

diabetes mellitus occurring as a complication of chronic hepatic diseases associated with HCV infection was higher than that of other chronic hepatic diseases, and that anti-HCV antibody-positive patients aged 40 years or more had a 3.77-fold higher risk of becoming diabetic than anti-HCV antibody-negative patients.⁶⁸ In addition, it has been demonstrated that complication by diabetes mellitus is both a risk factor for hepatocellular carcinoma⁶⁹ and a prognostic factor in cirrhosis patients.⁷⁰ These reports suggest a correlation between HCV infection and type 2 diabetes. Increased insulin resistance and insulin secretory deficiency are considered to be highly involved in the pathogenesis of type 2 diabetes.⁷¹ Petit et al.⁷² reported that insulin resistance increases even in chronic hepatitis C patients with slight hepatic impairment and that the index of impairment (HOMA-IR) correlates with the severity of the liver tissue disorder. Tumor necrosis factor (TNF)- α , which closely correlates with hepatic inflammation and fibrillation in chronic hepatitis C,⁷³ is considered to enhance glucose uptake in peripheral tissue and to promote gluconeogenesis in the liver, leading to the induction of insulin resistance.⁷⁴ Shintani et al.⁷⁵ confirmed that in transgenic mice with the 1b HCV core genotype, tyrosyl phosphorylation of the insulin receptor substrate 1 in the insulin signal transduction pathway is disrupted and that this disruption causes gluconeogenesis inhibition by insulin in the liver, leading to the induction of marked insulin resistance. These transgenic mice exhibited a high anti-TNF- α antibody level, and insulin resistance was improved by the administration of an anti-TNF- α antibody. These results indicate a close relationship between HCV infection and the pathogenesis of diabetes mellitus. The relationship between HCV infection and hepatocyte fat modification has also attracted attention.⁷⁶ Moriya et al.⁷⁷ suggested the possible direct involvement of HCV core protein in hepatocyte fat modification, because they observed hepatocyte fat deposits in transgenic mice expressing the HCV core gene. In summary, the above-described findings strongly indicate that hepatitis C has the characteristics of a metabolic disease, and nutritional management is also considered important in the treatment of chronic hepatitis C.

Malignant lymphoma

HCV reproduces in lymphocytes, and studies of a short-term HCV culture system using lymphocytes have been reported.^{78,79} Infected lymphocytes may undergo malignant transformation, leading to the development of malignant lymphoma. HCV infection is considered to be associated with the development of malignant lymphoma, particularly in association with the pathogenesis

of non-Hodgkin B-cell lymphoma, and many reports suggest a relationship between HCV infection and malignant lymphoma.⁸⁰⁻⁸⁸ It has been assumed that some cryoglobulinemia patients develop non-Hodgkin B-cell lymphoma in association with *myc* gene mutation.⁸⁹ The anti-HCV antibody positivity rates in patients with non-Hodgkin B-cell lymphoma range from 0% to 33%.⁸⁰⁻⁸⁸ These differences in HCV antibody positivity rates are considered to relate to regional differences in the HCV infection rate. The HCV antibody prevalence tends to be higher in Japan and Italy but lower in Britain and Canada. Studies indicating a relationship between HCV infection and malignant lymphoma have been reported by Ferri et al.⁹⁰ and De Vita et al.⁹¹ Ferri et al.⁹⁰ reported that 14 of 500 patients with chronic hepatitis C were complicated with non-Hodgkin B-cell lymphoma, and they detected HCV RNA in peripheral blood lymphocytes in all of these patients. De Vita et al.⁹¹ detected positive-strand and negative-strand HCV RNAs in the parotid glands of patients with parotid non-Hodgkin B-cell lymphoma associated with HCV infection, and confirmed the presence of HCV in the parotid gland by *in situ* hybridization.⁹¹ As shown by these findings, many patients with HCV-associated non-Hodgkin B-cell lymphoma show involvement of extranodal sites such as the liver and salivary glands.⁹²

Treatment of HCV-associated malignant lymphoma is similar to that of HCV-associated non-Hodgkin B-cell lymphoma; however, recently, IFN monotherapy or IFN and ribavirin combination therapy have been reported to be effective.⁹³⁻⁹⁶ Vallisa et al.⁹⁶ reported that administration of both pegylated IFN and ribavirin to 13 patients with HCV-associated non-Hodgkin B-cell lymphoma achieved a complete response in seven of these patients. It is interesting that IFN-based antiviral therapy has been demonstrated to be useful for malignant lymphoma associated with HCV-associated non-Hodgkin B-cell lymphoma in addition to conventional chemotherapy.

Autoimmune thyroid disease

The relationship between HCV infection and thyroid disease has been analyzed in many studies,^{97,100} and a causal relationship between HCV infection and autoimmune thyroid disease has been particularly suggested.⁹⁸⁻¹⁰⁰ Antonelli et al.⁹⁸ assessed the incidence of thyroid dysfunction in 630 chronic hepatitis C patients without cirrhosis or hepatocellular carcinoma who had not been treated with IFN by recruiting 389 patients from an iodine-deficient area, 268 patients from an iodine-sufficient area, and 86 patients with chronic hepatitis B aged 40 years or older as study subjects. The chronic hepatitis C patients exhibited a higher thyroid-

stimulating hormone level and lower free thyroxine and triiodothyronine levels than the controls. In addition, the chronic hepatitis C patients exhibited hypothyroidism and tended to have antithyroglobulin antibodies and anti-thyroid peroxidase antibodies. These findings suggest a relationship between HCV infection and thyroid disorder.⁹⁸ A possible relationship between HCV infection and thyroid cancer has also attracted attention recently.⁹⁹ The mechanism underlying the pathogenesis of thyroid disease associated with HCV infection has not yet been elucidated, but a relationship with liver/kidney microsomal antibody type 1 has been suggested.⁹⁹ Many patients with thyroid disorder caused by HCV infection are asymptomatic, requiring no special treatment. Thyroid disorder is also known to be an adverse reaction to IFN- α therapy for chronic hepatitis C.^{99,101-104} Thyroid hypofunction caused by the administration of IFN- α is usually transient, and the patient recovers spontaneously after the end of the therapy. Hence, discontinuation of IFN- α is not required in many cases.¹⁰³

Idiopathic interstitial pneumonitis

Recently, viral infection has been suggested to be a cause of idiopathic interstitial pneumonitis.¹⁰⁴ With regard to the relationship between HCV infection and idiopathic interstitial pneumonitis, Ueda et al.¹⁰⁶ reported in 1992 that the anti-HCV antibody positivity rate in 66 patients with idiopathic interstitial pneumonitis determined by enzyme-linked immunosorbent assay was 28.8%, which was significantly higher than that in 9464 normal subjects serving as controls.¹⁰⁶ It has not yet been clarified how HCV infection is associated with the pathogenesis of idiopathic interstitial pneumonitis. Kubo et al.¹⁰⁷ suggested that activated T lymphocytes and eosinophils are related to the pathogenesis of idiopathic interstitial pneumonitis associated with HCV infection, because they observed increased activated T-lymphocyte and eosinophil counts in the bronchoalveolar fluid of 13 chronic hepatitis C patients, despite their having the same total cell counts as normal subjects. On the other hand, studies disagree regarding the relationship between HCV infection and idiopathic interstitial pneumonitis,¹⁰⁸ and in-depth studies of this issue are expected. Idiopathic interstitial pneumonitis is also reported to be an adverse reaction to IFN therapy in chronic hepatitis C patients.¹⁰⁹ Such patients often have a high pretreatment KL-6 level, and the potential of their developing idiopathic interstitial pneumonitis is suggested. Recovery from IFN therapy-induced idiopathic interstitial pneumonitis is achieved by the discontinuation of the therapy,¹⁰⁹ but steroid administration is required in some cases.

Rheumatoid arthritis

HCV-associated rheumatoid arthritis complicated by cryoglobulinemia or Sjögren's syndrome has been reported.^{10,39} For further information, please refer to the cited references.

Mooren's ulcer

Mooren's ulcer is a progressive ulcer associated with congestion and pain around the cornea.¹¹⁰ HCV infection has been suggested to contribute to the development of this disease.¹¹¹⁻¹¹³ The effectiveness of IFN therapy for HCV-associated Mooren's ulcer has been reported,^{110,111} but the exacerbation of ocular pain following the discontinuation of IFN therapy has also been observed; hence, caution is required.¹¹¹ Systemic corticoid administration has also been reported to be effective.¹¹² However, other investigators reported a negative correlation between HCV infection and Mooren's ulcer.¹¹⁴⁻¹¹⁶ It is hoped that further detailed studies will clarify this issue.

Conclusion

It is necessary to consider possible complications associated with extrahepatic diseases in the treatment of HCV-infected patients.

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PPAR α activation is essential for HCV core protein–induced hepatic steatosis and hepatocellular carcinoma in mice

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Transgenic mice expressing HCV core protein develop hepatic steatosis and hepatocellular carcinoma (HCC), but the mechanism underlying this process remains unclear. Because PPAR α is a central regulator of triglyceride homeostasis and mediates hepatocarcinogenesis in rodents, we determined whether PPAR α contributes to HCV core protein–induced diseases. We generated PPAR α -homozygous, -heterozygous, and -null mice with liver-specific transgenic expression of the core protein gene (*Ppara*^{+/+}:HCVcpTg, *Ppara*^{+/-}:HCVcpTg, and *Ppara*^{-/-}:HCVcpTg mice. Severe steatosis was unexpectedly observed only in *Ppara*^{+/+}:HCVcpTg mice, which resulted 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 tumors were not observed in the other genotypes. These phenomena were found to be closely associated with sustained PPAR α activation. In *Ppara*^{+/-}:HCVcpTg mice, PPAR α activation and the related changes did not occur despite the presence of a functional *Ppara* allele. However, long-term treatment of these mice with clofibrate, a PPAR α activator, induced HCC with mitochondrial abnormalities and hepatic steatosis. Thus, our results indicate that persistent activation of PPAR α 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, whereas 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 shown to have 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; on further aging, these symptoms worsened and hepatic adenomas developed in approximately 30% of mice between 16 and 18 months of age (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), which suggested that the HCV core protein itself induces hepatic steatosis and HCC independently of hepatitis.

Several studies support the contention that hepatic steatosis promotes the 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.

PPARs are ligand-activated nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily; 3 isoforms designated as α , β/δ , and γ exist, all of which are involved in lipid homeostasis (15). PPAR α regulates constitutive transcription of genes encoding fatty acid–metabolizing enzymes (16) and is associated with the maintenance of fatty acid transport and metabolism, primarily in liver, kidney, and heart. Administration of PPAR α agonists, such as the widely prescribed fibrate drugs clofibrate, gemfibrozil, and fenofibrate, ameliorate hyperlipidemia in humans (17) and hepatic steatosis in mice (18).

On the other hand, long-term administration of PPAR α ligands to rodents causes accelerated hepatocyte proliferation, increased ROS generation, and development of HCC (19, 20). Disruption of the PPAR α gene was shown to prevent the development of HCC caused by long-term exposure to PPAR α activators (21). Interestingly, accumulation of fatty acids/triglycerides in hepatocytes

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

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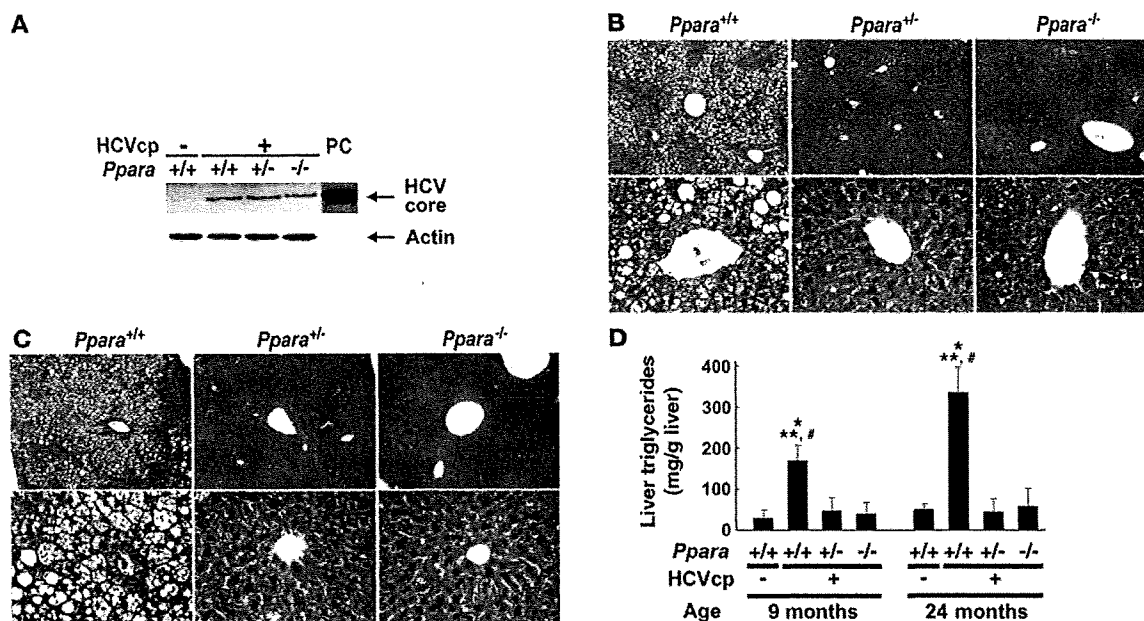


Figure 1

Phenotype changes in transgenic mouse liver. (A) Immunoblot analysis of HCV core protein expression in livers of 9-month-old mice. Because no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver prepared from each mouse ($n = 6$ /group) was mixed and homogenized. Whole-liver lysate (50 μ g protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of 4 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 ($\times 40$) and higher ($\times 400$) 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 ($\times 40$) and higher ($\times 400$) 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 liver. (D) Content of liver triglycerides. Results are expressed as the mean \pm SD ($n = 6$ /group) and compared between genotypes at the same age. * $P < 0.05$ compared with *Ppara*^{+/+} nontransgenic mice; ** $P < 0.05$ compared with *Ppara*^{+/-}:HCVcpTg mice; # $P < 0.05$ compared with *Ppara*^{-/-}:HCVcpTg mice.

could lead to continuous PPAR α activation because of the presence of fatty acid metabolites that serve as natural PPAR α 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 PPAR α activation, and development of hepatic adenoma and HCC by 15 months of age (22). These results suggest a strong contribution of activated PPAR α to liver tumorigenesis.

On the basis of several lines of evidence, we hypothesized that PPAR α might contribute to hepatocarcinogenesis in HCV core protein-expressing transgenic (HCVcpTg) mice. To explore this possibility, PPAR α -homozygous (*Ppara*^{+/+}), PPAR α -heterozygous (*Ppara*^{+/-}), and PPAR α -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 PPAR α 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 sex had only a minor influence on the hepatic expression of HCV core protein.

Requirement of homozygous PPAR α for the 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 showed macrovesicular and microvesicular steatosis with no apparent inflammation or hepatocyte necrosis (Figure 1B), in agreement with previous reports (7, 9). Biochemical analysis of liver extracts showed marked hepatic accumulation of triglycerides (Figure 1D). In contrast, livers of 9-month-old *Ppara*^{+/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice showed neither histological abnormalities nor accumulation of triglycerides (Figure 1, B and D). Hepatic levels of free fatty acids in *Ppara*^{+/+}:HCVcpTg mice were approximately 3 times those in *Ppara*^{+/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg 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). Apparent inflammation, hepatocyte

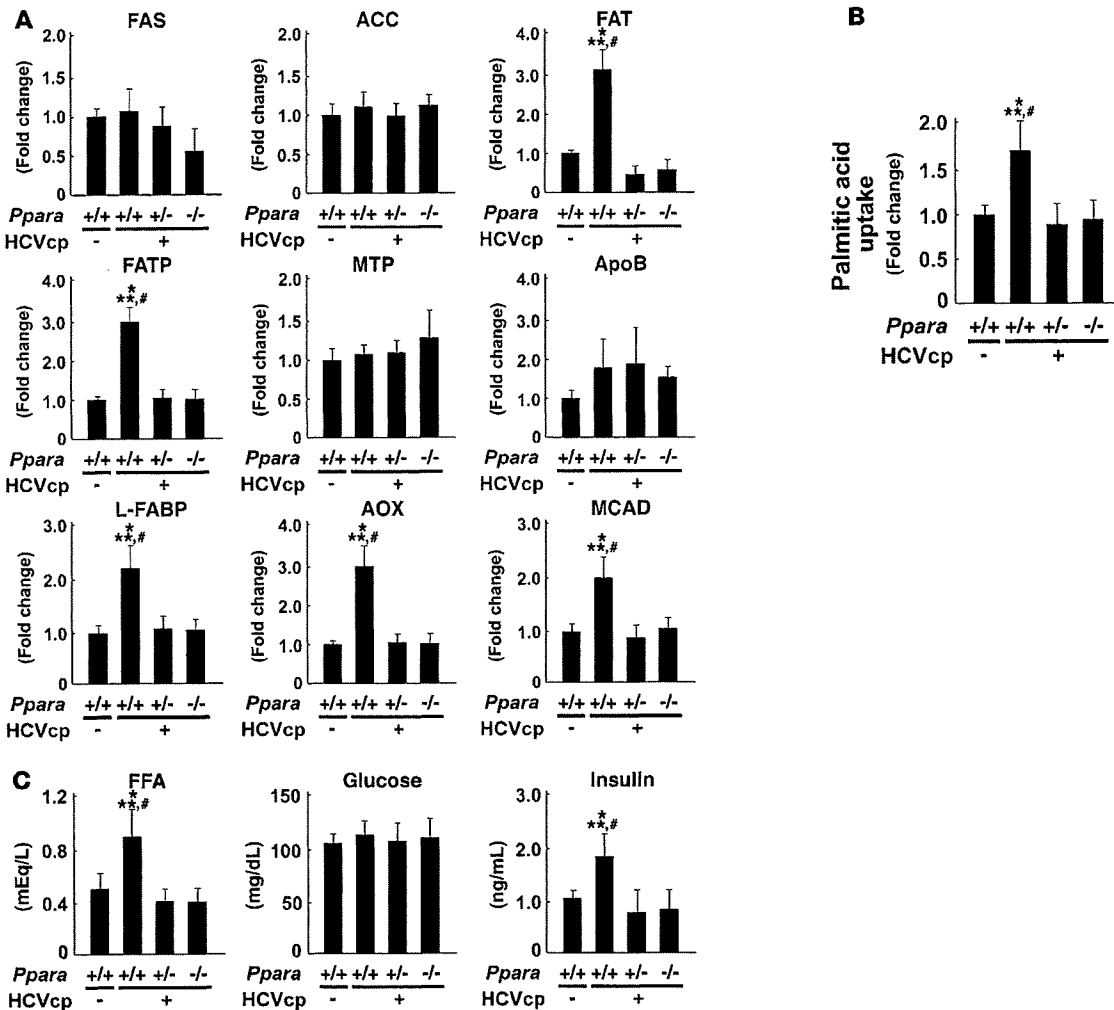


Figure 2

Analyses of factors associated with hepatic fatty acid and triglyceride metabolism. (A) Expression of genes associated with fatty acid and triglyceride metabolism in 9-month-old mouse livers. Total RNA was extracted from each mouse liver, and mRNA levels were determined by RT-PCR. mRNA levels were normalized by those of GAPDH and subsequently normalized by those in *Ppara*^{+/+} nontransgenic mice. Results are expressed as the mean ± SD (*n* = 6/group). **P* < 0.05 compared with *Ppara*^{+/+} nontransgenic mice; ***P* < 0.05 compared with *Ppara*^{-/-}:HCVcpTg mice; #*P* < 0.05 compared with *Ppara*^{-/-}:HCVcpTg mice. (B) Uptake of fatty acids in 9-month-old mouse livers. Liver slices obtained from 3 mice in each group were incubated in medium containing 0.8 mM [1-¹⁴C]palmitic acid for 7 h. Fatty acid uptake ability was estimated by the sum of palmitic acid converted to CO₂ and ketone bodies with that incorporated into total cellular lipids after incubation. The experiment was repeated 3 times. Results were normalized by those of *Ppara*^{+/+} nontransgenic mice and expressed as the mean ± SD. (C) Plasma concentrations of free fatty acids, glucose, and insulin. After an overnight fast, blood was obtained from each mouse and the above variables were determined. Results are expressed as the mean ± SD (*n* = 6/group).

degeneration and necrosis, and fibrosis were not detected. On the other hand, *Ppara*^{-/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice showed no steatosis (Figure 1, C and D). These results indicate that hepatic steatosis develops in *Ppara*^{+/+}:HCVcpTg mice, but not in *Ppara*^{-/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice.

Hepatic fatty acid and triglyceride metabolism in transgenic mice. To investigate the mechanism responsible for the development of severe steatosis in *Ppara*^{+/+}:HCVcpTg mice, the expression of genes associated with fatty acid and triglyceride metabolism in the livers of 9-month-old mice was analyzed using the quantitative RT-PCR method. As shown 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 VLDL (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 the 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

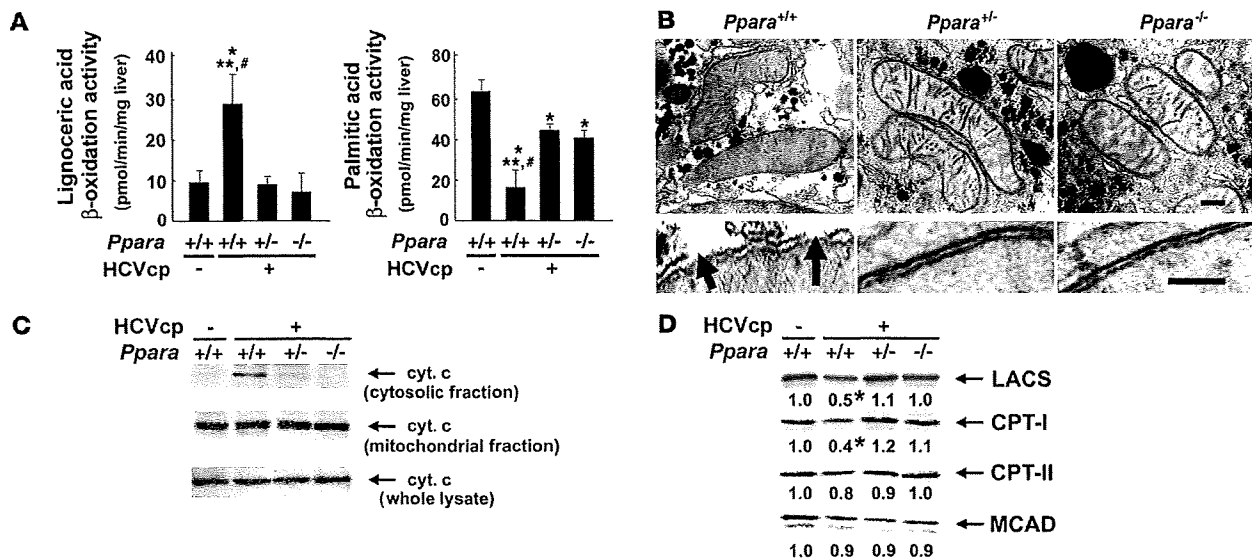


Figure 3

Analyses of mitochondrial abnormalities. (A) Lignoceric and palmitic acid β -oxidation activities in 9-month-old mice. Results are expressed as the mean \pm SD ($n = 6$ /group). * $P < 0.05$ compared with *Ppara*^{+/+} nontransgenic mice; ** $P < 0.05$ compared with *Ppara*^{+/+}:HCVcpTg mice; # $P < 0.05$ compared with *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) and amorphous inner structures were observed. In *Ppara*^{+/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice, mitochondria appeared normal; the scale bars represent 200 nm (top) and 30 nm (bottom), respectively. (C) Immunoblot analysis of cytochrome *c* in 9-month-old mice. Whole-liver lysate, mitochondrial fraction, or cytosolic fraction (50 μ g protein) was loaded in each well. Results are representative of 4 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 μ g protein) was loaded in each well. Results are representative of 4 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. LACS, long-chain acyl-CoA synthase; CPT, carnitine palmitoyl-CoA transferase.

dehydrogenase (MCAD), a rate-limiting enzymes in the peroxisomal and mitochondrial β -oxidation pathways, respectively, were significantly increased in *Ppara*^{+/-}:HCVcpTg mice. When fatty acid uptake ability was measured in 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 than in mice in the other groups. Although there were no differences in fasting plasma glucose levels between the groups, hyperinsulinemia was observed only in *Ppara*^{+/+}:HCVcpTg mice (Figure 2C), in agreement with the previous observation that significant insulin resistance developed in these mice (8). Similar results were obtained from 24-month-old mice (data not shown). These results combined show that the increased plasma fatty acid levels, which were likely due to enhanced peripheral fatty acid release caused by insulin resistance, and the increase in 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 had 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 lower in *Ppara*^{+/-}:HCVcpTg mice than 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) 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 showed that the expression levels of long-chain acyl-CoA synthase and carnitine palmitoyl-CoA transferase-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 PPAR α 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, which suggested that these changes were not caused by the core protein itself.

Table 1
Incidence of HCC in 24-month-old mice

HCV core protein	<i>Ppara</i>	Mice (n)	Mice with HCC (n)	Incidence (%)
-	+/+	20	0	0
-	+/-	18	0	0
-	-/-	20	0	0
+	+/+	17	6	35.3 ^A
+	+/-	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, *P* < 0.05 compared with *Ppara*^{+/-}:HCVcpTg mice, *P* < 0.05 compared with *Ppara*^{-/-}:HCVcpTg mice.

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 those found in *Ppara*^{+/-}:HCVcpTg mice (Table 1). Microscopic examination showed 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. Because sustained acceleration of hepatocyte proliferation relative to apoptosis may promote the 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 (Figure 4, A and B). In contrast, the number of TUNEL-positive hepatocytes and the hepatic caspase 3 activity, indicators of hepatocyte apoptosis, remained similar among the 3 mouse strains (Figure 4, C 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 protooncogenes (c-Fos and c-Myc), cell-cycle regulators (cyclin D1, cyclin-dependent kinase [CDK] 4, and PCNA), and phosphorylated ERK 1 and 2, all of which are associated with hepatocyte proliferation, were elevated in *Ppara*^{+/-}:HCVcpTg mice but not in other genotypes (Figure 4, E 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, which results in the initiation of hepatocarcinogenesis, and can also injure organelles, which can result in disorders in their

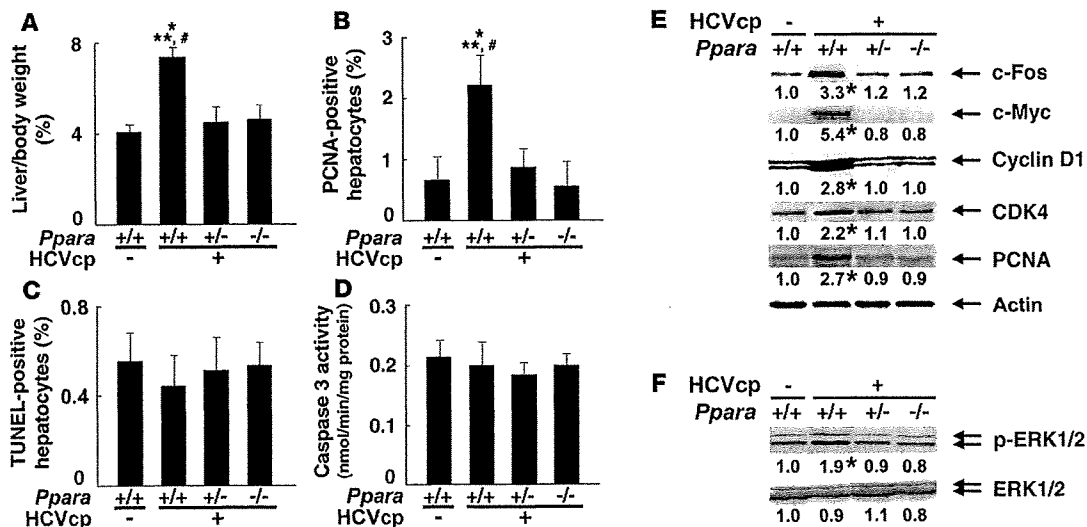


Figure 4

Increased hepatocyte proliferation in *Ppara*^{+/-}:HCVcpTg mice at 24 months of age. (A) Liver-to-body-weight ratio. Results are expressed as the mean ± SD (*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 expressed as the mean ± SD (*n* = 6/group). For A and B, comparisons are designated as follows: **P* < 0.05 compared with *Ppara*^{+/+} nontransgenic mice; ***P* < 0.05 compared with *Ppara*^{+/-}:HCVcpTg mice; #*P* < 0.05 compared with *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 from each mouse. Results are expressed as the mean ± SD (*n* = 6/group). (D) Caspase 3 activity. Results are expressed as the mean ± SD (*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 4 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. (F) Immunoblot analysis of phosphorylated ERK1/2 and total ERK1/2. The same samples in Figure 4E (50 μg protein) were used.

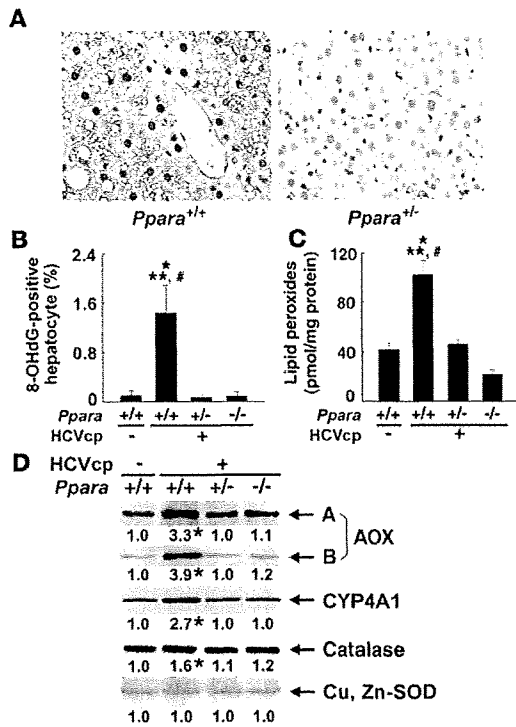


Figure 5

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, ×400. (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 ± SD (*n* = 6/group). (C) Hepatic content of lipid peroxides. Results are expressed as the mean ± SD (*n* = 6 /group). **P* < 0.05 compared with *Ppara*^{+/+} nontransgenic mice; ***P* < 0.05 compared with *Ppara*^{+/-}:HCVcpTg mice; #*P* < 0.05 compared with *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 4 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 *Ppara*^{+/+} nontransgenic mice.

function. 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 (Figure 5, A 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 the 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-SOD) was examined. Immunoblot analysis showed marked increases in the 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 the 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). 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 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, and MCAD (Figure 2); c-Myc, cyclin D1, CDK4, and 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 *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 with an active *Ppara* allele do not exhibit the hallmarks of PPARα activation and do not develop HCC. To address this issue, the nuclear PPARα content was analyzed. Immunoblot analysis for PPARα showed that the amount of nuclear PPARα protein in *Ppara*^{+/+}:HCVcpTg mice was approximately 2- to 3-fold that of *Ppara*^{+/-} nontransgenic mice, which was disproportionate to the higher PPARα mRNA levels (approximately 1.2- to 1.6-fold) (Figure 6, A 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 than in *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 (Figure 6, A 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 was significantly longer (*P* < 0.05) in *Ppara*^{+/-}:HCVcpTg mice (11.5 ± 2.3 h) than in *Ppara*^{+/+} nontransgenic mice (5.8 ± 1.4 h) (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 that the stability of nuclear PPARα was increased as a result of HCV core protein expression. Because it is known that the core protein interacts with retinoid X receptor α (RXRα) (27) and that

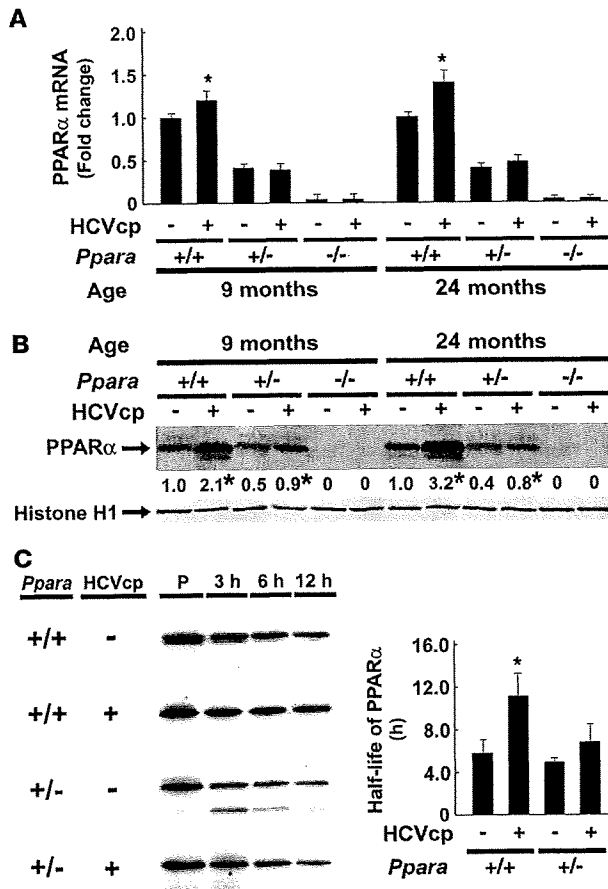


Figure 6

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 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 ± SD (*n* = 6 /group). (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 6 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 expressed under each band. **P* < 0.05 compared with nontransgenic mice of the same age and *Ppara* genotype. (C) Pulse-chase experiments for 3, 6, and 12 h and pulse-label (P) experiments for nuclear PPARα using isolated mouse hepatocytes. Left: labeled PPARα bands on x-ray film. Pulse-label and pulse-chase experiments were performed as described in Methods. Results are representative of 4 independent experiments. Right: 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 4 independent experiments are expressed as the mean ± SD. **P* < 0.05 compared with nontransgenic mice in the same *Ppara* genotype.

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 with 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 (Figure 7, A and B). Similar to our observations in *Ppara*^{+/-}:HCVcpTg mice not treated with clofibrate, aberrant mitochondria with discontinuous outer membranes and decreased palmitic acid β-oxidation activity were found only in the clofibrate-treated *Ppara*^{+/-}:HCVcpTg mice (Figure 7, A and C). In addition, levels of MCAD mRNA; AOX, and CYP4A1 proteins; PPARα mRNA; and nuclear PPARα protein were higher in the clofibrate-treated *Ppara*^{+/-}:HCVcpTg mice than in the clofibrate-treated *Ppara*^{+/-} mice (Figure 7, D–F), which suggests 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 not treated with clofibrate. Finally, the incidence of HCC after clofibrate treatment was higher in *Ppara*^{+/-}:HCVcpTg mice (25%; 5 in 20 mice) than in *Ppara*^{+/-} mice (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 were 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 VLDL secretion from hepatocytes (30). In the present study, we revealed 2 novel mechanisms of steatogenesis in the transgenic mice, i.e., an impairment of mitochondrial β-oxidation due to the 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 between 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 the 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

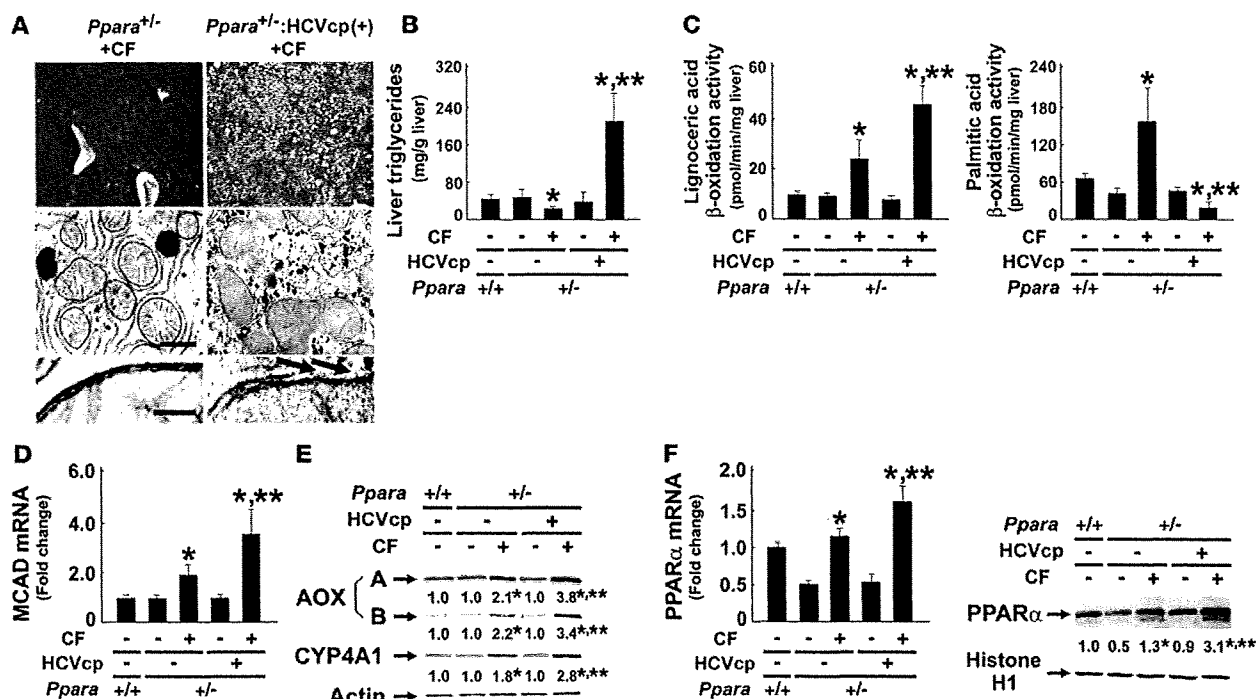


Figure 7

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 diet containing 0.05% (w/w) clofibrate for 24 months (CF). Top: Histological appearance of H&E-stained liver sections. Magnification, ×40. Microvesicular and macrovesicular steatosis were detected only in clofibrate-treated *Ppara*^{+/-}:HCVcpTg mice. Middle and bottom: Electron microscopic features of hepatic mitochondria. Some C-shaped mitochondria showing discontinuance of outer membranes (arrows) were found in clofibrate-treated *Ppara*^{+/-}:HCVcpTg mice. Scale bars: 400 nm (middle), 30 nm (bottom). (B and C) Content of liver triglycerides and lignoceric and palmitic acid β-oxidation activities. (D) MCAD mRNA levels. mRNA levels were normalized to those of GAPDH and subsequently normalized to those in *Ppara*^{+/+} nontransgenic mice. (E) Immunoblot analysis of AOX and CYP4A1. Whole-liver lysate (20 μg protein) was loaded in each lane. Actin was used as a loading control. Results are representative of 6 independent experiments. (F) PPARα mRNA levels and nuclear PPARα contents. Left: PPARα mRNA levels. The same samples used in D were adopted. Right: Immunoblot analysis of nuclear PPARα. Nuclear fraction obtained from each mouse (100 μg protein) was loaded in each well. Histone H1 was used as a loading control. In E and F, the mean value of the fold changes is shown under each band. Results are representative of 6 independent experiments. Band intensity was quantified densitometrically, normalized to that of the loading control, and subsequently normalized to that in *Ppara*^{+/+} nontransgenic mice. **P* < 0.05 compared with untreated mice of the same genotype; ***P* < 0.05 compared with clofibrate-treated *Ppara*^{+/-} mice without core protein gene. Results are expressed as mean ± SD (*n* = 6/group).

showed that, in isolated mitochondria, the core protein directly increased Ca²⁺ 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 the PPARα-RXRα complex through a direct interaction with cyclic-AMP responsive element binding protein-binding protein (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, because 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, which leads to increased

production of ROS and dicarboxylic acids. These toxic compounds can damage mitochondrial outer membranes, which impairs the mitochondrial β-oxidation system. These damages directly induce the accumulation of long-chain fatty acids in hepatocytes. Furthermore, PPARα activation increases the expression of FAT and FATP, which promotes 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, which further damages the outer membranes of mitochondria. Fatty acids and their derivatives function as natural ligands of PPARα, which results in the activation of PPARα and the induction of FAT, FATP, AOX, and CYP4A1, which further accelerates mitochondrial damage, the reduction of mitochondrial β-oxidation activity, and the accumulation of fatty acids in a vicious cycle.

Persistent PPARα activation increases oxidative DNA damage because of 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



Table 2
Primer pairs used for RT-PCR

Gene	GeneBank accession number	Primer sequence	Product (bp)
ACC	NM_133360	F 5'-GGGCACAGACCGTGGTAGTT-3' R 5'-CAGGATCAGCTGGGATACTGAGT-3'	105
ApoB	NM_009693	F 5'-TCACCCCGGGATCAAG-3' R 5'-TCCAAGGACACAGAGGGCTTT-3'	85
AOX	NM_015729	F 5'-TGGTATGGTGTCGTAAGTGAATGAC-3' R 5'-AATTTCTACCAATCTGGCTGCAC-3'	145
FAS	NM_007988	F 5'-ATCCTGGAACGAGAACAGATCT-3' R 5'-AGAGACGTGCTACTCTGGACTT-3'	140
FAT	NM_007643	F 5'-CCAAATGAAGATGAGCATAGGACAT-3' R 5'-GTTGACCTGCAGTCGTTTTGC-3'	87
FATP	NM_011977	F 5'-ACCACCGGGCTTCCTAAGG-3' R 5'-CTGTAGGAATGGTGCCAAAG-3'	80
GAPDH	M32599	F 5'-TGCACCACCACTGCTTAG-3' R 5'-GGATGCAGGGATGATGTTCTG-3'	177
L-FABP	NM_017399	F 5'-GCAGAGCCAGGAGAACTTTGAG-3' R 5'-TTTGATTTTCTCCCTTCATGCA-3'	121
MCAD	NM_007382	F 5'-TGCTTTGATAGAACCAGCTACAGT-3' R 5'-CTTGTGCTCCACTAGCAGCTT-3'	128
MTP	NM_008642	F 5'-GAGCGGTCTGGATTACAACG-3' R 5'-GTAGGTAGTGAGAGATGTGGCTTTTG-3'	72
PPAR α	NM_011144	F 5'-CCTCAGGGTACCCTACTACGGAGT-3' R 5'-GCCGAATAGTTCGCCGAA-3'	69

F, forward sequence; R, reverse sequence.

cell division, as revealed by the expression of cell cycle regulators such as cyclin D1 and CDK4. Furthermore, there is little change in apoptosis, which, 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 PPAR α 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), the 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 in that they have normal mitochondrial organization, increased mitochondrial β -oxidation activity, and no steatosis (16, 36). AOX-null mice are also different from *Ppara*^{+/+}:HCVcpTg mice with respect to mitochondrial structure (22). These detailed comparisons between the 3 mouse models reveal the importance of mitochondrial abnormalities in the pathogenesis of HCV-related diseases.

PPAR α is known to regulate the hepatic expression of many proteins associated with fatty acid and triglyceride metabolism, cell division and apoptosis, oxidative stress generation and degradation, and so forth (15, 16, 20, 21, 24–26); therefore, complete deletion of the PPAR α 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, which demonstrates that the presence of functional PPAR α itself is not a 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 important fact that the core protein-dependent pathological changes do not appear without significant activation of PPAR α . Thus, it is not the presence of PPAR α per se, but rather a high level of PPAR α 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, long-term clofibrate treatment caused a certain level of persistent PPAR α activation and a low incidence of HCC. Interestingly, in *Ppara*^{+/+}:HCVcpTg mice, clofibrate treatment induced more intensive PPAR α activation and HCC at a much higher incidence, accompanied by 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 of the *Ppara*^{+/+}:HCVcpTg mice not treated with clofibrate. Therefore, these findings further

support the concept that a long-term and high level of PPAR α activation is necessary for steatogenesis and hepatocarcinogenesis in HCVcpTg mice and emphasize the significant role of the HCV core protein as a PPAR α coactivator in vivo.

A pulse-chase experiment showed that PPAR α was stabilized in hepatocyte nuclei in mice expressing the HCV core protein. Many nuclear receptors, including PPAR α and RXR α , 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 shown conflicting result, i.e., that PPAR α was downregulated in the livers of chronic hepatitis C patients (39, 40). Although the association between PPAR α function and chronic HCV infection remains a matter of controversy in humans, the changes observed in the transgenic mice resemble in many ways the clinicopathological features of chronically HCV-infected patients; 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 the 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 PPAR α activation might be beneficial in the prevention of HCC. To clarify the exact relationship between PPAR α activation and HCV-induced hepatocarcinogenesis in humans, further



experiments using noncancerous liver tissues obtained from HCV-related HCC patients and using mice carrying human PPAR α and HCV core protein genes are needed.

In conclusion, we clarified for the first time that persistent and potent PPAR α 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 PPAR α in the mechanism of steatogenesis mediated by the core protein. Our results offer clues in the search for novel therapeutic and nutritional management options, especially with respect to neutral lipids, for chronically HCV-infected patients.

Methods

Mice. The 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 as *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: 5'-GCCACAGGACGTTAAGTTC-3' and 5'-TAGTTCACGCC-GTCCCTCCAG-3' for the HCV core gene (7) and 5'-CAGAGCAACCATCCAGATGA-3' and 5'-AAACGCAACGTAGAGTGCTG-3' for the PPAR α gene (24). Amplified alleles for HCV core and PPAR α genes were 460 and 472 base pairs, respectively. Five mice per cage were fed a routine diet and were kept free of specific pathogens according to institutional guidelines. For the clofibrate treatment experiments, 2-month-old male *Ppara*^{-/-} and *Ppara*^{-/-}:HCVcpTg mice were randomly divided into 2 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) for 24 months. All mice were killed by cervical dislocation before their livers were excised. If a hepatic tumor was present, the tumor was removed and subjected to histological analysis, and the remaining liver tissues were used for biochemical analyses. 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 tissue was minced on ice and transferred to 10% (w/v) isolation buffer (250 mM sucrose in 10 mM Tris-HCl [pH 7.4] and 0.5 mM EGTA and 0.1% bovine serum albumin [pH 7.4]). The samples were gently homogenized by 10–20 strokes with a chilled Dounce homogenizer (Wheaton) and loose-fitting pestle. The homogenate was centrifuged at 500 \times g for 5 min at 4°C. The supernatant was retained, and the resulting pellet was resuspended with isolation buffer and centrifuged at 4,500 g for 10 min at 4°C. The pellet fraction was suspended again and centrifuged at 20,000 \times 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 \times g for 10 min at 4°C to obtain a crude mitochondria pellet. This pellet was resuspended with isolation buffer, centrifuged at 7,800 \times g for 10 min at 4°C, and used as the mitochondrial fraction. Finally, all supernatant fractions were collected and centrifuged at 20,000 \times g for 30 min at 4°C, and the resulting supernatant was used as the cytosolic fraction.

Immunoblot analysis. Protein concentrations were measured colorimetrically with a BCA Protein Assay kit (Pierce). For the analysis of fatty acid-metabolizing enzymes, hepatocyte mitochondrial fractions or whole-liver lysates (20 μ g protein) were subjected to 10% SDS-PAGE (16). For analysis of PPAR α , nuclear fractions (100 μ g protein) were used. For analysis of other

proteins, whole lysates or cytosolic fractions (50 μ g protein) were subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody and then with alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. Antibodies against HCV core protein, fatty acid-metabolizing enzymes, CYP4A1, catalase, and PPAR α were described previously (9, 16, 24, 50). Antibodies against other proteins were purchased commercially: cytochrome *c* antibody from BD Transduction Laboratories and other antibodies from Santa Cruz Biotechnology. 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), and cDNA was generated by SuperScript II reverse transcriptase (Gibco BRL). Quantitative RT-PCR was performed using a SYBR green PCR kit and an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The primer pairs used for RT-PCR 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 μ m 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 μ m 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). Sections were immunostained using EnVision+ kit, with 3,3'-diaminobenzidine as a substrate (DAKO). Hepatocytes positive for PCNA or 8-OHdG were examined in 10 randomly selected \times 400 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 a MEBSTAIN Apoptosis Kit II (Medical & Biological Laboratories). 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 experiments. 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 3 times by means of differential centrifugation and the dead cells were removed by density-gradient centrifugation at 500 g for 3 min at 4°C on Percoll (Amersham Pharmacia Biotech). The live hepatocytes were washed and suspended in William's E medium containing 5% FBS. 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 FBS for 1 h at 37°C. The medium was replaced with the same medium containing 300 mCi/ml of [³⁵S]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 at 800 g for 5 min at 4°C to obtain a crude nucleus pellet. This pellet was resuspended with isolation buffer and centrifuged at 20,000 g for 1 h at 4°C 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, which suggested that the [³⁵S]methionine uptake capacity in the former hepatocytes was 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, and 10 mg/ml aprotinin). The lysate was incubated for 3 h at 4°C with purified anti-PPAR α 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, 3 mice in each group were fasted overnight. Livers were removed quickly, rinsed in ice-cold saline solution, and cut into 500- μ m thick slices with an Oxford Vibratome (Oxford Laboratories). Approximately 150 mg of fresh liver (6–8 liver slices) was placed on stainless steel grids positioned in a 25-ml flask equipped with suspended plastic center wells (Kontes) and incubated in RPMI-1640 medium (Sigma-Aldrich) devoid of fatty acids for 2 h at 37°C. The medium was then replaced with fresh RPMI-1640 medium supplemented with an antibiotic-antimycotic cocktail and 0.8 mM [14 C]palmitic acid (4 mCi/mmol) (American Radiolabeled Chemicals) complexed to BSA (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 μ l) and liver homogenates (250 μ l) were treated with ice-cold perchloric acid to make final concentrations of 200 mM and were centrifuged at 3,000 g for 20 min at 4°C. Aliquots of the supernatant containing the ketone bodies were introduced into the 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, and evaporated to dryness under an air stream; radioactivity was then counted. The experiment was repeated 3 times, and palmitic acid uptake ability was expressed as fold changes relative to that of Ppara $^{-/-}$ nontransgenic mice.

Other methods. To determine the 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 (malondialdehyde and 4-hydroxyalkenals) were measured using an LPO-586 kit (OXIS International). 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 a mouse insulin ELISA kit (U-type, AKRIN-031; Shibayagi), respectively.

Statistics. Statistical analysis was performed with a 2-tailed Student's *t* test for quantitative variables or with a chi-square test for qualitative variables. Quantitative data are expressed as the mean \pm SD. *P* < 0.05 was considered to be statistically significant.

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Review

Experimental models of hepatocellular carcinoma[☆]

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Hepatocellular carcinoma (HCC) is a common and deadly cancer whose pathogenesis is incompletely understood. Comparative genomic studies from human HCC samples have classified HCCs into different molecular subgroups; yet, the unifying feature of this tumor is its propensity to arise upon a background of inflammation and fibrosis. This review seeks to analyze the available experimental models in HCC research and to correlate data from human populations with them in order to consolidate our efforts to date, as it is increasingly clear that different models will be required to mimic different subclasses of the neoplasm. These models will be instrumental in the evaluation of compounds targeting specific molecular pathways in future preclinical studies.

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1. Introduction

Hepatocellular carcinoma is one of the world's deadliest cancers, ranking third among all cancer-related mortalities. Most cases occur in Asia and sub-Saharan Africa, where viral hepatitis is endemic. The incidence is rising in the West, likely due to the increase in patients infected with hepatitis C during the latter half of the last century [1]. The liver, unique in its capacity for regeneration following injury, also gives rise to this malignancy commonly associated with the inflammatory state of advanced fibrosis, or cirrhosis. Potentially curative therapies can be offered to approximately 30% of patients, but are complicated by a high rate of recurrence [2].

Encouraging progress has been made in understanding the molecular pathogenesis of cancer [1,2]. The discoveries of the signal transduction pathways, cascades of protein–protein interactions transmitting information from the cell surface to the nucleus, and of their link to tumor biology, are particularly impressive.

Several key mouse models have been instrumental in defining the pathogenesis of HCC by introducing genetic

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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; TKR, tyrosine kinase receptor; HBV, hepatitis B virus; TSG, tumor suppressor gene; TSP, tissue specific promoter; Tg, transgene.

alterations into one or more aetiological pathways that can be targeted exclusively to the liver. Moreover, these programmed manipulations can be introduced systematically, not only in this specific organ but also at defined times during development, growth and aging of the liver.

Nonetheless, substantial challenges persist in modeling liver diseases whose natural history requires a chronic inflammatory milieu. For example, infectious (hepatitis C virus), toxic (alcohol), metabolic (non-alcoholic steatohepatitis), or congenital (hemochromatosis) diseases share inflammation and fibrosis as precursors to cancer, yet none is easily mimicked in animals. There are few rodent models of HCC arising spontaneously within a background of regenerative nodules and cirrhosis, and most depend on the administration of hepatotoxic and/or carcinogenic agents to recreate the injury–fibrosis–malignancy cycle seen in chronic human liver diseases.

Comparative genomic studies in human HCC samples have begun to identify molecular subgroups with characteristic mutations, gene expression profiles and chromosomal gains and losses [3]. Moreover, since there is no single dominant molecular pathology underlying all HCCs, it is increasingly clear that different models will be required to mimic different subclasses of the neoplasm. These models will be instrumental as pre-clinical tools to evaluate compounds targeting specific molecular pathways.

With these challenges in mind, the objective of this review is to assemble and evaluate the available models of both cirrhosis and HCC, to provide a blueprint for understanding the pathogenesis of HCC and for optimizing preclinical models for drug testing.

2. Experimental models in cancer research

Although many experiments focusing on liver physiology have been conducted in rats due to their propensity for the development of fibrosis, the laboratory mouse (*Mus musculus*) is considered among the best model systems for cancer because of the availability of gene targeting methods, as well as the animal's size and breeding capacity, its lifespan of 3 years, and its physiologic and molecular similarities to human biology [4]. Significant advances have been made in modeling cancer genetics in mice, along a spectrum that ranges from simple xenograft models to more complex, genetically modified mice. Examples of each of the following are illustrated in Table 1.

2.1. Xenograft models

The demonstration that concentrated cancer cells grown *in vitro* could form tumors when implanted sub-


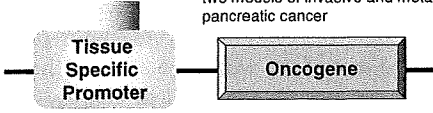
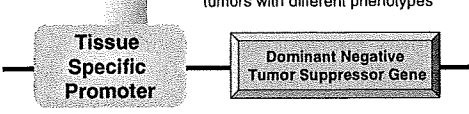
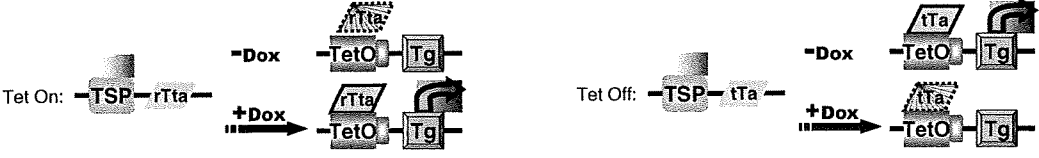
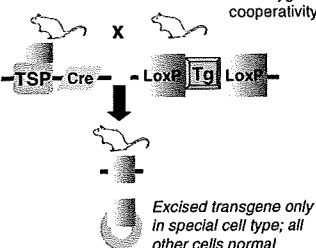
cutaneously into an immunocompromised mouse was first established in 1969 [5]. This xenograft model has since demonstrated several advantages that explain its persistence as the mainstay of pre-clinical studies of anti-neoplastic drugs *in vivo*: the tumors are rapidly and easily induced, and their subcutaneous location enables direct measurement of tumor growth. More recently, however, several critical differences between xenograft- and patient-derived specimens have become apparent, as discussed below. In addition, cancer is now appreciated as a complex disease dependent upon the interaction between transformed cells harboring oncogenic mutations, referred to as the 'cell autonomous compartment', and their surrounding tumor environment, the 'non-cell autonomous constituents' made up of normal cells, stromal cells, and immune cells [4], features that are not part of the xenograft approach.

Mouse models of cancer were first introduced over 60 years ago. Shortly after its inception in 1955, the Developmental Therapeutics Program at the National Cancer Institute (NCI) adopted the use of three transplanted rodent models of sarcoma, carcinoma, and leukemia, for the purposes of selecting agents for clinical use in cancer patients. Thousands of molecules were tested in mice bearing murine leukemias during the first decades of modern cancer drug development, circa 1945–1969 [6]. This tumor panel was later expanded to include human tumor xenografts, with the intention to study drug activity against solid tumors [7]. In 1990, the NCI focused on the development of *in vitro* assays in 60 different cell lines in order to screen pharmaceutical agents for their potency and their selective activity against either a particular disease category or specific cell line [8,9], the most promising of which were to be subsequently evaluated in the nude mouse xenograft model.

The validity of xenografts as a predictive indicator of probable clinical activity is limited, with the most success seen in cytotoxic agents. A retrospective analysis performed by the NCI for 39 compounds in which both xenograft testing and Phase II clinical data were available showing that less than 50% of agents with activity in more than one-third of xenografts showed clinical activity ($p = 0.04$) [6]. The same study demonstrated that activity in a particular histology in a tumor model did not closely correlate with activity in the same human cancer histology [10], with the exception of non-small cell lung and ovarian cancer [11].

There are several variables inherent to the xenograft experiments which may impact on the divergent outcomes compared to human disease, including growth properties and size at initiation of treatment of xenograft tumor, ectopic versus orthotopic location of tumor, local versus metastatic disease [12], tolerance for high doses of chemotherapeutic agents in mice [13],

Table 1
Available mouse models in cancer research

	Technical Method	Advanced Mouse Models of Cancer	Current Models in HCC	Future Prospects: Wish List for HCC
Xenograft		<p>COLON, BREAST, PROSTATE: Surgical orthotopic implantation: intact fragments of human cancer, including tumors taken directly from the patient, transplanted into the corresponding organ of immunodeficient rodents¹⁶</p>	<p>Orthotopic xenograft model in which hepatoma 129 cells originating from C3H mice are injected into fibrotic livers of mice pretreated with TAA and EtOH¹⁶⁵</p>	<p>Mouse HCC cell line derived from GEM tumor with specific molecular pathway dysregulated, with immunofluorescent marker, injected into fibrotic liver of immunocompetent mice</p>
Transgenic GEM	<p>Constitutive Transgenic</p> 	<p>PANCREATIC: Kras^{G12V} and chronic pancreatitis¹⁶⁸, Trp53^{R172H} and Kras^{G12D} double transgenic driven by insulin promoter¹⁷⁰, two models of invasive and metastatic pancreatic cancer</p>	<p>Mouse C-myc/Human E2F-1 overexpression driven by albumin promoter¹⁷¹; HCC at 6–8 months</p>	<p>Double transgenic overexpressing profibrotic gene combined with liver-specific oncogene</p>
	<p>Dominant Negative Transgenic</p> 	<p>PITUITARY: Rb and p27Kip1 Cdk inhibitor tissue specific knockout mice develop pituitary tumors with different phenotypes¹⁷²</p>	<p>Mdr-2 knockout mice are unable to secrete phospholipids into bile, and develop cholangitis and HCC at 6–12 months¹⁶⁶</p>	<p>Double transgenic liver-specific E-cadherin knockout and β-catenin overexpression</p>
	<p>Inducible Transgenic</p> 	<p>MELANOMA: Double transgenic combining Tet-induced overexpression of mutated Hras^{V12G} and Ink4a knockout¹⁷³</p>	<p>Tet-inducible Met expression under albumin promoter: 60% HCC at 12 months; tumors regressed when transgene (Tg) was inactivated⁹¹</p>	<p>Tet-induced, liver-specific overexpression of known oncogene in fibrotic mice</p>
	<p>Conditional Gene Targeting</p>  <p>Excised transgene only in special cell type; all other cells normal</p>	<p>PROSTATE: Double transgenic Cre-mediated PTEN^{-/-} homozygous loss and p19Arf^{+/+}; cooperativity in cancer development¹⁷⁴</p>	<p>Cre-mediated liver specific PTEN^{-/-} knockout: 66% HCC at 8 months¹⁰⁸</p>	<p>Cre-mediated, liver-specific knockout of known tumor suppressor gene in fibrotic mice</p>

and variability in selected endpoints. These variables can be minimized if given due consideration in the design of preclinical cancer drug experiments. However, the greatest discrepancies between success of cancer therapies in xenograft models and in human clinical trials are likely due to critical differences in both the tumor cells and their microenvironment. Natural tumor progression is a micro-evolutionary process during which increasingly

aggressive clones, generated through genetic instability, emerge from an initially monoclonal lesion. Autochthonous tumors, those that evolve *in situ* from normal cells, tend to have a diminished genetic heterogeneity compared to tumor xenografts, although selective pressures of cell culture or tissue explantation can cause a rapid expansion of a certain clonal constituent of polyclonal tumors [14,15].