRE) motif in its promotor region, which is consistent with the observed increase in ACOT12 activity upon treatment with the PPARα agonist CPIB (clofibrate) in rats' hepatocytes. However, in human ACOT12, the PPRE motif is found outside the putative promoter region in the introns and studies have suggested that human ACOT12 may not be responsive towards activation by PPAR activators, which is further validated by our findings. Finally, we found that farnesol-mediated upregulation of HADHA, HADHB and ACAA2 was attenuated by the PPARα antagonist GW6471 in OA-loaded HepaRG cells, demonstrating that farnesol's effects on these mitochondrial β-oxidation genes are mediated thorough PPARα.

In our study, OA-treatment decreased the expression of CYP1A2, CYP2B6, and CYP3A4 and increased CYP2E1 expression. Changes in the expression of various P450s are routinely seen in the livers of NAFLD patients, resulting in altered drug metabolism (Merrell and Cherrington, 2011). CYP2E1 is also directly involved in NAFLD pathogenesis because of its role in lipid peroxidation that increases oxidative stress, one of the factors responsible for NAFLD progression (Chalasani et al., 2003). All of the OA-induced changes in P450 expression were attenuated by farnesol co-treatment. Farnesol has been previously reported to modulate expression of rat P450s and to interact with mammalian P450s as a substrate and/or inhibitor. Specifically, farnesol has been shown to increase CYP2B mRNA levels in primary cultured rat hepatocytes (Kocarek and Mercer-Haines, 2002), induce several monooxygenase activities in rat liver (Horn et al., 2005), inhibit monooxygenase activities in rabbit liver microsomes (Raner et al., 2002), and be a substrate for ω-hydroxylation by rabbit and human CYP2E1 (DeBarber, 2004 #248). Our findings suggest that farnesol can potentially normalize

fatty acid-induced changes in the hepatic expression of several human drug-metabolizing enzymes.

In conclusion, our results suggest that farnesol can exert potentially beneficial effects on human hepatic lipid and drug metabolism. Farnesol can reverse fatty acid-induced steatosis in human hepatic cells by increasing the expression of genes responsible for mitochondrial β-oxidation, primarily through activation of the nuclear receptor PPARα. Farnesol's ability to normalize expression of human P450s might be an effective approach to ameliorate altered metabolism of drugs during treatment of NAFLD, but this possibility requires further investigation. Additionally, farnesol has been shown to function as a dual human PPARα/γ activator (Takahashi et al., 2002). Although we did not include PPARγ in this study, since PPARα is the major PPAR that is expressed in human liver, it would be interesting to determine whether PPARγ contributes to any of farnesol's effects using selective antagonists, such as GW9662 (Zhang et al., 2015).

CHAPTER 3: ROLE OF PHOSPHATIDIC ACID PHOSPHATASE DOMAIN CONTAINING 2 (PPAPDC2) IN SQUALESTATIN 1-MEDIATED ACTIVATION OF THE CONSTITUTIVE ANDROSTANE RECEPTOR IN PRIMARY CULTURED RAT HEPATOCYTES.

Published: Pant A and Kocarek TA (2015) Role of Phosphatidic Acid Phosphatase Domain Containing 2 (PPAPDC2) In Squalestatin 1-Mediated Activation of the Constitutive Androstane Receptor in Primary Cultured Rat Hepatocytes. *Drug Metab Dispos*.

3.1 Introduction

The mevalonate pathway comprises the first portion of the cholesterol biosynthetic pathway, and its rate-limiting enzyme, 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGCR), is the target of the statin class of cholesterol-lowering drugs. The mevalonate pathway is a highly conserved process whereby acetyl-CoA is converted to a series of progressively elongated isoprenoids that include the branch-point metabolite, farnesyl pyrophosphate (FPP, also known as farnesyl diphosphate). While FPP is normally converted mainly to squalene by squalene synthase in the first committed step of sterol biosynthesis, it is also metabolized to several non-sterol isoprenoids including ubiquinone (coenzyme Q), dolichol, dolichyl phosphate, heme O, heme A, and isopentenyl tRNA (Goldstein and Brown, 1990). Also, FPP and its metabolite geranylgeranyl pyrophosphate (GGPP) are substrates for protein prenylation reactions, which are required for cellular signaling by small GTPases (Zhang and Casey, 1996). FPP and GGPP are also precursors to the isoprenols, farnesol and geranylgeraniol (Edwards and Ericsson, 1999). Under normal physiological conditions, little FPP is dephosphorylated to farnesol, but when squalene synthase is inhibited, FPP accumulates, leading to massive production of farnesol and its progressively oxidized metabolites (collectively known as farnesoids), farnesal, farnesoic acid, and a series of dicarboxylic acids that are excreted in urine (Bostedor et al., 1997; Vaidya et al., 1998).

Our lab has previously reported that treatment of primary cultured rat hepatocytes with the squalene synthase inhibitor, squalestatin 1 (SQ1), increases CYP2B1 expression by causing accumulation of an endogenous isoprenoid(s) that activates the constitutive androstane receptor (CAR; NR1I3) (Kocarek and Mercer-Haines, 2002; Jackson and Kocarek, 2008). Additional evidence implied that this effect required the conversion of FPP to farnesol, since direct treatment with farnesol caused CYP2B induction in cultured rat hepatocytes (Kocarek and Mercer-Haines, 2002) and rat liver (Horn et al., 2005a). However, this possibility was not specifically addressed since the enzyme(s) responsible for converting FPP to farnesol was not known. Later, phosphatidic acid phosphatase domain containing 2 (PPAPDC2) was identified as a lipid phosphate phosphohydrolase that could convert presqualene diphosphate to presqualene monophosphate (Fukunaga et al., 2006). Subsequently PPAPDC2 was shown to hydrolyze FPP and GGPP preferentially over several other phospholipid substrates *in vitro* and to deplete FPP levels when overexpressed in cells (Miriyala et al., 2010). These data suggested that PPAPDC2 could be an important component of the pathway for synthesis of isoprenols. Therefore, the objective of the current investigation was to determine the impact of PPAPDC2 activity on SQ1-mediated activation of CAR in primary cultured rat hepatocytes.

3.2 Materials and methods

Materials

SQ1 was a gift from Bristol-Myers Squibb Co. (Stamford, CT) and pravastatin from GlaxoSmithKline (Research Triangle Park, NC). Phenobarbital (PB) was purchased from Sigma-Aldrich (St. Louis, MO). Matrigel was purchased from Corning Life Sciences (Tewksbury, MA), PureCol from Advanced BioMatrix (San Diego, CA), and recombinant human insulin (Novolin R) from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ).

Williams' Medium E and Lipofectamine 2000 reagent were purchased from Invitrogen (Life Technologies; Grand Island, NY). SYBR Green PCR master mix was purchased from Applied Biosystems (Foster City, CA). Additional materials were obtained from the sources indicated below.

Primary culture of rat hepatocytes

Adult male Sprague-Dawley rats (200–250 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility with free access to food and water for one week. Hepatocytes were isolated by two-step collagenase perfusion as described previously (Kocarek and Reddy, 1996). Freshly isolated hepatocytes were plated onto PureCol-coated plates at a density of 1.6 million hepatocytes per well for 6-well plates (for RNA isolation) or 0.6 million hepatocytes per well for 12-well plates (for transfection experiments) and maintained at 37°C under a humidified atmosphere of 95% air/5% CO_2 in Williams' Medium E supplemented with 0.25 U/ml insulin, 0.1 μM triamcinolone acetonide, 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture medium was renewed every 24 hr and drug treatments were begun 48 hr after plating as described in the individual figure legends. Drugs were prepared as 1000X stock solutions in sterile water.

PPAPDC2 overexpression by transient transfection in primary cultured rat hepatocytes

A CAR-responsive firefly luciferase reporter plasmid containing ~2.4 Kb of the CYP2B1 5'-flanking region has been previously described (Kocarek et al., 1998). An expression plasmid (pExpress-1) containing the rat PPAPDC2 cDNA sequence (Mammalian Gene Collection Clone ID 7125531) and a cloning plasmid (pBluescript) containing the human PPAPDC2 cDNA (Mammalian Gene Collection Clone ID 5268486) were purchased from GE

Healthcare (Pittsburgh, PA). An expression plasmid for human PPAPDC2 was prepared as follows. PCR was performed using the plasmid containing human PPAPDC2 cDNA as template, *Pfu* polymerase (Stratagene Cloning Systems; La Jolla, CA), and the following primer pairs: forward 5'-GCG AGCGGCCGCGCCACCATGCCAAGTCCCCGGAGGA-3' and reverse 5'-GCGAGGATCCTCATCGTTGACTCCACAGT-3' to amplify the coding region of human PPAPDC2 (nt 83 to 970 of NCBI Reference Sequence NM_203453.3). The PCR product was digested with *Not*I and *Bam*HI and ligated into the corresponding sites of the pE1.1 vector (O.D. 260 Inc.; Boise, ID), into which the cytomegalovirus promoter and bovine growth hormone polyadenylation sequences of pcDNA3.1 had been pre-ligated at the *Bgl*II and *Acc*65I/*Kpn*I sites, respectively. The sequence of this clone (and others described below that were derived by PCR) was verified by the Applied Genomics Technology Center at Wayne State University.

Twenty-four hr after plating, cell culture medium was replaced and hepatocytes were transiently transfected with Williams' Medium E containing 0.2ml of OptiMEM (Life Technologies; Grand Island, NY) and a premixed complex of Lipofectamine 2000 (4 μL), the CYP2B1 reporter plasmid (1.2 μg), rat or human PPAPDC2 expression plasmid (50 ng), pRL-CMV *Renilla* luciferase reporter plasmid (1 ng), and pBlueScript II KS^+ (350 ng) (Agilent Technologies; Santa Clara, CA) to adjust total DNA content to 1.6 μg. Five hr following transfection, medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. Drug treatments were begun the following day and repeated once after 24 hr. Hepatocytes were harvested 48 hr after initial treatment for measurement of luciferase activities using the Dual Luciferase Reporter Assay System and a GloMax luminometer (Promega Corporation; Madison, WI) according to the manufacturer's instructions. Luciferase data were calculated as

firefly/*Renilla* ratios and presented as described in the figure legends. Experiments were repeated in four independent rat hepatocyte preparations.

PPAPDC2 knockdown by transient transfection in primary cultured rat hepatocytes

Four plasmids expressing 29-mer shRNAs targeting rat PPAPDC2 and a non-targeting shRNA plasmid (TR30012) were purchased from Origene Technologies (Rockville, MD). A validation vector (rPPAPDC2-Luc) was additionally prepared to evaluate knockdown of rat PPAPDC2. To prepare this vector, the rat PPAPDC2 expression plasmid described above was used as a template to amplify the rat PPAPDC2 cDNA using the forward primer 5'-GCGAGCGGCCGCGCCACC CGGAGGACTATCGAGGGAC-3' and reverse primer 5'-GCGATAGCGGCCGC CTGGATGGCTCTGGCTTAGG-3' and *Pfu* polymerase to amplify the coding region of rat PPAPDC2 (nt 64 to 1014 of NCBI Reference Sequence NM_001034854.1). The PCR product was digested with *Not*I and ligated into the corresponding site of the pCMV-LUC validation vector (TR30004; Origene Technologies), which is located downstream of the firefly luciferase coding region.

To evaluate the abilities of the different shRNA constructs to knock down PPAPDC2, primary cultured rat hepatocytes were incubated in Williams' Medium E containing 0.2 ml of OptiMEM containing a premixed complex of 4 µl Lipofectamine 2000 with 50 ng PPAPDC2 validation vector, 200 ng of a PPAPDC2-targeting or non-targeting shRNA expression plasmid, 1 ng of pRL-CMV *Renilla* luciferase reporter plasmid, and 1350 ng of pBlueScript II KS^+. Five hr following transfection, culture medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. The cells were then harvested 48 hr after transfection, and luciferase activities were measured as described above. The shRNA construct that produced

the largest reduction in reporter activity compared to non-targeting control was TI713339, and this construct was used for further experiments.

To evaluate the effect of PPAPDC2 knockdown on SQ1-mediated CAR activation, primary cultured rat hepatocytes were transfected as described above, but using 1.2 μg of CYP2B1 reporter plasmid, 200 ng of shRNA construct (PPAPDC2-targeting or non-targeting), and 200 ng pBluescript II KS^{+}. After transfection and overnight incubation with Williams' Medium E containing Matrigel, drug treatments were performed as described in the individual figure legends (three wells per treatment group), repeated once after 24 hr. Forty-eight hr following initial treatment, cells were harvested for measurement of luciferase activities.

PPAPDC2 overexpression by adenoviral transduction in primary cultured rat hepatocytes

The generation of a recombinant adenovirus for expression of rat PPAPDC2 (Ad5.CMV-PPAPDC2) was performed as described below. The expression plasmid for rat PPAPDC2 described above was used as template to amplify rat PPAPDC2 cDNA using the following primers: forward primer 5'- GCGAGCGGCCGCGAAGCCTGTCTCCGGTCTG -3' and reverse primer 5'-GCGGGATCCGGATGGCTCTGGCTTAGGT -3' and *Pfu* polymerase to amplify the coding region of rat PPAPDC2 (nt 2 to 1012 of NCBI Reference Sequence NM_001034854.1). The PCR product was ligated into the *Not*I and *Bam*HI sites of the pE1.1 shuttle vector, containing the cytomegalovirus promoter and bovine growth hormone polyadenylation sequences, as described above. This shuttle plasmid was then provided to O.D. 260 Inc., who prepared the recombinant adenovirus expressing rat PPAPDC2 (Ad5.CMV-PPAPDC2). The titer of the recombinant adenovirus as well as of the control adenovirus (Ad-375; O.D. 260 Inc.) was determined using the QuickTiter Adenovirus Titer ELISA Kit (Cell

Biolabs, Inc.; San Diego, CA). Twenty-four hr following plating, primary cultured rat hepatocytes were transduced with Ad5.CMV-PPAPDC2 or control adenovirus without insert (Ad-375) at a multiplicity of infection of 5. After 5 hr of infection, medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. Drug treatments were begun 24 hr after infection (two wells per treatment group), and treatments were repeated once after 24 hr. Forty-eight hr following the initial treatments, cells were harvested for RNA or protein extraction as described below.

Quantitative reverse-transcription polymerase chain reaction analysis

Primary cultured rat hepatocytes were harvested and total RNA was extracted and column purified using the Purelink RNA isolation kit (Ambion; Carlsbad, CA). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), following the manufacturer's instructions. Primers used to detect CYP2B1 (Forward – 5'-CAACCCTTGATGACCGCAGTA-3' and Reverse – 5'- TTCAGTGTTCTTGGGAAGCAG -3') and primers for the TATA box-binding protein (TBP; Assay ID Rn.PT.51.24118050) were purchased from Integrated DNA Technologies (IDT; Coralville, IA). CYP2B1 mRNA levels were measured using Power SYBR Green Master Mix in the StepOne Plus Real Time PCR System (Applied Biosystems; Foster City, CA). The concentration of each primer was 150 nM, and the real-time cycling conditions were: Initial activation step at 95 ºC (15 min) and 40 cycles of melting (95 ºC, 15 s) and annealing/extension (60 ºC, 1 min). Relative changes in mRNA levels were quantified using the comparative CT (ΔΔCT) method (User bulletin no.2, Applied Biosystems). All assays were performed in duplicate in four independent experiments.

Western blot analysis

After 48 hr of treatment, cultured hepatocytes were incubated with gentle agitation on ice for 60 min with ice-cold phosphate-buffered saline (PBS) containing 5 mM EDTA to dissolve the Matrigel. The cells were then scraped into tubes and centrifuged briefly at 1000 rpm for 5 min at 4º C. Supernatants were aspirated and cells washed twice with ice-cold PBS. Following the final wash, cells were lysed in ice-cold RIPA buffer [50 mM Tris, 150 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA] containing Halt Protease Inhibitor Cocktail (ThermoFisher Scientific; Rockford, IL) by passing the suspension through a 24 gauge needle several times. After extensive vortexing, the lysates were centrifuged at 14,000 x *g* for 15 min at 4 °C and the protein concentrations of the supernatants were determined using the bicinchoninic acid assay (Sigma-Aldrich; St. Louis, MO) with bovine serum albumin as the standard. Lysate samples (20 µg protein) were diluted in Laemmli Sample Buffer, denatured at 100º C for 5 min, resolved on SDS-polyacrylamide gels (10% acrylamide), and electrophoretically transferred onto polyvinylidene difluoride membranes (BioRad; Hercules, CA). Following transfer, membranes were incubated in blocking buffer [5% nonfat dry milk diluted in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 (TBST)] for 1 hr at room temperature and then incubated overnight at 4º C with gentle rocking in blocking buffer containing rabbit polyclonal PPAPDC2 antibody (TA306886; Origene Technologies) diluted 1:15,000. The following day, blots were washed 3 times with TBST and then incubated 1 hr at room temperature in blocking buffer containing horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology; Dallas, TX) diluted 1:20,000. Following washing, immunoreactive bands were visualized using enhanced chemiluminescence

according to the manufacturer's instructions (GE Healthcare) and a FluorChem E detection system (ProteinSimple, San Jose, CA). Blots were subsequently incubated in stripping buffer (0.1 mM glycine, 1% SDS, 0.05% Tween-20, pH 2.2), blocked, and re-developed with a mouse monoclonal β-actin antibody (Sigma-Aldrich) diluted 1:250 in blocking buffer followed by anti-mouse IgG secondary antibody diluted 1:20,000 (Santa Cruz Biotechnology). Band densities were quantified using ImageJ software (Rasband, 2012).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software; San Diego California USA) to perform two-way analysis of variance with the Newman-Keuls correction for multiple comparisons. $P<0.05$ was considered significantly different. All results are presented as mean ± S.E.M.

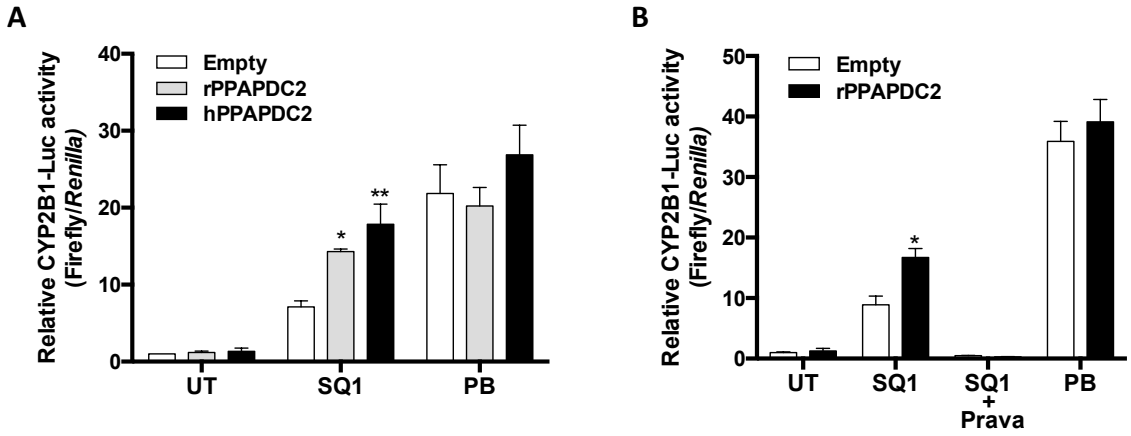
3.3 Results

3.3.1 Overexpression of rat or human PPAPDC2 enhances squalestatin 1-mediated activation of a CAR-responsive reporter.

Based on the reported ability of PPAPDC2 to dephosphorylate FPP to farnesol (Miriyala et al., 2010), we hypothesized that overexpression of PPAPDC2 would enhance SQ1-mediated CAR activation in primary cultured rat hepatocytes. Therefore, rat hepatocyte cultures were co-transfected with an expression plasmid for PPAPDC2 (rat or human) or empty vector and a reporter plasmid containing the CAR-responsive region of *CYP2B1* (Kocarek et al., 1998) and then treated with 0.1 μM SQ1 or 100 μM PB, the prototype CAR activator. SQ1 and PB treatment significantly increased CYP2B1 reporter activity by 7.1- and 22-fold, respectively, compared to untreated control in hepatocytes transfected with empty vector (Figure 3.1A). Therefore, rat and human PPAPDC2, which share 89% amino acid sequence

similarity, were functionally comparable in their abilities to enhance SQ1-inducible CAR activation. PPAPDC2 overexpression did not affect reporter activity in either untreated or PB-treated hepatocytes, indicating that PPAPDC2 had no direct effect on CAR activity, but rather mediated its effect through the mevalonate pathway. Co-treatment of rat hepatocytes with the HMGCR inhibitor pravastatin (30 μM), which blocks production of mevalonate (and therefore FPP), abolished the effect of SQ1, without or with PPAPDC2 overexpression, on CAR activity (Figure 3.1B), further demonstrating the dependence of the PPAPDC2-enhanced CAR activation on the mevalonate pathway.

Figure 3.1



3.1. Effect of PPAPDC2 overexpression on SQ1-mediated CAR activation in primary cultured rat hepatocytes. (A) Twenty-four hr old primary cultures of rat hepatocytes were transiently transfected with a CAR-responsive reporter plasmid (CYP2B1-Luc) and either an empty expression plasmid (Empty) or an expression plasmid for rat (rPPAPDC2) or human PPAPDC2 (hPPAPDC2). Then, 24 hr after transfection, cultures were incubated in medium alone (UT) or containing 0.1 μM SQ1 or 100 μM PB. Each bar represents the mean ± S.E.M. of the mean normalized (firefly/*Renilla*) luciferase measurements from four independent experiments (n=4). All values are normalized to the Empty:UT group. *Significantly different from Empty:SQ1 group, $p<0.05$; **$p<0.01$. **(B)** Rat hepatocytes were co-transfected with CYP2B1-Luc and either empty vector (Empty) or rPPAPDC2 and treated with medium alone (UT), 0.1 μM SQ1, 0.1 μM SQ1 and 30 μM pravastatin (Prava), or 100 μM PB. Forty-eight hr after drug treatment, transfected cultures were harvested for measurement of luciferase activity. Each bar represents the mean ± S.E.M. of mean normalized (firefly/*Renilla*) luciferase measurements from three independent experiments (n=3). All values are normalized to the Empty:UT group. *Significantly different from the Empty:SQ1 group, $p<0.05$.

3.3.2 Adenovirus-mediated overexpression of rat PPAPDC2 increases squalestatin 1-inducible CYP2B1 mRNA expression in primary rat hepatocytes.

As a complementary approach to plasmid-mediated overexpression of PPAPDC2, we used a recombinant adenovirus to overexpress rat PPAPDC2 (Ad5.CMV-PPAPDC2) in primary cultured rat hepatocytes and measured CYP2B1 mRNA levels as a marker of CAR activation. Transduction with PPAPDC2 at a multiplicity of infection of 5 resulted in a ~2.2-fold increase in PPAPDC2 immunoreactive protein content relative to that measured in empty adenoviral control vector-transduced cells after 48 hr (Figure 3.2A). SQ1 treatment increased CYP2B1 mRNA content by ~27-fold in hepatocytes transduced with control vector and ~38-fold in PPAPDC2-transduced cells (Figure 3.2B), for a significant 1.4-fold enhancement of SQ1-mediated CYP2B1 mRNA induction.

3.3.3 Selection of the most effective PPAPDC2 shRNA to achieve maximal rat PPADPC2 knockdown.

The above data show that supplementing the rat hepatocyte's capacity to convert FPP to farnesol increased the ability of a drug that causes FPP accumulation to activate CAR, suggesting that FPP dephosphorylation to farnesol is a necessary step in the mechanism by which SQ1 treatment activates CAR. However, these data do not address whether PPAPDC2 is a major enzyme that normally performs this function in hepatocytes. For this, we decided to knock down PPAPDC2 using shRNA expression plasmids (non-targeting and PPAPDC2-targeting) in primary rat hepatocytes. In order to select the best shRNA to achieve maximum knockdown efficiency, we first co-transfected primary rat hepatocytes with either of the four rat PPAPDC2-targeting shRNA or a non-targeting shRNA along with the rat PPAPDC2 validation vector as described in methods. As seen in the Figure 3.3, transfection with the all

four shRNAs suppressed the luciferase reporter activity compared to the empty control with shrPPAPDC2-3 producing the maximal effect. Therefore, this shRNA was selected to perform the knock down experiments.

Figure 3.2

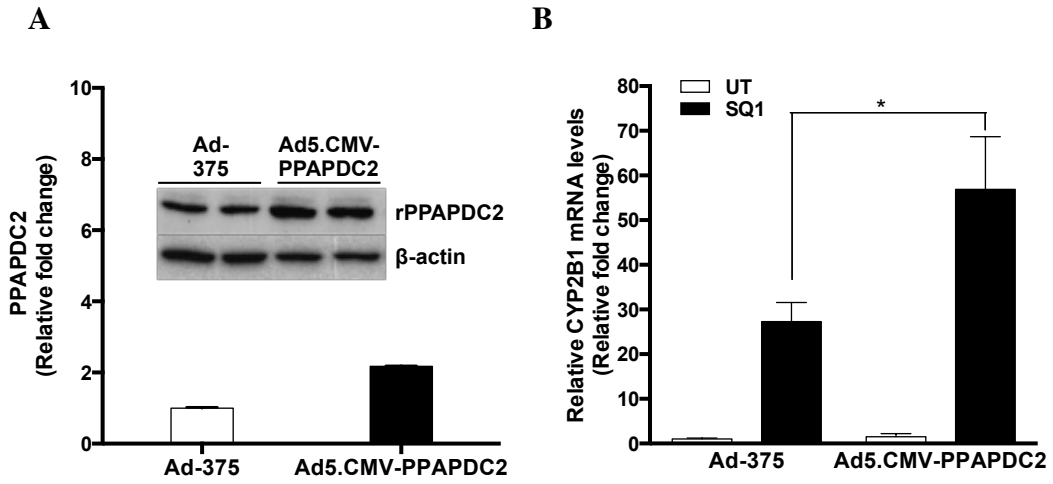


Figure 3.2 Effect of adenovirus-mediated overexpression of rat PPAPDC2 on SQ1-mediated CAR activation in primary cultured rat hepatocytes

Rat hepatocytes were transduced with empty adenoviral vector (Ad-375) or adenoviral vector expressing rPPAPDC2 (Ad5.CMV-PPAPDC2) and harvested after 48 hr for measurement of either rat PPAPDC2 or β-actin protein. The bar graph shows band intensity quantification of PPAPDC2 levels normalized to β-actin. **(D)** Rat hepatocytes were transduced with empty adenoviral vector (Ad-375) or adenoviral vector expressing rPPAPDC2 (Ad5.CMV-PPAPDC2), treated for 48 hr with medium alone (UT) or 0.1 μM SQ1, and harvested for measurement of CYP2B1 mRNA levels. Each bar represents the mean CYP2B1 levels ± S.E.M from four independent experiments normalized to the Ad-375:UT group (n=4). * Significantly different from Ad-375:SQ1 group, $p<0.05$.

Figure 3.3

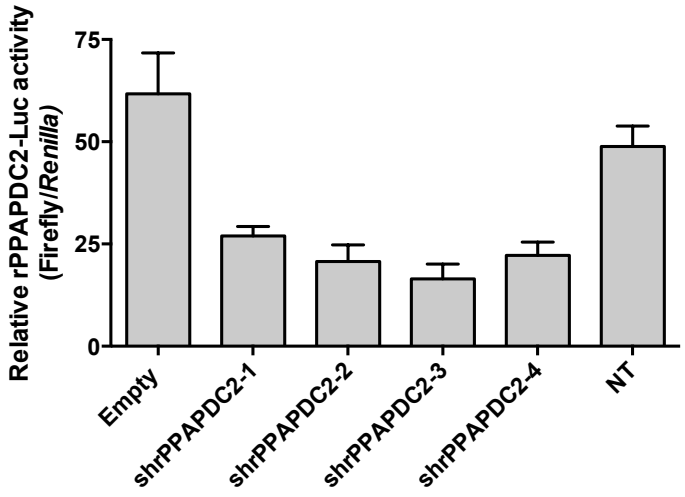


Figure 3.3 Selection of the PPAPDC2 shRNA producing maximal rat PPADPC2 knockdown. Twenty-four hr after plating, primary cultured rat hepatocytes were transiently transfected with a validation reporter vector for rat PPAPDC2 (rPPAPDC2-Luc) and either a non-targeting control shRNA (shNT) or one of the four shRNAs targeting rat PPAPDC2 (shrPPAPDC2-1, shrPPAPDC2-2, shrPPAPDC2-3 or shrPPAPDC2-4). After 48 hr, hepatocytes were harvested for measurement of luciferase activity. Each bar represents the mean ± S.E.M. of mean normalized (firefly/*Renilla*) luciferase measurements from three independent experiments.

3.3.4 PPAPDC2 knockdown attenuates squalestatin 1-mediated activation of the CAR-responsive reporter.

We evaluated the impact of knocking down PPAPDC2 on SQ1-mediated CAR activation by performing co-transfections with shRNA expression plasmids (non-targeting and PPAPDC2-targeting) and the CYP2B1 reporter plasmid. Transfection with the shRNA that produced the greatest knockdown of PPAPDC2 in a validation vector reporter assay (~62% reduction, Figure 3.4A) significantly attenuated SQ1-inducible CYP2B1 reporter activation (~80% reduction, Figure 3.4B), indicating that PPAPDC2 plays an essential role in SQ1-mediated CAR activation in primary cultured rat hepatocytes.

Figure 3.4

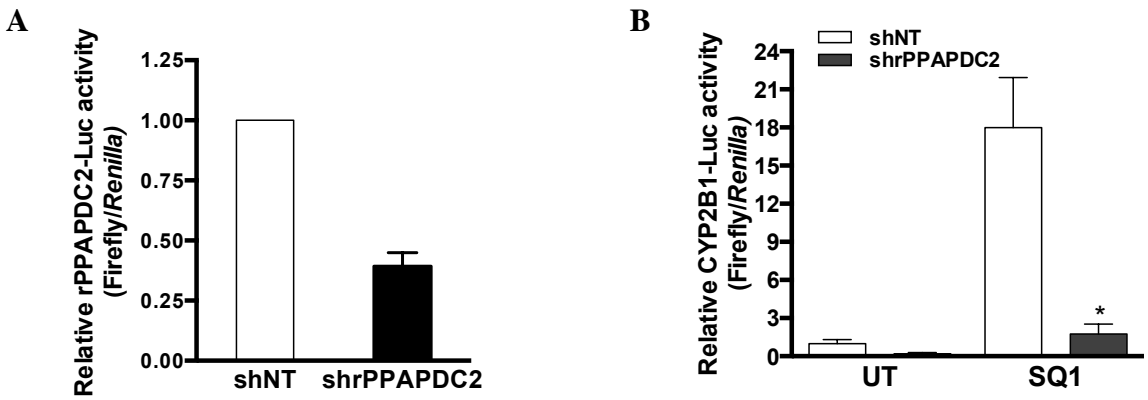


Figure 3.4 Effect of PPAPDC2 knockdown on SQ1-mediated CAR activation in primary cultured rat hepatocytes. (A) Validation of rPPAPDC2 knockdown: Twenty-four hr after plating, primary cultured rat hepatocytes were transiently transfected with a validation reporter vector for rat PPAPDC2 (rPPAPDC2-Luc) and either a non-targeting control shRNA (shNT) or a shRNA targeting rat PPAPDC2 (shrPPAPDC2). After 48 hr, hepatocytes were harvested for measurement of luciferase activity. Each bar represents the mean ± S.E.M. of mean normalized (firefly/*Renilla*) luciferase measurements from three independent experiments (n=3). **(B)** Rat hepatocytes were transiently transfected with the CYP2B1-Luc reporter and either the shNT or shrPPAPDC2 plasmid. Then, 24 hr after transfection cultures were incubated for 48 hr in medium alone (UT) or containing 0.1 μM SQ1 and harvested for measurement of luciferase activity. Each bar represents the mean ± S.E.M. of mean normalized (firefly/*Renilla*) luciferase measurements from three independent experiments (n=3). All values are normalized to the shNT:UT group. *Significantly different than the shNT:SQ1 group**,** $p<0.05$.

3.4 Discussion

The existence of one or more hepatic enzymes capable of catalyzing FPP dephosphorylation is apparent since squalene synthase inhibition causes accumulation of farnesol and farnesol-derived metabolites (Bostedor et al., 1997; Vaidya et al., 1998). In seeking such enzyme, Christophe and Popjack first described a metal ion-independent prenyl pyrophosphatase in rat liver microsome (Christophe and Popják, 1961). Subsequent studies in rat microsomes (Bansal and Vaidya, 1994) and in rice seedlings (Nah et al., 2001) identified two of these allyl pyrophosphates; farnesyl pyrophosphatase (FPPase) and geranylgeradenyl pyrophosphatase (GPPase) that could potentially dephosphorylate FPP to farnesol and GGPP to geranylgeranol respectively. Later, phosphatidic acid phosphatase domain containing 2 (PPAPDC2/PDP1) protein was identified through *in vitro* assays as the first lipid phosphate phosphohydrolase that metabolized presqualene diphosphate (PDSP) to presqualene monophosphate and FPP to farnesol with a substrate preference of PSDP > FDP > phosphatidic acid (Fukunaga et al., 2006). Further analysis by these investigators showed that the diphosphate phosphatase activity of PPAPDC2 was $Mg2^{+}$-independent, contained a consensus catalytic motif for lipid phosphate phosphohydrolase, and had widespread expression in human tissues.

Our data demonstrate that PPAPDC2 plays an essential role in the mechanism by which SQ1 treatment causes CAR activation in primary cultured rat hepatocytes, most likely by catalyzing the conversion of FPP to farnesol, which is then itself or a precursor of the active endogenous CAR activator. Miriyala et al. (Miriyala et al., 2010) established that PPAPDC2 catalyzes FPP dephosphorylation and that its overexpression can modulate FPP-dependent cellular processes. Overexpression of PPAPDC2 in yeast or mammalian cells decreased FPP

levels and caused cytotoxicity. In HEK293 cells, overexpression of PPAPDC2 but not a catalytically defective mutant caused cytostasis and accumulation of multinucleate cells. In mouse aortic vascular smooth muscle cells, PPAPDC2 overexpression affected processes associated with Rho family GTPase function, which is dependent on protein prenylation. Our data are the first to link PPAPDC2 to a signaling mechanism in normal hepatocytes.

Non-sterol isoprenoids play important roles in a variety of biological processes, and the control of their cellular levels is therefore an important therapeutic approach for several pathologies (Buhaescu and Izzedine, 2007; Oldfield, 2010; Li et al., 2012). For example, the nitrogen-containing bisphosphonate class of drugs (e.g., alendronate, ibandronate) are first line treatments for osteoporosis that produce their therapeutic effects by inhibiting FPP synthase, and therefore production of FPP and its metabolites, including GGPP. Therefore, the bisphosphonates reduce geranylgeranylation of small GTPase proteins in the osteoclast, which leads to osteoclast apoptosis (Drake et al., 2015).

The isoprenols farnesol and geranylgeraniol themselves have been implicated in several biological processes; for example, farnesol as a non-sterol regulator of HMGCR, which is a rate-limiting enzyme of mevalonate pathway for sterol biosynthesis. (Correll et al., 1994; Meigs and Simoni, 1997), and these alcohols can be converted back to FPP and GGPP through sequential monophosphorylation reactions (Thai et al., 1999). Therefore, the mechanisms controlling the interconversion of FPP and farnesol are likely important determinants of isoprenoid-regulated cellular processes, and our findings provide further insight into the role of PPAPDC2 as a modulator of hepatic physiology. It would be interesting to explore the effect of PPAPDC2 on other isoprenoids-regulated cellular mechanisms. Recent discovery novel

PPAPDC family lipid phosphatases inhibitors may provide additional tools to analyze additional functional role of PPAPDC2 in the future (Subramanian et al., 2014).

CHAPTER 4: CONCLUSIONS

Farnesol, one of the sesquiterpene isoprenols, is produced endogenously from dephosphorylation of farnesyl pyrophosphate (FPP), which is one of the intermediary metabolite of the mevalonate pathway for cholesterol synthesis (Goldstein and Brown, 1990). While it was shown recently that the enzyme PPAPDC2 is capable of dephosphorylating FPP to produce farnesol, its physiological significance has not been well understood (Miriyala et al., 2010). Therefore, one of the objectives of this project was to determine whether the enzyme PPAPDC2 has any effects on farnesol-mediated cellular processes that require conversion of FPP to farnesol.

FPP is metabolized to squalene by the enzyme squalene synthase (SS) in the first committed step towards cholesterol synthesis. Based on previous data from our lab, which showed that treatment with SQ1 results in the activation of the constitutive androstane receptor (CAR), a physiological response that requires accumulation of FPP and farnesol on primary cultured rat hepatocytes (Kocarek and Mercer-Haines, 2002), we hypothesized that overexpression of PPAPDC2 would enhance SQ1-mediated induction of the CAR target gene, CYP2B1. Our hypothesis was validated by our data, which showed that overexpression of PPAPDC2 in primary cultured rat hepatocytes results in significantly increased CAR activity as well as increased expression of the CYP2B1 mRNA. Also, knockdown of endogenous PPAPDC2 suppressed the SQ1-mediated induction of CAR activity demonstrating that PPAPDC2 plays an important role in SQ1-induced CAR activity in rat hepatocytes, most probably by mediating the conversion of FPP to farnesol. This finding provides a physiological significance to PPAPDC2 and its catalytic ability to dephosphorylate FPP to farnesol. Measurement of farnesol levels in PPAPDC2-overexpressed rat hepatocytes is recommended

for further analysis to show that PPAPDC2 is increasing farnesol levels in primary cultured rat hepatocytes.

Based on the published findings that farnesol regulates lipid metabolism in rodents and reduces hepatic accumulation of triglycerides through activation of peroxisome proliferator receptor alpha (PPARα) and farnesol X receptor (FXR), we hypothesized that farnesol has similar effects on human hepatocytes and that these effects occur through any or all of the nuclear receptors, PPARα, FXR and CAR. In order to test our hypothesis, we established a human hepatic cellular model for steatosis by incubating HepaRG cells with oleic acid (OA), a known inducer of steatosis. Treatment with farnesol (100μM) significantly reduced the OA-induced accumulation of TGs in HepaRG cell cultures. Farnesol increased the activity of PPARα and CAR in HepaRG cells and had no effect on FXR activity.

Customized array analysis of 85 genes involved in the regulation of human hepatic lipid metabolism showed that farnesol changes expression of several hepatic lipid-metabolizing enzymes, a majority of which belonged to the fatty acid oxidation pathway. Few other enzymes that regulate lipogenesis, fatty acid uptake and transport and export were also changed. Activation of CAR had no substantial effect on the expression of genes studied; while activation of FXR changed expression of genes including PPARγ and PCK1, whose primary functions are adipocyte differentiation and glucose homeostasis respectively. However, treatment with the PPARα agonist changed expression of several genes, most of which were involved in fatty acid oxidation, transport, uptake and export. Additionally, most of the farnesol-mediated changes in gene expression in control or OA-overloaded HepaRG cells were similar to that achieved through activation of PPARα. Further analysis revealed that farnesol activated fatty acid oxidation in HepaRG cells under steatogenic conditions. Farnesol also

increased the mRNA levels of genes involved in mitochondrial fatty acid oxidation, which was mediated through PPARα. Additionally, farnesol attenuated OA-induced alteration in the expression of drug-metabolizing enzymes.

Isoprenols, such as farnesol are natural compounds that are ubiquitously present in higher plants and in addition to lipid metabolism, dietary intake of several isoprenoids have been implicated in several other physiological processes, such as inhibition of tumor proliferation, initiation of apoptosis and cellular differentiation. Here, we provide evidence that farnesol can potentially ameliorate OA-induced TG accumulation in human hepatocytes and could be beneficial in reversing changes in the expression of hepatic enzymes involved in drug metabolism. While it is too early to recommend intake of farnesol or its dietary sources as a potential therapeutic approach against metabolic diseases, such as non-alcoholic fatty liver disease where hepatic TG accumulation is the major symptom or cause for disease pathogenesis, our data as well as data from previously published animal models warrants a more in-depth investigation in order to further elucidate any potential beneficial effects of farnesol and other isoprenoids.

APPENDIX

Appendix A. List of lipid metabolizing genes in the custom RT^2 Profilor^{TM} PCR Array.

Pathway	Symbol	Gene Name
Triglyceride Biosynthesis	AGPAT1	1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, alpha)
	AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta)
	AGPAT6	1-acylglycerol-3-phosphate O-acyltransferase 6 (lysophosphatidic acid acyltransferase, zeta)
	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9
	DGAT1	Diacylglycerol O-acyltransferase 1
	DGAT2	Diacylglycerol O-acyltransferase 2
	GPAT2	Glycerol-3-Phosphate Acyltransferase 2, Mitochondrial
	LPIN1	Lipin 1
	LPIN2	Lipin 2
	LPIN3	Lipin 3
Fatty acid synthesis	ACAA2	Acetyl-CoA acyltransferase 2
	ACACA	Acetyl-CoA carboxylase alpha
	ACACB	Acetyl-CoA carboxylase beta
	ACLY	ATP citrate lyase
	FASN	Fatty acid synthase
	SCD	Stearoyl-CoA desaturase
	MECR	Mitochondrial Trans-2-Enoyl-CoA Reductase
	ELOVL3	ELOVL fatty acid elongase 3
	ELOVL1	ELOVL fatty acid elongase 1
	ELOVL2	ELOVL fatty acid elongase 2
	ELOVL5	ELOVL fatty acid elongase 5
	MCAT	Malonyl CoA:ACP Acyltransferase (Mitochondrial)
	ELOVL4	ELOVL fatty acid elongase 4
	ELOVL6	ELOVL fatty acid elongase 6
	TECR	Trans-2,3-Enoyl-CoA Reductase
	SCD5	Stearoyl-CoA desaturase 5
	SREBP-1c	Sterol regulatory element-binding transcription factor 1c

Pathway	Symbol	Gene Name
Fatty acid transport	CPT1A	Carnitine palmitoyltransferase 1A (liver)
	CPT2	Carnitine palmitoyltransferase 2
	FABP1	Fatty acid binding protein 1, liver
	SLC27A1	Solute carrier family 27 (fatty acid transporter), member 1
	Scp2	Sterol Carrier Protein 2
	SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2
Fatty acid oxidation	ACOT1	Acyl-CoA thioesterase 1
	ACOT12	Acyl-CoA thioesterase 12
	ACOT6	Acyl-CoA thioesterase 6
	ACOT7	Acyl-CoA thioesterase 7
	ACADL	Acyl-CoA dehydrogenase, long chain
Mitochondrial fatty acid β-oxidation	ACADM	Acyl-CoA dehydrogenase, C-4 to C-12 straight chain
	ACADS	Acyl-CoA dehydrogenase, C-2 to C-3 short chain
	ACADVL	Acyl-CoA dehydrogenase, very long chain
	ACSL3	Acyl-CoA synthetase long-chain family member 3
	ACSL4	Acyl-CoA synthetase long-chain family member 4
	ACSL5	Acyl-CoA synthetase long-chain family member 5
	HADH	Hydroxyacyl-CoA Dehydrogenase
	EHHADH	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase
	DECR1	2,4-Dienoyl CoA Reductase 1, Mitochondrial
	ECHS1	Enoyl CoA Hydratase, Short Chain, 1, Mitochondrial
	HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit
	SLC25a20	Solute Carrier Family 25 (Carnitine/Acylcarnitine Translocase), Member 20
	HADHB	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit
Perioxisomal β-oxidation	ACOT4	Acyl-CoA thioesterase 4
	ACOT8	Acyl-CoA thioesterase 8
	ACOX1	Acyl-CoA oxidase 1, palmitoyl
	ACOX2	Acyl-CoA oxidase 2, branched chain
	ECH1	Enoyl CoA Hydratase 1, Peroxisomal
	PECR	Peroxisomal Trans-2-Enoyl-CoA Reductase
Microsomal oxidation	CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11
	CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1
Lipoprotein Assembly	ApoB	Apolipoprotein B
	ApoC4	Apolipoprotein C-IV
	ApoE	Apolipoprotein E
	MTTP	Microsomal triglyceride transfer protein
	ApoA5	Apolipoprotein A-V

Pathway	Symbol	Gene Name
Carbohydrate metabolism	PEPCK-C	Phosphoenolpyruvate Carboxykinase 1 (Soluble) (PCK1)
	ChREBP	MLX Interacting Protein-Like
Inflammation	IL6	Interleukin-6
	TNFA	Tumor Necrosis Factor α
Nuclear receptors	PPARG	Peroxisome Proliferator-Activated Receptor Gamma
	PPARA	Peroxisome Proliferator-Activated Receptor Alpha
	CAR	Constitutive androstane receptor (NR1I3)
	FXR	Farnesol X receptor (NR1H4)
Positive targets	CYP2B6	Cytochrome P450, Family 2, Subfamily B, Polypeptide 6
	SHP	Short heterodimer partner (NR0B2)
	PLIN2	Perilipin 2
Internal Controls	TBP	TATA binding protein
	HPRT1	Hypoxanthine Phosphoribosyltransferase 1
	UBC	Ubiquitin C
	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase

Appendix B: Customized gene array analysis data to measure the changes in the expression of hepatic lipid metabolizing genes in response to drug treatment in HepaRG cells.

Gene	CITCO	GW4064	GW7674	FOH	OA	OA+ FOH
ACAA1	0.8799	0.8395	1.6479	0.8655	0.9721	1.2377
ACAA2	0.9347	1.1325	2.0272	1.3062	1.3093	1.7096
ACACA	0.9855	1.0217	0.9994	0.8224	0.796	1.0945
ACACB	1.2172	1.0401	1.4265	0.7267	0.9187	1.153
ACADL	0.8675	0.8112	0.9124	1.1088	0.8857	0.9287
ACADM	1.0541	0.923	1.5619	1.2027	1.1132	1.282
ACADS	1.5445	1.4987	2.6926	1.017	1.3195	2.1444
ACADVL	0.9145	1.0013	1.5042	0.9845	1.1023	1.0624
ACAT1	1.0067	0.8273	1.2435	1.286	1.0937	1.1318
ACAT2	0.9109	0.9704	0.8419	1.2942	1.118	1.0567
ACLY	0.983	1.1091	0.942	0.9378	0.8512	1.1155
ACOT1	1.2821	1.1718	1.5341	0.8993	1.0454	1.229
ACOT12	1.3941	0.8307	1.6284	1.5955	1.7735	3.627
ACOT4	0.8704	0.7416	0.8807	1.0631	0.8421	0.9859
ACOT6	0.7218	0.6051	0.8094	0.9515	0.9337	0.7654
ACOT7	0.9654	1.0757	0.8729	1.0072	0.9008	0.8302
ACOT8	1.2356	1.1937	1.1652	0.8135	0.8991	1.1978
ACOX1	1.077	1.0202	1.3298	1.0728	0.9511	1.2925
ACOX2	1.0382	0.3606	1.2547	1.6993	0.7377	0.8591
ACSL3	0.9464	1.3041	1.3998	1.233	0.8494	0.9014
ACSL4	1.1188	0.9977	1.5984	0.9094	0.9711	1.3526
ACSL5	1.0347	1.6047	1.6668	1.2421	1.0974	1.4553
AGPAT1	0.9936	1.2626	1.0302	0.8698	0.8427	0.7275
AGPAT2	1.3003	1.5271	1.8207	0.7344	1.3935	1.1894
AGPAT6	1.0296	1.2749	1.1236	0.8133	0.9999	1.359
AGPAT9	1.2439	1.3468	1.3221	0.7875	1.3372	1.2009
APOA5	0.9963	1.6219	1.8338	1.3167	2.526	4.2487
CD36	0.7472	0.9812	3.3505	1.084	1.5526	1.1457
COX10	1.034	0.9851	1.0598	0.7541	0.9051	1.1201
CPT1A	1.193	1.1223	1.8485	0.9746	1.1953	1.6018
CPT2	1.0877	0.9595	1.507	0.9741	0.9289	1.0363
CYP2B6	8.2174	0.545	0.8278	1.9403	0.6128	2.056
CYP2E1	1.107	0.1995	1.0143	0.6968	1.2519	1.8636
CYP4A11	0.7328	0.4687	1.6903	0.9773	0.8708	0.9523
DGAT1	0.9348	0.5121	1.0708	0.777	0.8172	0.9472
DGAT2	1.1081	0.7129	1.196	0.8543	1.0491	1.2013
DHDDS	1.0441	1.2125	1.393	0.8875	1.1887	1.1632
EHHADH	1.0448	0.8354	1.288	0.9608	0.9764	1.2051
ELOVL3	2.993	1.5148	1.1878	1.4067	1.258	2.7722
ELOVL4	0.1089	0.1152	0.1193	1.4714	0.0834	0.096
ELOVL6	1.0914	1.0433	1.006	0.9558	1.0966	1.3819
FABP1	1.1298	0.7115	3.2373	1.8436	0.7531	1.2149
FASN	1.2928	1.3251	1.2404	0.9336	0.8057	1.2358
FDFT1	0.9353	1.0537	0.9788	1.3802	0.9781	0.9908

Gene	CITCO	GW4064	GW7674	FOH	OA	OA+ FOH
FDPS	0.8407	0.8519	0.9092	1.2214	0.8785	0.8394
FNTA	1.0159	0.8528	1.1345	0.8509	0.8971	1.233
FNTB	1.0901	1.1414	1.0968	0.7507	0.9045	1.1704
GGPS1	1.0164	1.1227	1.0335	1.1131	0.8738	0.9109
GPAT2	0.9084	0.859	0.8897	1.143	0.6411	0.7161
HADHA	0.8736	0.8788	1.8146	1.0476	1.178	1.315
HADHB	1.0243	1.0516	2.4506	1.2409	1.553	1.4498
HMGCR	0.9768	1.1588	0.9515	1.1232	1.1961	1.3589
IL6	0.9854	1.0259	0.7691	0.8693	0.52	1.052
LIPC	0.9222	0.4976	1.2883	1.3079	0.8627	0.9827
LPIN1	0.9997	0.8405	1.0866	0.8298	0.9478	1.2515
LPIN2	1.0402	0.8091	1.1049	0.8216	0.8378	1.1673
LPIN3	0.9026	0.8195	0.9344	0.7582	0.7753	1.061
NR0B2	0.7507	3.6372	0.4787	0.7553	0.7336	0.5794
NR1H4	1.0429	0.7239	1.14	1.0094	0.9533	1.1958
NR1I3	1.1957	0.7971	0.7753	0.7791	0.6958	0.9681
PCK1	0.9622	0.1389	2.4761	0.6907	1.7774	3.5014
PDSS1	1.051	0.9585	1.1836	1.3663	1.1598	1.0276
PLIN2	0.7819	1.0473	3.0052	1.2674	1.4752	1.7641
PPAPDC2	1.0382	1.1565	1.0054	0.9442	0.847	1.1158
PPARA	1.3218	1.3823	1.1067	0.9419	1.0846	1.5178
PPARG	1.0456	2.0577	1.2685	1.428	1.1001	1.2477
RABGGTA	0.9339	1.0157	0.9041	0.932	0.8326	0.7335
RABGGTB	0.8281	0.8807	0.9288	1.1707	0.8291	0.7658
SCD	0.9835	0.6528	1.3653	1.1431	0.4927	0.718
SCD5	0.8981	1.0085	0.9269	0.7609	0.6957	0.7802
SLC27A1	1.1283	1.2223	1.0891	0.6898	0.8428	1.1979
SLC27A2	1.0645	1.177	1.9246	1.6574	0.7996	1.0796
SREBF1	1.1633	0.9704	1.2816	0.6645	0.6338	0.9466

Appendix C. Primer sequences used for RT-qPCR.

Gene	Name	Forward primer	Reverse Primer
HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	GGAGGACTTGAGGTTGCCAT	TAAGGCCCCCAGC AAAACTT
HADHB	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	CCCCAGCTGTCCAGACCAAA	CCGATGCAACAAA CCCGTAA
ACAA2	Acetyl-CoA acyltransferase 2	CAGGGAATGCATCGGGTGTA	GCCCACAATTCTT GCCAGTG
ACOT12	Acyl-CoA thioesterase 12	TGCTCAAGTGGATCGACACC	TCCAACTCTAGCT GTCTCCTCA
EHHADH	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	AAAAGTGGTTTGCCTGACGG	AAGTCGTACTGAT CGCGTTG
ACOX1	Acyl-CoA oxidase 1, palmitoyl	CTTCAACCCGGAGCTGCTTA	ATGTTCTCGATCC GGCG
ACOX2	Acyl-CoA oxidase 2, branched chain	ACAGAGGGGAGCCAGGTTCTT	ATCCCCCAATGAC ACTCGGT
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	AGCACCTGCCTCTACAGTTGG	TTTTCAGGCCTTTG GGGACC
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4	TACCCAATAAGGCACCACCC	AATGTGCAGGAAA GCATCTGA
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	ACTCCCTGGCTCCAGCTTTA	TCTCTGTCCCCGCA AAGAAC
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6	AGCTTCATGACCGAGCCAAA	CTGTGTCCTTGGG GATGATGT
SHP	Small heterodimer partner (NR0B2)	GTGGCTTCATGCTGTCTGG	CTGGCACATCGGG GTTGA
PLIN2	Perilipin 2	GGCGAGGCGGGGTTTATAG	CCACACTCGGTTG TGGATCA
PCK1	Phosphoenolpyruvate Carboxykinase 1 (Soluble) (PEPCK1)	ATCCCCAAAACAGGCCTCAG	ACGTACATGGTGC GACCTTT
ACADS	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	CCAGGGATGGGCTTCAAGAT	TGTCTGCCAACTT GAACTGGA
SCD	Stearoyl-CoA desaturase	TTCCCGACGTGGCTTTTTCT	AGCCAGGTTTGTA GTACCTCC
SREBP1c	Sterol regulatory element-binding transcription factor 1c	GTAACGACCACTGTGACCTCG	CACTCACCAGGGT CGGCAAA

REFERENCES

Adany I, Yazlovitskaya EM, Haug JS, Voziyan PA, and Melnykovych G (1994) Differences in sensitivity to farnesol toxicity between neoplastically- and non-neoplastically-derived cells in culture. *Cancer letters* **79:**175-179.

Adorini L, Pruzanski M, and Shapiro D (2012) Farnesoid X receptor targeting to treat nonalcoholic steatohepatitis. *Drug Discovery Today* **17:**988-997.

Albuquerque P and Casadevall A (2012) Quorum sensing in fungi – a review. *Medical mycology* **50:**337-345.

Amin D, Rutledge RZ, Needle SN, Galczenski HF, Neuenschwander K, Scotese AC, Maguire MP, Bush RC, Hele DJ, Bilder GE, and Perrone MH (1997) RPR 107393, a potent squalene synthase inhibitor and orally effective cholesterol-lowering agent: comparison with inhibitors of HMG-CoA reductase. *The Journal of pharmacology and experimental therapeutics* **281:**746-752.

Ammerschlaeger M, Beigel J, Klein K-U, and Mueller SO (2004) Characterization of the Species-Specificity of Peroxisome Proliferators in Rat and Human Hepatocytes. *Toxicological Sciences* **78:**229-240.

and JB and Moller DE (2002) The Mechanisms of Action of PPARs. *Annual Review of Medicine* **53:**409-435.

Anderson N and Borlak J (2008) Molecular Mechanisms and Therapeutic Targets in Steatosis and Steatohepatitis. *Pharmacological Reviews* **60:**311-357.

Angulo P (2007) GI epidemiology: nonalcoholic fatty liver disease. *Alimentary pharmacology & therapeutics* **25:**883-889.

Aninat C, Piton A, Glaise D, Le Charpentier T, Langouet S, Morel F, Guguen-Guillouzo C, and Guillouzo A (2006) Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos* **34:**75-83.

Anstee QM, Daly AK, and Day CP (2011) Genetic modifiers of non-alcoholic fatty liver disease progression. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1812:**1557-1566.

Anthérieu S, Rogue A, Fromenty B, Guillouzo A, and Robin M-A (2011) Induction of vesicular steatosis by amiodarone and tetracycline is associated with up-regulation of lipogenic genes in heparg cells. *Hepatology* **53:**1895-1905.

Arun J, Clements RH, Lazenby AJ, Leeth RR, and Abrams GA (2006) The prevalence of nonalcoholic steatohepatitis is greater in morbidly obese men compared to women. *Obesity surgery* **16:**1351-1358.

Au-Yeung KK, Liu PL, Chan C, Wu WY, Lee SS, and Ko JK (2008) Herbal isoprenols induce apoptosis in human colon cancer cells through transcriptional activation of PPARgamma. *Cancer investigation* **26:**708-717.

Bansal VS and Vaidya S (1994) Characterization of 2 Distinct Allyl Pyrophosphatase Activities from Rat-Liver Microsomes. *Archives of Biochemistry and Biophysics* **315:**393-399.

Baxter A, Fitzgerald BJ, Hutson JL, McCarthy AD, Motteram JM, Ross BC, Sapra M, Snowden MA, Watson NS, and Williams RJ (1992) Squalestatin 1, a potent inhibitor of squalene synthase, which lowers serum cholesterol in vivo. *Journal of Biological Chemistry* **267:**11705-11708.

Bedogni G, Nobili V, and Tiribelli C (2014) Epidemiology of fatty liver: An update. *World Journal of Gastroenterology : WJG* **20:**9050-9054.

Blachier M, Leleu H, Peck-Radosavljevic M, Valla D-C, and Roudot-Thoraval F (2013) The burden of liver disease in Europe: a review of available epidemiological data. *Journal of hepatology* **58:**593-608.

Bostedor RG, Karkas JD, Arison BH, Bansal VS, Vaidya S, Germershausen JI, Kurtz MM, and Bergstrom JD (1997) Farnesol-derived dicarboxylic acids in the urine of animals treated with zaragozic acid A or with farnesol. *Journal of Biological Chemistry* **272:**9197-9203.

Brown MV, Compton SA, Milburn MV, Lawton KA, and Cheatham B (2013) Metabolomic signatures in lipid-loaded HepaRGs reveal pathways involved in steatotic progression. *Obesity (Silver Spring, Md)* **21:**10.1002/oby.20440.

Browning JD and Horton JD (2004) Molecular mediators of hepatic steatosis and liver injury. *The Journal of clinical investigation* **114:**147-152.

Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, and Hobbs HH (2004) Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **40:**1387-1395.

Buhaescu I and Izzedine H (2007) Mevalonate pathway: A review of clinical and therapeutical implications. *Clinical Biochemistry* **40:**575-584.

Burke YD, Ayoubi AS, Werner SR, McFarland BC, Heilman DK, Ruggeri BA, and Crowell PL (2002) Effects of the isoprenoids perillyl alcohol and farnesol on apoptosis biomarkers in pancreatic cancer chemoprevention. *Anticancer research* **22:**3127-3134.

Burke YD, Stark MJ, Roach SL, Sen SE, and Crowell PL (1997) Inhibition of pancreatic cancer growth by the dietary isoprenoids farnesol and geraniol. *Lipids* **32:**151-156.

Chalasani N, Gorski JC, Asghar MS, Asghar A, Foresman B, Hall SD, and Crabb DW (2003) Hepatic cytochrome P450 2E1 activity in nondiabetic patients with nonalcoholic steatohepatitis. *Hepatology* **37:**544-550.

Chawla A, Repa JJ, Evans RM, and Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science (New York, NY)* **294:**1866-1870.

Chen ZW, Chen LY, Dai HL, Chen JH, and Fang LZ (2008) Relationship between alanine aminotransferase levels and metabolic syndrome in nonalcoholic fatty liver disease. *Journal of Zhejiang University Science B* **9:**616-622.

Christophe J and Popják G (1961) Studies on the biosynthesis of cholesterol: XIV. the origin of prenoic acids from allyl Pyrophosphates in liver enzyme systems. *Journal of Lipid Research* **2:**244-257.

Correll CC, Ng L, and Edwards PA (1994) Identification of farnesol as the non-sterol derivative of mevalonic acid required for the accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Journal of Biological Chemistry* **269:**17390-17393.

Crick DC, Andres DA, and Waechter CJ (1995) Farnesol Is Utilized for Protein Isoprenylation and the Biosynthesis of Cholesterol in Mammalian Cells. *Biochemical and Biophysical Research Communications* **211:**590-599.

Crowell PL (1999) Prevention and therapy of cancer by dietary monoterpenes. *The Journal of nutrition* **129:**775s-778s.

Cui W, Chen SL, and Hu K-Q (2010) Quantification and mechanisms of oleic acid-induced steatosis in HepG2 cells. *American Journal of Translational Research* **2:**95-104.

Dietrich P and Hellerbrand C (2014) Non-alcoholic fatty liver disease, obesity and the metabolic syndrome. *Best Practice & Research Clinical Gastroenterology* **28:**637-653.

Dowman JK, Tomlinson JW, and Newsome PN (2010) Pathogenesis of non-alcoholic fatty liver disease. *QJM* **103:**71-83.

Drake MT, Clarke BL, and Lewiecki EM (2015) The Pathophysiology and Treatment of Osteoporosis. *Clinical Therapeutics* **37:**1837-1850.

Duncan RE and Archer MC (2008) Farnesol decreases serum triglycerides in rats: identification of mechanisms including up-regulation of PPARalpha and down-regulation of fatty acid synthase in hepatocytes. *Lipids* **43:**619-627.

Edwards PA and Ericsson J (1999) STEROLS AND ISOPRENOIDS: Signaling Molecules Derived from the Cholesterol Biosynthetic Pathway. *Annual Review of Biochemistry* **68:**157-185.

Eguchi Y, Hyogo H, Ono M, Mizuta T, Ono N, Fujimoto K, Chayama K, and Saibara T (2012) Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. *Journal of gastroenterology* **47:**586-595.

Fan JG (2013) Epidemiology of alcoholic and nonalcoholic fatty liver disease in China. *J Gastroenterol Hepatol* **28 Suppl 1:**11-17.

Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM, and Weinberger C (1995a) Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* **81:**687-693.

Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM, and Weinberger C (1995b) Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* **81:**687-693.

Fukunaga K, Arita M, Takahashi M, Morris AJ, Pfeffer M, and Levy BD (2006) Identification and Functional Characterization of a Presqualene Diphosphate Phosphatase. *Journal of Biological Chemistry* **281:**9490-9497.

George J and Liddle C (2008) Nonalcoholic Fatty Liver Disease: Pathogenesis and Potential for Nuclear Receptors as Therapeutic Targets. *Molecular Pharmaceutics* **5:**49-59.

Gerets HHJ, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, and Atienzar FA (2012) Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biology and Toxicology* **28:**69-87.

Goldstein JL and Brown MS (1990) Regulation of the mevalonate pathway. *Nature* **343:**425-430.

Gonzalez FJ and Shah YM (2008) PPARα: Mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. *Toxicology* **246:**2-8.

Goto T, Kim YI, Funakoshi K, Teraminami A, Uemura T, Hirai S, Lee JY, Makishima M, Nakata R, Inoue H, Senju H, Matsunaga M, Horio F, Takahashi N, and Kawada T (2011) Farnesol, an isoprenoid, improves metabolic abnormalities in mice via both PPARalpha-dependent and -independent pathways. *American journal of physiology Endocrinology and metabolism* **301:**E1022-1032.

Guillouzo A (1998) Liver cell models in in vitro toxicology. *Environmental Health Perspectives* **106:**511-532.

Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F, and Guguen-Guillouzo C (2007) The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-biological interactions* **168:**66-73.

Hanley K, Kömüves LG, Ng DC, Schoonjans K, He SS, Lau P, Bikle DD, Williams ML, Elias PM, Auwerx J, and Feingold KR (2000) Farnesol Stimulates Differentiation in Epidermal Keratinocytes via PPARα. *Journal of Biological Chemistry* **275:**11484-11491.

Hart SN, Li Y, Nakamoto K, Subileau E-a, Steen D, and Zhong X-b (2010) A Comparison of Whole Genome Gene Expression Profiles of HepaRG Cells and HepG2 Cells to Primary Human Hepatocytes and Human Liver Tissues. *Drug Metabolism and Disposition* **38:**988-994.

Hiyoshi H, Yanagimachi M, Ito M, Saeki T, Yoshida I, Okada T, Ikuta H, Shinmyo D, Tanaka K, Kurusu N, and Tanaka H (2001) Squalene synthase inhibitors reduce plasma triglyceride through a low-density lipoprotein receptor-independent mechanism. *European Journal of Pharmacology* **431:**345-352.

Hiyoshi H, Yanagimachi M, Ito M, Yasuda N, Okada T, Ikuta H, Shinmyo D, Tanaka K, Kurusu N, Yoshida I, Abe S, Saeki T, and Tanaka H (2003) Squalene synthase inhibitors suppress triglyceride biosynthesis through the farnesol pathway in rat hepatocytes. *J Lipid Res* **44:**128-135.

Horn TL, Long L, Cwik MJ, Morrissey RL, Kapetanovic IM, and McCormick DL (2005a) Modulation of hepatic and renal drug metabolizing enzyme activities in rats by subchronic administration of farnesol. *Chemico-biological interactions* **152:**79-99.

Horn TL, Long L, Cwik MJ, Morrissey RL, Kapetanovic IM, and McCormick DL (2005b) Modulation of hepatic and renal drug metabolizing enzyme activities in rats by subchronic administration of farnesol. *Chemico-biological interactions* **152:**79-99.

Jackson NM and Kocarek TA (2008) Suppression of CYP2B Induction by Alendronate-Mediated Farnesyl Diphosphate Synthase Inhibition in Primary Cultured Rat Hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **36:**2030-2036.

Joo JH and Jetten AM (2010) Molecular Mechanisms involved in Farnesol-Induced Apoptosis. *Cancer letters* **287:**123.

Joo JH, Liao G, Collins JB, Grissom SF, and Jetten AM (2007) Farnesol-induced apoptosis in human lung carcinoma cells is coupled to the endoplasmic reticulum stress response. *Cancer research* **67:**7929-7936.

Journe F, Laurent G, Chaboteaux C, Nonclercq D, Durbecq V, Larsimont D, and Body JJ (2008) Farnesol, a mevalonate pathway intermediate, stimulates MCF-7 breast cancer cell growth through farnesoid-X-receptor-mediated estrogen receptor activation. *Breast cancer research and treatment* **107:**49-61.

Kassam A, Winrow CJ, Fernandez-Rachubinski F, Capone JP, and Rachubinski RA (2000) The Peroxisome Proliferator Response Element of the Gene Encoding the Peroxisomal β-Oxidation Enzyme Enoyl-CoA Hydratase/3-Hydroxyacyl-CoA Dehydrogenase Is a Target for Constitutive Androstane Receptor β/9-cis-Retinoic Acid Receptor-mediated Transactivation. *Journal of Biological Chemistry* **275:**4345-4350.

Kawano Y and Cohen DE (2013) Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. *Journal of gastroenterology* **48:**434-441.

Keller RK, Zhao Z, Chambers C, and Ness GC (1996) Farnesol is not the nonsterol regulator mediating degradation of HMG-CoA reductase in rat liver. *Arch Biochem Biophys* **328:**324-330.

Khalid Q, Bailey I, Patel VB. (2015) Non-Alcoholic fatty liver disease: the effect of bile acids and farnesoid X receptor agonists on pathophysiology and treatment. Liver Res Open J. **1(2)**: 32-40.

Kiyosawa N, Kwekel JC, Burgoon LD, Dere E, Williams KJ, Tashiro C, Chittim B, and Zacharewski TR (2008) Species-specific regulation of PXR/CAR/ER-target genes in the mouse and rat liver elicited by o, p'-DDT. *BMC Genomics* **9:**487-487.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, and Lehmann JM (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. *Proceedings of the National Academy of Sciences* **94:**4318-4323.

Kocarek TA, Kraniak JM, and Reddy AB (1998) Regulation of Rat Hepatic Cytochrome P450 Expression by Sterol Biosynthesis Inhibition: Inhibitors of Squalene Synthase Are Potent Inducers of CYP2B Expression in Primary Cultured Rat Hepatocytes and Rat Liver. *Molecular Pharmacology* **54:**474-484.

Kocarek TA and Mercer-Haines NA (2002) Squalestatin 1-Inducible Expression of Rat CYP2B: Evidence That an Endogenous Isoprenoid Is an Activator of the Constitutive Androstane Receptor. *Molecular Pharmacology* **62:**1177-1186.

Kocarek TA and Reddy AB (1996) Regulation of cytochrome P450 expression by inhibitors of hydroxymethylglutaryl-coenzyme A reductase in primary cultured rat hepatocytes and in rat liver. *Drug Metabolism and Disposition* **24:**1197-1204.

Koo SH (2013) Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis. *Clinical and molecular hepatology* **19:**210-215.

LaBrecque DR, Abbas Z, Anania F, Ferenci P, Khan AG, Goh KL, Hamid SS, Isakov V, Lizarzabal M, Penaranda MM, Ramos JFR, Sarin S, Stimac D, Thomson ABR, Umar M, Krabshuis J, and LeMair A (2014) World Gastroenterology Organisation Global Guidelines Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. *J Clin Gastroenterol* **48:**467-473.

Li L, Zhang W, Cheng S, Cao D, and Parent M (2012) Isoprenoids and related pharmacological interventions: potential application in Alzheimer's disease. *Molecular neurobiology* **46:**64-77.

Li Y, Jadhav K, and Zhang Y (2013) Bile acid receptors in non-alcoholic fatty liver disease. *Biochemical Pharmacology* **86:**1517-1524.

Loomba R and Sanyal AJ (2013) The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* **10:**686-690.

López-Velázquez JA, Carrillo-Córdova LD, Chávez-Tapia NC, Uribe M, and Méndez-Sánchez N (2012) Nuclear Receptors in Nonalcoholic Fatty Liver Disease. *Journal of Lipids* **2012:**10.

Machado MV and Cortez-Pinto H (2014) Nuclear receptors: how do they position in non-alcoholic fatty liver disease treatment? *Liver International* **34:**1291-1294.

Mandard S, Müller M, and Kersten S (2004) Peroxisome proliferator-activated receptor α target genes. *CMLS, Cell Mol Life Sci* **61:**393-416.

Masarone M, Federico A, Abenavoli L, Loguercio C, and Persico M (2014) Non alcoholic fatty liver: epidemiology and natural history. *Reviews on recent clinical trials* **9:**126-133.

Mazzoccoli G, Vinciguerra M, Oben J, Tarquini R, and De Cosmo S (2014) Non-alcoholic fatty liver disease: the role of nuclear receptors and circadian rhythmicity. *Liver international : official journal of the International Association for the Study of the Liver* **34:**1133-1152.

Meigs TE and Simoni RD (1997) Farnesol as a Regulator of HMG-CoA Reductase Degradation: Characterization and Role of Farnesyl Pyrophosphatase. *Archives of Biochemistry and Biophysics* **345:**1-9.

Melnykovych G, Haug JS, and Goldner CM (1992) Growth inhibition of leukemia cell line CEM-C1 by farnesol: effects of phosphatidylcholine and diacylglycerol. *Biochem Biophys Res Commun* **186:**543-548.

Merrell MD and Cherrington NJ (2011) Drug metabolism alterations in nonalcoholic fatty liver disease. *Drug metabolism reviews* **43:**317-334.

Miriyala S, Subramanian T, Panchatcharam M, Ren H, McDermott MI, Sunkara M, Drennan T, Smyth SS, Spielmann HP, and Morris AJ (2010) Functional Characterization of the Atypical Integral Membrane Lipid Phosphatase PDP1/PPAPDC2 Identifies a Pathway for Interconversion of Isoprenols and Isoprenoid Phosphates in Mammalian Cells. *The Journal of Biological Chemistry* **285:**13918-13929.

Mishra A and Younossi ZM (2012) Epidemiology and Natural History of Non-alcoholic Fatty Liver Disease. *Journal of clinical and experimental hepatology* **2:**135-144.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods* **65:**55-63.

Moya M, José Gómez-Lechón M, Castell JV, and Jover R (2010) Enhanced steatosis by nuclear receptor ligands: A study in cultured human hepatocytes and hepatoma cells with a characterized nuclear receptor expression profile. *Chemico-biological interactions* **184:**376-387.

Musso G, Gambino R, and Cassader M (2009) Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Progress in lipid research* **48:**1-26.

Nah J, Song S-J, and Back K (2001) Partial Characterization of Farnesyl and Geranylgeranyl Diphosphatases Induced in Rice Seedlings by UV-C Irradiation. *Plant and Cell Physiology* **42:**864-867.

Naik A, Belič A, Zanger UM, and Rozman D (2013) Molecular Interactions between NAFLD and Xenobiotic Metabolism. *Frontiers in Genetics* **4:**2.

Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarathy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, and Doo E (2015) Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet (London, England)* **385:**956-965.

Nguyen P, Leray V, Diez M, Serisier S, Bloc'h JL, Siliart B, and Dumon H (2008) Liver lipid metabolism. *Journal of Animal Physiology and Animal Nutrition* **92:**272-283.

Nishimoto T, Amano Y, Tozawa R, Ishikawa E, Imura Y, Yukimasa H, and Sugiyama Y (2003) Lipid-lowering properties of TAK-475, a squalene synthase inhibitor, in vivo and in vitro. *British journal of pharmacology* **139:**911-918.

O'Brien ML, Rangwala SM, Henry KW, Weinberger C, Crick DC, Waechter CJ, Feller DR, and Noonan DJ (1996) Convergence of three steroid receptor pathways in the mediation of nongenotoxic hepatocarcinogenesis. *Carcinogenesis* **17:**185-190.

Oldfield E (2010) Targeting isoprenoid biosynthesis for drug discovery: bench to bedside. *Accounts of chemical research* **43:**1216-1226.

Ong JP, Pitts A, and Younossi ZM (2008) Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. *Journal of Hepatology* **49:**608-612.

Ong TP, Heidor R, de Conti A, Dagli ML, and Moreno FS (2006) Farnesol and geraniol chemopreventive activities during the initial phases of hepatocarcinogenesis involve similar actions on cell proliferation and DNA damage, but distinct actions on apoptosis, plasma cholesterol and HMGCoA reductase. *Carcinogenesis* **27:**1194-1203.

Parkash O and Hamid S (2013) Are we ready for a new epidemic of under recognized liver disease in South Asia especially in Pakistan? Non alcoholic fatty liver disease. *J Pak Med Assoc* **63:**95-99.

Pettinelli P, del Pozo T, Araya J, Rodrigo R, Araya AV, Smok G, Csendes A, Gutierrez L, Rojas J, Korn O, Maluenda F, Diaz JC, Rencoret G, Braghetto I, Castillo J, Poniachik J, and Videla LA (2009) Enhancement in liver SREBP-1c/PPAR-α ratio and steatosis in obese patients: Correlations with insulin resistance and n-3 long-chain polyunsaturated fatty acid depletion. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1792:**1080-1086.

Pineda Torra I, Claudel T, Duval C, Kosykh V, Fruchart JC, and Staels B (2003) Bile acids induce the expression of the human peroxisome proliferator-activated receptor alpha gene via activation of the farnesoid X receptor. *Molecular endocrinology (Baltimore, Md)* **17:**259-272.

Rakhshandehroo M, Hooiveld G, Müller M, and Kersten S (2009) Comparative Analysis of Gene Regulation by the Transcription Factor PPARα between Mouse and Human. *PLoS ONE* **4:**e6796.

Ramirez-Zacarias J, Castro-Munozledo F, and Kuri-Harcuch W (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* **97:**493-497.

Raner GM, Muir AQ, Lowry CW, and Davis BA (2002) Farnesol as an inhibitor and substrate for rabbit liver microsomal P450 enzymes. *Biochemical and biophysical research communications* **293:**1-6.

Rasband W (2012) ImageJ: Image processing and analysis in Java. *Astrophysics Source Code Library* **1:**06013.

Reddy JK and Sambasiva Rao M (2006) Lipid Metabolism and Liver Inflammation. II. Fatty liver disease and fatty acid oxidation. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **290:**G852-G858.

Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S, Lonardo A, Carulli N, and Loria P (2009) Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J Gastroenterol Hepatol* **24:**830-840.

Rioja A, Pizzey AR, Marson CM, and Thomas NS (2000) Preferential induction of apoptosis of leukaemic cells by farnesol. *FEBS Lett* **467:**291-295.

Rogue A, Anthérieu S, Vluggens A, Umbdenstock T, Claude N, de la Moureyre-Spire C, Weaver RJ, and Guillouzo A (2014) PPAR agonists reduce steatosis in oleic acid-overloaded HepaRG cells. *Toxicology and Applied Pharmacology* **276:**73-81.

Rogue A, Lambert C, Spire C, Claude N, and Guillouzo A (2012) Interindividual variability in gene expression profiles in human hepatocytes and comparison with HepaRG cells. *Drug Metab Dispos* **40:**151-158.

Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, and Hobbs HH (2008) Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* **40:**1461-1465.

Ross J, Plummer SM, Rode A, Scheer N, Bower CC, Vogel O, Henderson CJ, Wolf CR, and Elcombe CR (2010) Human constitutive androstane receptor (CAR) and pregnane X receptor (PXR) support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogens phenobarbital and chlordane in vivo. *Toxicol Sci* **116:**452-466.

Roth A, Looser R, Kaufmann M, and Meyer UA (2008) Sterol regulatory element binding protein 1 interacts with pregnane X receptor and constitutive androstane receptor and represses their target genes. *Pharmacogenetics and genomics* **18:**325-337.

Samanez CH, Caron S, Briand O, Dehondt H, Duplan I, Kuipers F, Hennuyer N, Clavey V, and Staels B (2012) The human hepatocyte cell lines IHH and HepaRG: models to study glucose, lipid and lipoprotein metabolism. *Archives Of Physiology And Biochemistry* **118:**102-111.

Sambasiva Rao M and Reddy JK (2004) PPARα in the pathogenesis of fatty liver disease. *Hepatology* **40:**783-786.

Schattenberg JM, Wang Y, Singh R, Rigoli RM, and Czaja MJ (2005) Hepatocyte CYP2E1 Overexpression and Steatohepatitis Lead to Impaired Hepatic Insulin Signaling. *Journal of Biological Chemistry* **280:**9887-9894.

Scheper MA, Shirtliff ME, Meiller TF, Peters BM, and Jabra-Rizk MA (2008) Farnesol, a fungal quorum-sensing molecule triggers apoptosis in human oral squamous carcinoma cells. *Neoplasia (New York, NY)* **10:**954-963.

Schupp M and Lazar MA (2010) Endogenous Ligands for Nuclear Receptors: Digging Deeper. *The Journal of Biological Chemistry* **285:**40409-40415.

Subramanian T, Ren H, Subramanian KL, Sunkara M, Onono FO, Morris AJ, and Spielmann HP (2014) Design and synthesis of non-hydrolyzable homoisoprenoid α-monofluorophosphonate inhibitors of PPAPDC family integral membrane lipid phosphatases. *Bioorganic & Medicinal Chemistry Letters* **24:**4414-4417.

Swarbrick CMD, Roman N, Cowieson N, Patterson EI, Nanson J, Siponen MI, Berglund H, Lehtiö L, and Forwood JK (2014) Structural Basis for Regulation of the Human Acetyl-CoA Thioesterase 12 and Interactions with the Steroidogenic Acute Regulatory Protein-related Lipid Transfer (START) Domain. *Journal of Biological Chemistry* **289:**24263-24274.

Tailleux A, Wouters K, and Staels B (2012) Roles of PPARs in NAFLD: Potential therapeutic targets. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1821:**809-818.

Takahashi N, Kawada T, Goto T, Yamamoto T, Taimatsu A, Matsui N, Kimura K, Saito M, Hosokawa M, Miyashita K, and Fushiki T (2002) Dual action of isoprenols from herbal medicines on both PPARγ and PPARα in 3T3-L1 adipocytes and HepG2 hepatocytes. *FEBS Letters* **514:**315-322.

Thai L, Rush JS, Maul JE, Devarenne T, Rodgers DL, Chappell J, and Waechter CJ (1999) Farnesol is utilized for isoprenoid biosynthesis in plant cells via farnesyl pyrophosphate formed by successive monophosphorylation reactions. *Proceedings of the National Academy of Sciences* **96:**13080-13085.

Tolman KG and Dalpiaz AS (2007) Treatment of non-alcoholic fatty liver disease. *Therapeutics and Clinical Risk Management* **3:**1153-1163.

Ugawa T, Kakuta H, Moritani H, Matsuda K, Ishihara T, Yamaguchi M, Naganuma S, Iizumi Y, and Shikama H (2000) YM-53601, a novel squalene synthase inhibitor, reduces plasma cholesterol and triglyceride levels in several animal species. *British journal of pharmacology* **131:**63-70.

Vaidya S, Bostedor R, Kurtz MM, Bergstrom JD, and Bansal VS (1998) Massive production of farnesol-derived dicarboxylic acids in mice treated with the squalene synthase inhibitor zaragozic acid A. *Archives of Biochemistry and Biophysics* **355:**84-92.

Videla LA and Pettinelli P (2012) Misregulation of PPAR Functioning and Its Pathogenic Consequences Associated with Nonalcoholic Fatty Liver Disease in Human Obesity. *PPAR Research* **2012:**14.

Wada T, Gao J, and Xie W (2009) PXR and CAR in energy metabolism. *Trends in endocrinology and metabolism: TEM* **20:**273-279.

Wagner M, Zollner G, and Trauner M (2011) Nuclear receptors in liver disease. *Hepatology* **53:**1023-1034.

Wang H, Chen J, Hollister K, Sowers LC, and Forman BM (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Molecular cell* **3:**543-553.

Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, and Auwerx J (2004) Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *The Journal of clinical investigation* **113:**1408-1418.

Weiss J, Rau M, and Geier A (2014) Non-alcoholic fatty liver disease: epidemiology, clinical course, investigation, and treatment. *Deutsches Arzteblatt international* **111:**447-452.

Wiegand S, Keller KM, Robl M, L'Allemand D, Reinehr T, Widhalm K, and Holl RW (2010) Obese boys at increased risk for nonalcoholic liver disease: evaluation of 16,390 overweight or obese children and adolescents. *International journal of obesity (2005)* **34:**1468-1474.

Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, and Harrison SA (2011) Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* **140:**124-131.

Willner IR, Waters B, Patil SR, Reuben A, Morelli J, and Riely CA (2001) Ninety patients with nonalcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. *Am J Gastroenterol* **96:**2957-2961.

Wiseman DA, Werner SR, and Crowell PL (2007) Cell cycle arrest by the isoprenoids perillyl alcohol, geraniol, and farnesol is mediated by p21(Cip1) and p27(Kip1) in human

pancreatic adenocarcinoma cells. *The Journal of pharmacology and experimental therapeutics* **320:**1163-1170.

Xiao L, Wang J, Jiang M, Xie W, and Zhai Y (2013) The emerging role of constitutive androstane receptor and its cross talk with liver X receptors and peroxisome proliferator-activated receptor A in lipid metabolism. *Vitamins and hormones* **91:**243-258.

Xu J-Y, Li Z-P, Zhang L, and Ji G (2014) Recent insights into farnesoid X receptor in non-alcoholic fatty liver disease. *World Journal of Gastroenterology : WJG* **20:**13493-13500.

Yang Q, Nagano T, Shah Y, Cheung C, Ito S, and Gonzalez FJ (2008) The PPARα-Humanized Mouse: A Model to Investigate Species Differences in Liver Toxicity Mediated by PPARα. *Toxicological sciences : an official journal of the Society of Toxicology* **101:**132-139.

Yang YJ and Huang SC (2010) Non-Alcoholic Fatty Liver Disease and Steatohepatitis in Taiwanese Children: The Prevalence and Serum Retinol Binding Protein 4 Level. *Pediatr Res* **68:**142-142.

Yang Z-X, Shen W, and Sun H (2010) Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease. *Hepatology International* **4:**741-748.

Younossi ZM, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, and Srishord M (2011) Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clinical gastroenterology and hepatology : the*

official clinical practice journal of the American Gastroenterological Association **9:**524-530.e521; quiz e560.

Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & Therapeutics* **138:**103-141.

Zelko I and Negishi M (2000) Phenobarbital-Elicited Activation of Nuclear Receptor CAR in Induction of Cytochrome P450 Genes. *Biochemical and Biophysical Research Communications* **277:**1-6.

Zhang FL and Casey PJ (1996) Protein prenylation: molecular mechanisms and functional consequences. *Annual review of biochemistry* **65:**241-269.

Zhang L, You W, Zhang H, Peng R, Zhu Q, Yao A, Li X, Zhou Y, Wang X, Pu L, and Wu J (2015a) PNPLA3 polymorphisms (rs738409) and non-alcoholic fatty liver disease risk and related phenotypes: a meta-analysis. *Journal of Gastroenterology and Hepatology* **30:**821-829.

Zhang W, Sargis RM, Volden PA, Carmean CM, Sun XJ, and Brady MJ (2012) PCB 126 and Other Dioxin-Like PCBs Specifically Suppress Hepatic PEPCK Expression via the Aryl Hydrocarbon Receptor. *PLoS ONE* **7:**e37103.

Zhang Y, Cui Y, Wang X-L, Shang X, Qi Z-G, Xue J, Zhao X, Deng M, and Xie M-L (2015b) PPARα/γ agonists and antagonists differently affect hepatic lipid metabolism, oxidative stress and inflammatory cytokine production in steatohepatitic rats. *Cytokine* **75:**127-135.

ABSTRACT

FARNESOL-MEDIATED REGULATION OF HEPATIC LIPID METABOLISM IN HepaRG CELLS

by

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Non-alcoholic fatty liver disease is emerging as one of the most common liver disorders worldwide and is characterized by accumulation of triglycerides (TGs) in liver. The endogenous isoprenoid farnesol reduces hepatic TG levels in rodents, and this effect appears to involve at least two nuclear receptors, peroxisome proliferator-activated receptor α and farnesoid X receptor (FXR). However, farnesol's effects on human hepatic lipid metabolism are currently unknown. The objective of this study is to evaluate how farnesol treatment would affect hepatic lipid accumulation and metabolism in a cellular model of human hepatic steatosis that was created by incubating the hepatocyte-like HepaRG cell line with oleic acid. Farnesol treatment suppressed the OA-induced TGs accumulation in HepaRG cells by 25%. Farnesol upregulated the activity of lipid-sensing nuclear receptors PPARα and constitutive androstane receptor (CAR), which was evident by the increase in the expression of their respective target genes, PLIN2 and CYP2B6; however, farnesol had no effect on FXR activity in HepaRG cells. Gene expression analysis through customized arrays revealed that farnesol modulated mRNA levels of several hepatic lipid- and drug-metabolizing enzymes in both control and OA-

overloaded HepaRG cells, and the expression pattern was similar to that achieved through activation of PPARα rather than through CAR or FXR.

In OA-overloaded HepaRG cells, farnesol treatment induced overall fatty acid oxidation rate, which was accompanied by upregulation in the mRNA levels of PPARα target genes involved in hepatic mitochondrial fatty acid oxidation, such as HADHB, ACADS and ACOT12. This effect was lost when cells were co-treated with the PPARα antagonist except for ACOT12. Farnesol had no effect on genes regulating peroxisomal oxidation. OA-induced changes in drug-metabolizing enzymes, such as CYP3A4, CYP2B6, CYP1A2 and CYP2E1 were also attenuated by co-treatment with farnesol. Our findings show for the first time that farnesol regulates human hepatic lipid metabolism and suppresses lipid accumulation in HepaRG cells under steatogenic conditions by upregulating mitochondrial fatty acid oxidation, primarily through PPARα pathway.

Farnesyl pyrophosphate (FPP) is a branch-point intermediate in the mevalonate pathway that is normally converted mainly to squalene by squalene synthase in the first committed step of sterol biosynthesis. Treatment with the squalene synthase inhibitor squalestatin 1 (SQ1) causes accumulation of FPP, its dephosphorylated metabolite farnesol, and several oxidized farnesol-derived metabolites. Also, SQ1 treatment of primary cultured rat hepatocytes increases CYP2B expression through a mechanism that requires FPP synthesis and activation of the constitutive androstane receptor (CAR). Because direct farnesol treatment also increases CYP2B expression, it seems likely that SQ1-mediated CAR activation requires FPP dephosphorylation to farnesol. The lipid phosphatase, phosphatidic acid phosphatase domain containing 2 (PPAPDC2), was recently reported to catalyze FPP dephosphorylation. We therefore determined the effect of overexpressing or knocking down PPAPDC2 on SQ1-

mediated CAR activation in primary cultured rat hepatocytes. Co-transfection of rat hepatocytes with a plasmid expressing rat or human PPAPDC2 enhanced SQ1-mediated activation of a CAR-responsive reporter by 1.7- or 2.4-fold over the SQ1-mediated activation that was produced when hepatocytes were co-transfected with empty expression plasmid. Similarly, transduction of rat hepatocytes with a recombinant adenovirus expressing PPAPDC2 enhanced SQ1-mediated CYP2B1 mRNA induction by 1.4-fold over the induction that was seen in hepatocytes transduced with control adenovirus. Co-transfection with a shRNA targeting PPAPDC2 reduced SQ1-mediated CAR activation by ~80% relative to the activation that occurred in hepatocytes transfected with non-targeting shRNA. These results indicate that PPAPDC2 plays an important role in SQ1-mediated CAR activation, most probably by catalyzing the conversion of FPP to farnesol.

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Peer Reviewed Publications

Pant A and Kocarek TA (2015) Role of Phosphatidic Acid Phosphatase Domain Containing 2 (PPAPDC2) In Squalestatin 1-Mediated Activation of the Constitutive Androstane Receptor in Primary Cultured Rat Hepatocytes. *Drug Metab Dispos*.

Rondini EA, Pant A, and Kocarek TA (2015) Transcriptional Regulation of Cytosolic Sulfotransferase 1C2 by Intermediates of the Cholesterol Biosynthetic Pathway in Primary Cultured Rat Hepatocytes. *The Journal of pharmacology and experimental therapeutics* **355:**429-441.

Pant A, Rondini EA, and Kocarek TA. Farnesol suppresses hepatic steatosis through regulation of lipid-metabolizing enzymes in HepaRG cells. (In preparation).