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Research Article

Cancer Prevention Research

Resveratrol Helps Recovery from Fatty Liver and Protects against Hepatocellular Carcinoma Induced by Hepatitis B Virus X Protein in a Mouse Model

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Abstract

Resveratrol is a natural polyphenol that has beneficial effects across species and various disease models. Here, we investigate whether resveratrol is effective against hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) using HBV X protein (HBx) transgenic mice. We found that resveratrol (30 mg/kg/d) has a therapeutic effect on HBx-induced fatty liver and the early stages of liver damage. Resveratrol decreased intracellular reactive oxygen species and transiently stimulated hepatocyte proliferation. Interestingly, resveratrol inhibited LXRα and downregulated the expression of the lipogenic genes, Srebp1-c and PPARγ. The decrease in Srebp1-c seems to further downregulate the expression of its target genes, Acc and Fas. In addition, resveratrol stimulated the activity of Ampk and SirT1. Thus, resveratrol has a pleiotropic effect on HBx transgenic mice in terms of the downregulation of lipogenesis, the promotion of transient liver regeneration, and the stimulation of antioxidant activity. Furthermore, at the later precancerous stages, resveratrol delayed HBx-mediated hepatocarcinogenesis and reduced HCC incidence from 80% to 15%, a 5.3-fold reduction. Resveratrol should be considered as a potential chemopreventive agent for HBV-associated HCC. *Cancer Prev Res; 5(7); 952–62. ©2012 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and has a poor prognosis and low survival rate (1). A large proportion of HCC cases occur in less-developed countries in Asia and Africa, and are typically associated with chronic hepatitis virus infection (mainly HBV and HCV). Interestingly, although the incidence of HCC in less-developed areas is decreasing because of vaccination, the incidence of HCC in well-developed countries, including the United States and Europe, has increased in the last 20 years (2, 3). The etiology of this increase in HCC in developed countries remains to be elucidative, but it is likely to involve HCV and metabolic factors such as obesity and diabetes (4, 5). However, there are currently limited therapeutic regimens available for the effective treatment of HCC. The fact that HCC exhibits a high recurrent rate after resection and is resistant to conventional chemotherapy and radiotherapy renders the disease a very serious health problem at the current time.

In addition, hepatic steatosis (fatty liver), which manifested as an excess accumulation of lipids in hepatocytes, is associated with hepatitis virus infection, various drugs, nutritional factors, and multiple genetic defects in energy metabolism. Fatty liver is a vulnerability factor that can promote liver damage and inflammation, which results in a further progression to cirrhosis and HCC (6). To treat fatty liver and protect against the development of more severe forms of end-stage liver disease and cancer, the discovery and development of chemopreventive agents for HCC is of paramount importance.

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a polyphenol found in a wide variety of plant species. Resveratrol has been shown to exert beneficial effects across species and various disease models; it can prevent or slow the progression of a wide variety of illnesses, including cancer, cardiovascular disease, diabetes and metabolic disease, as well as enhance stress resistance (7, 8). For liver diseases, previous studies have showed the protective effects of resveratrol against alcohol-induced fatty liver and liver injury in mice (9, 10). This protective action of resveratrol in preventing the development of alcoholic fatty liver seems to be associated with an upregulation of the SIRT1 and AMPK signaling pathways in the livers of the ethanol-fed mice (11). The

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beneficial effect of resveratrol has also been shown in a rodent model of nonalcoholic fatty liver disease where the disease is induced by a protocol involving a high carbohydrate-fat free modified diet and fasting (12).

The anticancer potential of resveratrol in HCC has been investigated in xenografted nude mice and in rodent models carrying transplanted hepatome cells; in addition, the chemopreventive effect of resveratrol has also been evaluated in chemical carcinogen-induced HCC in rats (13). Bishayee and Dhir (14) applied a 2-stage protocol of rat hepatocarcinogenesis involving a single intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg) followed by promotion with phenobarbital (PB, 0.05%) in the drinking water. Suppression of oxidative stress and the inflammatory response as well as alternations in hepatic proinflammatory cytokines have been implicated in the chemopreventive actions of resveratrol in the DEN-initiated and PB-promoted HCC model (15, 16). However, neither DEN nor PB is epidemiologically associated with HCC in humans. Accordingly, the DEN/PB carcinogen-induced HCC model may not faithfully parallel the normal physiologic and pathologic situations in terms of the etiologic tissue microenvironments of those cells later become cancerous. Thus, this model may not recapitulate the spontaneous carcinogenesis progress toward the developed cancerous status. Therefore, an animal model of hepatocarcinogenesis mimicking the spontaneous progression of HCC development in human patients is required, and this has not yet been explored.

In this study, we investigate the therapeutic effects of resveratrol on HBV-associated liver damage and fatty liver during the early stages of pathogenesis, and evaluate the potential chemopreventive activity of resveratrol on HBV-associated HCC at a later precancerous stage. This was done using a transgenic mouse model that expresses the HBV X protein (HBx) specifically in the hepatocytes. The HBx transgenic mice spontaneously develop HCC at between 13 and 16 months of age. The HCC that develops in these HBx transgenic mice exhibits a well-differentiated morphology of the trabecular pattern, which is similar to that observed in human HCC (17). The HBx transgenic mice thus provide an animal model for evaluating new chemopreventive and therapeutic agents for HBV-associated HCC under physiologic conditions (18). In addition, we also examined the changes in the hepatic gene expression profile and signaling pathways before and after resveratrol treatment at various time points, and compared these between the HBx transgenic and wild-type (WT) mice. This allowed us to explore the potential molecular mechanisms through which the protective effects of resveratrol may work.

Materials and Methods

HBx transgenic mice

We have previously generated 4 lines of HBx transgenic mice, namely A105, A106, A110, and A112, in the C57BL/6 background (17). All of the HBx transgenic lines develop HCC. In this study, all the animal experiments used male mice of the line A106; this line develops HCC faster than the other 3 transgenic lines (17). All of the mice were housed in a specific pathogen free facility. All of the animal protocols are consistent with the recommendations outlined in the "Guide for the Care and Use of Laboratory Animals" (Washington, DC, National Academy Press). The Institutional Animal Care and Use Committees of the National Yang-Ming University (Taipei, Taiwan) had specifically approved this study (approval number 981207).

Resveratrol administration

To study the therapeutic effect of resveratrol on the fatty liver and early stage of liver pathogenesis, 4-week-old HBx transgenic male mice and their WT male littermates were randomly assigned into different groups. Resveratrol (Sigma R5010; 30 mg/kg/d) was dissolved in H_2O and delivered to the mice by oral administration using a feeding needle once a day. Mice were sacrificed at 2, 3, 7, and 14 days after resveratrol administration. To study the chemopreventive effect of resveratrol on the precancerous stage of liver carcinogenesis, 12-month-old HBx transgenic male mice and their WT male littermates were used. In this case resveratrol (Sigma R5010) was mixed with powdered chow at a concentration of 3g/12.5 kg of food to provide a dose of 30 mg/kg/d for a mouse (average body weight 30 g, eating 4 g of chow daily), and pellets were then reconstituted; this special diet was prepared by Research Diets, Inc. Mice were sacrificed at 16-month-old after resveratrol supplementation to the chow for 4 months. Liver tissues and sera were collected for pathologic and biochemical analysis.

Pathologic analysis

The number and size of liver nodules were measured at mouse sacrifice. The livers were collected, fixed, with formalin and embedded in paraffin. Liver sections were subjected to hematoxylin–eosin (H&E) staining. Fat accumulation was showed by oil red-O staining of cryostat frozen sections (19). Ultrastructural changes in the liver were examined by transmission electron microscopy (TEM; ref. 20).

Intracellular reactive oxygen species and glutathione levels

Primary hepatocytes were isolated from mouse livers using the 2-step collagenase perfusion method (21). Intracellular reactive oxygen species (ROS) levels were determined using an oxidative sensitive fluorescence dye, dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes; ref. 18). Intracellular glutathione (GSH) levels were determined using a cell-permeable nonfluorescent dye monochlorobimane (MCB; Molecular Probes) that becomes highly fluorescent after reaction with intracellular GSH (18).

RNA analysis

Total RNA was isolated from mouse tissues using TRIzol Reagent (Life Technology). Slot blot hybridization was conducted as previously described (22). We execute reverse transcription with 2 μg of total RNA using oligo-d(T) as

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primer and Superscript III reverse transcriptase (Invitrogen Life Technologies). The real-time quantitative PCR was carried out on a Roche LightCycler 480 instrument using a TaqMan probe. All amplifications were carried out in triplicate for each RNA sample and primer set, and all measurements were done using RNA samples from 3 individual mice. The amount of total input cDNA was normalized using hypoxanthine phosphoribosyltransferase (HPRT) as an internal control.

Western blotting and immunohistochemical analysis

Western blotting was conducted as described previously (23) and detected using Visualizer Kit (Upstate 64-201BP). The following antibodies were used: p-Ampk (Cell Signaling 2535, 1:1,000); Ampk (Cell Signaling 2532, 1:1,000); p-Akt (Upstate 05-736, 1:2,000); Akt (Upstate 05-591, 1:2,000); β-actin (Sigma A5441, 1:5,000); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore MAB374, 1:5,000). IHC detection of the Ki67 protein was conducted using monoclonal Ki67 antibody (B56; BD Pharmingen, 1:100) and visualized by the Chemicon IHC Select System (DAB150) according to the manufacturer's instructions.

Statistical analysis

The results are presented as mean ± SD from at least 3 independent experiments. Comparisons between 2 groups were done using a Student *t* test. A *P* value of <0.05 was considered significant.

Results

Resveratrol promoted recovery from fatty liver and the early stages of liver damage

The animal protocol for resveratrol treatment is shown in Fig. 1A; resveratrol was used at a concentration of 30 mg/kg/d, which is a feasible daily dose for human (24). Without resveratrol, at an early stage of the HBx-mediated liver pathogenesis, namely 4 to 6 weeks of age, liver pathology including fatty changes (microsteatosis), pleomorphic and bizarre nuclei, ballooning of the hepatocytes and abnormal arrangements of the sinusoid, all of which were clearly detectable in the HBx transgenic mice [Fig. 1B (i)]. Interestingly, oral administration of resveratrol (30 mg/kg/d) from 4 to 6 weeks of age reduced liver damage and regressed the histopathology of the HBx transgenic mice in a time-dependent manner [Fig. 1B (ii–iv)]. The liver pathology of

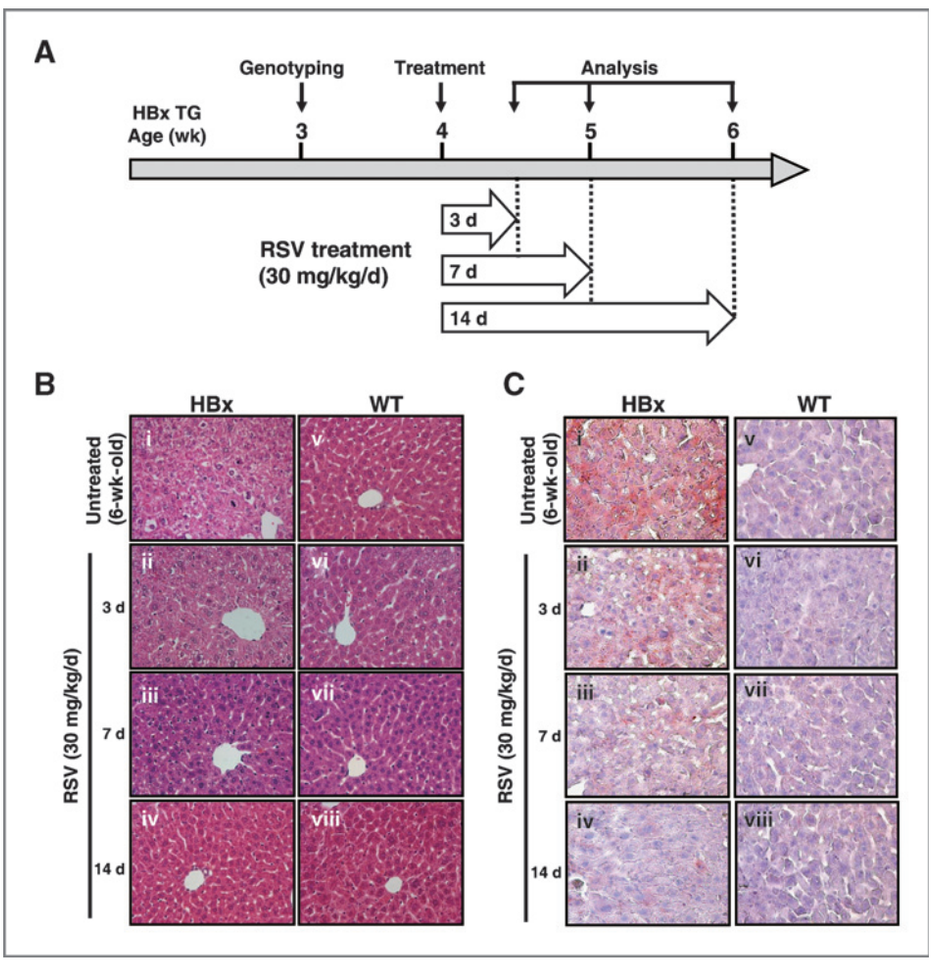

Figure 1. Treatment with resveratrol (RSV) reversed fatty liver and early-stage liver damage in the HBx transgenic mice. A, the treatment protocol used with resveratrol. Resveratrol (30 mg/kg/d) and vehicle (H_2O) were orally administrated to the HBx transgenic (TG) and WT mice (4-week-old). The mice were sacrificed and analyzed after resveratrol administration for 3, 7, and 14 days. Six to 10 mice per group were used. B, i, H&E staining of a liver section without any treatment from an HBx transgenic mouse (6-week-old). ii–iv, H&E staining of liver sections from HBx transgenic mice treated with resveratrol for 3, 7, and 14 days. v, H&E staining of a liver section without any treatment from a WT mouse (6-week-old). vi–viii, H&E staining of liver sections from WT mice treated with resveratrol for 3, 7, and 14 days. C, i, oil red-O staining of liver section without any treatment from an HBx transgenic mouse (6-week-old). ii–iv, oil red-O staining of liver sections from HBx transgenic mice treated with resveratrol for 3, 7, and 14 days. v, oil red-O staining of a liver section without any treatment from a WT mouse (6-week-old). vi–viii, oil red-O staining of liver sections from WT mice treated with resveratrol for 3, 7, and 14 days. Original magnification, ×400.

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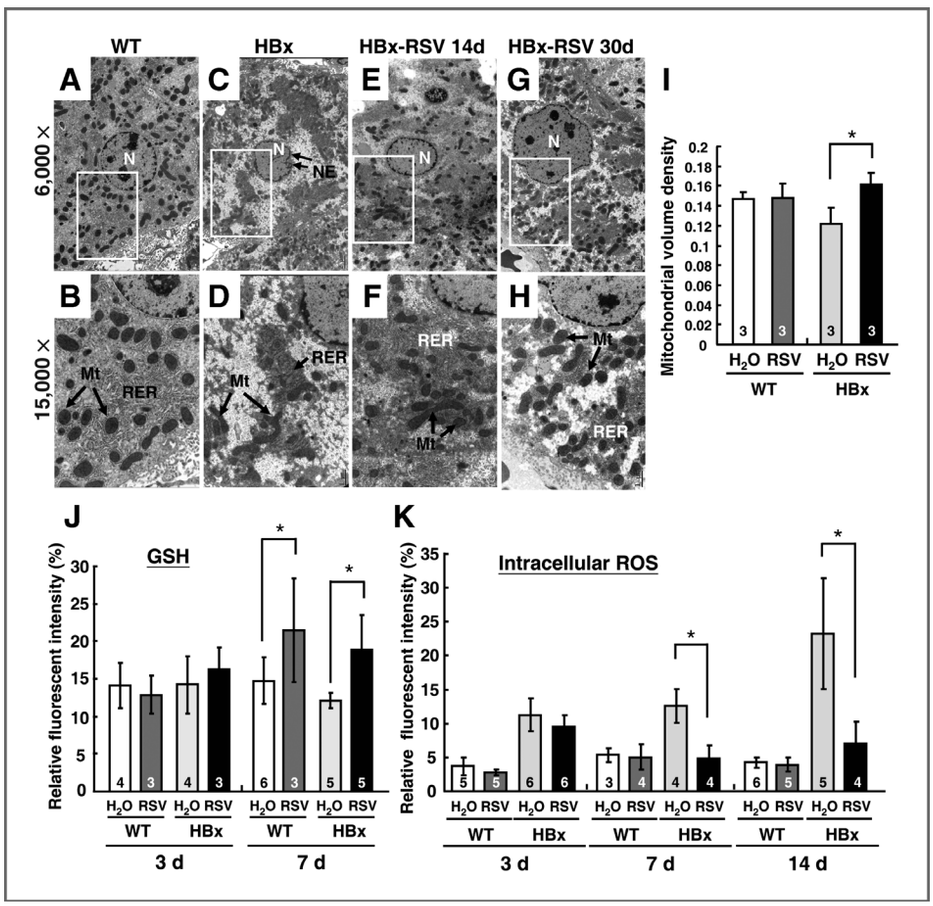

Figure 2. Resveratrol (RSV) helped recover hepatocyte ultrastructure, increased GSH, and decreased ROS in the HBx transgenic (TG) livers. A and B, TEM of hepatocytes of WT mice treated with vehicle (H_2O) for 14 days. C and D, TEM of hepatocytes of HBx transgenic mice treated with vehicle (H_2O) for 14 days. E and F, TEM of hepatocyte of HBx transgenic mice treated with resveratrol for 14 days. G and H, TEM of hepatocyte of HBx transgenic mice treated with resveratrol for 30 days. Photomicrographs shown in B, D, F, and H are at the magnification of the boxed area in A, C, E, and G, respectively. Mt, mitochondria; N, nucleus; NE, nuclear envelope; RER, rough endoplasmic reticulum. I, comparison of mitochondrial volume density after resveratrol or vehicle (H_2O) treatment for 30 days. J, intracellular GSH levels of hepatocytes after resveratrol or vehicle (H_2O) treatment for 3 and 7 days. K, intracellular ROS levels of hepatocytes after resveratrol or vehicle (H_2O) treatment for 3, 7, and 14 days. *, $P < 0.05$. Numbers in bars are mice (I–K); bars represent mean ± SD.

the HBx transgenic mice recovered significantly after receiving resveratrol for 7 days [Fig. 1B (iii)] and recovered to normal morphology after receiving resveratrol for 14 days [Fig. 1B (iv)] compared with the WT control [Fig. 1B (v–viii)]. Oil-red O staining of liver cryosections further revealed that fatty liver had completely disappeared in the HBx transgenic mice after resveratrol receiving for 14 days (Fig. 1C). There was no obvious difference in the body weight and ratio of liver to body weight in the HBx transgenic or WT mice with or without resveratrol (Supplementary Fig. S1). However, the value for serum ALT in the HBx transgenic mice was significantly reduced at day 14 after receiving resveratrol. Importantly, no obvious difference in the serum ALT values could be detected in the WT mice with or without resveratrol treatment, indicating that resveratrol has no toxic effect on the liver (Supplementary Fig. S2). Thus, our results showed that resveratrol treatment results in recovery from fatty liver and that resveratrol exerted therapeutic effects during the early stages of liver pathogenesis in the HBx transgenic mice.

Resveratrol produced a significant recovery in hepatocyte ultrastructure, increased GSH levels, and decreased ROS levels in the HBx transgenic livers

To study the efficacy of resveratrol on hepatocyte ultrastructure, HBx transgenic livers after receiving resveratrol were examined by transmission electron microscopy. In the WT mice, no ultrastructural abnormalities were detected with or without resveratrol treatment (Fig. 2A and B). In the HBx transgenic mice without resveratrol, severe ultrastructural alterations were observed in the hepatocytes, including disorganization of rough ER and degeneration of the nuclear envelope and mitochondria (Fig. 2C and D). After receiving resveratrol, most of the ultrastructural abnormalities were absent at 14 days (Fig. 2E and F) and seem to have completely recovered at 30 days (Fig. 2G and H). Quantification further supported a recovery in mitochondrial volume density among the HBx transgenic hepatocytes after receiving resveratrol when compared with WT hepatocytes (Fig. 2I; 25). Previously we have showed that there are persistently increased levels of ROS during liver carcinogenesis of HBx transgenic mice (18). To study the antioxidant activity of resveratrol in the liver, the intracellular GSH and ROS levels of the hepatocytes were monitored. Indeed, the intracellular GSH levels were significantly increased (Fig. 2J), whereas the intracellular ROS levels were significantly reduced after receiving resveratrol for 7 days (Fig. 2K). These results clearly showed that resveratrol exhibits antioxidant activity in the liver and that it can efficiently reduce the intracellular ROS levels induced by HBx during HBV-associated carcinogenesis.

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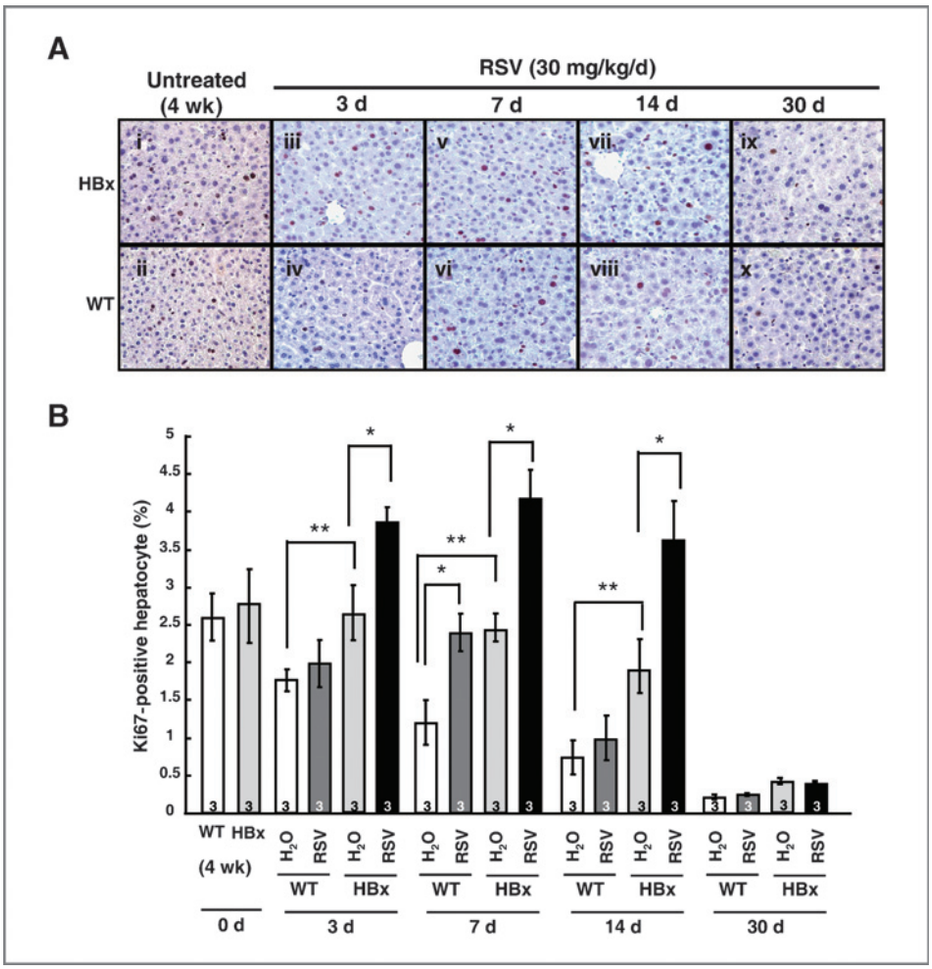

Figure 3. Resveratrol (RSV) transiently stimulated hepatocyte proliferation in the HBx transgenic (TG) mice. A, i and ii, IHC staining of the Ki67 cell proliferation marker in liver sections prepared from 4-week-old HBx transgenic and WT mice without resveratrol treatment. iii and iv, IHC staining of Ki67 protein in liver sections prepared from HBx transgenic and WT mice treated with resveratrol for 3 days. v and vi, IHC staining of Ki67 protein in liver sections prepared from HBx transgenic and WT mice treated with resveratrol for 7 days. vii and viii, IHC staining of Ki67 protein in liver sections prepared from HBx transgenic and WT mice treated with resveratrol for 14 days. ix and x, IHC staining of Ki67 protein in liver sections prepared from HBx transgenic and WT mice treated with resveratrol for 30 days. Original magnification, ×400. B, quantification of hepatocyte proliferation as monitored by Ki67-positive staining. Between 600 and 1,000 hepatocytes from each mouse were examined for the presence of Ki67-positive staining. The mean for each group is expressed as a percentage of total hepatocytes counted. *, $P < 0.05$; **, $P < 0.005$. Numbers in bars are mice (B); bars represent mean ± SD.

Resveratrol transiently stimulated hepatocyte proliferation, which helps to replace damaged cells in the HBx transgenic liver

To study whether hepatocyte proliferation and liver regeneration were affected by resveratrol, the Ki67 cell proliferation marker was examined by IHC staining of liver sections. Our results indicated that Ki67-positive cells were obviously increased after resveratrol administration (Fig. 3A). In WT mice, proliferation of hepatocyte decreased gradually from 4- to 8-week-old during maturation (Fig. 3B): 4-week-old (2.64% ± 0.16%) → 5-week-old (1.2% ± 0.17%; H_2O 7d) → 6-week-old (0.74% ± 0.13%; H_2O 14d) → 8-week-old (0.21% ± 0.03%; H_2O 30d). In the WT mice treated with resveratrol, hepatocyte proliferation was not affected after receiving resveratrol for 3 days; however, there was a lower but significant stimulation after receiving resveratrol for 7 days. Furthermore, the phenomenon of enhanced proliferation disappeared after receiving resveratrol for 14 days (Fig. 3B). In the HBx transgenic mice, quantification revealed that after receiving resveratrol for 3, 7, and 14 days, there was a significant increase in the number of Ki67-positive hepatocytes found in treated transgenic mice compared with the untreated transgenic mice (Fig. 3B). Importantly, the proliferation of hepatocytes in the HBx transgenic mice went back to a basal level after receiving resveratrol for 30 days; this is the time when the animal has completely recovered from all the liver pathology and ultrastructure abnormalities after treatment with resveratrol (Fig. 3B). These results suggested that the enhanced hepatocyte proliferation and liver regeneration induced by resveratrol helps to replace damaged cells in the HBx transgenic mice and this may contribute in part to the chemotherapeutic effect of resveratrol on fatty liver.

Resveratrol inhibited lipogenic gene expression in the HBx transgenic livers

To investigate whether HBx gene expression is affected by resveratrol and whether this might contribute to the regression of morbid liver pathology after resveratrol administration, expression of the HBx gene was examined. Our results revealed that the mRNA level of the HBx gene was not inhibited by resveratrol in the HBx transgenic mice (Fig. 4A and Supplementary Fig. S3A).

To dissect the molecular mechanisms underlying the beneficial effects of resveratrol on HBx-mediated fatty liver and histopathology at the early stage of liver damage (4- to

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6-week-old), we examined the expression of lipogenic genes and the genes related to lipid metabolism in liver. Previous studies have shown that HBx induces lipid accumulation and fatty liver through transcriptional activation of Srebp1-c (sterol regulatory element binding protein 1, isoform c), and PPARγ (26, 27). Srebp1-c is a key regulator of lipogenic genes in liver (28). Interestingly, there was an age-dependent decrease of Srebp1-c expression in the liver of WT mice. However, the level of Srebp1-c mRNA was significantly increased in the HBx transgenic mice at around 3-month-old and thereafter, compared with age- and sex-matched WT mice (Fig. 4B). Although there was no obvious difference in the expression levels of Srebp1-c between WT and HBx transgenic mice from 4- to 6-week-old, our result revealed that specifically in the HBx transgenic mice, resveratrol significantly reduced Srebp1-c mRNA expression as early as 2 days after receiving the resveratrol (Fig. 4C). Subsequently, expression levels of the downstream target genes of Srebp1-c, namely acetyl-CoA carboxylase (Acc) and fatty acid synthase (Fas), were found to be reduced after the expression of Srebp1-c was lowered (Fig. 4D and E). However, expression of the Srebp1-c target gene stearoyl-CoA desaturase 1 (Scd1) was not affected by resveratrol (Supplementary Fig. S3B). In addition to Srebp1-c, PPARγ is suggested to be a key regulator for lipid uptake and synthesis in liver (29, 30). Our results revealed that resveratrol also decreased the expression of PPARγ after resveratrol had been given for 3, 7, and 14 days in the HBx transgenic mice (Fig. 4F).

Inhibition of LXRα seems to be the early event upstream affecting Srebp1-c in the HBx transgenic liver after receiving resveratrol

Previously studies have shown that HBx induces expression of liver X receptor (LXR) and its lipogenic target genes, including Srebp1-c and PPAR, in HBx transgenic mice (27, 31). Our results revealed that resveratrol reduced the expression of LXRα mRNA as early as 2 days after receiving resveratrol (Fig. 4G); however, resveratrol had no effect on the expression of LXRβ in either WT or HBx transgenic mice (Supplementary Fig. S3C). Moreover, we also examined whether resveratrol affects the activation of Akt by phosphorylation, which has been implicated in the HBx-mediated survival signaling (32) and the activation of Srebp1-c (26). Our results did not show a significant difference in the p-Akt/Akt ratios of HBx transgenic mice with or without resveratrol treatment (Supplementary Fig. S4).

Furthermore, because Srebp1-c is also modulated by AMP-activated protein kinase (Ampk) during control of lipid metabolism in the liver (33, 34), we monitored the effect of resveratrol on Ampk activity by examining the protein levels of phosphorylated Ampk (pAmpk) and total Ampk. We detected a significant increase in the pAmpk/Ampk ratio at day 3, but not at day 2, after the mice had received resveratrol in both the WT and HBx transgenic mice (Fig. 4H and I). This activation was 1 day after the decrease in Srebp1-c in the resveratrol treated HBx transgenic mice (Fig. 4C), which suggests that Ampk signaling is unlikely to be the upstream regulator responsible for the resveratrol-mediated Srebp1-c inhibition in the HBx transgenic mice.

Moreover, because resveratrol is an activator of SirT1 (35); we sought to examine the effect of resveratrol on the hepatic SirT1 activity and gene expression in the resveratrol treated HBx transgenic mice. Our results revealed that there was a significant increase in SirT1 enzymatic activity (1.5- to 2-fold) after receiving resveratrol for 7 and 14 days in both the WT and HBx transgenic mice (Supplementary Fig. S5A). We further examined the SirT1 protein level in the various livers, and found a similar magnitude of change in enzymatic activity after receiving resveratrol (Supplementary Fig. S5B and S5C); this result indicated that resveratrol activated SirT1 in liver mainly by upregulating its protein expression.

In summary (Fig. 5), our results reveal that resveratrol helps the recovery of HBx-induced fatty liver in a coordinating manner by affecting multiple lipid metabolism signaling pathways, which in turn produces reduced lipid synthesis, and prevents the accumulation of hepatic lipids in mice. Specifically, resveratrol inhibits LXRα and downregulates the expression of its lipogenic target genes, Srebp1-c and PPARγ; the decrease in Srebp1-c further downregulates the expression of its target genes, Acc and Fas, both of which are lipogenic-associated enzymes. In addition, our results also show that resveratrol stimulates the activity of Ampk and SirT1 in the HBx transgenic liver. The combined effects of these multiple pathway changes seem to be associated directly or indirectly with lipid metabolism. Furthermore, it seems that resveratrol can transiently induce liver regeneration; this likely helps with the replacement of damaged cells in the HBx transgenic mice. Moreover, resveratrol exhibits antioxidant activity, which is accompanied by an increase in the GSH level and a decrease in the ROS level. Taken together, our results indicate that resveratrol functions as a pleiotropic chemotherapeutic agent and acts by regulating lipogenesis, promoting transient regeneration, and stimulating antioxidant activity in the liver. These activities together may contribute to the recovery of fatty livers and a reversing of the liver histopathology found in the HBx transgenic mice.

Resveratrol delayed HBx-mediated hepatocarcinogenesis and significantly reduced HCC incidence at the precancerous stage

Because resveratrol has a beneficial (therapeutic) effect during the early stages of liver pathogenesis, we further tested the preventive effect of resveratrol on the later stages of HBx-mediated HCC development. Resveratrol (30 mg/kg/d) was orally administrated to HBx transgenic and WT mice from 12- to 16-month-old (Fig. 6A). In the precancerous mice at 12 months of age and before receiving resveratrol, hyperplastic nodules measuring between 0.5 to 2.5 mm in diameter could be detected

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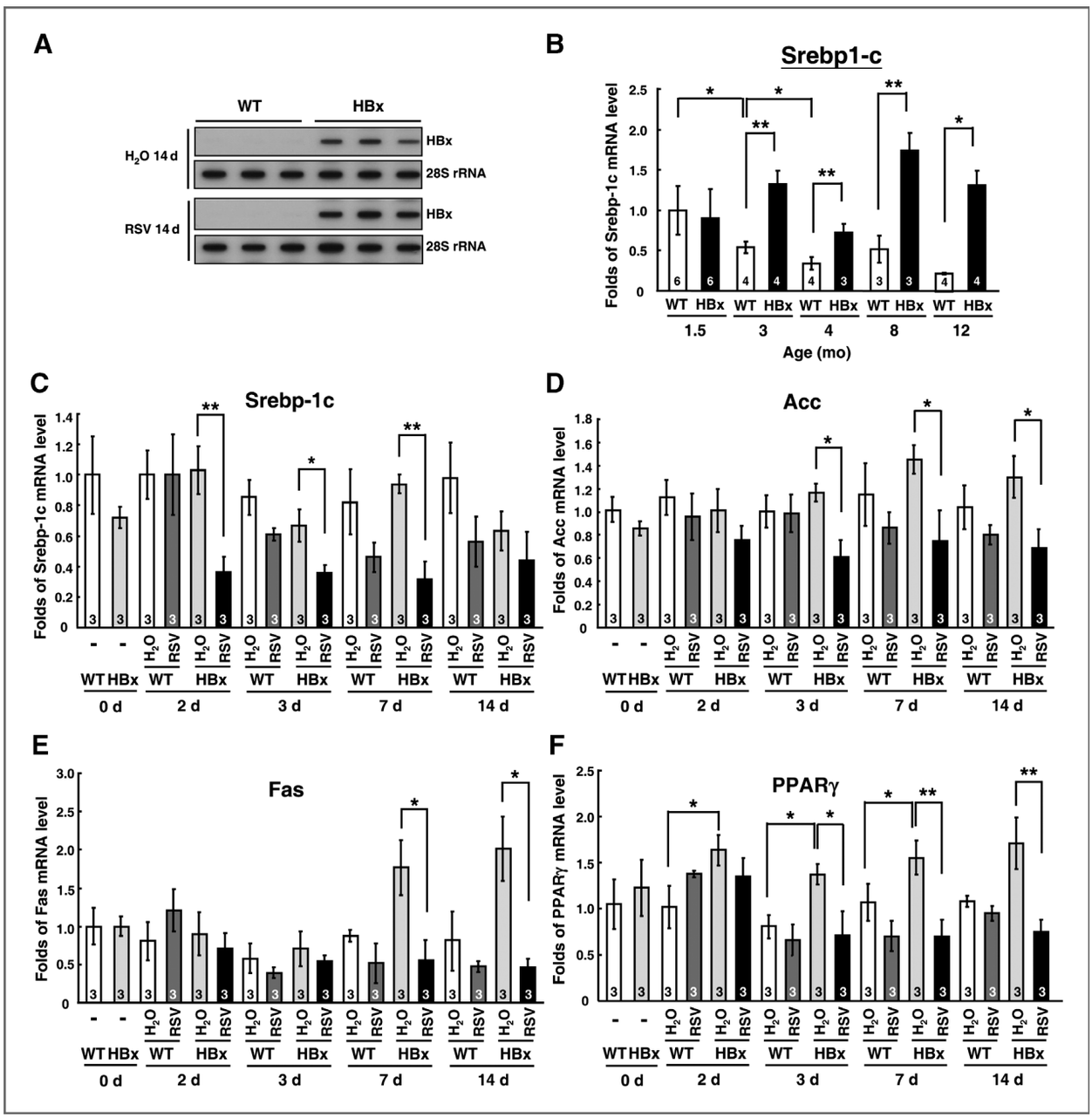

Figure 4. Expression of HBx and the genes associated with lipogenesis in the HBx transgenic (TG) livers. Resveratrol (RSV) inhibited LXRα expression while enhanced the level of p-Ampk in the HBx transgenic livers. A, HBx mRNA levels in the livers of 6-week-old HBx transgenic mice with or without resveratrol treatment for 14 days were detected by slot blot hybridization. HBx gene expression was not inhibited by resveratrol treatment. The 28S rRNA was used as an internal control for RNA loading. B, expression of the Srebp1-c mRNA was activated in the HBx transgenic livers in an age-dependent manner without any treatment. C, a significant decrease in Srebp1-c mRNA level was detected as early as 2 days after resveratrol treatment in the HBx transgenic mice and this preceded the inhibition of the Acc and Fas mRNA expression. D, a significant decrease in Acc mRNA level was detected in the HBx transgenic mice after resveratrol treatment for 3, 7, and 14 days. E, a significant decrease in Fas mRNA level was detected in the HBx transgenic mice after resveratrol treatment for 7 and 14 days. F, a significant decrease in PPARγ mRNA level was detected in the HBx transgenic mice after resveratrol treatment for 3, 7, and 14 days. The relative mRNA levels of the Srebp1-c, Acc, Fas, and PPARγ were measured by real-time quantitative reverse transcriptase (RT)-PCR; the amount of total input cDNA was normalized using HPRT as an internal control.

in about 67% of the HBx transgenic mice (Supplementary Fig. S6A). Furthermore, there was an 80% incidence of HCC in the 16-month-old HBx transgenic mice without any treatment (Fig. 6B and Supplementary Fig. S6B; ref. 17). In WT mice, our results showed that there was no detectable toxicity after receiving resveratrol for 4 months and there was also no difference in body weight and serum ALT level (a liver damage marker) between

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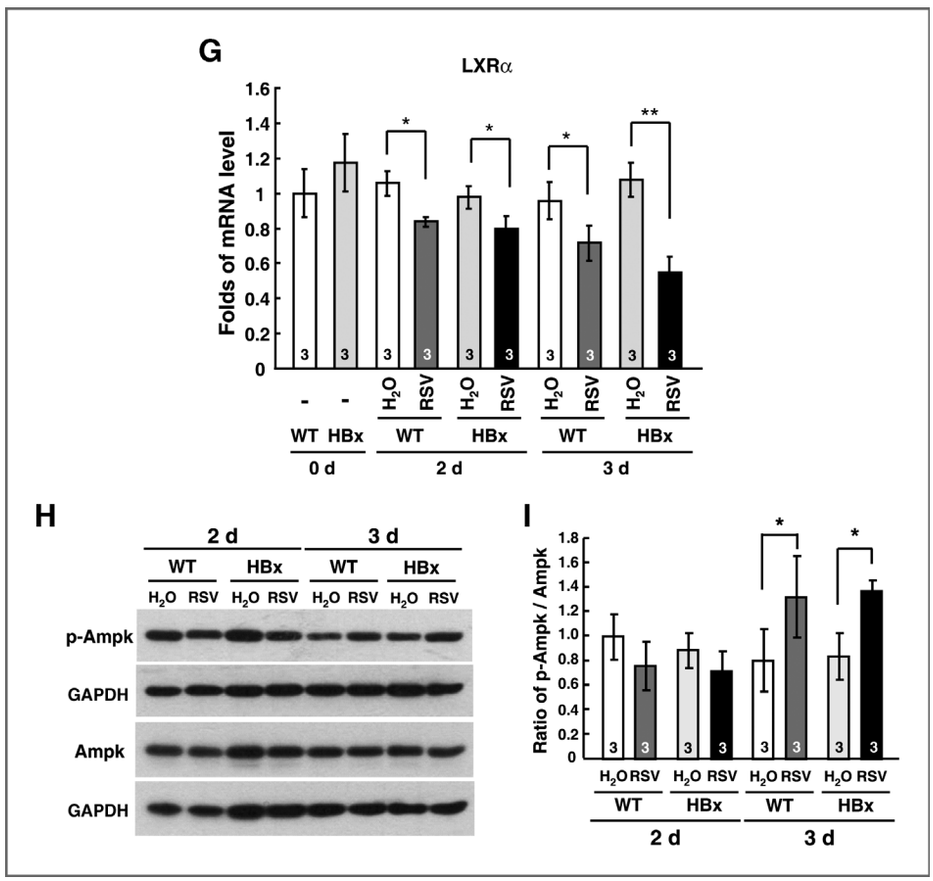

Figure 4. (*Continued*) G, resveratrol reduced the expression of LXRα mRNA as early as 2 days after the RSV treatment. H, a representative Western blot analysis of the p-Ampk and total Ampk protein. I, the p-Ampk/Ampk ratio increased significantly at day 3 after resveratrol treatment, but not at day 2. *, $P < 0.05$; **, $P < 0.005$. Numbers in bars are mice (B–G and I); bars represent mean ± SD.

the mice treated with resveratrol and the control group (Fig. 6C and Supplementary Fig. S7). Notably, in the HBx transgenic mice, there was a significant delay in liver carcinogenesis and a remarkable decrease in HCC incidence after receiving resveratrol for 4 months. Specifically, no grossly identifiable nodules could be detected in 15% (3/20) of the precancerous HBx transgenic mice, whereas 55% (11/20) of the mice contained only small 0.5 to 2.5 mm hyperplastic nodules and 15% (3/20) of the mice contained 3 to 6 mm hyperplastic nodules that were later pathologically confirmed to be benign tumors (Fig. 6D and Supplementary Fig. S8). Out of all the precancerous HBx transgenic mice, only 15% (3/20) developed HCC. This is a significant reduction after resveratrol treatment compared with the incidence of HCC in HBx transgenic mice that have not been treated with resveratrol, namely 15% compared with 80%, respectively, which is a 5.3-fold reduction of HCC in the resveratrol treated HBx transgenic mice compared with the control mice.

Discussion

The central finding in this work is that resveratrol has therapeutic effects on the early stages of HBx-mediated liver damage, reversing fatty changes, and producing a recovery in liver histopathology. Moreover, this study provides evidence for the first time that resveratrol at 30 mg/kg/d exerts a significant chemopreventive effect on HBx-mediated HCC. Specifically, resveratrol exhibits anticarcinogenesis properties by significantly decreasing cancer incidence and delaying the progression of spontaneous HCC in the HBx transgenic mice.

The molecular mechanism underlying the chemotherapeutic effects of resveratrol on HBx-induced fatty liver and liver damage seems to be attributable to the pleiotropic actions of resveratrol (summarized in Fig. 5). These actions involve the following. First, resveratrol inhibits lipogenesis by decreasing LXRα-Srebp1c signaling and, thereby, decreases the expression of its downstream target genes, Acc and Fas; this decrease in the LXRα-Srebp1c signaling is an early event observed 2 days after receiving resveratrol in the HBx transgenic mice. The inhibition of lipogenesis may also be attributable to an increase in the activities of Ampk and SirT1, which can be detected 7 days after receiving resveratrol in the HBx transgenic mice. Previously, the resveratrol-mediated increase Ampk and SirT1 activity has been documented to be associated with the alleviation of alcoholic fatty liver in mice (11). Second, resveratrol treatment leads to a transient stimulation of liver regeneration that helps to replace damaged hepatocytes in HBx transgenic mice. Importantly, the proliferation of hepatocytes in the HBx transgenic mice returned to the basal level of a resting adult liver when the animal had completely recovered from all the pathology and ultrastructure

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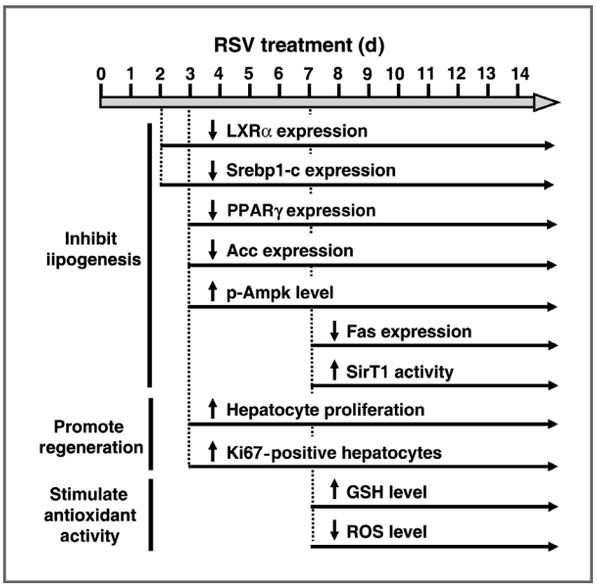

Figure 5. Summary of lipogenesis, liver regeneration, and antioxidant activity in HBx transgenic (TG) mice after resveratrol (RSV) treatment. The levels of gene expression related to lipogenesis, the results related to hepatocyte proliferation, and the level of antioxidant activity are summarized as a function of time after resveratrol treatment from 4- to 6-week-old.

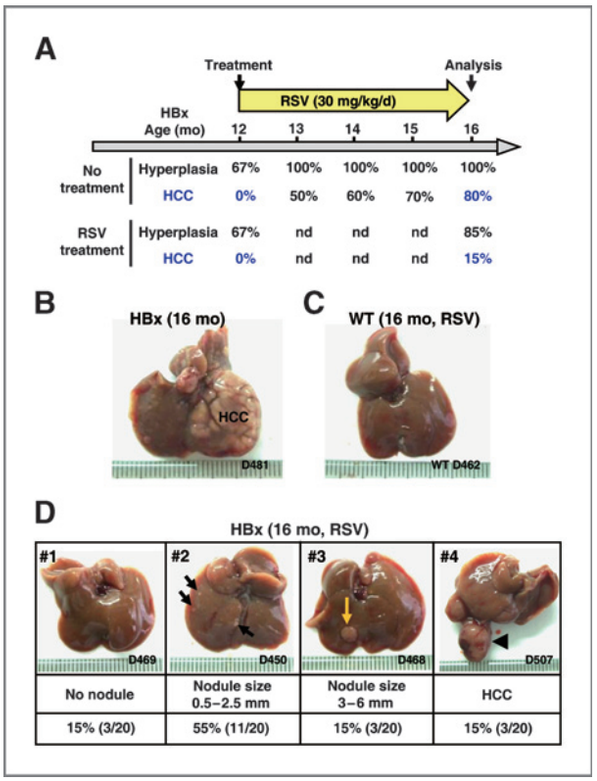

Figure 6. Resveratrol (RSV) delayed hepatocarcinogenesis and reduced HCC incidence in the precancerous stage of HBx transgenic (TG) mice. A, summary of the incidence of hyperplasia nodules and HCC in the HBx transgenic mice with or without resveratrol treatment. nd, not determined. B, a representative liver of an HBx transgenic mouse (16-month-old) without any treatment C, a representative liver of a WT mouse (16-month-old) with resveratrol treatment for 4 months. D, the 16-month-old HBx transgenic mice after resveratrol treatment for 4 months can be divided into 4 groups: group #1, no grossly identifiable nodules detected; group #2, livers contain small 0.5 to 2.5 mm hyperplastic nodules; group #3, livers contain 3 to 6 mm hyperplastic nodules; group #4, livers with HCCs. All of the hyperplastic nodules and HCCs were pathologically confirmed. Arrows indicate hyperplastic nodules; arrowhead indicates HCC.

abnormalities; this was after receiving resveratrol for 30 days. Third, resveratrol enhances antioxidant activity in the liver by, at least in part, increasing intracellular level of GSH. The increased GSH and decreased lipid content in the hepatocytes of HBx transgenic mice may both contribute to the reduction in the intracellular ROS after resveratrol treatment. Previous studies have revealed that resveratrol has antioxidative properties that can increase hepatic GSH and protect the liver against oxidative stress induced by partial hepatectomy (36) and CCl_4 intoxication (37). Here, we provided further evidence that resveratrol also protects the liver from oxidative damage mediated by HBx protein in mice.

It is well established that HCC develops in the presence of chronic liver diseases and is typically associated with fatty liver, fibrosis, cirrhosis from hepatitis virus infection (HBV and HCV), and/or alcoholic liver disease. In addition, there is increasing evidence to support the idea that fatty liver is one of the risk factors promoting the development of HCC in association with HBV and HCV (38). Accordingly, eliminating the risk factor of fatty changes and histopathologic damage by treating with resveratrol at an early stage should help to protect the liver against HBx-mediated carcinogenesis, and retard the progression to advanced liver disease and subsequent HCC at the later stage.

The overall safety of resveratrol has been documented in several *in vivo* studies. Resveratrol is well-tolerated and nontoxic in rodents from low doses (20 mg/kg/d) to high doses (up to 750 mg/kg/d) in a 28- or 90-day studies (39–41). Only at a very high dose (3,000 mg/kg/d) of resveratrol, which is at least 30 times the routine human dose (the dose as high as 7.5 g/d has been suggested for humans, which is equivalent to a dose of 100 mg/kg/d for a 75 kg person; ref. 7), did rats exhibit clinical signs of toxicity as well as a reduced body weight and food consumption after 4 weeks of receiving resveratrol. In addition, renal toxicity and nephropathy were also observed in these rats (42). In this study, our results revealed that oral administration of resveratrol at 30 mg/kg/d over a period of 4 months seems to have no obvious negative effect that is detrimental to the whole organism. In WT mice, the body weight and serum ALT level did not differ between mice treated with resveratrol and the control group over the whole treatment period. Moreover, histopathologic examination of the organs obtained at autopsy did not reveal any detectable alterations in the treated WT mice. These results provide *in vivo* evidence that chronic oral consumption of resveratrol at 30 mg/kg/d for up to 4 months does not adversely affect physiologic functioning.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Writing, review, and/or revision of the manuscript: T.-F. Tsai
Study supervision: C.-H. Kao, T.-F. Tsai
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Conducted part of the histopathology work and gene expression analyses: W.-H. Hsu
Conducted part of the mouse works and gene expression analyses and helped to prepare the figures and supplementary materials: Y-F. Chen
Conducted the gene expression analyses: C.-W. Yang
Conducted TEM analyses and helped to design the experiments: C.-H. Kao

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Cancer Prevention Research

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Hsiu-Ching Lin, Yi-Fan Chen, Wen-Hsin Hsu, et al.

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