

# Chemopreventive Effect of Silymarin on Liver Pathology in HBV X Protein Transgenic Mice

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## Abstract

There are currently limited therapeutic regimens available for effective treatment of hepatocellular carcinoma (HCC). Silymarin is a naturally derived polyphenolic antioxidant with hepatoprotective properties and is very widely used in clinical application; however, effect of silymarin on spontaneous HCC has not been studied. Silymarin was evaluated for its efficacy against spontaneous carcinogenesis using the HBV X protein (HBx) transgenic model. Silymarin was p.o. given to the HBx transgenic mice from 4 to 6 weeks of age. Our data indicated that silymarin has therapeutic effects on the early stages of liver damage, reversing fatty changes and recovering liver histopathology in a dose-dependent manner. To study the chemopreventive effects on the later stages of carcinogenesis, the mice at 13 months were split into a precancerous group and a group with significant liver carcinogenesis. After silymarin was given to the precancerous mice from 13 to 16 months of age, in contrast to an 80% incidence of HCC development in the untreated transgenic mice, no HCC was detected in any of these mice. Nonetheless, small hyperplastic nodules were detected in 86% of these precancerous mice. In the second group with notable HCC, silymarin was unable to block cancer progression. Although silymarin did not affect HBx expression, intracellular reactive oxygen species levels were decreased, cell proliferation was stimulated, and hepatocyte ultrastructure was found to significantly recover. In conclusion, silymarin exerts beneficial effects on the early stages of liver pathogenesis, preventing and delaying liver carcinogenesis. This drug should be considered as a potential chemopreventive agent for HBV-related hepatocarcinogenesis. [Cancer Res 2008;68(6):2033–42]

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common and recurrent malignancies worldwide. Many risk factors, including HBV infection, play a major role in the etiology of HCC (1). However, there are limited therapeutic regimens available for the effective treatment of liver cancer, and HCC is a disease with a very poor prognosis. The fact that HCC exhibits a high recurrent rate

after resection and is resistant to conventional chemotherapy and radiotherapy has rendered the disease a very serious health problem due to the lack of effective therapeutic options for HCC treatment at the current time (2–4). To reduce the morbidity and mortality of HCC, the discovery and development of chemopreventive and therapeutic agents for HCC is of paramount importance.

Oxidative stress is one of the key factors in tumorigenesis of many cancer types, including HCC. Reactive oxygen species (ROS) within cells act as second messengers in the signaling cascades that induce and maintain the oncogenic phenotypes of cancer cells (5). In addition, ROS can damage proteins, DNA, and RNA, as well as oxidize fatty acids in cell membrane, and these changes lead to an increased risk of mutation (6). Previous studies have shown that overexpression of the HBV large surface antigen (LsAg) and HBV X protein (HBx) leads to HCC development in transgenic mice (7, 8). Both LsAg and HBx induce oxidative stress in liver cells. Overexpressed LsAg accumulates in the endoplasmic reticulum, inducing high levels of oxidative endoplasmic reticulum stress and thus causes oxidative DNA damage and genomic instability (9, 10). In addition, previous studies have indicated that HBx protein directly interacts with mitochondrial membrane proteins and alters mitochondrial transmembrane potential in liver cells (11). Waris et al. further showed that, as a consequence of the association of protein with the mitochondria, HBx constitutively activates transcriptional factors STAT-3 and nuclear factor- $\kappa$ B via oxidative stress (12). The levels of intrahepatic mitochondrial ROS and lipid peroxide production are thus increased in hepatoma cell lines and transgenic mice expressing HBx protein. This indicates that oxidative injury occurs as a direct result of HBx expression (13). Accordingly, the induction of oxidative stress by these two viral proteins contributes to the liver pathogenesis associated with HBV infection (14) and, thus, can provide an experimental model for the investigation of antioxidant therapy.

Antioxidants are used to counteract the reactivity of ROS. The cellular oxidative stress can, therefore, be resolved by antioxidants. Silymarin, extracted mainly from the seeds of milk thistle (*Silybum marianum*), is a naturally derived polyphenolic flavonoid with hepatoprotective and antihepatotoxic properties (15). Silymarin is composed of five major flavonolignans: silybinin, isosilybinin, silychristin, silydianin, and taxifoline. Silybinin is the major component of silymarin, and many people have regarded this component as the most biologically active constituent of silymarin (16, 17). Silymarin, the mixture of flavonolignans, is a very widely used herbal medicine and dietary supplement in the treatment of alcoholic liver disease, acute and chronic viral hepatitis, and toxin-induced liver damage (18). As such, the effectiveness of silymarin as a treatment is highly important. Previous studies have indicated

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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that silymarin has strong antioxidative properties and acts as a free radical scavenger reducing free radicals, ROS, and lipid peroxidation in patients with alcoholic cirrhosis (19). Similar results have been found with diethylnitrosamine and ethanol-induced hepatotoxicity in rodents (20–22). Extensive analysis has shown that silymarin can suppress cancer cell proliferation and has anticarcinogenic effects on a range of cancers, such as prostate, breast, skin, colon, and bladder cancers, using cancer cell lines, xenograft, or carcinogen-induced tumors in rodents (23). However, although silymarin has been used in the treatment of liver diseases for a long time, its anticarcinogenic effects on HCC have not yet been well studied.

In the present study, we investigate the chemopreventive and therapeutic effects of silymarin on the hepatocarcinogenesis using the HBx transgenic mouse model, which was previously established in our laboratory. HBx transgenic mice spontaneously develop HCC at 13 to 16 months of age. The HCC developed in the HBx transgenic mice exhibits a well-differentiated morphology of the trabecular pattern. Fibrosis, bizarre nuclei, cytoplasmic lipid droplets and hyaline globules can be observed in the HBx-induced HCC samples similar to those observed in human HCC (24). The HBx transgenic mice thus provide an animal model for evaluating chemopreventive and new therapeutic agents for HCC under physiologic conditions.

## Materials and Methods

**HBx transgenic mice.** There are four lines of HBx transgenic mice, namely A105, A106, A110, and A112, that were generated in our laboratory in the C57BL/6 background (24). All of the HBx transgenic lines develop HCC. The chemoprevention experiments used male mice of the line A106, and the results were confirmed using the line A110. 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" (NIH publication 86-23 revised 1985) and strictly follow the rules issued by Institutional Animal Care and Use Committees of National Yang-Ming University.

**Silymarin administration.** Silymarin (Sigma) was dissolved in 0.5% sodium carboxymethylcellulose (Sigma). Various doses of silymarin, including 0 (carboxymethylcellulose vehicle control), 30, 100, and 300 mg/kg/d, were delivered to the mice daily through p.o. administration using a feeding needle. To study the therapeutic effects of the treatments on the early stage of liver pathogenesis, 4-wk-old HBx transgenic male mice and their wild-type male littermates were randomly assigned into different groups. Mice were sacrificed at 7 and 14 d after silymarin administration. To study the therapeutic effects of the treatments on the precancerous stage of liver carcinogenesis, 13-mo-old HBx transgenic male mice and their wild-type male littermates were used. Mice were p.o. given with silymarin (300 mg/kg/d) daily. Mice were sacrificed at 16-mo-old after silymarin administration for 3 mo. 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. Histopathologic and ultrastructural changes were examined by light and electron microscopy. The livers were collected, fixed with formalin, and embedded in paraffin. Liver sections were subjected to H&E staining (25). Ultrastructural changes in the liver were examined by transmission electron microscopy (TEM; ref. 26).

**Alanine aminotransferase assay.** Blood samples were obtained at various stages of the silymarin treatment. Serum alanine aminotransferase (ALT) values were analyzed by DRI-CHEM 3500s (FUJIFILM).

**Intracellular ROS and glutathione levels.** Primary hepatocytes were isolated from mouse livers using the two-step collagenase perfusion method (27). Intracellular ROS levels were determined by the use of an oxidative-sensitive fluorescence dye, dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes). Primary hepatocytes ( $1 \times 10^6$ ) were incubated with

DCF-DA in PBS, washed, and resuspended in PBS. The intensities of fluorescence in cells were analyzed using the FACS Vantage system at a wavelength of 526 nm. Intracellular glutathione levels were determined using a cell-permeable nonfluorescent dye monochlorobimane (Molecular Probes) that becomes highly fluorescent after reaction with intracellular glutathione. Primary hepatocytes ( $1 \times 10^6$ ) were incubated with monochlorobimane in PBS, washed, and resuspended in PBS. The intensities of fluorescence in cells were analyzed using a spectofluorometer with an excitation wavelength of 390 nm and emission wavelength of 460 nm.

**RNA analysis.** Total RNA was isolated from mouse tissues using TRIzol reagent (Life Technology). Slot blot hybridization was performed, as previously described (28). The hybridization signal was standardized against the intensity of the 28S rRNA. Real-time quantitative reverse transcription-PCR (RT-PCR) was performed using the Assays-on-Demand Gene Expression Product System from Applied Biosystems.

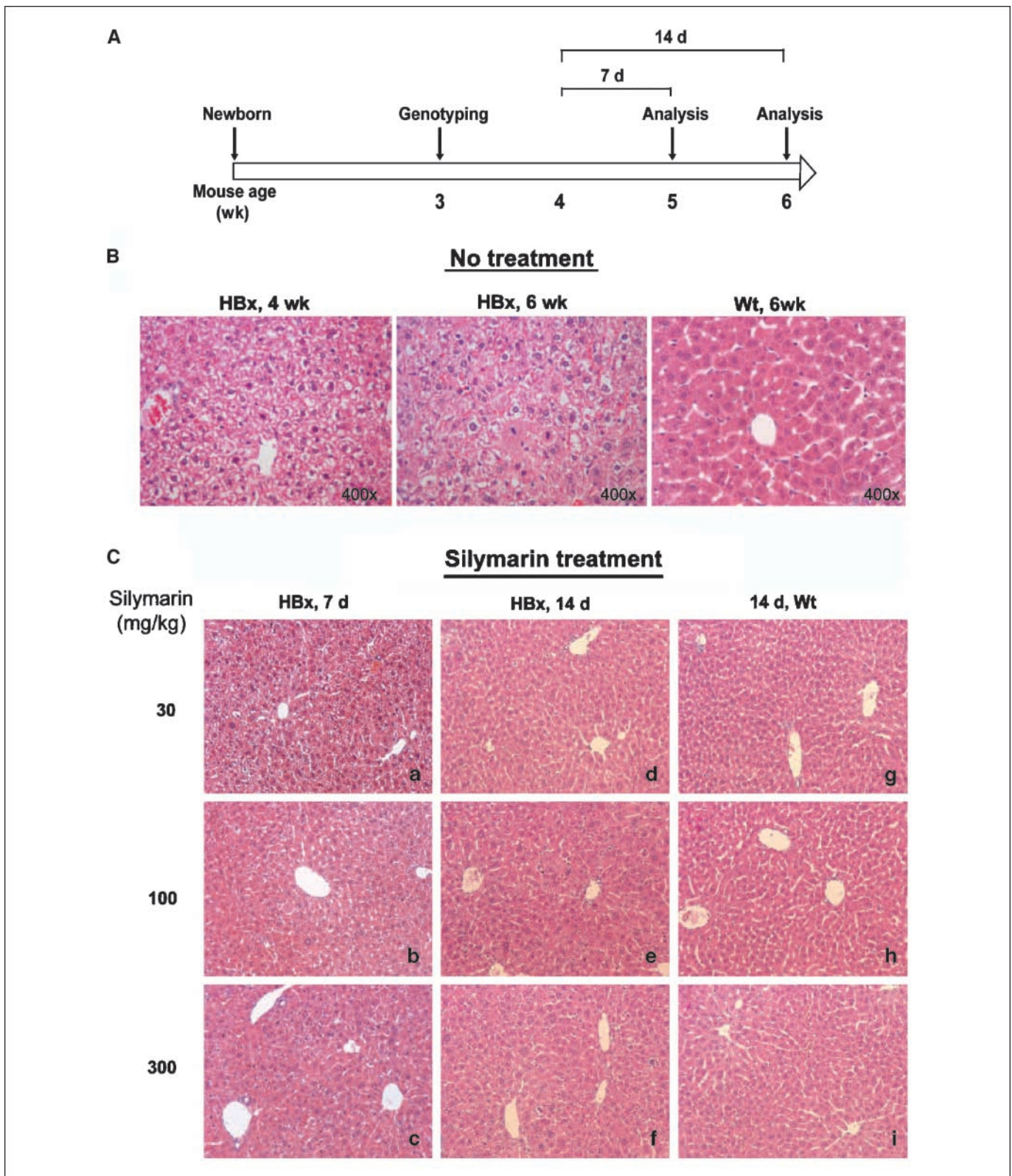
**Immunohistochemistry.** Immunohistochemistry detection of the HBx protein was performed using HBx polyclonal antibody (24). Monoclonal Ki67 antibody (B56) was purchased from BD PharMingen. Paraffin-embedded liver sections (3  $\mu$ m) were incubated with the primary antibody and detected with a LSABTM kit (DakoCytomation) according to the manufacturer's instructions.

**Statistical analysis.** Results are presented as mean  $\pm$  SD from at least three independent experiments. Differences among multiple groups were analyzed by a one-way ANOVA (SPSS 14.0 statistical software). Comparisons between two groups were done using a Student's *t* test. A *P* value of <0.05 was considered significant.

## Results

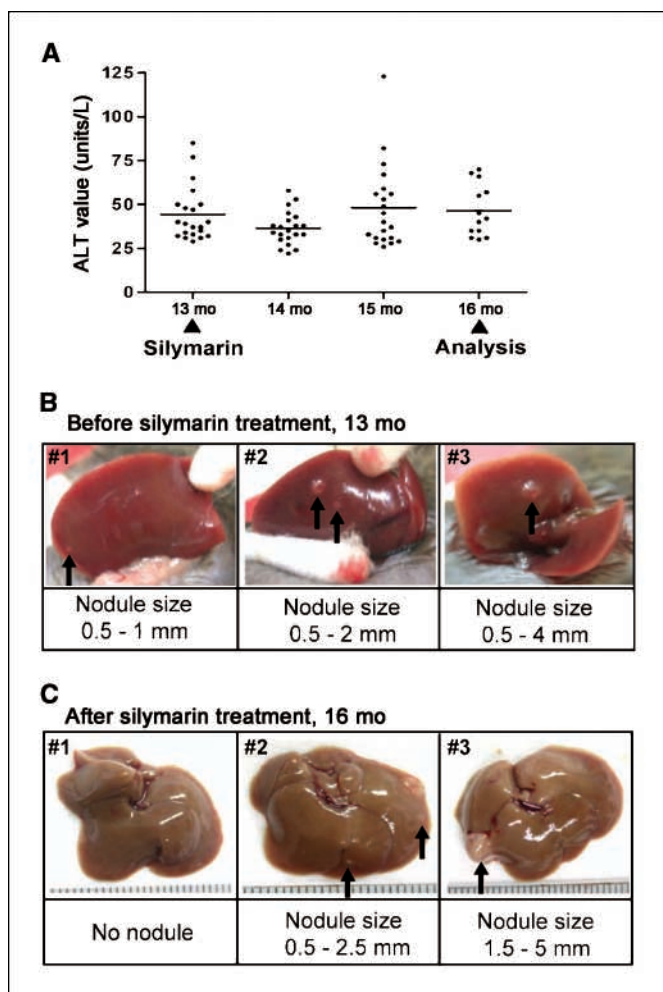
**Silymarin exerted beneficial effects on the early stages of liver damage and fatty changes.** The animal protocol for silymarin treatment is shown in Fig. 1A. Without any treatment, at an early stage of the HBx-mediated liver pathogenesis, namely 4 to 6 weeks of age, liver degeneration including fatty changes (microsteatosis), pleomorphic and bizarre nuclei, ballooning of the hepatocytes, and abnormal arrangement of the sinusoid are detectable in the HBx transgenic mice (Fig. 1B). Interestingly, p.o. administration of silymarin of various doses (0, 30, 100, and 300 mg/kg/d) from 4 to 6 weeks of age reduced liver damage and regressed the histopathology of the HBx transgenic mice in a dose-dependent manner (Fig. 1C). The HBx transgenic livers recovered to normal morphology, with either a high dose of silymarin (300 mg/kg/d) treatment for 7 days (Fig. 1C, *a-c*) or a low dose of silymarin (30 mg/kg/d) treatment for 14 days (Fig. 1C, *d-f*) compared with the wild-type control (Fig. 1C, *g-i*). The mean values of serum ALT observed for both wild-type and HBx transgenic mice are all in the basal level range (20–50 units/L) at this early stage. No obvious difference in the serum ALT values was detected in both HBx transgenic and wild-type mice with or without silymarin treatment (Supplementary Fig. S1), indicating that silymarin has no toxic effect on liver. Thus, our results showed that silymarin exerted therapeutic effects at an early stage of liver pathogenesis in the HBx transgenic mice.

**Silymarin regressed liver histopathology and prevented HCC formation during the precancerous stage of HBx-mediated carcinogenesis.** Because silymarin was found to have therapeutic effect on the early stage of liver pathogenesis, we further tested the preventive and/or therapeutic effects of silymarin on the later stage of HBx-mediated liver carcinogenesis. Silymarin (300 mg/kg/d) was p.o. given to HBx transgenic (line A106) and wild-type mice from 13 to 16 months of age. At 13 months of age, the HBx transgenic mice can be divided into two groups: one group (21 mice) had precancerous stage liver pathology and a low serum ALT value, and the other group (seven mice) had already developed HCC and

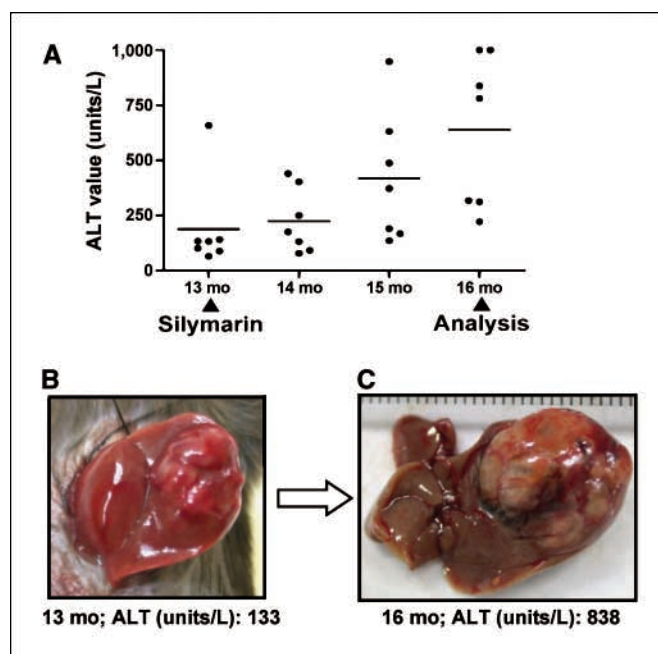


**Figure 1.** Beneficial effects of silymarin on early stages of liver damage and fatty changes. *A*, treatment protocol used with silymarin. Various doses (0, 30, 100, and 300 mg/kg/d) of silymarin were p.o. given to the HBx transgenic and wild-type (*Wt*) mice at 4 wk of age. The mice were sacrificed and analyzed after silymarin administration for 7 and 14 d. Six to ten mice per group were used. *B*, H&E staining of liver sections without any treatment for the HBx transgenic mice at 4 and 6 wk of age, as well as the wild-type control. Original magnification, 400 $\times$ . *C*, *a-c*, H&E staining of liver sections for the HBx transgenic mice given with 30, 100, and 300 mg/kg/d of silymarin for 7 d, respectively. *d-f*, H&E staining of liver sections for the HBx transgenic mice given with 30, 100, and 300 mg/kg/d of silymarin for 14 d, respectively. *g-i*, H&E staining of liver sections for the wild-type mice given with 30, 100, and 300 mg/kg/d of silymarin for 14 d, respectively. Original magnification, 200 $\times$ . The representative photomicrographs were prepared from HBx transgenic line A106.

relatively high ALT values. Among the precancerous mice with low-serum ALT values ( $44.3 \pm 15.5$ ,  $n = 21$ ) before silymarin treatment (Fig. 2A), the hyperplastic nodules resembling liver adenomas and measuring between 0.5 and 4 mm in diameter were detected in 100% of the mice in this precancerous group (Fig. 2B). Our previous data has revealed that there was an 80% incidence of HCC



**Figure 2.** Silymarin regressed the liver histopathology and prevented HCC formation during the precancerous stage of HBx-mediated carcinogenesis. **A**, serum ALT values of the HBx transgenic mice before and after silymarin administration. Silymarin (300 mg/kg/d) was p.o. given to the precancerous stage of HBx transgenic (line A106) and wild-type mice at 13 mo of age. Mice were sacrificed and analyzed after silymarin administration for 3 mo. The ALT values for the precancerous stage of HBx transgenic mice are  $44.3 \pm 15.5$  at 13 mo of age before silymarin treatment. The ALT values are  $36.5 \pm 9.5$ ,  $48.1 \pm 23.6$ , and  $46.5 \pm 14.9$  at 14, 15, and 16 mo of age after silymarin treatment for 1, 2, and 3 mo, respectively;  $n = 21$ . **B**, gross view of representative livers of the precancerous HBx transgenic mice at 13 mo of age before silymarin treatment. The gross liver morphology of the precancerous HBx transgenic mice and age-matched wild-type mice was examined after surgery before the start of silymarin treatment. The photographs show the size of hyperplastic nodules present in the HBx transgenic livers before silymarin treatment. **C**, gross view of representative livers of the precancerous HBx transgenic mice at 16 mo of age after silymarin treatment for 3 mo. The photographs show the size of hyperplastic nodules present in the HBx transgenic livers after silymarin treatment. No grossly identifiable nodules could be detected in 14% (3 of 21) of the HBx mice after treatment (representative liver #1); 81% (17 of 21) of the HBx transgenic mice contained small 0.5-mm to 2.5-mm hyperplastic nodules after treatment (representative liver #2); only one mouse (of 21; 5%) contained a hyperplastic nodule after treatment that was later pathologically confirmed as a benign tumor measuring 5 mm in diameter (representative liver #3). Arrows, hyperplastic nodules.



**Figure 3.** Silymarin is unable to block cancer progression after notable HCC nodule formation. **A**, serum ALT values of the HBx transgenic mice with HCC nodules at 13 mo of age before silymarin treatment. The ALT values for these HBx transgenic mice are  $171.8 \pm 199.6$  at 13 mo of age before silymarin treatment. The ALT values are  $224.6 \pm 146.4$ ,  $419.1 \pm 296.4$ , and  $638.7 \pm 342.6$  at 14, 15, and 16 mo of age after silymarin treatment (300 mg/kg/d) for 1, 2, and 3 mo, respectively;  $n = 7$ . **B**, gross view of a representative liver from one of the HBx transgenic mice with HCC nodules at 13 mo of age before silymarin treatment. The gross liver morphology of the HBx transgenic mice was examined after surgery before the start of silymarin treatment. **C**, gross view of the representative liver of the same mouse, as in **B**, at 16 mo of age after silymarin treatment (300 mg/kg/d) for 3 mo.

development in the HBx transgenic mice (line A106) at 16 months of age without any treatment (24). Interestingly, our results here showed that the serum ALT values (a measure of liver damage) did not increase over the period of treatment with silymarin (Fig. 2A) and that there was no HCC detected in any of the 21 precancerous mice at 16 months of age after silymarin treatment for 3 months (Fig. 2C). No grossly identifiable nodules could be detected in 14% (3 of 21) of the precancerous mice, whereas 81% (17 of 21) of these mice contained small (0.5–2.5 mm) hyperplastic nodules. Out of all the precancerous mice, only one (of 21; 5%) contained a hyperplastic nodule that was later pathologically confirmed as a benign tumor, measuring 5 mm in diameter (Fig. 2C). Histologic examination of these liver sections further confirmed that silymarin administration did indeed cause a regression of the liver morbid morphology and prevented abnormal histopathology in the HBx transgenic mice (Supplementary Fig. S2).

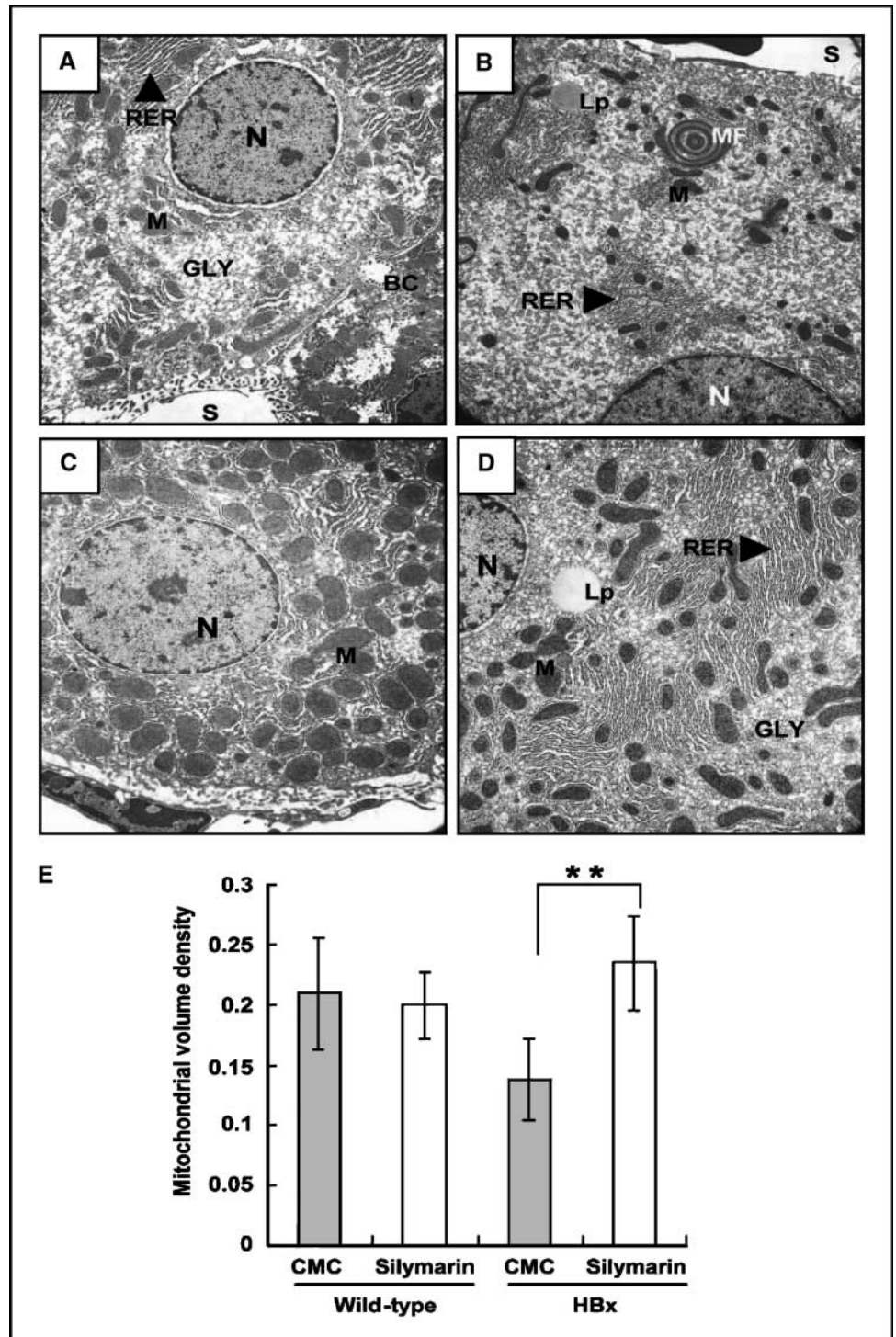
**Silymarin is unable to block cancer progression after notable HCC nodule formation.** For the mice with notable HCC nodule formation at 13 months of age, a relatively high-serum ALT value ( $171.8 \pm 199.6$ ,  $n = 7$ ) was detected (Fig. 3A), indicating significant liver damage. To test whether silymarin has therapeutic effects on liver carcinogenesis after HCC formation, silymarin (300 mg/kg/d) was p.o. given to these HBx transgenic mice (line A106) from 13 to 16 months of age. The serum ALT values gradually increased over the period of treatment (Fig. 3A) and the HCC nodules progressed to a larger size after 3 months of silymarin

treatment (Fig. 3B and C). These results indicated that silymarin has neither preventive nor therapeutic effects on liver carcinogenesis after notable HCC nodule formation.

**Hepatocyte ultrastructure was significantly recovered after silymarin treatment.** To study the efficacy of silymarin on hepatocytes ultrastructure, transgenic livers with 2 weeks of silymarin treatment (300 mg/kg/d) from 4 to 6 weeks of age were examined by TEM. In the wild-type mice, no ultrastructural abnormality was detected after treatment with vehicle for 2 weeks

(Fig. 4A). In the transgenic mice, without any treatment or treated with vehicle for 2 weeks, severe ultrastructural alterations were observed in the hepatocytes, including disorganization of rough endoplasmic reticulum, degeneration of mitochondria, and detection of myelin figures, which are the membranous debris of mitochondrion degeneration (Fig. 4B). After treatment with silymarin for 2 weeks, all the ultrastructural abnormalities were absent (Fig. 4C and D). Quantification further supported the recovery of mitochondrial volume density among the transgenic

**Figure 4.** Significant recovery of hepatocyte ultrastructure of the 6-week-old HBx transgenic mice given with silymarin (300 mg/kg/d) for 2 wk. **A**, TEM of hepatocytes of wild-type mice treated with vehicle for 2 wk. **B**, TEM of hepatocytes of HBx transgenic mice treated with vehicle for 2 wk. Severe ultrastructural alterations were observed in the HBx hepatocytes, including disorganization of rough endoplasmic reticulum (RER), degeneration of mitochondria (M), and detection of myelin figures (MF), which is the membranous debris of mitochondrion degeneration. **C**, TEM of hepatocyte of wild-type mice treated with silymarin for 2 wk. **D**, TEM of hepatocyte of HBx transgenic mice treated with silymarin for 2 wk. All the ultrastructural abnormalities of HBx transgenic hepatocytes were absent after silymarin treatment. *N*, nucleus; *GLY*, glycogen; *BC*, bile canaliculus; *S*, sinusoid; *Lp*, lipid. The representative data was prepared from HBx transgenic line A106. Original magnification, 12,000 $\times$ . **E**, recovery of the mitochondrial volume density of the HBx transgenic hepatocytes after silymarin treatment when compared with wild-type hepatocytes. Three to six mice per group were used for TEM examination. \*\*,  $P < 0.005$ .

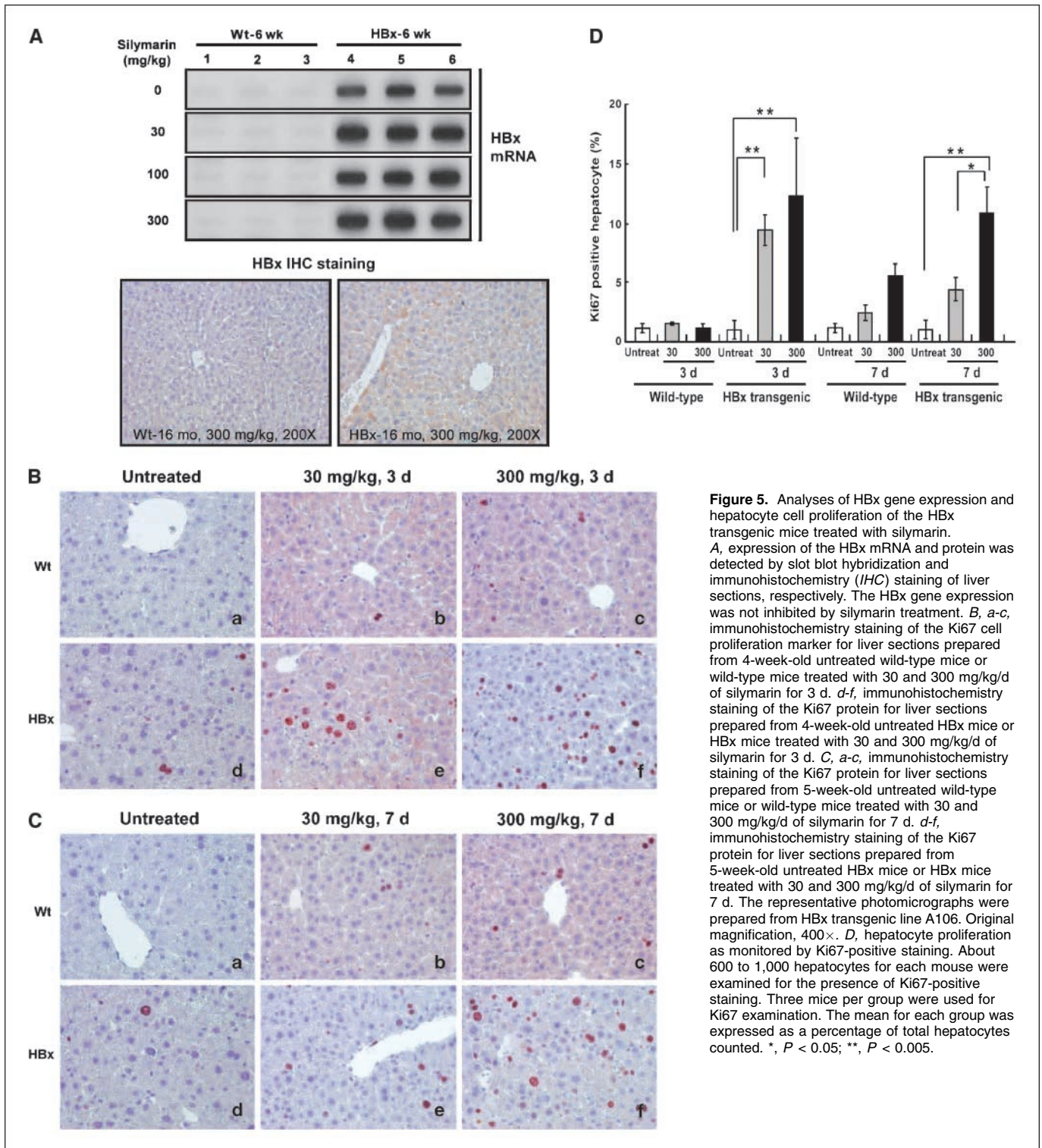


hepatocytes after silymarin treatment when compared with wild-type hepatocytes (Fig. 4E).

**HBx gene expression was not inhibited by silymarin.** To investigate whether HBx gene expression is affected by silymarin and whether this might contribute to the regression of morbid liver pathology after silymarin administration, the HBx mRNA and protein expression levels were detected by slot blot hybridization

and immunohistochemistry staining of liver sections, respectively. Our data revealed that expression of the HBx gene was not inhibited by silymarin either at an early stage (4–6 weeks of age) or at a precancerous stage (13–16 months of age) of the HBx transgenic mice (Fig. 5A).

**Silymarin stimulated hepatocyte cell proliferation leading to cell replacement in the HBx transgenic liver.** To study whether



**Figure 5.** Analyses of HBx gene expression and hepatocyte cell proliferation of the HBx transgenic mice treated with silymarin. *A*, expression of the HBx mRNA and protein was detected by slot blot hybridization and immunohistochemistry (IHC) staining of liver sections, respectively. The HBx gene expression was not inhibited by silymarin treatment. *B*, *a-c*, immunohistochemistry staining of the Ki67 cell proliferation marker for liver sections prepared from 4-week-old untreated wild-type mice or wild-type mice treated with 30 and 300 mg/kg/d of silymarin for 3 d. *d-f*, immunohistochemistry staining of the Ki67 protein for liver sections prepared from 4-week-old untreated HBx mice or HBx mice treated with 30 and 300 mg/kg/d of silymarin for 3 d. *C*, *a-c*, immunohistochemistry staining of the Ki67 protein for liver sections prepared from 5-week-old untreated wild-type mice or wild-type mice treated with 30 and 300 mg/kg/d of silymarin for 7 d. *d-f*, immunohistochemistry staining of the Ki67 protein for liver sections prepared from 5-week-old untreated HBx mice or HBx mice treated with 30 and 300 mg/kg/d of silymarin for 7 d. The representative photomicrographs were prepared from HBx transgenic line A106. Original magnification, 400 $\times$ . *D*, hepatocyte proliferation as monitored by Ki67-positive staining. About 600 to 1,000 hepatocytes for each mouse were examined for the presence of Ki67-positive staining. Three mice per group were used for Ki67 examination. The mean for each group was expressed as a percentage of total hepatocytes counted. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ .

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cell proliferation and liver regeneration were affected by silymarin, the Ki67 cell proliferation marker was examined by immunohistochemistry staining of liver sections prepared from 4-week-old to 5-week-old mice with silymarin treatment for 3 and 7 days. Our data indicated that Ki67-positive cells were obviously increased after silymarin administration (Fig. 5B and C). Quantification revealed that after silymarin treatment for 3 days, there was a 9-fold (transgenic mice treated with 30 mg/kg/d silymarin,  $9.37 \pm 1.3\%$ ) and 12-fold (transgenic mice treated with 300 mg/kg/d silymarin,  $12.21 \pm 5.0\%$ ) increase in the number of Ki67-positive hepatocytes of the HBx transgenic mice compared with the untreated transgenic mice ( $1.01 \pm 0.74\%$ ; Fig. 5D). After silymarin treatment for 7 days, there was a 4-fold (transgenic mice treated with 30 mg/kg/d silymarin,  $4.44 \pm 0.98\%$ ) and 11-fold (transgenic mice treated with 300 mg/kg/d silymarin,  $10.8 \pm 2.22\%$ ) increase in the number of Ki67-positive hepatocytes of the HBx transgenic mice compared with the untreated transgenic mice ( $1.01 \pm 0.74\%$ ; Fig. 5D). In wild-type mice, hepatocyte cell proliferation was not affected after silymarin treatment for 3 days, but there was a lower but significant stimulation after silymarin treatment for 7 days (Fig. 5D). These results suggested that the enhanced cell proliferation and liver regeneration induced by silymarin help to replace damaged cells in the HBx transgenic mice and this may contribute in part to the chemopreventive effect of silymarin on liver pathogenesis.

**Silymarin treatment caused a dose-dependent decrease in the ROS levels.** To study the antioxidative effect of silymarin on liver redox status, the intracellular ROS levels of the hepatocytes were monitored. Elevated level of ROS was detected at an early stage (5 weeks of age) of HBx-mediated liver carcinogenesis (Supplementary Fig. S3). The level of intracellular ROS in hepatocytes was even higher at 13.5 months of age (Supplementary Fig. S3). Our data clearly indicated that there are persistently increased levels of ROS during liver carcinogenesis of the HBx transgenic mice. The intracellular ROS levels of hepatocytes were examined at 6 weeks of age after 2 weeks of various doses (carboxymethylcellulose vehicle control, 30, 100, and 300 mg/kg/d) of silymarin treatment. Our results for two independent HBx transgenic lines, A106 and A110, clearly showed that silymarin, indeed, can efficiently reduce intracellular ROS levels in a dose-dependent manner under physiologic conditions (Fig. 6A).

**Differential expression of enzymes related to glutathione metabolism and liver functions after silymarin treatment.** RNA samples from 6-week-old HBx transgenic and wild-type mice with or without silymarin (300 mg/kg/d) treatment for 2 weeks were systematically compared for changes in gene expression by Affymetrix microarray. Genes related to antioxidation and liver functions were identified based on KEGG pathway database.<sup>7</sup> Previous reports indicated that silymarin exhibits antioxidant activity partly through restoration of intracellular glutathione levels and activation of antioxidation-related enzymes involved in drug-induced and carcinogen-induced hepatotoxicity (20, 29, 30). Indeed, the intracellular glutathione levels of hepatocytes were significantly increased after silymarin treatment (Fig. 6B). Furthermore, our real-time quantitative RT-PCR analysis revealed that transcripts of the enzymes involved in the glutathione metabolism, such as isocitrate dehydrogenase 2 (Idh2), Gst2, Gsto1, Gstk1, and Mgst3, were up-regulated by silymarin (Fig. 6C). Genes involved in

the glutamate metabolism (e.g., Got1, Gpt1, Gpt2, and Gls2), citrate cycle (e.g., Idh3a, Acl, Mdh2, and Fh1), and urea cycle (e.g., Asl and Arg1) were also up-regulated (Fig. 6C). All three of these pathways are indirectly related to the level of glutathione (Fig. 6D). Thus, the combined effects of the multiple pathway changes were related directly or indirectly to glutathione metabolism and liver functions, and these may all contribute to the reversion of liver pathophysiology and chemopreventive effect of silymarin on spontaneous carcinogenesis of HCC in the HBx transgenic mice.

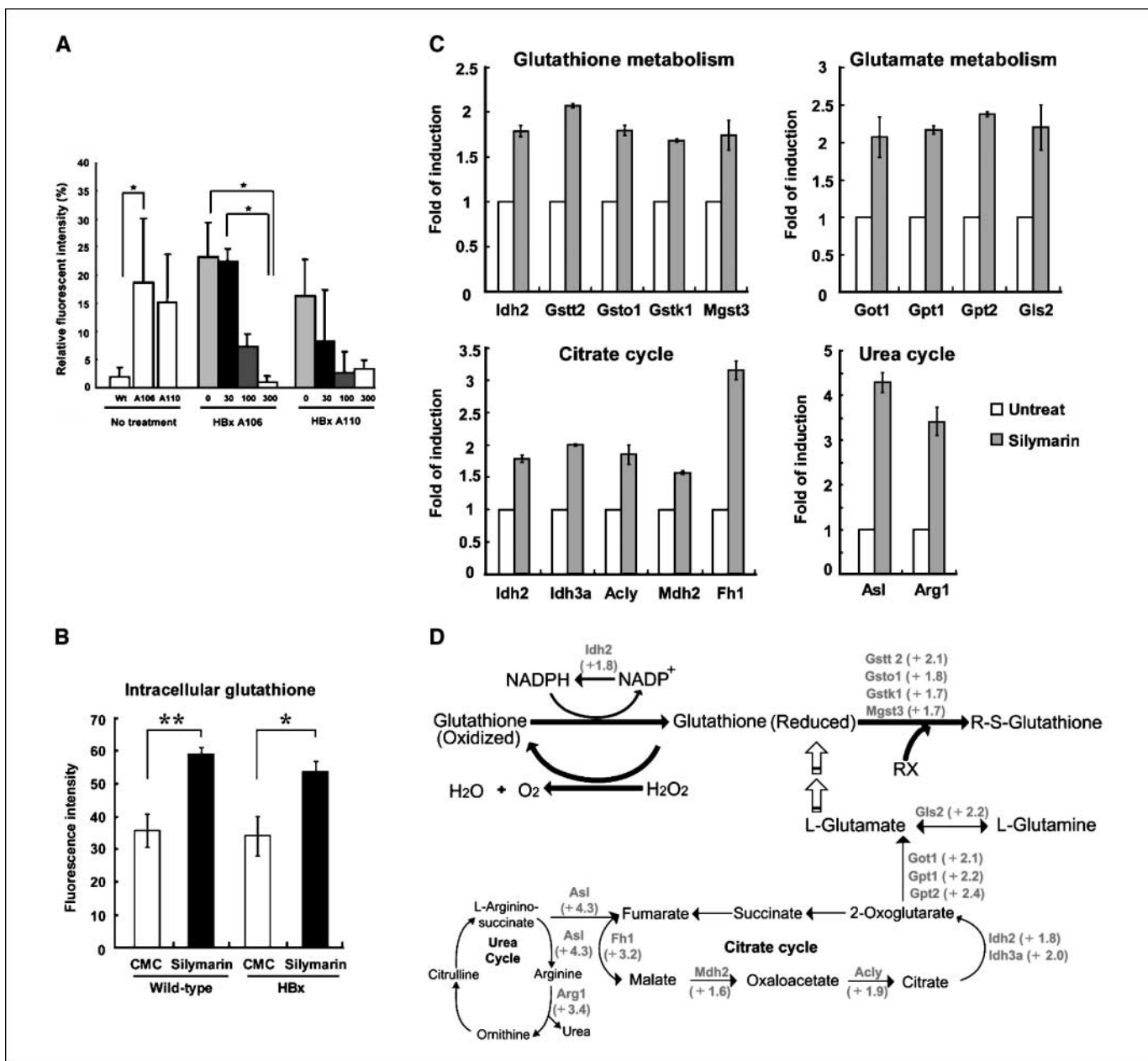
## Discussion

The potential benefits of silymarin in the clinical treatment of liver diseases remained a controversial issue. Our current studies provide *in vivo* evidence demonstrating that silymarin has therapeutic effects on the early stages of liver damage, involving the reversal of fatty changes and a regression of the liver morbid histopathology. Silymarin is composed mainly of silibinin and smaller amounts of a number of other stereoisomers (16). The silymarin used in the present study contains 55.1% silibinin, as analyzed by high-performance liquid chromatography (data not shown). Previous studies have indicated comparable effects for silymarin and silibinin on the antioxidative properties and growth inhibition of cancer cells (17, 31, 32). It is not clear at present whether one, more than one, or various combinations of silymarin components are the active agent of the *in vivo* efficacy of the mixture. We are currently working on the purification of individual components that are not commercially available, such as isosilybinin, silychristin, and silydianin from silymarin. Based on the results presented here showing that the mixture present in silymarin is active, it is important to test the efficacy of individual components and their different combinations using the HBx transgenic mouse model to study their individual chemopreventive effect on spontaneous HCC. The widespread medical use of silymarin as a chemopreventive agent creates a need to identify the active ingredient(s) of this useful mixture flavonolignans.

Regarding the antioxidative and radical scavenging properties, in addition to silibinin, there are other active constituents of silymarin, such as silydianin that can act as a free radical scavenger. On treatment with silydianin, decreased superoxide radical release in activated neutrophils, and lipid peroxidation in mouse spleen microsomes has been reported (33). Sersen et al. reported that silymarin, silychristin, and silydianin exhibit relatively good antioxidant ability against phenylglyoxylic ketyl radicals. The most effective scavengers of these free radicals were silymarin and silychristin, whereas silibinin was ineffective (34). The combined effectiveness of multiple antioxidants during the chemoprevention and therapy of the liver pathology may explain the efficacy of silymarin, which is a mixture of flavonolignans, including silychristin, silydianin, silibinin, and isosilybinin. It is possible that due to the variation in the free radical scavenging properties of the chemicals making up silymarin, the mixture may have synergic properties.

In the present study, we have shown that there are persistently increased levels of ROS during hepatocarcinogenesis of the HBx transgenic mice (Supplementary Fig. S3), and this may damage protein, DNA, and RNA leading to chronic cell death and induce liver regeneration. In addition, our previous study revealed that HBx protein blocks G<sub>1</sub>-S transition of hepatocyte cell cycle progression in the regenerating liver of the HBx transgenic mice (24). Accordingly, chronic hepatocyte cell death may cause repeated local liver repair leading to the acquisition of mutations

<sup>7</sup> <http://www.genome.jp/kegg/pathway.html>



**Figure 6.** Silymarin treatment caused a dose-dependent decrease of the ROS levels in the hepatocytes of the HBx transgenic mice. **A**, intracellular ROS levels of hepatocytes for HBx transgenic lines A106, A110, and wild-type mice at 6 wk of age after 2 wk of various doses (0, 30, 100, and 300 mg/kg/d) of silymarin treatment. Three to five mice per group were used for intracellular ROS examination. \*,  $P < 0.05$ . **B**, intracellular glutathione levels of hepatocytes for HBx transgenic (line A106) and wild-type mice at 5 wk of age after silymarin (300 mg/kg/d) or carboxymethylcellulose (vehicle) treatment for 3 d. Three mice per group were used for intracellular glutathione examination. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . **C**, differential expression of enzymes related to glutathione metabolism and liver functions after silymarin treatment. Real-time quantitative RT-PCR of genes related to glutathione metabolism, glutamate metabolism, citrate cycle, and urea cycle was performed using RNA isolated from 6-wk-old HBx transgenic mice with or without silymarin (300 mg/kg/d) treatment for 2 wk. **D**, summary of the up-regulated genes based on KEGG pathway database. Up-regulation of Idh2 facilitates the generation of NADPH and conversion of glutathione from oxidized to reduced form. Increase of reduced glutathione further facilitates the removal of  $H_2O_2$  from cells. Up-regulation of several glutathione *S*-transferases (Gst2, Gsto1, Gstk1, and Mgst3), which are involved in the metabolism of xenobiotic compounds, enhances the catalysis of conjugation of reduced glutathione to a wide variety of electrophilic substrates (RX organic compounds carrying a sulfate, nitrile, or halide group), leading to higher levels of water-soluble and removable derivatives. The urea cycle enzymes, Asl and Arg1, were up-regulated after silymarin treatment. These two enzymes will facilitate the generation of fumarate and urea. Fumarate further enters the citrate cycle and, together with the up-regulated levels of citrate cycle enzymes Fh1, Mdh2, Acly, Idh2, and Idh3a, leads to increased level of 2-oxoglutarate. The up-regulation of Got1, Gpt1, and Gpt2 further enhances the conversion of 2-oxoglutarate to glutamate and indirectly links to the glutathione metabolism. Mgst3, microsomal glutathione *S*-transferase 3; Gsto1, glutathione *S*-transferase  $\omega$ 1; Gstk1, glutathione *S*-transferase  $\kappa$ 1; Gst2, glutathione *S*-transferase  $\theta$ 2; Asl, argininosuccinate lyase; Arg1, arginase 1; Fh1, fumarate hydratase 1; Mdh2, malate dehydrogenase 2; Acly, ATP citrate lyase; Got1, glutamate oxaloacetate transaminase 1; Gpt1, glutamic pyruvic transaminase 1; Gpt2, glutamic pyruvate transaminase 2.

and enhancement of hepatocarcinogenesis. The present animal study clearly showed that silymarin can efficiently reduce intracellular ROS levels of hepatocytes, thus preventing oxidative stress-induced cellular damage. Furthermore, hepatocyte cell

proliferation was found to be stimulated after silymarin treatment for 3 and 7 days, suggesting that enhanced liver regeneration may help replace the damaged liver cells and contribute, at least in part, to the chemopreventive effect of silymarin.



Silymarin have been shown to inhibit cell growth and DNA synthesis in many types of cancer cells using mostly cell culture system (35, 36). However, the correlations between *in vitro* and *in vivo* systems have always been controversial. It is not clear whether some of the phenomena observed in simple cell culture systems can be related to what actually occurs *in vivo* under physiologic conditions. In addition, cell lines established from cancers with an accumulation of various genetic mutations and epigenetic alternations that cause uncontrolled cell proliferation may affect the response of cells to silymarin treatment when compared with noncancerous or precancerous cells when studied in a spontaneous hepatocarcinogenic animal model.

Regarding the anticarcinogenic effect of silymarin on HCC, previously, Varghese et al. reported the efficacy of silybinin against two human HCC cell lines (37). In their study, silybinin strongly inhibited growth of both Hep G<sub>2</sub> and Hep 3B cells. Silybinin also caused G<sub>1</sub> arrest in Hep G<sub>2</sub> and both G<sub>1</sub> and G<sub>2</sub>-M arrest in Hep 3B cells. Mechanistic studies revealed that silybinin induced Kip/p27 but decreased cell cycle-associated gene expression (37). Our *in vivo* efficacy study using the mouse model indicated that silymarin possesses effective cancer-preventive effects on spontaneous HCC and affords strong anticarcinogenic effects before HCC formation. However, silymarin is unable to block HCC progression and inhibit cancer cell proliferation after accumulation of multiple irreversible genetic alternations/mutations *in vivo*. A similar animal study using silymarin involving stage-specific phenomenon has been reported for a mouse skin carcinogenesis model (38, 39). Agarwal's group reported that the anticancer effect of silymarin is primarily targeted against stage I tumor promotion in mouse skin with significant inhibition of TPA-induced lipid peroxidation. In terms of any preventive effect on stage II tumor promotion, silymarin shows only a very limited effect on the tumor incidence of carcinogen-induced skin cancers (38, 39). In the *N*-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis model, low effectiveness was observed in rats posttreated with silymarin after administration of NDEA for 10 weeks compared with rats pretreated with silymarin in terms of the number and size of the HCC tumors (40), suggesting that silymarin may not block/inhibit the later stages of HCC progression.

Previous studies have revealed that HBV infection is associated with an increased production of ROS within the liver and that this is responsible for the oxidation of intracellular molecules and activation of genes related to oxidative stress. Valgimigli et al. reported that ROS levels in HBV chronic hepatitis patients were higher than in healthy controls by radical probe electron paramagnetic resonance measurements of human liver biopsy specimens (41). In addition, induction of HBV replication in human hepatoma HepAD38 cells, in which HBV production is under the

control of a tetracycline-regulated promoter, led to up-regulation of genes related to oxidative stress (42). The fact that the liver histopathology (Fig. 1C) and ROS levels (Fig. 6A) were decreased in a dose-dependent manner in the silymarin-treated HBx transgenic mice provides solid evidences for the antioxidative function of silymarin during HBV-associated pathogenesis. Our real-time quantitative RT-PCR analysis revealed that the molecular mechanism of silymarin-mediated antioxidative effect may involve genes related directly or indirectly to glutathione metabolism (Fig. 6C and D). Up-regulation of *Idh2* facilitates the generation of NADPH and conversion of glutathione from its oxidized to its reduced form. The increase in reduced glutathione further facilitates the removal of H<sub>2</sub>O<sub>2</sub> from cells. In addition, up-regulation of several glutathione *S*-transferases (*Gstt2*, *Gsto1*, *Gstk1*, and *Mgst3*), whose functions are involved in the conjugation reaction of glutathione to diverse electrophilic substrates, reveals the presence of a higher concentration of reduced glutathione. In addition, genes up-regulated by silymarin and involved in the glutamate metabolism, urea cycle, and citrate cycle may all indirectly contribute to the increase of glutathione level and reversion of liver pathophysiology.

Milk thistle is the dietary supplement taken most frequently by patients with chronic liver diseases. This supplement is one of the most widely used herbal medicines and has been for a long time. Both milk thistle and its active ingredient silymarin are pharmacologically safe and well tolerated (23, 43, 44). The present study further provides *in vivo* evidence demonstrating that silymarin is a safe and effective antioxidant drug in an animal model system. Furthermore, the fact that silymarin effectively regresses morbid liver pathology and recovers liver structure and hepatocyte ultrastructure, as well as prevents and delays HBx-mediated carcinogenesis, suggests that silymarin is a potential chemopreventive agent for HBV-related hepatocarcinogenesis. The effectiveness of the mixture of flavonolignans known as silymarin on HCC prevention as modeled using HBx transgenic mice in this study is an important step forward in identifying how and why silymarin works.

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