PPARα activation is essential for severe hepatic steatosis and hepatocellular carcinoma induced by HCV core protein

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Nonstandard abbreviations used in this paper: HCC, hepatocellular carcinoma; AOX, acyl-CoA oxidase; HCVcpTg, HCV core protein-expressing transgenic; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; MTP, microsomal transfer protein; FAT, fatty acid translocase; FATP, fatty acid transport protein; L-FABP, liver fatty acid-binding protein; MCAD, medium-chain acyl-CoA dehydrogenase; LACS, long-chain acyl-CoA dehydrogenase; CPT-I, carnitine palmitoyltransferase-I; PCNA, proliferating cell nuclear antigen; CDK, cyclin-dependent kinase; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CYP4A1, cytochrome P450 4A1; SOD, superoxide dismutase; RXR, retinoid X receptor

Abstract

Transgenic mice expressing HCV core protein develop hepatic steatosis and hepatocellular carcinoma (HCC), but the mechanism underlying this process remains unclear. PPARα is a central regulator of triglyceride homeostasis, and mediates hepatocarcinogenesis in rodents. To determine whether PPARα contributes to HCV core protein-induced diseases, PPARα-homozygous, PPARα-heterozygous, and PPARα-null mice bearing the core protein gene (*Ppara*$^{+/+}$:HCVcpTg, *Ppara*$^{+/-}$:HCVcpTg, and *Ppara*$^{-/-}$:HCVcpTg mice) were generated. Despite the presence of considerable PPARα activation, severe steatosis was unexpectedly observed only in *Ppara*$^{+/+}$:HCVcpTg mice, resulting from enhanced fatty acid uptake and decreased mitochondrial β-oxidation due to breakdown of mitochondrial outer membranes. Interestingly, HCC developed in approximately 35% of 24-month-old *Ppara*$^{+/+}$:HCVcpTg mice, but not in the other genotypes. These phenomena were found to be closely associated with sustained PPARα activation. In *Ppara*$^{+/-}$:HCVcpTg mice, PPARα activation and the related changes did not occur despite the presence of functional PPARα and HCV core protein, which seemed to reflect low nuclear PPARα levels. However, long-term treatment of these mice with clofibrate, a PPARα activator, induced HCC with mitochondrial abnormalities and hepatic steatosis. Thus, our results indicate that not the presence of PPARα but its persistent activation is essential for the pathogenesis of hepatic steatosis and HCC induced by HCV infection.

Introduction

HCV is one of the major causes of chronic hepatitis, while patients with persistent HCV infection have a high incidence of hepatocellular carcinoma (HCC) (1, 2). Occurrence of HCC associated with chronic HCV infection has increased over the past 2 decades (3-5), and chronic HCV infection is recognized as a serious debilitating disease. However, the mechanism in which chronic HCV infection mediates hepatocarcinogenesis remains unclear.

HCV core protein was demonstrated to possess oncogenic potential (6). To examine how HCV core protein participates in HCV-related hepatocarcinogenesis, transgenic mouse lines were established in which HCV core protein is expressed constitutively in liver at cellular levels similar to those found in chronic HCV-infected patients (7). These mice exhibited hepatic steatosis (7) and insulin resistance (8) as early as 3 months of age; upon further aging, these symptoms worsened and between 16 and 18 months of age, hepatic adenomas developed in approximately 30% of mice (9). Finally, HCC was found within hepatic adenomas in a classic "nodule-in-nodule" pathology (9). Interestingly, no hepatic inflammation or fibrosis was found in these mice throughout the course of HCC development (9), suggesting that HCV core protein itself induces hepatic steatosis and HCC independently of hepatitis.

A number of studies support the contention that hepatic steatosis promotes development of HCC (10). Epidemiologic data have identified hepatic steatosis as a major accelerating factor of hepatocarcinogenesis in chronic HCV-infected patients (11). Moreover, increases in ROS production that can cause oxidative DNA damage, mitochondrial abnormalities, and accelerated hepatocyte proliferation were observed in the steatotic livers (12-14). Thus, an intriguing possibility has emerged that alteration of fatty acid metabolism in hepatocytes may be central to the pathogenesis of HCC induced by HCV core protein.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily; three isoforms designated α, β/δ, and γ, exist, all of which are involved in lipid homeostasis (15). PPARα regulates constitutive transcription of genes encoding fatty acid-metabolizing enzymes (16), and is associated with maintenance of fatty acid transport and metabolism, primarily in liver, kidney and heart. Administration of PPARα agonists such as the widely prescribed fibrate drugs, clofibrate, gemfibrozil, and fenofibrate ameliorates hyperlipidemia in humans (17) and hepatic steatosis in mice (18).

On the other hand, long-term administration of PPARα ligands to rodents causes accelerated hepatocyte proliferation, increased ROS generation, and development of

HCC (19, 20). Disruption of the PPARα gene prevented the development of HCC caused by long-term exposure to PPARα activators (21). Interestingly, accumulation of fatty acids/triglycerides in hepatocytes could lead to continuous PPARα activation, due to the presence of fatty acid metabolites that serve as natural PPARα ligands. For example, mice lacking expression of the peroxisomal acyl-CoA oxidase (AOX) gene showed massive accumulation of very-long-chain fatty acids in hepatocytes, severe microvesicular steatosis, chronic PPARα activation, and development of hepatic adenoma and HCC by 15 months of age (22). These results suggest a strong contribution of activated PPARα to liver tumorigenesis.

Based on several lines of evidence, we hypothesized that PPARα might contribute to hepatocarcinogenesis in HCV core protein-expressing transgenic (HCVcpTg) mice. To explore this possibility, PPARα-homozygous (*Ppara*$^{+/+}$), PPARα-heterozygous (*Ppara*$^{+/-}$), and PPARα-null (*Ppara*$^{-/-}$) mice bearing the HCV core protein gene, designated *Ppara*$^{+/+}$:HCVcpTg, *Ppara*$^{+/-}$:HCVcpTg, and *Ppara*$^{-/-}$:HCVcpTg mice, were generated, and phenotypic changes were examined. Surprisingly, we found that severe hepatic steatosis and HCC induced by HCV core protein developed only in *Ppara*$^{+/+}$ mice, which were related to persistent PPARα activation.

Results

Expression of HCV core protein in transgenic mice

Ppara$^{-/-}$:HCVcpTg and *Ppara*$^{+/-}$:HCVcpTg mice appeared healthy, and body weight in both genotypes was similar to that of *Ppara*$^{+/+}$:HCVcpTg and *Ppara*$^{+/+}$ mice without the transgene. When hepatic expression of HCV core protein in 9-month-old transgenic mice was examined by immunoblot analysis, it was similar among *Ppara*$^{+/+}$:HCVcpTg, *Ppara*$^{+/-}$:HCVcpTg, and *Ppara*$^{-/-}$:HCVcpTg mice (Figure 1A), and was also similar to expression in HCVcpTg mice reported previously (7, 9). Age and gender had only a minor influence on hepatic expression of HCV core protein.

Requirement of homozygous PPARα for development of hepatic steatosis in transgenic mice

Livers of 9-month-old male HCVcpTg mice with or without the *Ppara* allele were examined. Those of *Ppara*$^{+/+}$:HCVcpTg mice were soft, slightly enlarged, and light in color, and histologically demonstrated macrovesicular and microvesicular steatosis with no apparent inflammation or hepatocyte necrosis (Figure 1B), in agreement with previous reports (7, 9). Biochemical analysis using liver extracts demonstrated marked hepatic accumulation of triglycerides (Figure 1D). In contrast, livers of 9-month-old *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice demonstrated neither histological abnormalities nor accumulation of triglycerides (Figures 1B and D). The hepatic levels of free fatty acids in *Ppara*$^{+/+}$:HCVcpTg mice were approximately three times compared with those in *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice, or *Ppara*$^{+/+}$ mice not expressing the HCV core protein.

In 24-month-old *Ppara*$^{+/+}$:HCVcpTg mice, hepatic steatosis was found (Figure 1C), and the hepatic levels of triglycerides were further increased (Figure 1D). No apparent inflammation, hepatocyte degeneration and necrosis, or fibrosis was detected. On the other hand, *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice showed no steatosis (Figures 1C and D). These results demonstrate that hepatic steatosis develops in *Ppara*$^{+/+}$:HCVcpTg mice, but not in *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice.

Hepatic fatty acid/triglyceride metabolism in transgenic mice

To investigate the mechanism of development of severe steatosis in *Ppara*$^{+/+}$:HCVcpTg mice, the expression of genes associated with fatty acid/triglyceride metabolism in the livers of 9-month-old mice was analyzed using the quantitative RT-PCR method. As demonstrated in Figure 2A, the mRNA levels of genes related to de novo lipogenesis [fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC)] and

secretion of very-low-density lipoprotein [microsomal transfer protein (MTP) and apoB)] were constant in all groups. The mRNA levels of fatty acid translocase (FAT) and fatty acid transport protein (FATP), which are associated with uptake of fatty acids into hepatocytes, were significantly increased only in *Ppara*$^{+/+}$:HCVcpTg mice, but the mRNA levels of hepatic triglyceride lipase, another contributor to fatty acid uptake, remained unchanged (data not shown). The mRNA levels of liver fatty acid binding protein (L-FABP) were also elevated only in *Ppara*$^{+/+}$:HCVcpTg mice. Surprisingly, the mRNA levels of AOX and medium-chain acyl-CoA dehydrogenase (MCAD), a rate-limiting enzyme in the peroxisomal and mitochondrial β-oxidation pathways, respectively, were significantly increased in *Ppara*$^{+/+}$:HCVcpTg mice. When fatty acid uptake ability was measured using fresh liver slices, it was significantly enhanced only in *Ppara*$^{+/+}$:HCVcpTg mice (Figure 2B). Additionally, plasma free fatty acid levels were higher in these mice compared with those in the other groups. Although there were no differences in fasting plasma glucose levels among the groups, hyperinsulinemia was observed only in *Ppara*$^{+/+}$:HCVcpTg mice (Figure 2C), in agreement with the previous observations that significant insulin resistance developed in these mice (8). Similar results were obtained from 24-month-old mice (data not shown). Together, these results demonstrate that the increased plasma fatty acid levels, likely due to enhanced peripheral fatty acid release caused by insulin resistance, and the elevated fatty acid uptake ability are consistent with steatogenesis in *Ppara*$^{+/+}$:HCVcpTg mice.

Decreased mitochondrial β-oxidation in transgenic mice

Although the transcriptional activities of major β-oxidation enzymes were markedly increased, *Ppara*$^{+/+}$:HCVcpTg mice demonstrated severe steatosis. To explore this discrepant result, peroxisomal and mitochondrial β-oxidation activities were measured using lignoceric and palmitic acids as substrates, respectively. The lignoceric acid degrading capacity was increased only in *Ppara*$^{+/+}$:HCVcpTg mice, where it corresponded to an increase in AOX expression. However, the capacity for palmitic acid degradation, which occurs particularly in mitochondria, was significantly reduced in *Ppara*$^{+/+}$:HCVcpTg mice compared with that in *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice (Figure 3A). Thus, decreased mitochondrial β-oxidation ability was considered to be another important mechanism for the development of steatosis induced by the core protein.

We further evaluated mitochondrial abnormalities. In electron microscopic examination, discontinuous outer membranes (Figure 3B, arrows in left lower panel) and lack of an internal structure were observed in some mitochondria of

Ppara$^{+/+}$:HCVcpTg mouse livers, in agreement with the previous report (9). However, these abnormalities were not seen in *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice (Figure 3B). Immunoblot analysis showed that cytochrome c, which is usually localized in the mitochondrial intermembrane space, was present in the cytosolic fractions of *Ppara*$^{+/+}$:HCVcpTg mice (Figure 3C). Moreover, immunoblot analysis using mitochondrial fractions revealed that the expression levels of long-chain acyl-CoA synthase (LACS) and carnitine palmitoyltransferase-I (CPT-I), which are enzymes indispensable to the initial step of mitochondrial β-oxidation and are localized mainly in mitochondrial outer membranes, were significantly decreased only in *Ppara*$^{+/+}$:HCVcpTg mice (Figure 3D).

Overall, these results suggest that homozygous PPARα is essential to the pathogenesis of hepatic steatosis induced by the HCV core protein, which results from a decrease in mitochondrial fatty acid degradation capacity caused by the breakdown of mitochondrial outer membranes and a disproportionate increase in the uptake of fatty acids. Interestingly, steatosis and the related changes did not occur in *Ppara*$^{+/-}$ and *Ppara*$^{-/-}$ mice expressing the HCV core protein, suggesting that these changes are not caused by the core protein itself.

Requirement of homozygous PPARα for hepatic tumor development in transgenic mice

At 9 months of age, hepatic nodules were not observed at all in transgenic mice, whereas, at 24 months, approximately 35% of *Ppara*$^{+/+}$:HCVcpTg mice had macroscopically evident hepatic nodules (Table 1). Microscopically, these nodules had the appearance of well-differentiated HCC with trabecular features, which was consistent with the previous report (9). Surprisingly, *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice of the same ages developed no evidence of hepatic tumors, despite the expression of HCV core protein at similar levels to that found in *Ppara*$^{+/+}$:HCVcpTg mice (Table 1). Microscopic examination demonstrated that there were no dysplastic cells or precancerous lesions throughout the livers in *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice (Figure 1C). These results provide strong evidence that homozygous PPARα is essential for hepatic tumorigenesis induced by HCV core protein.

Increased hepatocyte proliferation only in Ppara*$^{+/+}$*:HCVcpTg mice

Since sustained acceleration of hepatocyte proliferation relative to apoptosis may promote development of HCC, these opposing processes were quantified in the livers of

24-month-old mice. Both the liver-to-body weight ratio and the number of hepatocytes expressing proliferating cell nuclear antigen (PCNA) were increased only in *Ppara*$^{+/+}$:HCVcpTg mice (Figures 4A and B). In contrast, the number of TUNEL-positive hepatocytes and the hepatic caspase 3 activity, indicators of hepatocyte apoptosis, remained similar among the three mouse strains (Figures 4C and D). Interestingly, despite the presence of HCV core protein, the amounts of these proliferative and apoptotic markers in *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice were similar to those in *Ppara*$^{+/+}$ nontransgenic mice. Expression levels of several proteins such as proto-oncogenes (c-Fos and c-Myc), cell-cycle regulators [cyclin D1, cyclin-dependent kinase (CDK) 4, and PCNA], and phosphorylated ERK 1 and 2, all associated with hepatocyte proliferation, were increased in *Ppara*$^{+/+}$:HCVcpTg mice, but not in other genotypes (Figures 4E and F).

Increased oxidative stress and DNA damage only in Ppara$^{+/+}$:HCVcpTg mice

HCV core protein is associated with increased production of ROS (23). Enhanced ROS production induces nuclear DNA damage resulting in the initiation of hepatocarcinogenesis, and can also injure organelles, resulting in their functional disorders. The number of hepatocytes positive for 8-hydroxy-2'-deoxyguanosine (8-OHdG), an indicator of oxidative damage to nuclear DNA, was increased only in 24-month-old *Ppara*$^{+/+}$:HCVcpTg mice (Figures 5A and B). Lipid peroxides were slightly increased in the livers of 9-month-old *Ppara*$^{+/+}$:HCVcpTg mice (data not shown), and were more abundant in livers of 24-month-old *Ppara*$^{+/+}$:HCVcpTg mice than in those of *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice or *Ppara*$^{+/+}$ nontransgenic mice (Figure 5C). Expression of typical ROS-generating enzymes [AOX and cytochrome P450 4A1 (CYP4A1)] and ROS-eliminating enzymes [catalase and Cu, Zn-superoxide dismutase (SOD)] was examined. Immunoblot analysis showed marked increases in expression of AOX and CYP4A1 and mild increases in that of catalase only in *Ppara*$^{+/+}$:HCVcpTg mice. No changes in Cu, Zn-SOD were found in all subgroups of transgenic mice (Figure 5D). These results suggest that enhanced oxidative stress causes damage in nuclear DNA and probably in mitochondria in the *Ppara*$^{+/+}$:HCVcpTg mice.

Persistent and spontaneous PPARα activation in Ppara$^{+/+}$:HCVcpTg mice

Liver tumorigenesis induced by long-term exposure to peroxisome proliferators and the related changes such as sustained hepatocyte proliferation and increased oxidative stress are associated with persistent PPARα activation (19-21). To examine the activation of PPARα, we quantified the level of PPARα mRNA, which is induced

by PPARα activation (24, 25). The PPARα mRNA levels were higher in 9-month-old *Ppara*$^{+/+}$:HCVcpTg mice than in *Ppara*$^{+/+}$ nontransgenic mice (Figure 6A). These increases were more pronounced at 24 months of age. However, there were no differences in the PPARα mRNA levels between *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{+/-}$ nontransgenic mice at either 9 or 24 months of age. The expression levels of typical PPARα target genes (16, 25, 26), such as FAT, FATP, L-FABP, AOX, MCAD (Figure 2), c-Myc, cyclin D1, CDK4, PCNA (Figure 4), and CYP4A1 (Figure 5), were simultaneously and synchronously increased in *Ppara*$^{+/+}$:HCVcpTg mice, but not in *Ppara*$^{+/-}$:HCVcpTg or *Ppara*$^{-/-}$:HCVcpTg mice. These results confirm that persistent activation of PPARα occurs only in the *Ppara*$^{+/+}$:HCVcpTg mice. Various changes observed in *Ppara*$^{+/+}$:HCVcpTg mice, i.e. increased fatty acid uptake, mitochondrial abnormalities, steatosis, ROS overproduction, accelerated hepatocyte proliferation, and hepatocarcinogenesis, were considered to be closely linked with sustained PPARα activation.

Nuclear PPARα content

The results described above suggest that persistent PPARα activation is critical to the steatogenesis and hepatocarcinogenesis induced by the HCV core protein. A question arises as to why *Ppara*$^{+/-}$:HCVcpTg mice having an active *Ppara* allele do not exhibit the hallmarks of PPARα activation and do not develop HCC. To address this issue, nuclear PPARα content was analyzed. Immunoblot analysis for PPARα showed that the amount of nuclear PPARα protein in *Ppara*$^{+/+}$:HCVcpTg mice was increased approximately 2- to 3-fold compared with that in *Ppara*$^{+/+}$ nontransgenic mice, which was disproportionate to the increase in PPARα mRNA levels (approximately 1.2- to 1.6-fold) (Figures 6A and B). The level of nuclear PPARα in *Ppara*$^{+/-}$:HCVcpTg mice was significantly lower than that in *Ppara*$^{+/+}$:HCVcpTg mice, and was similar to that in *Ppara*$^{+/+}$ nontransgenic mice (Figure 6B). Thus, the lower amount of nuclear PPARα in *Ppara*$^{+/-}$:HCVcpTg mice relative to that *Ppara*$^{+/+}$:HCVcpTg mice might have heightened the threshold of expression required for long-term spontaneous PPARα activation.

The degree of an increase in nuclear PPARα levels was evidently higher than the degree of an increase in PPARα mRNA levels in HCVcpTg mice (Figures 6A and B). To investigate this disparity, the stability of nuclear PPARα was evaluated by pulse-chase experiments using isolated hepatocytes obtained from these mice. The half-life of nuclear PPARα in *Ppara*$^{+/+}$:HCVcpTg mice was significantly longer than that in *Ppara*$^{+/+}$ nontransgenic mice (11.5 $\pm$ 2.3 vs 5.8 $\pm$ 1.4 h, $P<0.05$; Figure 6C). The

half-life of nuclear PPARα in *Ppara*$^{+/-}$:HCVcpTg mice tended to be prolonged compared with that in *Ppara*$^{+/-}$ nontransgenic mice (Figure 6C). These results suggest the stability of nuclear PPARα was increased as a result of HCV core protein expression. Since it is known that the core protein interacts with retinoid X receptor (RXR) α (27) and that PPARα influences the stability of RXRα (28), it is plausible that the core protein would affect its action in nuclei through an interaction with the PPARα-RXRα heterodimer and stabilization of PPARα.

Development of hepatic steatosis and HCC by long-term clofibrate treatment in Ppara$^{+/-}$:HCVcpTg mice

To further confirm the significance of persistent PPARα activation on core protein-induced pathological changes, *Ppara*$^{+/-}$ and *Ppara*$^{+/-}$:HCVcpTg mice were fed a standard diet containing 0.05% clofibrate for 24 months. Interestingly, hepatic steatosis appeared in the clofibrate-treated *Ppara*$^{+/-}$:HCVcpTg mice, but not in the *Ppara*$^{+/-}$ mice under the same treatment conditions (Figures 7A and B). Similar to our observations in *Ppara*$^{+/+}$:HCVcpTg mice without clofibrate treatment, aberrant mitochondria having discontinuous outer membranes and decreased palmitic acid β-oxidation activity were found only in the clofibrate-treated *Ppara*$^{+/-}$:HCVcpTg mice (Figures 7A and C). In addition, levels of MCAD mRNA, AOX and CYP4A1 proteins, PPARα mRNA, and nuclear PPARα protein, respectively, were higher in the clofibrate-treated *Ppara*$^{+/-}$:HCVcpTg mice than in the clofibrate-treated *Ppara*$^{+/-}$ mice (Figures 7D, E and F), suggesting that the degree of PPARα activation in the former group was greater than that in the latter group and similar to that in *Ppara*$^{+/+}$:HCVcpTg mice without clofibrate treatment. Finally, the incidence of HCC after the clofibrate treatment was higher in *Ppara*$^{+/-}$:HCVcpTg mice than that in *Ppara*$^{+/-}$ mice [25% (5 in 20 mice) vs. 5% (1 in 20 mice)]. Therefore, these results corroborate the importance of constant PPARα activation to the pathogenesis of hepatic steatosis and HCC in the transgenic mice.

Discussion

A novel and striking finding in this study is the absolute requirement of persistent PPARα activation for the development of HCV core protein-induced steatosis and HCC. Our data also show that the HCV core protein alone cannot induce steatosis and HCC in transgenic mice.

Mechanisms of development of steatosis in HCVcpTg mice have been previously explained as an enhancement of de novo synthesis of fatty acids (29) and a decrease in MTP expression, the latter of which results in insufficient very-low-density lipoprotein secretion from hepatocytes (30). In this study, we revealed two novel mechanisms of steatogenesis in the transgenic mice, i.e. an impairment of mitochondrial β-oxidation due to breakdown of mitochondrial outer membranes and an increase in fatty acid uptake into hepatocytes, associated with PPARα activation. PPARα activation, mitochondrial dysfunction, and hepatic steatosis appeared in 9-month-old *Ppara*$^{+/+}$:HCVcpTg mice and continued until 24 months of age, clearly preceding development of HCC. These findings thereby indicate a correlation among PPARα activation, hepatic steatosis, and HCC.

We obtained the novel and rather paradoxical finding that significant PPARα activation, which generally is expected to reduce hepatic triglyceride levels, is essential for development of severe steatosis induced by HCV core protein. According to the results of this study, the following hypothesis concerning the development of steatosis in *Ppara*$^{+/+}$:HCVcpTg mice is proposed. First, the HCV core protein localizes partly in mitochondria (9). A recent study demonstrated that, in isolated mitochondria, the core protein directly increased Ca^{2+} influx, inhibited electron transport complex I activity, and induced ROS production (31), all of which can increase the fragility of mitochondria and depress mitochondrial function. In addition, the HCV core protein also localizes in nuclei (9) and can coexist in PPARα-RXRα heterodimer through a direct interaction with the DNA binding domain of RXRα, which enhances the transcriptional activity of PPARα target genes, such as AOX, despite the absence of PPARα ligands in cultured cells (27). The HCV core protein can also be involved in PPARα-RXRα complex through a direct interaction with cyclic-AMP responsive element binding protein-binding protein (CBP/p300) (32), which is able to bind to PPARα (33). Thus, the core protein probably serves as a coactivator and stabilizer of PPARα in vivo, which was further confirmed in this study. Moreover, since it is also known that the core protein itself activates ERK1/2 and p38 mitogen-activated protein kinase (34), these activations might phosphorylate PPARα and thereby transactivate it (35). The core protein-induced PPARα activation enhances the basal expression of AOX

and CYP4A1, leading to increased production of ROS and dicarboxylic acids. These toxic compounds can damage mitochondrial outer membranes, impairing the mitochondrial β-oxidation system. These damages directly induce accumulation of long-chain fatty acids in hepatocytes. Furthermore, PPARα activation increases the expression of FAT and FATP, promoting the influx of fatty acids from blood. Long-chain fatty acids and their CoA esters accumulated in hepatocytes are likely to act as potent detergents, further damaging mitochondrial outer membranes. Fatty acids and their derivatives function as natural ligands of PPARα, resulting in activation of PPARα and induction of FAT, FATP, AOX, and CYP4A1, which further accelerates mitochondrial damage, reduction of mitochondrial β-oxidation activity, and accumulation of fatty acids in a vicious cycle.

Persistent PPARα activation increases oxidative DNA damage due to a disproportionate increase in ROS-generating enzymes relative to the levels of degrading enzymes such as catalase and SOD, which can predispose hepatocytes to malignant transformation. In addition, persistent PPARα activation leads to increased cell division, as revealed by the expression of cell cycle regulators such as cyclin D1 and CDK4. Furthermore, there is little change in apoptosis that, under normal circumstances, would remove damaged cells capable of undergoing transformation. Thus, under these conditions, it is plausible that some aberrant hepatocytes do not undergo apoptosis and develop into HCC.

It is well known that chronic activation of PPARα is associated with hepatocarcinogenesis in mice exposed to peroxisome proliferators or in mice lacking AOX expression. The common clinicopathological characteristics of HCC in these mice are multicentric HCC (20, 22, 36, 37), well-differentiated appearance of HCC including trabecular features and often a “nodule-in-nodule” pattern (22, 36, 37), and no evidence of fibrosis or cirrhosis in the nonneoplastic liver parenchyma (22, 36), similar to that observed in *Ppara*$^{+/+}$:HCVcpTg mice. However, mice chronically exposed to peroxisome proliferators are clearly distinct from *Ppara*$^{+/+}$:HCVcpTg mice with regard to normal mitochondrial organization, increased mitochondrial β-oxidation activity, and absence of steatosis (16, 36). AOX-null mice are also different from *Ppara*$^{+/+}$:HCVcpTg mice with respect to mitochondrial structure (22). These detailed comparisons among the three mouse models reveal the importance of mitochondrial abnormalities in the pathogenesis of HCV-related diseases.

PPARα is known to regulate the hepatic expression of a great number of proteins associated with fatty acid/triglyceride metabolism, cell division/apoptosis, oxidative stress generation/degradation, and so forth (15, 16, 20, 21, 24-26); therefore, complete

deletion of the PPARα gene from mice might cause hitherto unknown influences on the pathways involved in the development of hepatic steatosis and HCC. To consider these unknown effects, *Ppara*$^{+/-}$:HCVcpTg mice were adopted in the current study. Surprisingly, almost all results from *Ppara*$^{+/-}$:HCVcpTg mice were similar to those from *Ppara*$^{-/-}$:HCVcpTg mice, demonstrating that the presence of functional PPARα itself is not prerequisite for the occurrence of steatosis and HCC induced by the HCV core protein. Moreover, a comparison between *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{+/+}$:HCVcpTg mice uncovered an unexpected and quite important fact that the core protein-dependent pathological changes do not appear without significant activation of PPARα. Thus, it is not the presence of PPARα per se, but rather a high level of PPARα activation that seems to be essential for the development of HCV core protein-induced steatosis and HCC.

To reinforce the above-mentioned hypothesis, *Ppara*$^{+/-}$ and *Ppara*$^{+/-}$:HCVcpTg mice were treated with an exogenous PPAR agonist, clofibrate, for 24 months. In *Ppara*$^{+/-}$ mice, the long-term clofibrate treatment caused a certain level of persistent PPARα activation and a low incidence of HCC. Interestingly, in *Ppara*$^{+/-}$:HCVcpTg mice, the treatment induced more intensive PPARα activation and HCC at a much higher incidence, accompanied with damaged mitochondrial outer membranes, severe steatosis, and decreased mitochondrial β-oxidation activity. The results from the clofibrate-treated *Ppara*$^{+/-}$:HCVcpTg mice were similar to those from *Ppara*$^{+/+}$:HCVcpTg mice without such treatment. Therefore, these findings further support the concept that a long-term and high level of PPARα activation is necessary for steatogenesis and hepatocarcinogenesis in HCVcpTg mice, and emphasize the significant role of the HCV core protein as a PPARα coactivator in vivo.

A pulse-chase experiment revealed that PPARα was stabilized in hepatocyte nuclei in mice expressing the HCV core protein. Many nuclear receptors, including PPARα and RXRα, are known to be degraded by the ubiquitin-proteasome system (38), which plays an important role in modulating the activity of nuclear receptors. Further studies will be needed to clarify whether the core protein influences the ubiquitin-proteasome pathway.

Recent studies have demonstrated a conflicting result that PPARα was down-regulated in the livers of chronic hepatitis C patients (39, 40). Although the association between PPARα function and chronic HCV infection remains a matter of controversy in humans, the changes observed in the transgenic mice resemble the clinicopathological features of chronically HCV-infected patients in several ways; both show a high frequency of accompanying steatosis (10, 40, 41), increased accumulation

of carbon 18 monounsaturated fatty acids in the liver (42), mitochondrial dysfunction (43), increased insulin resistance (44) and oxidative stress (45, 46), male-preferential (2) and multicentric occurrence of HCC (47, 48), and well-differentiated appearance of HCC including trabecular features and often a “nodule-in-nodule” pattern (47, 48). Thus, it is postulated that the mechanism of steatogenesis and hepatocarcinogenesis we proposed may partially apply to patients with chronic HCV infection. If so, therapeutic interventions to alleviate persistent and excessive PPARα activation might be beneficial for prevention of HCC. To clarify the exact relationship between PPARα activation and HCV-induced hepatocarcinogenesis in humans, further experiments using noncancerous liver tissues obtained from HCV-related HCC patients, and using mice carrying human PPARα and HCV core protein genes, are needed.

In conclusion, we clarified for the first time that persistent and potent PPARα activation is absolutely required for the development of severe steatosis and HCC induced by HCV core protein. In addition, we uncovered paradoxical and specific functions of PPARα in the mechanism of steatogenesis mediated by the core protein. Our results would offer clues to find novel therapeutic and nutritional management options, especially with respect to neutral lipids, for chronically HCV-infected patients.

Methods

Mice

Generation of HCVcpTg mice and *Ppara*$^{-/-}$ mice was described previously (7, 24, 49). Male HCVcpTg mice and female *Ppara*$^{-/-}$ mice were mated, and F1 mice bearing the HCV core protein gene were intercrossed to produce F2 mice. *Ppara*$^{+/+}$, *Ppara*$^{+/-}$, and *Ppara*$^{-/-}$ mice bearing the HCV core protein gene, designated *Ppara*$^{+/+}$:HCVcpTg, *Ppara*$^{+/-}$:HCVcpTg, and *Ppara*$^{-/-}$:HCVcpTg mice, in the F4 generation were subjected to serial analyses. Because HCC develops preferentially in male HCVcpTg mice (9), male mice were analyzed. Age-matched male *Ppara*$^{+/+}$ mice without the core protein gene were used as controls. For identifying genotypes, genomic DNA was isolated from mouse tails and amplified by PCR. Primer pairs were designed as described elsewhere: these were 5'-GCCCACAGGACGTTAAGTTC-3' and 5'-TAGTTCACGCCGTCCTCCAG-3' for the HCV core gene (7), and 5'-CAGAGCAACCATCCAGATGA-3' and 5'-AAACGCAACGTAGAGTGCTG-3' for the PPARα gene (24). Amplified alleles for HCV core and PPARα genes were 460 and 472 base pairs, respectively. Five mice per cage were fed routine diet and were kept free of specific pathogens according to institutional guidelines. For the clofibrate treatment experiments, two-month-old male *Ppara*$^{+/-}$ and *Ppara*$^{+/-}$:HCVcpTg mice were randomly divided into two groups (n = 20 in each group), and were fed either a routine diet or one containing 0.05% (w/w) clofibrate (Wako Pure Chemicals Industries, Osaka, Japan) for 24 months. All mice were killed by cervical dislocation before livers were excised. When hepatic tumor was present, the tumor was removed and the remaining liver tissues were used for biochemical analyses. Hepatic tumor was subjected to histological analysis. All animal experiments were conducted in accordance with animal study protocols outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and approved by the Shinshu University School of Medicine.

Preparation of nuclear, mitochondrial, and cytosolic fractions

Approximately 400 mg of liver tissues was minced on ice and transferred to 10% (w/v) isolation buffer (250 mM sucrose in 10 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 0.1% bovine serum albumin, pH 7.4). The samples were gently homogenized by 10-20 strokes with a chilled Dounce homogenizer (Wheaton, Milliville, NJ, USA) and loose-fitting pestle. The homogenate was centrifuged at 500 x *g* for 5 min at 4^{o}C. The supernatant was retained, and the resulting pellet was resuspended with isolation buffer and centrifuged. The pellet fraction was suspended again and centrifuged at 20,000 x *g*

for 1 h at 4°C, and the resulting pellet was used as the nuclear fraction. The combined supernatant fractions were centrifuged at 7,800 x *g* for 10 min at 4°C to obtain a crude mitochondria pellet. This pellet was resuspended with isolation buffer, centrifuged at 7,800 x *g* for 10 min at 4°C, and used as the mitochondrial fraction. Finally, all supernatant fractions were collected and centrifuged at 20,000 x *g* for 30 min at 4°C, and the resulting supernatant was used as the cytosolic fraction.

Immunoblot analysis

Protein concentration was measured colorimetrically by a BCA™ Protein Assay kit (Pierce, Rockford, IL, USA). For analysis of fatty acid-metabolizing enzymes, hepatocyte mitochondrial fractions or whole liver lysates (20 μg protein) were subjected to 10% SDS-PAGE (16). For analysis of PPARα, nuclear fractions (100 μg protein) were used. For analysis of other proteins, whole lysates or cytosolic fractions (50 μg protein) were subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. Antibodies against HCV core protein, fatty acid-metabolizing enzymes, CYP4A1, catalase, and PPARα were described previously (9, 16, 24, 50). Antibodies against other proteins were purchased commercially (BD Transduction Laboratories, San Diego, CA, USA for cytochrome C antibody, and Santa Cruz Biotechnology, Santa Cruz, CA, USA for others, respectively). The band of actin or histone H1 was used as the loading control. The band intensity was measured densitometrically, normalized to that of actin or histone H1, and subsequently expressed as fold changes relative to that of *Ppara*$^{+/+}$ nontransgenic mice.

Analysis of mRNA

Total liver RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), and cDNA was generated by SuperScript II reverse transcriptase (Gibco BRL, Paisley, Scotland). Quantitative RT-PCR was performed using a SYBR green PCR kit and an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primer pairs used are shown in Table 2. The mRNA level was normalized to the GAPDH mRNA level, and subsequently expressed as fold changes relative to that of *Ppara*$^{+/+}$ nontransgenic mice.

Light microscopy and immunohistochemical analysis

Small blocks of liver tissue from each mouse were fixed in 10% formalin in

phosphate-buffered saline, and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin. For immunohistochemical localization of PCNA and 8-OHdG, other small blocks of liver tissue were fixed in 4% paraformaldehyde in phosphate-buffered saline. Sections (4 μm thick) then were affixed to glass slides and incubated overnight with mouse monoclonal antibodies against PCNA (1:100 dilution; Santa Cruz) or 8-OHdG (1:10 dilution; Japan Institute for the Control of Aging, Shizuoka, Japan). Sections were immunostained using EnVision+ kit, with 3,3'-diaminobenzidine as a substrate (DAKO, Glostrup, Denmark). Hepatocytes positive for PCNA or 8-OHdG were examined in 10 randomly selected x400 microscopic fields per section. Two-thousand hepatocytes were examined for each mouse, and the number of immunostained hepatocyte nuclei was expressed as a percentage.

Assessment of hepatocyte apoptosis

TUNEL assay was performed using MEBSTAIN Apoptosis Kit II (Medical & Biological Laboratories, Nagoya, Japan). Two-thousand hepatocytes were examined for each mouse, and the number of TUNEL-positive hepatocytes was expressed as a percentage.

Pulse-label and pulse-chase experiment

Parenchymal hepatocytes were isolated by the modified *in situ* perfusion method (51). After perfusion with 0.05% collagenase solution (Wako), the isolated hepatocytes were washed three times by means of differential centrifugation and the dead cells removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Buckinghamshire, UK). The live hepatocytes were washed and suspended in William's E medium containing 5% fetal bovine serum. When the viability of the isolated hepatocytes exceeded 85% as determined by the trypan blue exclusion test, the following experiments were conducted. The isolated hepatocytes were washed twice and incubated in methionine-free medium containing 5% dialyzed fetal bovine serum for 1 h at 37^{o}C. The medium was replaced with the same medium containing 300 mCi/ml of [^{35}S]methionine (Amersham Pharmacia Biotech). After a 3 h incubation, the labeled medium was exchanged for the standard medium and the preparation was chased for 3, 6, or 12 h. The labeled cells were washed, homogenized and centrifuged to prepare the nuclear fraction. The levels of radioactivity in the homogenates of the pulse-labeled preparations were similar between the transgenic and the nontransgenic mice, suggesting that the [^{35}S]methionine uptake capacity in the former hepatocytes is

similar to that in the latter. The nuclear fraction was lysed in RIPA buffer [10 mM Tris-HCl, pH 7.4, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.1% SDS, 0.25 mM PMSF, 10 mg/mL aprotinin]. The lysate was incubated for 3 h at 4^{o}C with purified anti-PPARα antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed in RIPA buffer, the labeled proteins were resolved by 10% SDS-PAGE and visualized by autoradiography.

Analysis of fatty acid uptake ability

Assays for fatty acid uptake were carried out according to a method reported by Graulet et al. (52) with minor modifications. Briefly, three mice in each group were fasted overnight. Livers were removed quickly, rinsed in ice-cold saline solution, and cut into 500 μm thick slices by means of an Oxford Vibratome (Oxford Laboratories, Foster City, CA, USA). Approximately 150 mg of fresh liver (6-8 liver slices) were placed on stainless steel grids positioned in a 25 ml flask equipped with suspended plastic center wells (Kontes, Vineland, NJ, USA) and incubated in RPMI-1640 medium (Sigma Chemicals, St Louis, MO, USA) devoid of fatty acids for 2 h at 37 ^{o}C. The medium was then replaced with fresh RPMI-1640 medium supplemented with an antibiotic-antimycotic cocktail and 0.8 mM [1-^{14}C]palmitic acid (4 mCi/mmol) (American Radiolabeled Chemicals, St Louis, MO, USA) complexed to bovine serum albumin (palmitic acid:albumin molar ratio of 4:1). After a 7 h incubation, the medium was collected and slices were washed with 2 ml of saline solution and homogenized in Tris buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl). Fatty acid uptake ability was calculated as the sum of palmitic acid converted to CO_2 and ketone bodies with that incorporated into total cellular lipids after incubation. For measurement of CO_2 production by the liver slices, the center wells were placed into scintillation vials containing 4 ml of scintillation cocktail, and radioactivity was counted. For measurement of ketone body generation, aliquots of medium (500 μl) and liver homogenates (250 μl) were treated with ice-cold perchloric acid to make final concentrations of 200 mM and were centrifugated at 3,000 x *g* for 20 min at 4^{o}C. Aliquots of the supernatant containing the ketone bodies were introduced into scintillation vials, and radioactivity was counted. Total cellular lipids were extracted from the liver homogenates according to a modified method developed by Folch et al. (53), collected into scintillation vials, evaporated to dryness under an air stream, and then radioactivity was counted. The experiment was repeated three times, and palmitic acid uptake ability was expressed as fold changes relative to that of *Ppara*$^{+/+}$

nontransgenic mice.

Other methods

To determine hepatic content of lipids and lipid peroxides, lipids were extracted according to a method by Folch et al. (53). Triglycerides and free fatty acids were measured with a Triglyceride E-test kit and a NEFA C-test kit (Wako), respectively. Lipid peroxides (malondialdeyde and 4-hydroxyalkenals) were measured using an LPO-586 kit (OXIS International, Portland, OR, USA). Hepatic β-oxidation activity was determined as described previously (16). Hepatic caspase 3 activity was measured as described elsewhere (54). Plasma glucose and insulin levels were determined using a Glucose CII-test kit (Wako) and mouse insulin ELISA kit (U-type) (AKRIN-031, Shibayagi, Gunma, Japan), respectively.

Statistical analysis

Statistical analysis was performed by two-tailed Student's *t* test for quantitative variables or chi-squared test for qualitative variables. Quantitative data are expressed as the mean $\pm$ standard deviation. A probability value of less than 0.05 was considered to be statistically significant.

References

1. Kiyosawa, K., Sodeyama, T., Tanaka, E., Gibo, Y., Yoshizawa, K., Nakano, Y., Furuta, S., Akahane, Y., Nishioka, K., Purcell, R.H., et al. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* **12**: 671-675.
2. Kiyosawa, K., Umemura, T., Ichijo, T., Matsumoto, A., Yoshizawa, K., Gad, A., and Tanaka, E. 2004. Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* **127**: S17-S26.
3. Tanaka, Y., Hanada, K., Mizokami, M., Yeo, A.E., Shih, J.W., Gojobori, T., and Alter, H.J. 2002. Inaugural Article: A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc. Natl. Acad. Sci. USA.* **99**: 15584-15589.
4. Okuda, K., Fujimoto, I., Hanai, A., and Urano, Y. 1987. Changing incidence of hepatocellular carcinoma in Japan. *Cancer Res.* **47**: 4967-4972.
5. El-Serag, H.B., and Mason, A.C. 1999. Rising incidence of hepatocellular carcinoma in the United States. *New Engl. J. Med*. **340**: 745-750.
6. Shimotohno, K. 2000. Hepatitis C virus and its pathogenesis. *Semin. Cancer Biol.* **10**: 233-240.
7. Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., and Koike, K. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* **78**: 1527-1531.
8. Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Tsukamoto, K., Kimura, S., Moriya, K., and Koike, K. 2004. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* **126**: 840-848.
9. Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., and Koike, K. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* **4**: 1065-1068.
10. Powell, E.E., Jonsson, J.R., and Clouston, A.D. 2005. Steatosis: co-factor in other liver diseases. *Hepatology* **42**: 5-13.
11. Ohata, K., Hamasaki, K., Toriyama, K., Matsumoto, K., Saeki, A., Yanagi, K., Abiru S., Nakagawa, Y., Shigeno, M., Miyazoe, S., et al. 2003. Hepatic steatosis is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C virus infection. *Cancer* **97**: 3036-3043.

12. Browning, J.D., and Horton, J.D. 2004. Molecular mediators of hepatic steatosis and liver injury. *J. Clin. Invest.* **114**: 147-152.
13. Le, T.H., Caldwell, S.H., Redick, J.A., Sheppard, B.L., Davis, C.A., Arseneau, K.O., Iezzoni, J.C., Hespenheide, E.E., Al-Osaimi, A., and Peterson, T.C. 2004. The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology* **39**: 1423-1429.
14. Yang, S., Lin, H.Z., Hwang, J., Chacko, V.P., and Diehl, A.M. 2001. Hepatic hyperplasia in noncirrhotic fatty livers: is obesity-related hepatic steatosis a premalignant condition? *Cancer Res.* **61**: 5016-5023.
15. Desvergne, B., and Wahli, W. 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr. Rev*. **20**: 649-688.
16. Aoyama, T., Peters, J.M., Iritani, N., Nakajima, T., Furihata, K., Hashimoto, T., and Gonzalez, F.J. 1998. Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor α (PPARα). *J. Biol. Chem.* **273**: 5678-5684.
17. Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., and Fruchart, J.C. 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation* **98**: 2088-2093.
18. Harano, Y., Yasui, K., Toyama, T., Nakajima, T., Mitsuyoshi, H., Mimani, M., Hirasawa, T., Itoh, Y., and Okanoue, T. 2006. Fenofibrate, a peroxisome proliferator-activated receptor α agonist, reduces hepatic steatosis and lipid peroxidation in fatty liver Shionogi mice with hereditary fatty liver. *Liver Int.* **26**: 613-620.
19. Yeldandi, A.V., Rao, M.S., and Reddy, J.K. 2000. Hydrogen peroxide generation in peroxisome proliferator-induced oncogenesis. *Mutat. Res*. **448**: 159-177.
20. Yu, S., Rao, M.S., and Reddy, J.K. 2003. Peroxisome proliferator-activated receptors, fatty acid oxidation, steatohepatitis and hepatocarcinogenesis. *Curr. Mol. Med*. **3**: 561-572.
21. Peters, J.M., Cattley, R.C., and Gonzalez, F.J. 1997. Role of PPARα in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. *Carcinogenesis* **18**: 2029-2033.
22. Fan, C.Y., Pan, J., Usuda, N., Yeldandi, A.V., Rao, M.S., and Reddy, J.K. 1998. Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor α natural ligand metabolism. *J. Biol. Chem*. **273**: 15639-15645.

23. Moriya, K., Nakagawa, K., Santa, T., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Miyazawa, T., Ishibashi, K., Horie, T., et al. 2001. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res*. **61**: 4365-4370.
24. Lee, S.S., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H., and Gonzalez, F.J. 1995. Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol. Cell. Biol*. **15**: 3012-3022.
25. Mandard, S., Muller, M., and Kersten, S. 2004. Peroxisome proliferator-activated receptor α target genes. *Cell. Mol. Life Sci*. **61**: 393-416.
26. Peters, J.M., Aoyama, T., Cattley, R.C., Nobumitsu, U., Hashimoto, T., and Gonzalez, F.J. 1998. Role of peroxisome proliferator-activated receptor α in altered cell cycle regulation in mouse liver. *Carcinogenesis* **19**: 1989-1994.
27. Tsutsumi, T., Suzuki, T., Shimoike, T., Suzuki, R., Moriya, K., Shintani, Y., Fujie, H., Matsuura, Y., Koike, K., and Miyamura, T. 2002. Interacton of hepatitis C virus core protein with retinoid X receptor α modulates its transcriptional activity. *Hepatology* **35**: 937-946.
28. Tanaka, N., Hora, K., Makishima, H., Kamijo, Y., Kiyosawa, K., Gonzalez, F.J., and Aoyama, T. 2003. In vivo stabilization of nuclear retinoid X receptor α in the presence of peroxisome proliferator-activated receptor α. *FEBS Lett.* **543**: 120-124.
29. Moriishi, K., Mochizuki, R., Moriya, K., Miyamoto, H., Mori, Y., Abe, T., Murata, S., Tanaka, K., Miyamura, T., Suzuki, T., et al. 2007. Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA* **104**: 1661-1666.
30. Perlemuter, G., Sabile, A., Letteron, P., Vona, G., Topilco, A., Chretien, Y., Koike, K., Pessayre, D., Chapman, J., Barba, G., et al. 2002. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* **16**: 185-194.
31. Korenaga, M., Wang, T., Li, Y., Showalter, L.A., Chan, T., Sun, J., and Weinman, S.A. 2005. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J. Biol. Chem.* **280**: 37481-37488.
32. Gomez-Gonzalo, M., Benedicto, I., Carretero, M., Lara-Pezzi, E., Maldonado-Rodriguez, A., Moreno-Otero, R., Lai, M.M.C., and Lopez-Cabrera, M. 2004. Hepatitis C virus core protein regulates p300/CBP co-activation function.

Possible role in the regulation of NF-AT1 transcriptional activity. *Virology* **328**: 120-130.

33. Yu, S., and Reddy, J.K. 2007. Transcription coactivators for peroxisome proliferator-activated receptors. *Biochim. Biophys. Acta.* **1771**: 936-951.
34. Spaziani, A., Alisi, A., Sanna, D., and Balsano, C. 2006. Role of p38 MAPK and RNA-dependent protein kinase (PKR) in hepatitis C virus core-dependent nuclear delocalization of cyclin B1. *J. Biol. Chem.* **281**: 10983-10989.
35. Diradourian, C., Girard, J., and Pegorier, J.P. 2005. Phosphorylation of PPARs: from molecular characterization to physiological relevance. *Biochimie* **87**: 33-38.
36. Reddy, J.K., Rao, M.S., Azarnoff, D.L., and Sell, S. 1979. Mitogenic and carcinogenic effects of a hypolipidemic peroxisome proliferator, [4-chloro-6-(2,3-xylidino)-2- pyrimidinylthio]acetic acid (Wy-14,643), in rat and mouse liver. *Cancer Res.* **39**: 152-161.
37. Rao, M.S., and Reddy, J.K. 1996. Hepatocarcinogenesis of peroxisome proliferators. *Ann. N.Y. Acad. Sci*. **804**: 573-587.
38. Genini, D., and Catapano, C.V. 2006. Control of peroxisome proliferator-activated receptor fate by the ubiquitin-proteasome system. *J. Recept. Signal. Transduct. Res.* **26**: 679-692.
39. Dharancy, S., Malapel, M., Perlemuter, G., Roskams, T., Cheng, Y., Dubuquoy, L., Podevin, P., Conti, F., Canva, V., Philippe, D., et al. 2005. Impaired expression of the peroxisome proliferator-activated receptor alpha during hepatitis C virus infection. *Gastroenterology* **128**: 334-342.
40. Gottardi, A.D., Pazienza, V., Pugnale, P., Bruttin, F., Rubbia-Brandt, L., Juge-Aubry, C.E., Meier, C.A., Hadengue, A., and Negro, F. 2006. Peroxisome proliferator-activated receptor-α and -γ mRNA levels are reduced in chronic hepatitis C with steatosis and genotype 3 infection. *Alim. Pharmacol. Ther.* **23**: 107-114.
41. Lefkowitch, J.H., Schiff, E.R., Davis, G.L., Perrillo, R.P., Lindsay, K., Bodenheimer, H.C. Jr., Balart, L.A., Ortego, T.J., Payne, J., Dienstag, J.L., et al. 1993. Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. *Gastroenterology* **104**: 595-603.
42. Moriya, K., Todoroki, T., Tsutsumi, T., Fujie, H., Shintani, Y., Miyoshi, H., Ishibashi, K., Takayama, T., Makuuchi, M., Watanabe, K., et al. 2001. Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biochem. Biophys. Res. Commun.* **281**: 1207-1212.

43. Barbaro, G., Di Lorenzo, G., Asti, A., Ribersani, M., Belloni, G., Grisorio, B., Filice, G., and Barbarini, G. 1999. Hepatocellular mitochondrial alterations in patients with chronic hepatitis C: ultrastructural and biochemical findings. *Am. J. Gastroenterol.* **94**: 2198-2205.
44. Hui, J.M., Sud, A., Farrell, G.C., Bandara, P., Byth, K., Kench, J.G., McCaughan, G.W., and George, J. 2003. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. *Gastroenterology* **125**: 1695-1704.
45. Kato, J., Kobune, M., Nakamura, T., Kuroiwa, G., Takada, K., Takimoto, R., Sato, Y., Fujikawa, K., Takahashi, M., Takayama, T., et al. 2001. Normalization of elevated hepatic 8-hydroxy-2'-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. *Cancer Res.* **61**: 8697-8702.
46. Horiike, S., Kawanishi, S., Kaito, M., Ma, N., Tanaka, H., Fujita, N., Iwasa, M., Kobayashi, Y., Hiraku, Y., Oikawa, S., et al. 2005. Accumulation of 8-nitroguanine in the liver of patients with chronic hepatitis C. *J. Hepatol.* **43**: 403-410.
47. Takenaka, K., Adachi, E., Nishizaki, T., Hiroshige, K., Ikeda, T., Tsuneyoshi, M., and Sugimachi, K. 1994. Possible multicentric occurrence of hepatocellular carcinoma: a clinicopathological study. *Hepatology* **19**: 889-894.
48. Oikawa, T., Ojima, H., Yamasaki, S., Takayama, T., Hirohashi, S., and Sakamoto, M. 2005. Multistep and multicentric development of hepatocellular carcinoma: histological analysis of 980 resected nodules. *J. Hepatol.* **42**: 225-229.
49. Akiyama, T.E., Nicol, C.J., Fievet, C., Staels, B., Ward, J.M., Auwerx, J., Lee, S.S.T., Gonzalez, F.J., and Peters, J.M. 2001. Peroxisome proliferator-activated receptor-α regulates lipid homeostasis, but is not associated with obesity. *J. Biol. Chem.* **276**: 39088-39093.
50. Nakajima, T., Kamijo, Y., Tanaka, N., Sugiyama, E., Tanaka, E., Kiyosawa, K., Fukushima, Y., Peters, J.M., Gonzalez, F.J., and Aoyama, T. 2004. Peroxisome proliferator-activated receptor α protects against alcohol-induced liver damage. *Hepatology* **40**: 972-980.
51. Ni, R., Tomita, Y., Matsuda, K., Ichihara, A., Ishimura, K., Ogasawara, J., and Nagata, S. 1994. Fas-mediated apoptosis in primary cultured mouse hepatocytes. *Exp. Cell Res*. **215**: 332-337.
52. Graulet, B., Gruffat, D., Durand, D., and Bauchart, D. 1998. Fatty acid metabolism and very low density lipoprotein secretion in liver slices from rats and preruminant calves. *J. Biochem.* **124**: 1212-1219.
53. Folch, J., Lees, M., and Sloane Stanley, G.H. 1957. A simple method for the

isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**: 497-509.

54. Gurtu, V., Kain, S.R., and Zhang, G. 1997. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. *Anal. Biochem*. **251**: 98-102.

Figure Legends

Figure 1

Phenotype changes in transgenic mouse liver

(**A**) Immunoblot analysis of HCV core protein expression in livers of 9-month-old mice. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each mouse (n = 6 /group) was mixed and homogenized. Whole liver lysate (50 μg protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of four independent experiments. PC, lysate prepared from COS-1 cells overexpressing HCV core protein as a positive control.

(**B**) Histological appearance of hematoxylin and eosin-stained liver sections from 9-month-old HCVcpTg mice. Upper and lower rows show a lower (x40) and higher (x400) magnification, respectively. Microvesicular and macrovesicular steatosis was found only in *Ppara*$^{+/+}$:HCVcpTg mice. No inflammation or hepatocyte degeneration was evident in any of the genotypes.

(**C**) Histological appearance of hematoxylin and eosin-stained liver sections from 24-month-old HCVcpTg mice. Upper and lower rows show a lower (x40) and higher (x400) magnification, respectively. Hepatic steatosis was marked in *Ppara*$^{+/+}$:HCVcpTg mice, but not in other mice. Hepatic inflammation, fibrosis, and hepatocyte degeneration were not observed. In *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice, dysplastic hepatocytes and precancerous lesions were not detected throughout the entire livers.

(**D**) Content of liver triglycerides. Results are expressed as the mean $\pm$ standard deviation (n = 6 /group) and compared among genotypes in the same age. a, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice; b, $P<0.05$ compared with those in *Ppara*$^{+/-}$:HCVcpTg mice; c, $P<0.05$ compared with those in *Ppara*$^{-/-}$:HCVcpTg mice.

Figure 2

Analyses of factors associated with hepatic fatty acid/triglyceride metabolism

(**A**) Expression of genes associated with fatty acid/triglyceride metabolism in 9-month-old mouse livers. Total RNA was extracted from each mouse liver, and the mRNA levels were determined by the RT-PCR method. The mRNA levels were normalized by those of GAPDH, and subsequently normalized by those in *Ppara*$^{+/+}$ nontransgenic mice. Results are expressed as the mean $\pm$ standard deviation (n = 6 /group). a, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice; b, $P<0.05$ compared with those in *Ppara*$^{+/-}$:HCVcpTg mice; c, $P<0.05$ compared with those in

Ppara$^{-/-}$:HCVcpTg mice.

(**B**) Uptake of fatty acid in 9-month-old mouse livers. Liver slices obtained from three mice in each group were incubated in medium containing 0.8 mM [1-^{14}C]palmitic acid for 7 h. Fatty acid uptake ability was estimated by the sum of palmitic acid converted to CO_2 and ketone bodies with that incorporated into total cellular lipids after incubation. The experiment was repeated three times. Results were normalized by those of *Ppara*$^{+/+}$ nontransgenic mice, and expressed as the mean ± standard deviation. Abbreviations are identical to those in panel (**A**).

(**C**) Plasma concentrations of free fatty acids (FFA), glucose and insulin. After an overnight fast, blood was obtained from each mouse and the above parameters were determined. Results are expressed as the mean ± standard deviation (n = 6 /group). Abbreviations are identical to those in panel (**A**).

Figure 3

Analyses of mitochondrial abnormalities

(**A**) Lignoceric and palmitic acid β-oxidation activities in 9-month-old mice. Results are expressed as the mean ± standard deviation (n = 6 /group). a, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice; b, $P<0.05$ compared with those in *Ppara*$^{+/-}$:HCVcpTg mice; c, $P<0.05$ compared with those in *Ppara*$^{-/-}$:HCVcpTg mice.

(**B**) Electron microscopic features of hepatic mitochondria of 9-month-old HCVcpTg mice. Upper and lower rows show a lower and higher magnification, respectively. In *Ppara*$^{+/+}$:HCVcpTg mice, some mitochondria showing discontinuance of outer membranes (arrows in higher magnification picture) and amorphous inner structures were observed. In *Ppara*$^{+/-}$:HCVcpTg and *Ppara*$^{-/-}$:HCVcpTg mice, mitochondria appeared normal. The scale bars in the right upper and lower panels represent 200 and 30 nm, respectively.

(**C**) Immunoblot analysis of cytochrome C in 9-month-old mice. Whole liver lysate, mitochondrial fraction, or cytosolic fraction (50 μg protein) was loaded in each well. Results are representative of four independent experiments.

(**D**) Immunoblot analysis of representative mitochondrial β-oxidation enzymes using a mitochondrial fraction prepared from 9-month-old mouse livers. The mitochondrial fraction (20 μg protein) was loaded in each well. Results are representative of four independent experiments. The band intensity was quantified densitometrically and normalized by that in *Ppara*$^{+/+}$ nontransgenic mouse. The mean value of the fold changes is shown under the representative band. *, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice.

Figure 4

Increased hepatocyte proliferation in *Ppara*$^{+/+}$:HCVcpTg mice at 24 months of age

(**A**) Liver-to-body-weight ratio. Results are expressed as the mean ± standard deviation (n = 6 /group).

(**B**) Numbers of proliferating hepatocytes. Two-thousand hepatocytes were examined in each mouse, and hepatocyte nuclei positive for anti-PCNA antibody were counted. Results are shown as the mean ± standard deviation (n = 6 /group). For panels (**A**) and (**B**), comparisons are designated as follows: a, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice; b, $P<0.05$ compared with those in *Ppara*$^{+/-}$:HCVcpTg mice; c, $P<0.05$ compared with those in *Ppara*$^{-/-}$:HCVcpTg mice.

(**C**) Numbers of apoptotic hepatocytes. Liver sections were subjected to TUNEL staining, and TUNEL-positive hepatocyte nuclei were counted in 2,000 hepatocytes of each mouse. Results are expressed as the mean ± standard deviation (n = 6 /group).

(**D**) Caspase 3 activity. Results are shown as the mean ± standard deviation (n = 6 /group).

(**E**) Immunoblot analysis of oncogene products and cell cycle regulators. The same sample used in Figure 1A (whole liver lysate, 50 μg protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of four independent experiments. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in *Ppara*$^{+/+}$ nontransgenic mice. The mean value of the fold changes is expressed under each band. *, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice.

(**F**) Immunoblot analysis of phosphorylated ERK1/2 and total ERK1/2. The same samples in Figure 4E (50 μg protein) were used. Abbreviations are identical to those in Figure 4E.

Figure 5

Increased oxidative stress and DNA damage in *Ppara*$^{+/+}$:HCVcpTg mice at 24 months of age

(**A**) Immunohistochemical staining using antibody against 8-OHdG. In *Ppara*$^{+/+}$:HCVcpTg mice, some steatotic hepatocytes were positive for 8-OHdG. Original magnification is x400.

(**B**) Numbers of 8-OHdG-positive hepatocytes. Hepatocyte nuclei stained with anti-8-OHdG antibody were counted in 2,000 hepatocytes of each mouse. Results are expressed as the mean ± standard deviation (n = 6 /group).

(**C**) Hepatic content of lipid peroxides. Results are expressed as the mean ± standard deviation (n = 6 /group). For panel (**B**) and (**C**), comparisons are designated as follows: a, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice; b, $P<0.05$ compared with those in *Ppara*$^{+/-}$:HCVcpTg mice; c, $P<0.05$ compared with those in *Ppara*$^{-/-}$:HCVcpTg mice.

(**D**) Immunoblot analysis of AOX, CYP4A1, catalase, and Cu, Zn-SOD. The whole liver lysate used in the experiment in Figure 4E (20 μg protein for AOX and CYP4A1, and 50 μg for others) was loaded in each lane. The band of actin was used as the loading control. Results are representative of four independent experiments. A and B indicate full-length and truncated AOX, respectively. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in *Ppara*$^{+/+}$ nontransgenic mice. The mean value of the fold changes is expressed under each band. *, $P<0.05$ compared with those in *Ppara*$^{+/+}$ nontransgenic mice.

Figure 6

Persistent PPARα activation in *Ppara*$^{+/+}$:HCVcpTg mice

(**A**) PPARα mRNA levels. Total RNA was prepared from each mouse, and PPARα mRNA levels were determined by the RT-PCR, normalized by those of GAPDH, and subsequently normalized by those of 9-month-old *Ppara*$^{+/+}$ nontransgenic mice. Results are expressed as the mean ± standard deviation (n = 6 /group). *, $P<0.05$ compared with those in nontransgenic mice in the same age and *Ppara* genotype.

(**B**) Immunoblot analysis of nuclear PPARα. The nuclear fraction obtained from each mouse (100 μg protein) was loaded in each well. The band of histone H1 was used as the loading control. Results are representative of six independent experiments. The band intensity was quantified densitometrically, normalized by that of histone H1, and subsequently normalized by that in 9-month-old *Ppara*$^{+/+}$ nontransgenic mice. The mean value is demonstrated under each band. *, $P<0.05$ compared with those in nontransgenic mice in the same age and *Ppara* genotype.

(**C**) Pulse-label and pulse-chase experiments for nuclear PPARα using isolated mouse hepatocytes. (**Left panels**) Labeled PPARα bands on X-ray film. Pulse-label and pulse-chase experiments were performed as described in the Methods. Results are representative of four independent experiments. P, pulse-label; 3 h, 6 h, 12 h, pulse-chase for 3, 6, or 12 h, respectively. (**Right panel**) Half-life of PPARα. The band intensity was measured densitometrically and subsequently normalized by that of the pulse-label experiments. The percentage of the band intensity was plotted and the half-life of PPARα was calculated. Results obtained from four independent experiments

are expressed as the mean ± standard deviation. *, $P<0.05$ compared with those in nontransgenic mice in the same *Ppara* genotype.

Figure 7

Development of hepatic steatosis by long-term treatment of clofibrate in *Ppara*$^{+/-}$:HCVcpTg mice

(**A**) Histological examination of *Ppara*$^{+/-}$ and *Ppara*$^{+/-}$:HCVcpTg mice treated with a diet containing 0.05% (w/w) clofibrate (+CF) for 24 months. (**Upper panels**) Histological appearance of hematoxylin and eosin-stained liver sections at a low (x40) magnification. Microvesicular and macrovesicular steatosis was detected only in clofibrate-treated *Ppara*$^{+/-}$:HCVcpTg mice. (**Middle and lower panels**) Electron microscopic features of hepatic mitochondria. Some C-shaped mitochondria showing discontinuance of outer membranes (arrows in lower right panel) were found in clofibrate-treated *Ppara*$^{+/-}$:HCVcpTg mice. The scale bars in the left middle and lower pictures represent 400 and 30 nm, respectively.

(**B**) Content of liver triglycerides in clofibrate-treated *Ppara*$^{+/-}$ mice. Results are expressed as the mean ± standard deviation (n = 6 /group). CF, 0.05% (w/w) clofibrate treatment for 24 months; *, $P<0.05$ compared with those in clofibrate-untreated mice with the same genotype; **, $P<0.05$ compared with those in clofibrate-treated *Ppara*$^{+/-}$ mice without the core protein gene.

(**C**) Lignoceric and palmitic acid β-oxidation activities in *Ppara*$^{+/-}$ mice. Results are expressed as the mean ± standard deviation (n = 6 /group). Abbreviations used are the same as those in panel (**B**).

(**D**) MCAD mRNA levels. Total RNA was extracted from each mouse liver and mRNA levels were determined by the RT-PCR method. The mRNA levels were normalized by those of GAPDH, and subsequently normalized by those in *Ppara*$^{+/+}$ nontransgenic mice. Results are expressed as the mean ± standard deviation (n = 6 /group). Abbreviations are identical to those in panel (**B**).

(**E**) Immunoblot analysis of AOX and CYP4A1. Whole liver lysate (20 μg protein) was loaded in each lane. The band of actin was used as the loading control. Results are representative of six independent experiments. A and B indicate full-length and truncated AOX, respectively. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in *Ppara*$^{+/+}$ nontransgenic mice. The mean value of the fold changes is expressed under each band. CF, 0.05% (w/w) clofibrate treatment for 24 months; *, $P<0.05$ compared with those in clofibrate-untreated mice with the same genotype; **, $P<0.05$ compared with those in

clofibrate-treated *Ppara*$^{+/-}$ mice without the core protein gene.
(**F**) PPARα mRNA levels and nuclear PPARα contents. (**Left panel**) PPARα mRNA levels. The same samples used in panel (**D**) were adopted. The mRNA levels were normalized by those of GAPDH, and subsequently normalized by those in *Ppara*$^{+/+}$ nontransgenic mice. Results are expressed as the mean ± standard deviation (n = 6 /group). (**Right panel**) Immunoblot analysis of nuclear PPARα. The nuclear fraction obtained from each mouse (100 μg protein) was loaded in each well. The band of histone H1 was used as the loading control. Results are representative of six independent experiments. The band intensity was quantified densitometrically, normalized by that of histone H1, and subsequently normalized by that in *Ppara*$^{+/+}$ nontransgenic mice. The mean value of the fold changes is shown under each band. Abbreviations are identical to those in panel (**E**).

Table 1. Incidence of HCC in 24-month-old Mice

Genotype		Mice	Mice with HCC	Incidence
HCV core protein	PPARα	(n)	(n)	(%)
-	+/+	20	0	0
-	+/-	18	0	0
-	-/-	20	0	0
+	+/+	17	6	35.3 [a,b,c]
+	+/-	16	0	0
+	-/-	14	0	0

Mice were killed at 24 months of age for analysis. HCC was diagnosed according to histological findings. a, $P<0.05$ compared with *Ppara*$^{+/+}$ nontransgenic mice; b, $P<0.05$ compared with *Ppara*$^{+/-}$:HCVcpTg mice; c, $P<0.05$ compared with *Ppara*$^{-/-}$:HCVcpTg mice.

Table 2. Primer pairs used for the RT-PCR

Gene	GeneBank Accession Number		Primer Sequence	Product (bp)
ACC	NM_133360	F	5'-GGGCACAGACCGTGGTAGTT-3’	105
		R	5'-CAGGATCAGCTGGGATACTGAGT-3'	
ApoB	XM_137955	F	5'-TCACCCCCGGGATCAAG-3'	85
		R	5'-TCCAAGGACACAGAGGGCTTT-3'	
AOX	NM_015729	F	5'-TGGTATGGTGTCGTACTTGAATGAC-3'	145
		R	5'-AATTTCTACCAATCTGGCTGCAC-3'	
FAS	NM_007988	F	5'-ATCCTGGAACGAGAACACGATCT-3'	140
		R	5'-AGAGACGTGTCACTCCTGGACTT-3'	
FAT	NM_007643	F	5'-CCAAATGAAGATGAGCATAGGACAT-3'	87
		R	5'-GTTGACCTGCAGTCGTTTTGC-3'	
FATP	NM_011977	F	5'-ACCACCGGGCTTCCTAAGG-3'	80
		R	5'-CTGTAGGAATGGTGGCCAAAG-3'	
GAPDH	M32599	F	5'-TGCACCACCAACTGCTTAG-3'	177
		R	5'-GGATGCAGGGATGATGTTCTG-3'	
L-FABP	NM_017399	F	5'-GCAGAGCCAGGAGAACTTTGAG-3'	121
		R	5'-TTTGATTTTCTTCCCTTCATGCA-3'	
MCAD	NM_007382	F	5'-TGCTTTTGATAGAACCAGACCTACAGT-3'	128
		R	5'-CTTGGTGCTCCACTAGCAGCTT-3'	
MTP	NM_008642	F	5'-GAGCGGTCTGGATTTACAACG-3'	72
		R	5'-GTAGGTAGTGACAGATGTGGCTTTTG-3'	
PPARα	NM_011144	F	5'-CCTCAGGGTACCACTACGGAGT-3'	69
		R	5'-GCCGAATAGTTCGCCGAA-3'	

F, forward sequence; R, reverse sequence.