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The phosphorylated retinoid X receptor-α promotes diethylnitrosamine-induced hepatocarcinogenesis in mice through the activation of β-catenin signaling pathway

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Abstract

Previous studies have shown that phosphorylation of the retinoid X receptor-α (RXRα) is associated with the development of hepatocellular carcinoma (HCC). However, these findings were revealed using HCC cell lines that express phosphorylated-RXRα (p-RXRα) proteins; therefore, it remains unclear whether p-RXRα affects hepatocarcinogenesis *in vivo*. Therefore, to investigate the biological function of p-RXRα *in vivo*, we developed a doxycycline-inducible ES cell line and transgenic mouse, both of which overexpress the phosphomimetic mutant form of RXRα, *T82D/S260D*, in a doxycycline-dependent manner. We found that the development of liver tumors, especially high-grade adenoma and HCC, was enhanced in diethylnitrosamine (DEN)-treated *T82D/S260D*-inducible mice. Moreover, the increased incidence of liver tumors in the transgenic mice was attributable to the promotion of cell cycle progression. Interestingly, the expression of β-catenin protein and its target gene *cyclin D1* was elevated in the liver tumors of DEN-treated *T82D/S260D*-inducible mice, concurrent with increased cytoplasmic and nuclear β-catenin protein expression, indicating its stabilization and transcriptional activation. These results indicate that p-RXRα promotes DEN-induced hepatocarcinogenesis in mice through the activation of the β-catenin signaling pathway, suggesting that p-RXRα may serve as a possible therapeutic target for HCC.

Abbreviations: APC, adenomatous polyposis coli; DEN, diethylnitrosamine; DOX, doxycycline; HCC, hepatocellular carcinoma; LEF, lymphoid enhancer factor; PCNA, proliferating cell nuclear antigen; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; RARs, retinoic acid receptors; RXRα, retinoid X receptor-α; TCF, T-cell factor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide; more than half a million people are diagnosed with HCC annually (1). The development of HCC is generally associated with chronic liver inflammation and subsequent cirrhosis. Cirrhosis is a well-established major risk factor for HCC, irrespective of the underlying liver disease (1). Surveillance for patients at high risk of HCC has increased the possibility of early diagnosis and curative treatments, including surgical resection and percutaneous ablation, thereby providing long-term control for patients with early-stage HCC (2,3). In addition, treatment with molecularly targeted agents such as sorafenib, lenvatinib and regorafenib has been reported to improve the survival of patients with advanced HCC (4–6). Despite the progress in diagnosis and treatment strategies, the prognosis for patients with HCC remains poor owing to its high recurrence rate (70% of cases at 5 years), which is attributed to either intrahepatic metastasis or the development of *de novo* tumors (2). Moreover, there are currently no established agents for the curative treatment of this malignancy (2). Therefore, to improve the prognosis of patients with HCC, the underlying molecular mechanisms of hepatocarcinogenesis need to be clarified, and more effective chemopreventive and chemotherapeutic strategies are needed.

Retinoids, a derivative of vitamin A, are physiological signaling molecules that regulate cell proliferation, tissue differentiation and organism development primarily through two distinct nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which are composed of three subtypes (α, β and γ) (7). Notably, abnormalities in the expression and function of these receptors are associated with the development of various malignancies, including HCC (8). RXRα can bind to the enhancer element of HBV (9). In addition, the RARα gene is located near one of the integration sites of HBV and its expression is induced in HBV-related HCC (10). In addition, genetic variants of RXR have been associated with the risk of hepatitis C virus (HCV) infection chronicity among the Chinese population with a high risk of HCC (11). Thus, abnormal expression and mutations of RXR and RAR genes have been identified

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in HBV- or HCV-related HCC. Moreover, altered expression of RARs has been reported to be associated with malignant transformation of animal tissues or cultured cells (12,13). Our previous studies have reported that a malfunction of RXRα due to its aberrant phosphorylation plays a role in the development of HCC (14,15). In HCC cell lines, RXRα is constitutively phosphorylated at both threonine 82 and serine 260 owing to the activation of Ras/MAPK signaling (14), and it accumulates by avoiding ubiquitination and proteasome-mediated degradation (15). The accumulation of phosphorylated-RXRα (p-RXRα) protein interferes with the functioning of the remaining normal RXRα in a dominant-negative manner, thereby promoting the development of HCC cells (14). In contrast, acyclic retinoid, a synthetic retinoid that can inhibit the phosphorylation of RXRα and restore its receptor function (16), has been reported to inhibit the growth of human hepatoma cells (16–19). Thus, our previous *in vitro* studies have revealed the important role of p-RXRα in hepatocarcinogenesis.

Although our previous studies showed that p-RXRα is associated with the development of HCC cell lines, the effects of p-RXRα expression on hepatocarcinogenesis *in vivo* remain unknown. Therefore, in the current study, we established a phosphomimetic mutant form of human RXRα, *T82D/S260D*, transgenic mice using a doxycycline (DOX)-dependent expression system to investigate the role of *T82D/S260D* expression in hepatocarcinogenesis in mice.

Materials and methods

Molecular cloning and gene targeting in ES cells

A phosphomimetic mutant form of human RXRα cDNA, *T82D/S260D*, in which threonine 82 and serine 260 were mutated to aspartate, respectively, was generated as described previously (14), and then cloned into pcr2.1-TOPO. Sequence-verified *T82D/S260D* cDNA was subcloned into a unique EcoR1 site of the pBS31 prime vector (20,21). KH2 ES cells obtained from Open Biosystems, Huntsville, AL were used to insert a single copy of T82D/S260D by flippase recombination into the *Col1A1* locus under the control of a minimal CMV tetracycline-inducible promoter using a previously described method (20), and ES cells were selected for hygromycin resistance.

Mouse generation

Fertilized zygotes were isolated from the oviducts of day-0.5 pregnant B6D2F1 females and allowed to develop to the blastocyst stage in culture. Subsequently, 7-12 ES cells were injected into the blastocysts, which were then transferred into day-2.5 pseudo-pregnant females.

Doxycycline treatment

Mice were administered 2 mg/ml (wt/vol) DOX (D9891; Sigma–Aldrich, St. Louis, MO) in their drinking water, which was supplemented with 10 mg/ml sucrose. For culture cells, DOX was used at a concentration of 2 μg/ml (w/v).

RNA preparation and quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted using the RNAqueous-4PCR kit (Ambion, Carlsbad, CA) according to the manufacturer's instructions. Thereafter, cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis using the fluorescent SYBR green method was performed according to previously described protocols (22). The expression of each gene was normalized to that of β-actin using the standard curve method. Primer sequences are shown in Supplementary Table S1, available at *Carcinogenesis* Online.

Western blotting analysis

Western blotting analysis was performed as described previously (22). The following primary antibodies were used: anti-RXRα (rabbit IgG, 1:500 dilution, sc-553; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-β-catenin (mouse IgG, 1:8000 dilution, #610154; BD Transduction, San Jose, CA), anti-Rb (mouse IgG, 1:500 dilution, #554136; BD Pharmingen, San Jose, CA), anti-cyclin D1 (rabbit IgG, 1:1000 dilution, sc-753; Santa Cruz Biotechnology), anti-proliferating cell nuclear antigen (PCNA) (mouse IgG, 1:2000 dilution, #2586; Cell Signaling Technology, Danvers, MA) and anti-GAPDH (rabbit IgG, 1:2000 dilution, #2118; Cell Signaling Technology).

Animals and experimental procedures

Heterozygous *Rosa26::M2rtTA* mice with heterozygous *tetO-T82D/S260D* allele (*Rosa/+; Rxr/+*) were generated by breeding homozygous *Rosa26::M2rtTA* male mice with homozygous *tetO-T82D/S260D* allele (*Rosa/Rosa; Rxr/Rxr*) to C3H/HeN females without the *Rosa26::M2rtTA* allele or *tetO-T82D/S260D* allele (SLC Japan, Shizuoka, Japan). In contrast, heterozygous *Rosa26::M2rtTA* mice (*Rosa/+*), which served as a control for *Rosa/+; Rxr/+* mice, were generated by breeding homozygous *Rosa26::M2rtTA* (*Rosa/Rosa*) males to C3H/HeN females without *Rosa26::M2rtTA* or *tetO-T82D/S260D* allele (SLC Japan) (Supplementary Figure S1A, available at *Carcinogenesis* Online). First, to investigate the spontaneous development of liver tumors *in vivo*, *T82D/S260D*-inducible male mice (*Rosa/+; Rxr/+*) and control male mice (*Rosa/+*) were administered tap water containing 2 mg/ml (wt/vol) DOX (D9891; Sigma–Aldrich) starting at 4 weeks of age and killed at either 6 (Supplementary Figure S1B, available at *Carcinogenesis* Online) or 8 (Supplementary Figure S1C, available at *Carcinogenesis* Online) months of age for both macroscopic inspection and histological analysis. Next, to induce liver tumors, the liver carcinogen diethylnitrosamine (DEN) (442687; Sigma–Aldrich) dissolved in 0.9% saline was administered intraperitoneally (25 mg/kg of body weight) to 15-day-old male pups, which were obtained from each breeding pair described above. The DEN-treated *T82D/S260D*-inducible male mice (*Rosa/+; Rxr/+*) and DEN-treated control male mice (*Rosa/+*) were given tap water containing 2 mg/ml (wt/vol) DOX from 4 weeks to 6 months of age. At 6 months of age, both genotypes of mice were killed for both macroscopic inspection and histological analysis (Supplementary Figure S1D, available at *Carcinogenesis* Online). All mice received humane care and were housed at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. All animal experiments were approved by the Institutional Committee on Animal Experiments of Gifu University.

Macroscopic inspection, histopathologic analysis, immunohistochemical analysis and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assay

After killing the mice, the livers were immediately removed and weighed, and the number of hepatic tumors on the surface of the left lobe with diameter ≥1 mm was macroscopically counted. Maximum sagittal sections of three sublobes (left lateral, medial and right medial lobes) were histopathologically examined. Four-micrometer-thick sections of 10% buffered formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin for conventional histopathology. The numbers and the histological grades of liver tumors were evaluated microscopically according to previously described criteria by which liver tumors were classified as Grade 1, 2 or 3 adenomas and HCC depending on the atypical degree (23). Per these criteria (23), liver tumors were defined as Grade 1: the lesion showed a focal trabecular pattern with single cell plates; Grade 2: the lesion showed a prominent trabecular pattern with plates of two cell layers, slight cellular pleomorphism and increased cell size; Grade 3: the lesion was composed of prominent abnormal trabeculation with more than two cell layers, increased N/C ratio, high mitotic rates and marked cellular pleomorphism; and HCC: the lesion showed features similar to that of Grade 3, but necrosis was present with no visible remnant of adenoma (Supplementary Figure S2A, available at *Carcinogenesis* Online). In addition, foci of cellular alteration (FCA), which are hepatic preneoplastic lesions with a basophilic cytoplasm and hyperchromatic nuclei (24), were also evaluated (Supplementary Figure S2B, available at *Carcinogenesis* Online). Immunohistochemistry was performed using an avidin–biotin immunoperoxidase assay, according to a previously described protocol (22). The following primary antibodies were used: anti-PCNA (rabbit IgG, 1:100 dilution, ab2426; Abcam, Cambridge, MA), anti-cleaved caspase 3 (rabbit IgG, 1:100 dilution, #9661; Cell Signaling Technology) and anti-β-catenin (mouse IgG, 1:500 dilution, #610154; BD Transduction). Apoptotic cells in the liver were evaluated using the ApopTag peroxidase *in situ* apoptosis detection kit (#S7100; Millipore, Billerica, MA), which labels DNA strand breaks by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). The mean ± standard deviation (SD) was calculated for all parameters determined. Statistical significance was evaluated using either Student's *t*-test or Welch's *t*-test for paired samples. Statistical significance was set at $P < 0.05$.

Results

Inducible expression of a phosphomimetic mutant form of the human RXRα (*T82D/S260D*) in ES cells and mice

We generated DOX-inducible *T82D/S260D* ES cells, in which a phosphomimetic mutant form of the human RXRα gene, *T82D/S260D*, can be induced under the control of a tetracycline-responsive regulatory element (Figure 1A). Upon treatment of these ES cells with DOX, the expression of *T82D/S260D* mRNA was significantly increased compared with that in ES cells without DOX treatment (Figure 1B). Increased expression of T82D/S260D protein, which was evaluated using an anti-RXRα antibody, was also confirmed in DOX-treated ES cells (Figure 1C). Next, we administered DOX to *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+) to confirm the expression of the phosphomimetic mutant form of human RXRα, *T82D/S260D*, in the liver. As shown in Figure 1D and E, the administration of DOX induced a time-dependent increase in *T82D/S260D* mRNA and protein expression in the liver, indicating that the phosphomimetic mutant form of human RXRα was effectively induced in these transgenic mice by the administration of DOX.

T82D/S260D-inducible mice are highly susceptible to the development of DEN-induced liver tumors

We investigated the effects of a phosphomimetic mutant form of human RXRα, *T82D/S260D,* on the spontaneous development of liver tumors *in vivo*. Initially, both *T82D/S260D*-inducible male mice (*Rosa*/+; *Rxr*/+) and control male mice (*Rosa*/+) were administered DOX starting at 4 weeks of age and were killed at 6 months of age (Supplementary Figure S1B, available at *Carcinogenesis* Online). Macroscopic inspection and microscopic analysis revealed no development of liver tumors in both genotypes of mice, and histological findings of the liver were comparable between both genotypes (data not shown). We then extended DOX treatment up to 8 months of age (Supplementary Figure S1C, available at *Carcinogenesis* Online); however, the development of liver tumors was not observed in either group (data not shown). To induce liver tumors, we administered the liver carcinogen DEN (25 mg/kg of body weight) intraperitoneally to 15-day-old male pups of each group and started DOX treatment at 4 weeks of age, and killed the mice at 6 months of age (Supplementary Figure S1D, available at *Carcinogenesis* Online). As shown in Table 1 and Figure 2, the number and maximum size of macroscopic liver tumors were significantly increased in DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+) compared with those in DEN-treated control mice (*Rosa*/+). In addition, microscopic analysis revealed that the number of high-grade liver tumors, including grade 3 adenoma and HCC, was significantly increased in DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+) (Table 2). No signs of liver cirrhosis, such as pseudolobule formation and liver fibrosis, were observed in either mice genotype (data not shown). Thus, these results indicate that a phosphomimetic mutant form of human RXRα, *T82D/S260D,* by itself, does not initiate liver tumorigenesis, but promotes hepatocarcinogenesis following the administration of DEN.

Development of liver tumors in DEN-treated *T82D/S260D*-inducible mice is primarily attributed to the promotion of cell proliferation

The retinoid receptor RXRα plays a role in maintaining homeostasis by regulating fundamental cell activities, including cell proliferation and apoptosis (7,25). Previous studies have shown that RXRα loses its receptor function by undergoing phospho-modifications, which are associated with hepatocarcinogenesis *in vitro* (14,17–19,26,27). We then investigated the levels of cell proliferation and apoptosis in liver tumors developed in either DEN-treated

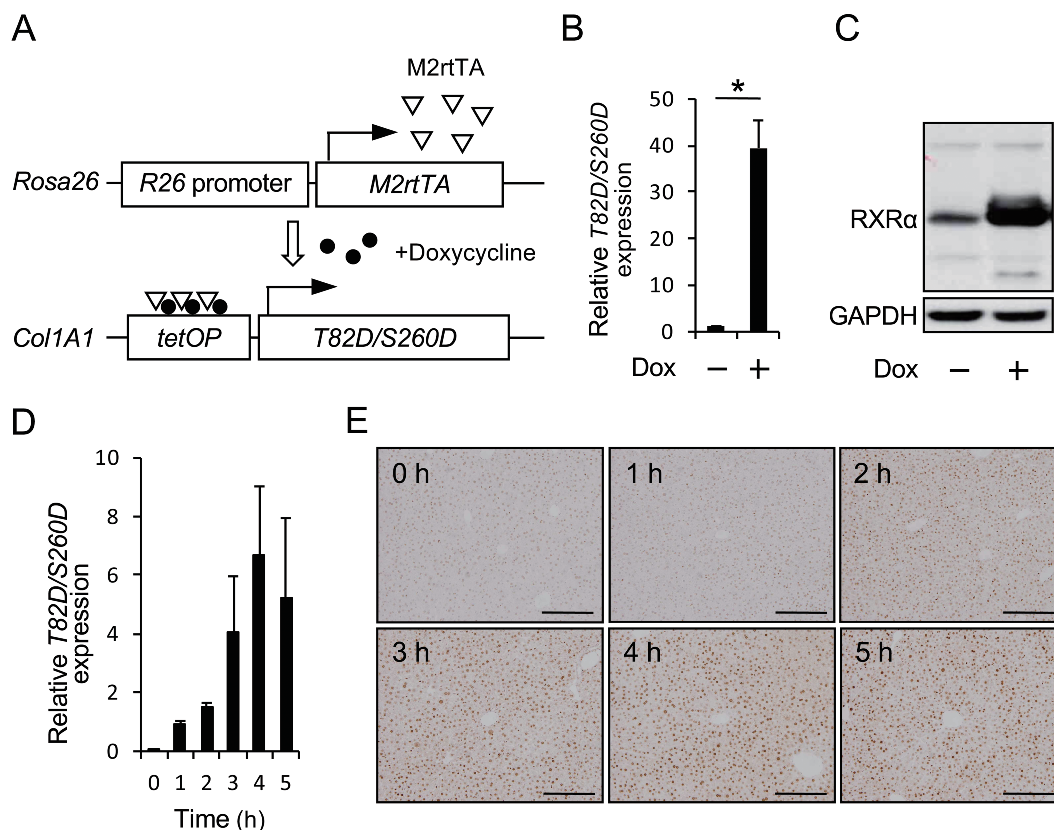

Figure 1. Inducible expression of *T82D/S260D*. (**A**) Schematic of the doxycycline-inducible *T82D/S260D* alleles. (**B**) The expression of *T82D/S260D* mRNA in *T82D/S260D*-inducible ES cells was detected by qRT-PCR using specific primers. The administration of doxycycline (2 µg/ml in culture medium) for 12 h significantly induced *T82D/S260D* mRNA expression in the ES cells. Transcript levels were normalized to that of β-*actin*. Data are presented as mean ± SD (n = 3). *P < 0.05, Student's t-test. (**C**) T82D/S260D protein expression in *T82D/S260D*-inducible ES cells was evaluated by western blotting analysis using an anti-RXRα antibody, as there is no specific antibody for the T82D/S260D protein. The administration of doxycycline (2 µg/ml in culture medium) for 24 h induced RXRα protein expression in the ES cells. GAPDH served as the loading control. Representative images of three independent experiments are shown. (**D**) The expression of *T82D/S260D* mRNA in the liver of *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) was detected by qRT-PCR using specific primers. The administration of doxycycline (2 mg/ml in drinking water) induced *T82D/S260D* mRNA expression in the liver in a time-dependent manner after starting the treatment. Transcript levels were normalized to that of β-*actin*. Data are presented as mean ± SD (n = 3). (**E**) The expression and localization of T82D/S260D protein in the liver of *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) were analysed by immunohistochemical analysis using an anti-RXRα antibody. The administration of doxycycline (2 mg/ml in drinking water) induced T82D/S260D protein expression in a time-dependent manner, and the protein was primarily localized in the nucleus of liver cells. Representative images of RXRα-stained liver sections are shown. Scale bar, 200 µm.

Table 1. Experimental data of macroscopic liver tumors observed in each genotype of mice

Genotype	No. of mice	Incidence (%)	Multiplicity[a]	Max. size (mm)	LW/BW (%)
Control mice (Rosa/+)	21	21/21 (100)	8.1 ± 7.7[b]	2.9 ± 1.2	5.0 ± 0.8
T82D/S260D-inducible mice (Rosa/+; Rxr/+)	19	19/19 (100)	33.6 ± 12.0[c]	5.4 ± 2.5[c]	6.9 ± 1.3[c]

BW, body weight; LW, liver weight; Max., maximum; No., number.
[a]Number of tumors per mouse.
[b]Mean ± SD.
[c]Significantly different from control mice (*Rosa*/+) by unpaired t-test with Welch's correction (P < 0.01).

T82D/S260D-inducible mice (*Rosa/+; Rxr/+*) or DEN-treated control mice (*Rosa/+*). Immunohistochemistry for PCNA showed that the percentage of PCNA-positive cells per liver tumor was significantly increased in DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) compared with that in DEN-treated control mice (*Rosa/+*) (Figure 3A). In contrast, the levels of apoptosis, as ascertained by immunohistochemical staining of tumor sections with TUNEL and cleaved caspase 3, were identical in both genotypes of mice (Figure 3B). The expression of the anti-apoptotic *Bcl-2* and *Bcl-xl* genes tended to increase in the liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*), but no difference was observed in the gene expression levels of pro-apoptotic *Bax* and *Bad* genes between the two genotypes (Figure 3C). Thus, our findings suggest that the increased liver tumors observed in DEN-treated *T82D/S260D*-inducible mice are primarily attributed to the promotion of cell proliferation.

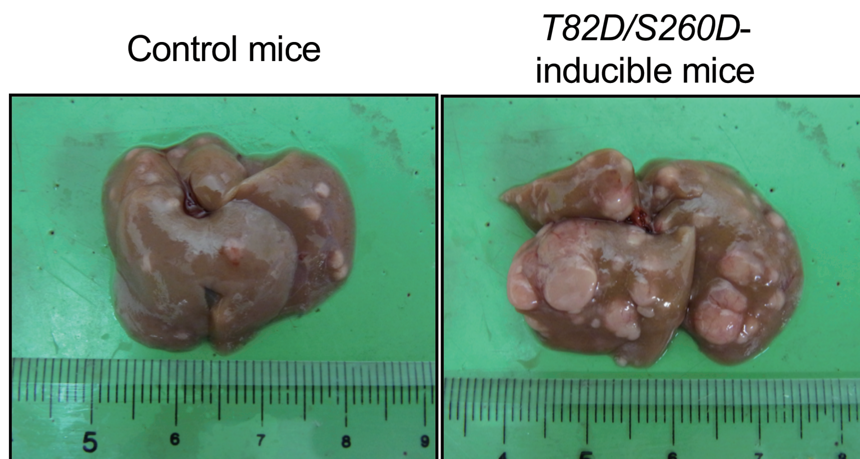

Figure 2. Representative macroscopic images of the livers from each genotype of mice.

Table 2. The number of FCA, adenoma and HCC observed microscopically in each genotype of mice

Genotype	No. of mice	Multiplicity[a]					
		FCA	G1	G2	G3	HCC	Total
Control mice (Rosa/+)	21	1.0 ± 0.8[b]	1.1 ± 0.9	3.0 ± 1.4	13.4 ± 3.9	1.6 ± 1.0	20.2 ± 5.3
T82D/S260D-inducible mice (Rosa/+; Rxr/+)	19	1.2 ± 0.9	1.2 ± 0.8	4.8 ± 2.1	21.5 ± 5.5[c]	13.5 ± 4.6[d]	42.1 ± 11.1[d]

FCA, foci of cellular alteration; G1, grade 1 adenoma; G2, grade 2 adenoma; G3, grade 3 adenoma; HCC, hepatocellular carcinoma; No., number.
[a]Number of tumors per mouse.
[b]Mean ± SD.
[c]Significantly different from control mice (*Rosa*/+) by unpaired Student's *t*-test ($P < 0.05$).
[d]Significantly different from control mice (*Rosa*/+) by unpaired *t*-test with Welch's correction ($P < 0.05$).

The β-catenin signaling pathway is activated in the liver tumors of DEN-treated *T82D/S260D*-inducible mice

We previously reported that an impaired receptor function of RXRα due to its phosphorylation is associated with the growth of HCC cells, which was attributed to either a decrease in RARβ and p27 or an increase in cyclin D1(17–19). Therefore, we examined the effect of *T82D/S260D* expression on the mRNA levels of these molecules. As shown in Figure 4A, the expression levels of *RARβ* and *p27* mRNA in liver tumors and non-tumorous liver tissues were comparable between DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+) and DEN-treated control mice (*Rosa*/+). In contrast, the expression levels of *cyclin D1* mRNA were significantly increased in the liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+). Cyclin D1 is known as a downstream target of β-catenin/Tcf transcription (28), and the activation of the Wnt/β-catenin pathway promotes the development of several types of cancer, including HCC (29–33). As shown in Figure 4B, the levels of β-catenin and cyclin D1 proteins in the liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+) were higher than in the liver tumors of DEN-treated control mice (*Rosa*/+). In addition, the level of phosphorylated Rb protein, which induces G1-S checkpoint transition of the cell cycle under the control of cyclin D1 (34,35), was also increased in the liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+). Consistent with these results, the protein levels of PCNA, which assists in DNA replication and is a well-known marker of cell proliferation (36), were also increased in the liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+).

To histologically evaluate the activation of the β-catenin signaling pathway, it is important to determine the subcellular and nuclear localization of β-catenin, because signal transduction via this protein involves its post-transcriptional stabilization and translocation into the nucleus (37). Therefore, we compared the localization of β-catenin protein in precancerous Grade 3 adenomas, defined according to previous criteria (23), between DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+) and DEN-treated control mice (*Rosa*/+). As shown in Figure 4C, the expression of β-catenin protein in DEN-treated control mice (*Rosa*/+) was primarily localized in the membrane of tumor cells. In contrast, relatively strong cytoplasmic expression of β-catenin protein in tumor cells was observed in DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+). β-catenin protein was localized in the nucleus in some tumor cells of DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+). Overall, these findings suggest that in DEN-treated *T82D/S260D*-inducible mice (*Rosa*/+; *Rxr*/+), β-catenin signaling pathway is activated even in precancerous lesions of HCC and may play a role in promoting hepatocarcinogenesis.

Reduced expression of Pleckstrin homology domain-containing family B member 1 (*Plekhb1*) mRNA may be associated with DEN-induced liver tumorigenesis in *T82D/S260D*-inducible mice

We also investigated other molecular targets that can be regulated by a phosphomimetic mutant form of human

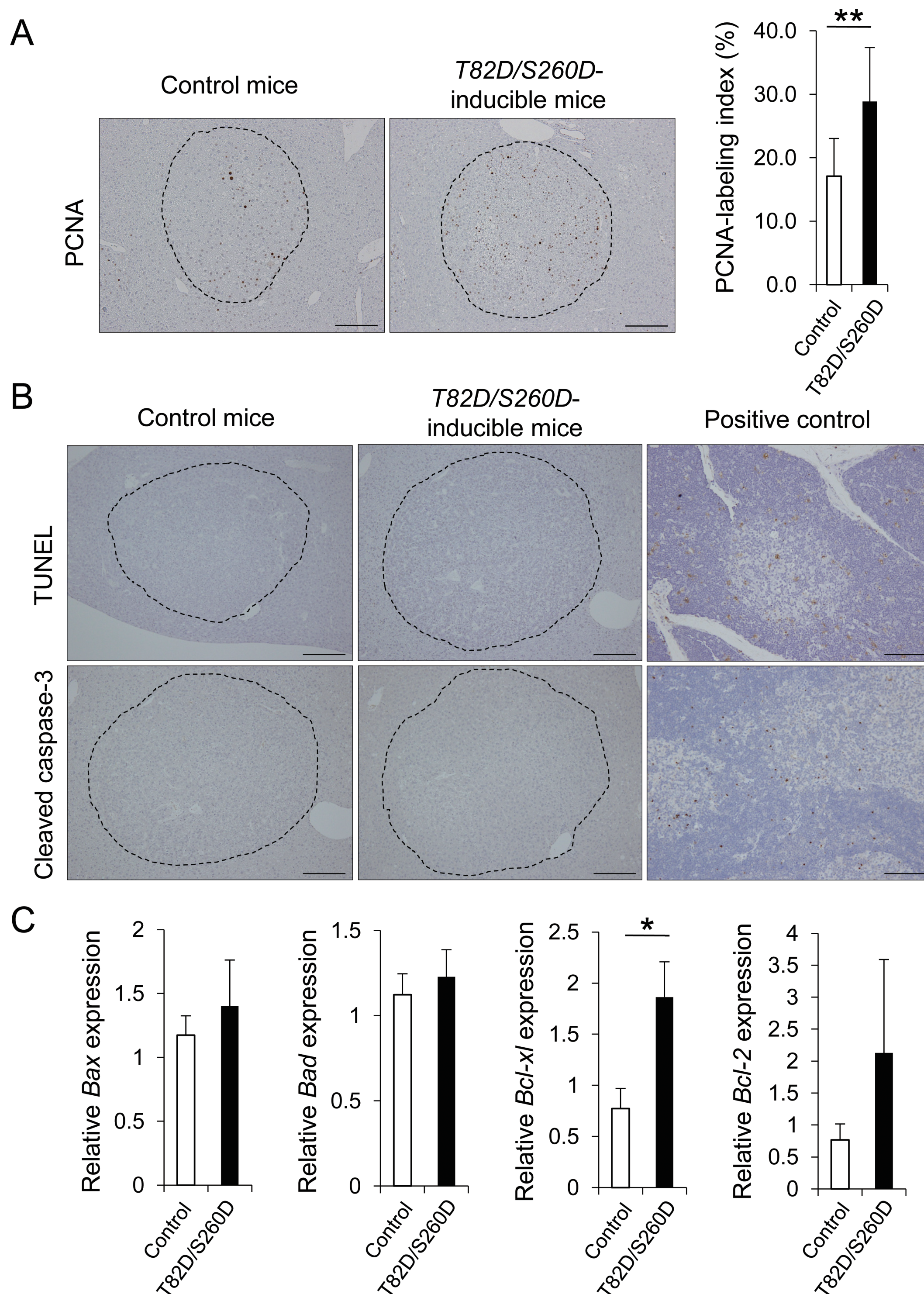

Figure 3. Effects of *T82D/S260D* expression on cellular proliferation and apoptosis in DEN-induced liver tumors. (**A**) Liver sections from DEN-treated control mice (*Rosa/+*) and DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) were stained with anti-PCNA antibody. Representative images from each group are shown in the left panels. PCNA-positive cells were counted and expressed as a percentage of the total number of cells per liver tumor. The positive cell indices are shown in the right panels. The dotted lines indicate the margin of liver tumors. Scale bar, 200 μm. (**B**) TUNEL and cleaved caspase 3-positive cells in liver tumors were evaluated using an apoptosis detection kit and immunohistochemical analysis, respectively. Representative images from each group are shown in the left panels. Sections of rat thymus were used as the positive control in each experiment. The dotted lines indicate the margin of liver tumors. Scale bar, 200 μm. (**C**) The mRNA expression levels of *Bax*, *Bad*, *Bcl-xl* and *Bcl-2* in liver tumors were detected by qRT-PCR using specific primers. Transcript levels were normalized to that of β-*actin*. Data are presented as mean ± SD (n = 3). *P < 0.05, Student's t-test.

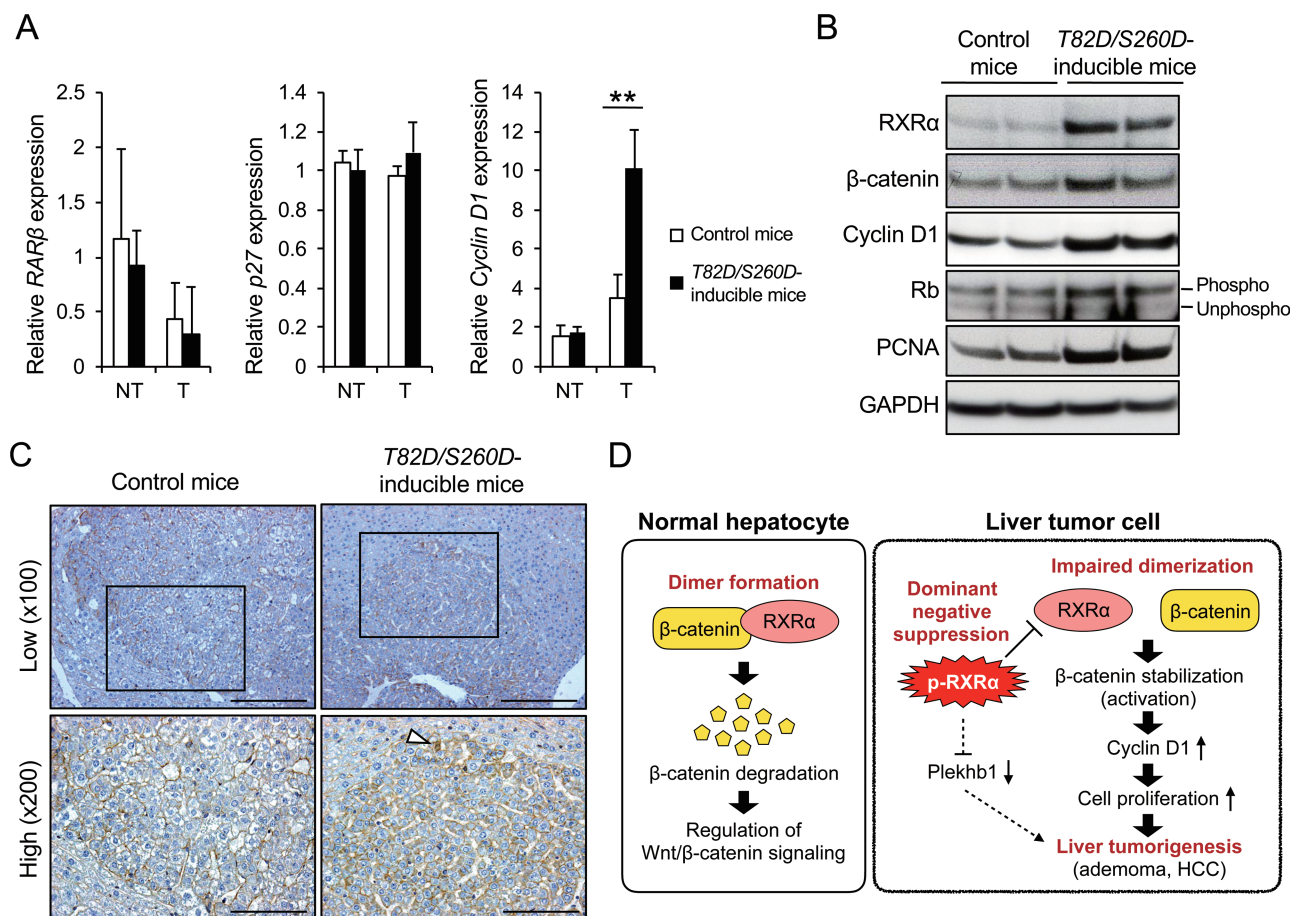

Figure 4. The β-catenin signaling pathway is activated in the liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*). (**A**) The mRNA expression levels of *RAR*β, *p27* and *cyclin D1* in the liver tumors (T) and adjacent non-tumor tissues (NT) of either DEN-treated control mice (*Rosa/+*) or DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) were detected by qRT-PCR using specific primers. Transcript levels were normalized to that of β-*actin*. Data are presented as mean ± SD ($n = 3$). $^{**}P < 0.01$, Student's *t*-test. (**B**) Total proteins were extracted from DEN-induced liver tumors of each genotype of mice, and the protein expression of RXRα, β-catenin, cyclin D1, Rb and PCNA were examined by western blotting analysis using specific antibodies. GAPDH served as the loading control. Representative images of three independent experiments are shown. (**C**) Liver sections from each genotype of mice were stained with anti-β-catenin antibody. Representative images from each group are shown. Lower panels indicate the enlarged images of the regions enclosed within the solid lines in the respective upper panels. Arrowhead indicates a tumor cell in which β-catenin is localized in the nucleus. Scale bar, 200 μm (upper panels), 100 μm (lower panels). (**D**) Proposed molecular mechanisms underlying the role of p-RXRα in promoting DEN-induced hepatocarcinogenesis in mice.

RXRα. Previously, *Plekhb1* was shown to be downregulated in human HCC (38). In addition, the *Plekhb1* gene was identified to have a canonical retinoic acid response element (RARE) in the promoter regions, and the levels of *Plekhb1* expression in germ cells were downregulated in RARα conditional knockout mice (39). Given that the overexpression of *T82D/S260D* inhibited transactivation through RARE in normal human hepatocytes (14), we estimated the association between the expression of *T82D/S260D* and *Plekhb1* mRNA. We then focused on validating gene expression in the liver of DOX-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) using qRT-PCR. In contrast with the significant increase in *T82D/S260D* mRNA levels after DOX treatment, the mRNA expression of *Plekhb1* was significantly reduced, suggesting a negative correlation between the expression of each gene (Supplementary Figure S3A, available at *Carcinogenesis* Online). Meanwhile, in DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*), the expression of *Plekhb1* mRNA was significantly reduced in liver tumors compared with non-tumorous liver tissues (Supplementary Figure S3B, available at *Carcinogenesis* Online). Notably, compared with DEN-treated control mice (*Rosa/+*), the mRNA level of *Plekhb1* in the liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) was significantly reduced (Supplementary Figure S3C, available at *Carcinogenesis* Online). In contrast, increased *T82D/S260D* mRNA expression was observed in liver tumors of DEN-treated *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) (Supplementary Figure S3C, available at *Carcinogenesis* Online). Collectively, these results suggest that the expression of *Plekhb1* mRNA may be negatively regulated by *T82D/S260D* expression, and the reduced expression of the *Plekhb1* gene may be associated with DEN-induced liver tumorigenesis in *T82D/S260D*-inducible mice (*Rosa/+; Rxr/+*) (Figure 4D).

Discussion

In the present study, our findings demonstrate that the *in vivo* expression of *T82D/S260D* promotes DEN-induced

hepatocarcinogenesis in mice through the activation of the β-catenin signaling pathway.

β-Catenin is a structural protein in the cadherin-mediated cell–cell adhesive system which plays a role in the differentiation and repair of normal tissues (40). It is also known to act as a mediator in the canonical Wnt signaling pathway; inappropriate activation of this pathway has been implicated in the development of several types of malignancies, including HCC (30–33). A previous study showed that overexpression of β-catenin accelerates liver tumorigenesis and progression to HCC following DEN exposure (41). In this study, a high prevalence of high-grade liver tumors and increased expression of β-catenin protein in tumor cells was observed in DEN-treated *T82D/S260D*-inducible mice (Table 2, Figure 4B and C), suggesting the involvement of the β-catenin signaling pathway in hepatocarcinogenesis in this experimental model.

Nuclear and cytoplasmic β-catenin expression has been shown to play an important role in tumor progression (30,42). The stabilized cytoplasmic β-catenin enters the nucleus by binding to the T-cell factor (TCF) and lymphoid enhancer factor (LEF) family of proteins, and induces the transcription of target genes, including cyclin D1. Cyclin D1 promotes the transition between the G1-S checkpoint of the cell cycle by influencing the activity of Rb protein and induces cell proliferation in the cell cycle (34,35). Indeed, cytoplasmic and nuclear accumulation of β-catenin in HCC tissues has been reported in previous clinical and experimental studies (13,29–31,43,44), and the amplification of cyclin D1 genes and its overexpression have been shown to be associated with aggressive forms of liver tumors, including HCC (45,46). In this study, β-catenin protein was localized in the cytoplasm and the nucleus of liver tumor cells in DEN-treated *T82D/S260D*-inducible mice, and the downstream proliferative signals, such as cyclin D1 and phosphorylated Rb proteins, were elevated in these liver tumors (Figure 4B and C), indicating the activation of the β-catenin signaling pathway in these mouse models. Our findings suggest that the acceleration of hepatocarcinogenesis observed in DEN-treated *T82D/S260D*-inducible mice is attributable to the activation of the β-catenin signaling pathway. In addition, as previously indicated (45,46), increased cyclin D1 expression may be associated with the high prevalence of high-grade liver tumors observed in this mouse model.

Previous studies have reported that retinoid receptors, including RARα and RXRα, directly interact with β-catenin and regulate the Wnt/β-catenin signaling pathway. Han *et al.* revealed that RXRα overexpression directly inhibited both β-catenin/TCF/LEF transcriptional activity and β-catenin protein levels in colorectal cancer cells, whereas downregulation of RXRα by small interfering RNA abolished these inhibitory effects and elevated both β-catenin protein levels and β-catenin/TCF/LEF transcriptional activity (47). In addition, a previous study using transgenic mice expressing the RARα-dominant negative form in hepatocytes showed that the reduction of the RARα/β-catenin complex caused an increase in the β-catenin/TCF complex, thus inducing the expression of cyclin D1 and leading to hepatocarcinogenesis (13). Thus, RXRα and RARα regulate free β-catenin protein levels by directly forming a complex with β-catenin, thereby inhibiting the Wnt/β-catenin/TCF function. Notably, the direct regulation of β-catenin by these retinoid receptors is perturbed by not only the reduced expression of retinoid receptors, but also their malfunction (13,29,43,47). *T82D/S260D*, the phosphomimetic mutant form of human RXRα used in this study, has been reported to interfere with the function of the remaining normal RXRα in a dominant-negative manner (14). Previous studies combined with our present findings may lead to the hypothesis that the *in vivo* expression of *T82D/S260D* may inhibit complex formation between normal RXRα and β-catenin, thereby causing an increase in the free levels of β-catenin, activating the Wnt/β-catenin signaling pathway, inducing overexpression of cyclin D1 and thereby contributing to hepatocarcinogenesis in DEN-treated *T82D/S260D*-inducible mice (Figure 4D).

The levels of free β-catenin are regulated by two adenomatous polyposis coli (APC)-dependent proteasomal degradation pathways: glycogen synthesis kinase-3β-regulated pathway involving the APC/Axin complex and a p53-inducible pathway involving Siah-1(48,49). Mutations in either the key components of the above two pathways, such as APC, Axin and p53, or β-catenin itself, have been reported to lead to dysregulation of β-catenin turnover in several malignancies, including HCC, subsequently resulting in its cytoplasmic and nuclear accumulation and abnormal activation of TCF/LEF-regulated genes that are involved in oncogenesis (43,50). To date, there have been no reports of an association between retinoid receptor malfunction and the above-mentioned mutations. However, given that the β-catenin signaling pathway was activated in the liver tumors of DEN-treated *T82D/S260D*-inducible mice, the possibility that the *in vivo* expression of *T82D/S260D* may be associated with these mutations cannot be ruled out. Further experiments are required to address this issue.

The levels of the *Plekhb1* gene were significantly reduced in liver tumors of DEN-treated *T82D/S260D*-inducible mice compared with those of DEN-treated control mice (Supplementary Figure S3C, available at *Carcinogenesis* Online). Given that the gene expression has been revealed to be downregulated in human HCC (38), it has been suggested that the reduced expression of *Plekhb1* gene may be associated with DEN-induced liver tumorigenesis in *T82D/S260D*-inducible mice (Figure 4D). Further experiments are necessary to elucidate the underlying molecular mechanisms.

In conclusion, our findings demonstrate that p-RXRα plays a role in chemically induced hepatocarcinogenesis in mice. The fact that the abnormal phosphorylation of RXRα is involved in liver carcinogenesis *in vivo* suggests that p-RXRα may serve as a possible therapeutic target for HCC.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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Conflict of Interest Statement

None declared.

Authors' contributions

H.S., Y.Y. and M.S. collaborated with the conception and design of the experiment. H.S., M.K. and Y.S. performed the experiments and acquired the data and images. H.S., Y.Y., K.I. and H.T. analyzed the data. H.S. wrote the main manuscript text and prepared all the figures and tables. A.H., Y.Y. and M.S. supervised the manuscript preparation. All authors reviewed the manuscript.

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