# c-Jun Mediates Hepatitis C Virus Hepatocarcinogenesis Through Signal Transducer and Activator of Transcription 3 and Nitric Oxide–Dependent Impairment of Oxidative DNA Repair

Keigo Machida,[1,4] Hidekazu Tsukamoto,[2,4,5] Jian-Chang Liu,[1] Yuan-Ping Han,[3] Sugantha Govindarajan,[2,4] Michael M. C. Lai,[1,7] Shizuo Akira,[6] and Jing-hsiung James Ou[1]

**Hepatocellular carcinoma (HCC) occurs in a significant number of patients with hepatitis C virus (HCV) infection. HCV causes double-strand DNA breaks and enhances the mutation frequency of proto-oncogenes and tumor suppressors. However, the underlying mechanisms for these oncogenic events are still elusive. Here, we studied the role of c-Jun, signal transducer and activator of transcription 3 (STAT3), and nitric oxide (NO) in spontaneous and diethylnitrosamine (DEN)-initiated and/or phenobarbital (Pb)-promoted HCC development using HCV core transgenic (Tg) mice. The viral core protein induces hepatocarcinogenesis induction as a tumor initiator under promotion by Pb treatment alone. Conditional knockout of *c-jun* and *stat3* in hepatocytes achieves a nearly complete, additive effect on prevention of core-induced spontaneous HCC or core-enhanced HCC incidence caused by DEN/Pb. Core protein induces hepatocyte proliferation and the expression of inflammatory cytokines (interleukin-6, tumor necrosis factor-α, interleukin-1) and inducible NO synthase (iNOS); the former is dependent on c-Jun and STAT3, and the latter on c-Jun. Oxidative DNA damage repair activity is impaired by the HCV core protein due to reduced DNA glycosylase activity for the excision of 8-oxo-2′-deoxyguanosine. This impairment is abrogated by iNOS inhibition or c-Jun deficiency, but aggravated by the NO donor or iNOS-inducing cytokines. The core protein also suppresses apoptosis mediated by Fas ligand because of c-Jun–dependent Fas down-regulation. *Conclusion:* These results indicate that the HCV core protein potentiates chemically induced HCC through c-Jun and STAT3 activation, which in turn, enhances cell proliferation, suppresses apoptosis, and impairs oxidative DNA damage repair, leading to hepatocellular transformation.** (HEPATOLOGY **2010;52:480-492**)

*Abbreviations: 8-oxodG, 8-oxo-2′-deoxyguanosine; AP-1, activator protein-1; BER, base excision repair; BHA, butylated hydroxyanisole; DEN, diethylnitrosamine; [$^{32}$P]dGTP, [$^{32}$P]deoxyguanosine triphosphate; iNOS, inducible nitric oxide synthase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JNK, c-Jun N-terminal kinase; NO, nitric oxide; Pb, phenobarbital; PCNA, proliferating cell nuclear antigen; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RNS, reactive nitrogen species; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; Tg, transgenic; WT, wild type.*

*From the Departments of [1]Molecular Microbiology and Immunology, [2]Pathology, and [3]Surgery, and [4]Southern California Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis, University of Southern California, Keck School of Medicine, Los Angeles, CA; [5]Department of Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA; [6]Osaka University, Osaka, Japan; and [7]National Chun Kung University, Tainan, Taiwan.*

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*Address reprint requests to: Keigo Machida, Ph.D., Department of Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, 211 Zonal Avenue, Los Angeles, CA 90033. E-mail: kmachida@usc.edu; fax: 323-442-1721.*



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Hepatitis C virus (HCV) causes chronic hepatitis and liver cirrhosis and greatly increases the risk for hepatocellular carcinoma (HCC).[1-3] In both HCC and chronic hepatitis, the transcription factor activator protein-1 (AP-1) is activated and implicated.[4] The ectopic expression of HCV core protein in cell cultures also activates AP-1 (c-Jun)[5] via the activation of c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase,[6,7] and HCV core transgenic (Tg) mice develop liver tumors,[8] suggesting the role of c-Jun in core-induced oncogenesis.

The transcription activator c-Jun is required for cell proliferation in postnatal hepatocytes.[9] Mice deficient in c-Jun die between embryonic days E12.5 and E13.5 from massive apoptosis of hepatoblasts, erythroblasts, and other cell types, indicating the requirement of c-Jun in normal liver development and hematopoiesis.[10,11] To rescue embryonic lethality, a "floxed" *c-jun* allele is deleted in a designated cell type upon expression of the Cre recombinase under the control of a cell-type–specific promoter. Using this conditional gene disruption, the requirement for *c-jun* is also shown for chemically-induced HCC in mice where c-Jun deficiency in hepatocytes reduces both the number and size of HCC after tumor initiation with diethylnitrosamine (DEN), while increasing apoptosis.[12]

HCV core protein induces reactive oxygen species (ROS), and HCV core Tg mice have higher hepatic levels of 8-oxo-2′-deoxyguanosine (8-oxodG), which is indicative of DNA damage by ROS.[13] In fact, HCV core Tg mice show increased mutation frequencies of tumor suppressor and proto-oncogenes.[13,14] ROS also activates c-Jun and signal transducer and activator of transcription 3 (STAT3).[15] Therefore, the core protein may increase the growth and survival of initiated tumor cells via activation of c-Jun and STAT3. However, the mechanisms by which c-Jun and STAT3 specifically contribute to liver oncogenesis induced by interactions of HCV core and environmental carcinogens remain to be elucidated. Furthermore, whether HCV core protein works as a tumor initiator or promoter has not been determined.[16] The present study demonstrates that the mitogenic and antiapoptotic effects mediated by c-Jun/AP-1 and STAT3 are both required for hepatocyte susceptibility to HCV core-initiated hepatocellular transformation, and that this is caused by fixation of genetic mutations induced by oxidative stress and impaired DNA repair, resulting from activation of c-Jun and nitric oxide (NO).

## Materials and Methods

***Mice.*** For animal studies, mice expressing the HCV core gene genotype 1b under control of the human elongation factor 1a promoter, were generated and bred at the University of Southern California transgenic mouse facility (8-13 and 8-20 lines). The *c-jun*$^{flox/flox}$ mice are a generous gift from Dr. Carter at Vanderbilt University, Nashville, TN. The *stat3*$^{flox/flox}$ mice were generated by standard procedures.

***Adenovirus Injection.*** The adenovirus which expresses *cre* recombinase under the albumin promoter was used to disrupt the c-jun gene. The removal of the neo gene was confirmed by polymerase chain reaction (PCR) or Southern blotting, demonstrating that the targeted *c-jun* allele contains the protein-coding sequence flanked by *loxP* sites.[17]

***Statistical Analysis.*** Statistical comparisons of the groups were made by one-way analysis of variance, and when they were statistically significant, each group was compared with others by Fisher's protected least significant difference test (Statview, version 4.0; Abacus Concept Inc., Berkeley, CA).

## Results

***HCV Core and DEN/Phenobarbital Synergistically Induce Liver Tumor.*** To determine whether HCV core promotes carcinogen-induced liver tumorigenesis, we injected HCV core Tg mice with the genotoxic carcinogen DEN as a tumor initiator at 6 weeks of age and administered the tumor promoter phenobarbital (Pb) in drinking water starting from 10 weeks of age, until 22 months of age (Fig. 1A). Mortality of core Tg mice given DEN and Pb became evident at 8 months old and continued to increase with time. By 20 months, the DEN/Pb treatment caused 42% mortality among core Tg mice as compared to 12% among wild-type (WT) mice ($P < 0.05$; Fig. 1B). Autopsy results confirmed that the lethality of the Tg mice was associated with primary liver tumors. Without DEN tumor induction, Tg mice developed spontaneous HCC at the rate of 14%, 28%, and 38% at 14, 18, and 22 months of age, respectively, whereas none of the WT littermates developed the tumor (Fig. 1C); the results are consistent with the previously reported finding.[8] The DEN/Pb treatment resulted in 22% liver tumor incidence in WT mice but 62% incidence with enhanced dysplastic changes in core Tg mice at 14 months of age ($P < 0.05$; Fig. 1C,D, panel f). At 18 and 22 months, the magnitude of the difference in the liver tumor incidence between WT and Tg mice diminished, although the latter animals still showed higher incidence (Fig. 1C).

Histological analysis revealed that the tumors were mainly adenoma and HCC with occasional angiosarcoma

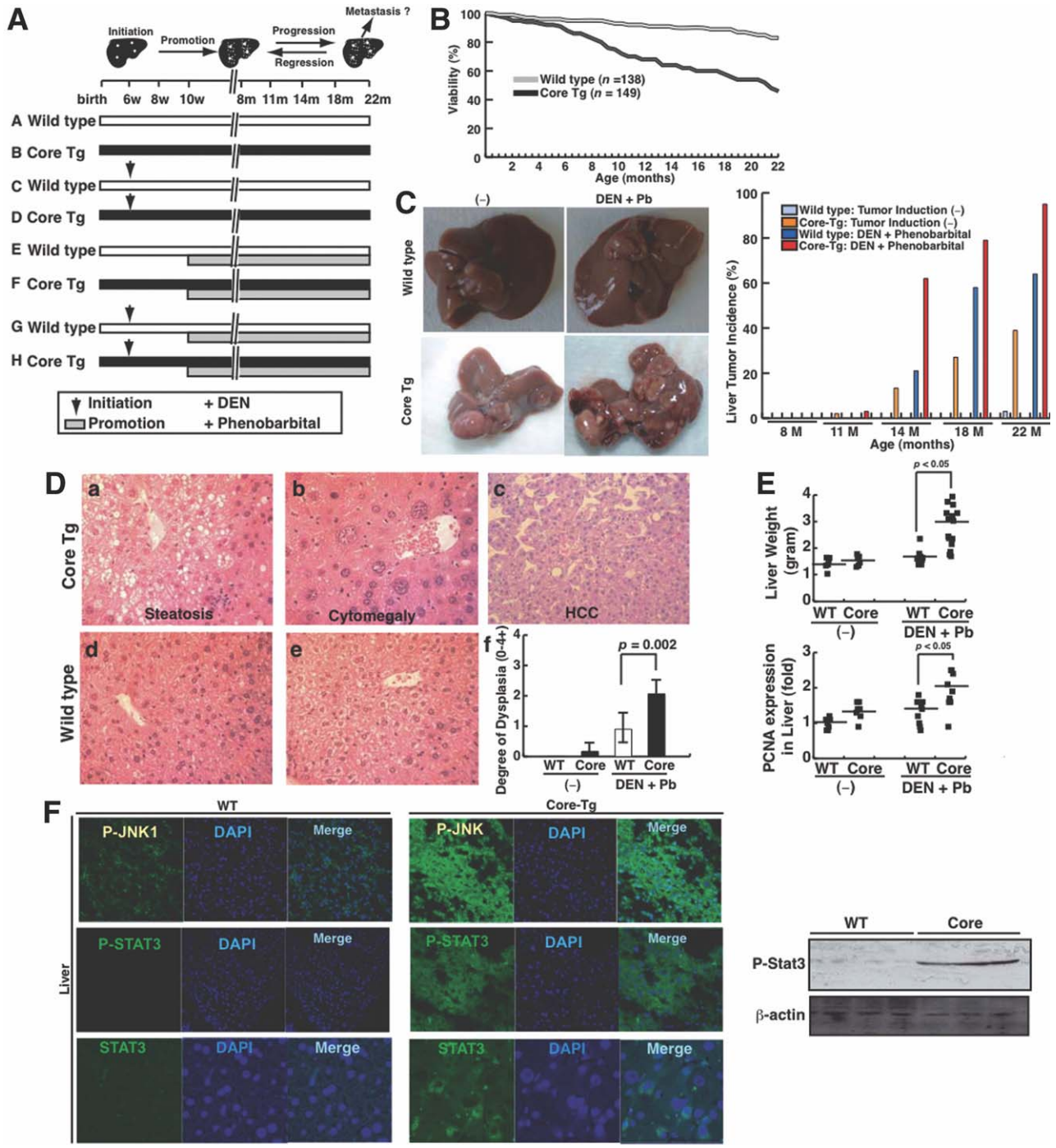



Fig. 1. Incidence of DEN-induced foci in transgenic mouse liver tissue. (A) Summary of the different experimental groups and design. Carcinogenic treatment was initiated at 6 weeks of age (arrow) by a single injection of DEN, and tumor promotion was started at 10 weeks with food containing the tumor promoter Pb. (B) Kaplan-Meier survival plots of mice treated with DEN and Pb in 14-month-old core Tg ($n = 52$) and wild-type (WT) ($n = 67$) animals. (C) Appearance of liver nodule (foci) in a 14-month-old mouse liver tissue section at a magnification of $\times 200$. Liver tumor incidence (more than 3 mm in diameter) was measured at the age of 14 months. (D) HCV core protein induces severe steatosis and HCC in hepatocytes after DEN/Pb treatment. These HCV *core* Tg mice spontaneously developed cytomegaly with a high frequency. The treatment of the mice with DEN and Pb induced more conspicuous steatosis, dysplasia, and HCC (panels a, b, and c, respectively). Pathologists blindly quantified the degree of dysplasia at the degree of 0 to +4 (f). (E) Liver weight and PCNA expression in liver from HCV Core Tg mice. (F) Representative immunohistochemical staining of phospho-JNK (P-JNK1), Phospho-STAT3 (P-STAT3), and total STAT3 in the livers from DEN-treated core Tg and WT mouse liver tissue sections (original magnification, $\times 200$). Phosphorylation of STAT3 is shown by immunoblots of livers from HCV core Tg mice.

(Fig. 1D, panel c). In most cases, multiple hepatocellular neoplasms of various sizes were present in the liver of these mice (Fig. 1C). The treatment of the core Tg mice with DEN and Pb induced more severe steatosis and dysplasia (Fig. 1D, panels a and f). Administration of DEN resulted in comparable increases in the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase, the markers for liver damage, in both WT and core Tg mice (data not shown) suggesting that the hepatotoxin induced comparable liver necrosis in both groups of mice.

***HCV Core Enhances Proliferation of Hepatocytes in Response to DEN/Pb.*** The extent of hepatocellular proliferation, as assessed by proliferating cell nuclear antigen (PCNA) staining and liver weight, was nearly two-fold higher in the HCV core Tg mice than in WT mice under DEN/Pb treatment (Fig. 1E), indicating that dysregulated hepatocyte proliferation may be the cause of increased hepatocellular transformation in Tg mice. This difference was not apparent in carcinogen-untreated mice (Fig. 1E). To test if the livers in core Tg mice had JNK and STAT3 activation, we stained tissue sections for phospho-STAT3 and phospho-JNK. The staining and phospho-STAT3 protein levels as determined by immunoblotting were clearly increased in DEN/Pb-treated Tg mice as compared to WT mice (Fig. 1F). These results indicate that increased liver tumor development in HCV core Tg mice is associated with enhanced hepatocyte proliferation and activation of JNK and STAT3.

***Disruption of c-Jun Abrogates HCV Core-Induced and Core-Promoted Liver Oncogenesis.*** To determine the possible role of *c-jun* in core-induced or core-enhanced liver oncogenesis, we bred core Tg mice with *c-jun* conditional knockout ($c\text{-}jun^{flox/flox}$) mice. The *c-jun* gene in this mouse line is flanked by the lox site, which will recombine to delete the *c-jun* gene in the presence of the Cre recombinase. We injected mice with a recombinant adenovirus that expresses Cre (A5CMVCre) to induce the deletion of the *c-jun* gene primarily in the liver. As a control, adenovirus expressing *lacZ* (Ad.LAcZ) was injected (see the experimental design in Fig. 2A). Immunoblot and quantitative reverse transcription PCR (qRT-PCR) of c-Jun demonstrated effective c-Jun deficiency in animals which received Ad5CMVCre (Fig. 2A, lower blots and graph). The mortality associated with DEN/Pb treatment in core Tg mice was significantly attenuated by c-Jun deficiency (Fig. 2B). Spontaneous HCC development (without DEN/Pb treatment) in core Tg mice was largely abrogated by c-Jun deficiency (Fig. 2C). The enhanced tumor incidence in the core-Tg mice treated with DEN/Pb was also reduced by 60% ($P < 0.001$) due to c-Jun deficiency (Fig. 2C,E), whereas a smaller 30% reduction was observed in c-Jun–deficient WT mice given DEN/Pb (Fig. 2C). The number of cells double-positive for CD133 and CD49f, which are markers for cancer stem cells, clearly increased in core Tg mice treated with DEN/Pb but not in c-Jun–deficient core Tg mice or WT mice treated with the carcinogens (Fig. 2F). Thus, our results demonstrate that HCV core protein not only serves as an independent tumor inducer, but also accentuates carcinogen-induced HCC development in a manner largely dependent on c-Jun.

***Core Acts as a Tumor Initiator Rather than a Promoter Mediated by c-Jun.*** HCV core enhanced DEN/Pb-induced hepatocarcinogenesis. However, we do not know whether this effect is due to the core protein's role as a tumor initiator or promoter. To address this question, WT and Tg mice were treated with Pb (a tumor promoter) or DEN (a tumor initiator) alone and compared for the effect of core on the number and size of liver tumors induced. As shown in Fig. 3B, even with Pb treatment alone, HCV core Tg mice developed more than three-fold larger and numerous liver tumors than did WT mice, and these increments resembled those seen with DEN/Pb treatment (Fig. 3A). Furthermore, c-Jun deficiency markedly abrogated these oncogenic effects in core Tg mice treated with DEN+Pb or PB alone. In contrast, HCV core Tg mice treated with DEN alone developed liver tumors with much smaller mass and fewer numbers than those treated with DEN+Pb or PB alone (Fig. 3A-C). These results indicate that HCV core initiates, but does not promote, hepatocarcinogenesis.

***Antioxidant Reduces the Tumor Incidence in HCV Core Tg Mice.*** Our previous *in vitro* data indicate that the HCV core protein induces DNA mutations via an increase in the production of ROS and reactive nitrogen species (RNS).[13,18] Thus, we investigated next whether the administration of an antioxidant reduces core-enhanced liver tumor development under DEN+Pb treatment. Butylated hydroxyanisole (BHA), an antioxidant that scavenges ROS and RNS, was administered via drinking water for 12 months (Fig. 3D). The treatment of BHA significantly reduced HCV core-induced enhancements of liver tumor size and number, indicating that ROS-mediated or RNS-mediated oncogenic mutation is important for the enhanced liver oncogenesis in core Tg mice given DEN/Pb (Fig. 3D). To make a mechanistic connection of hepatocarcinogenesis and DNA repair, DNA mutation frequency was determined by plasmid-based

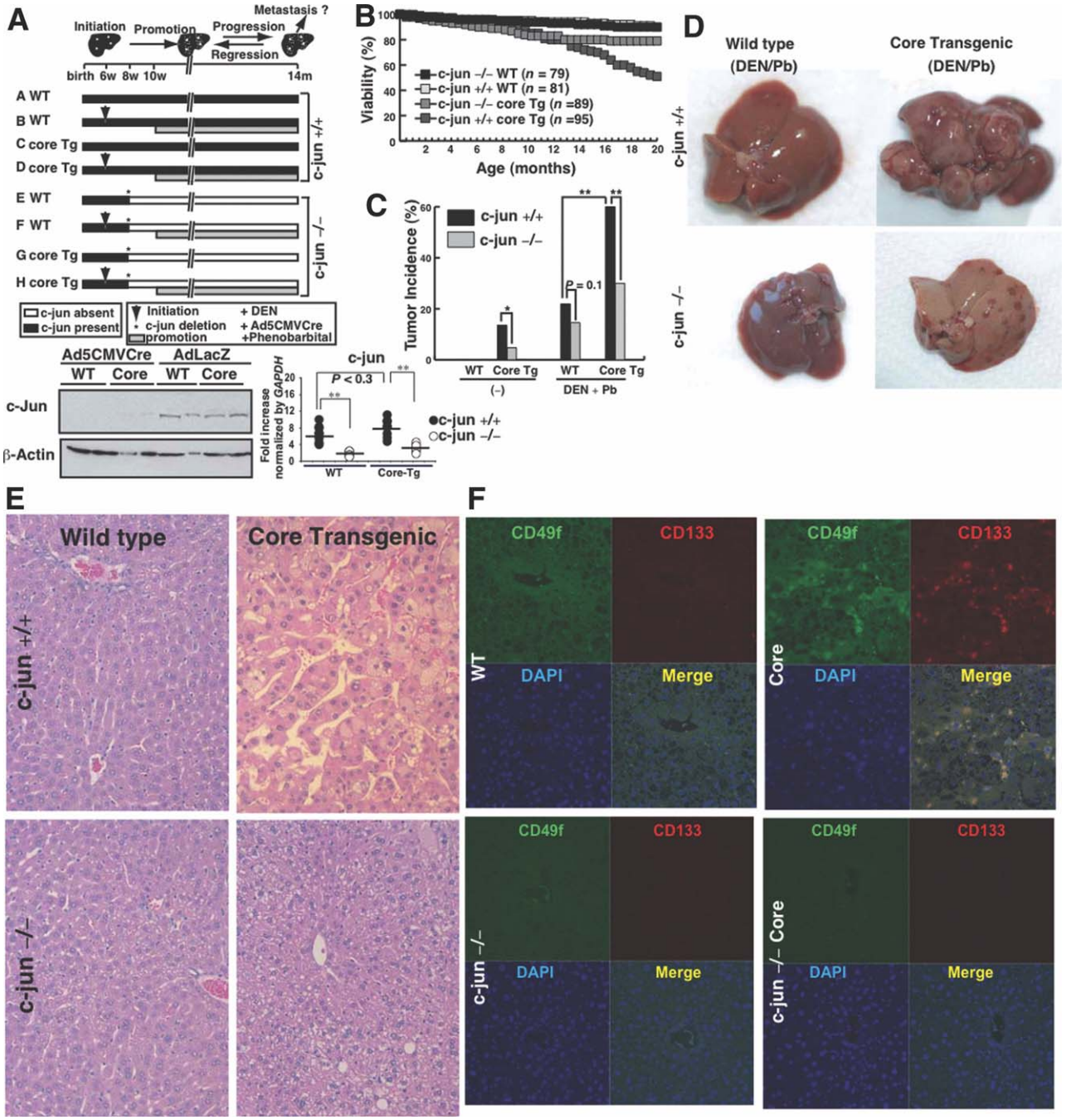
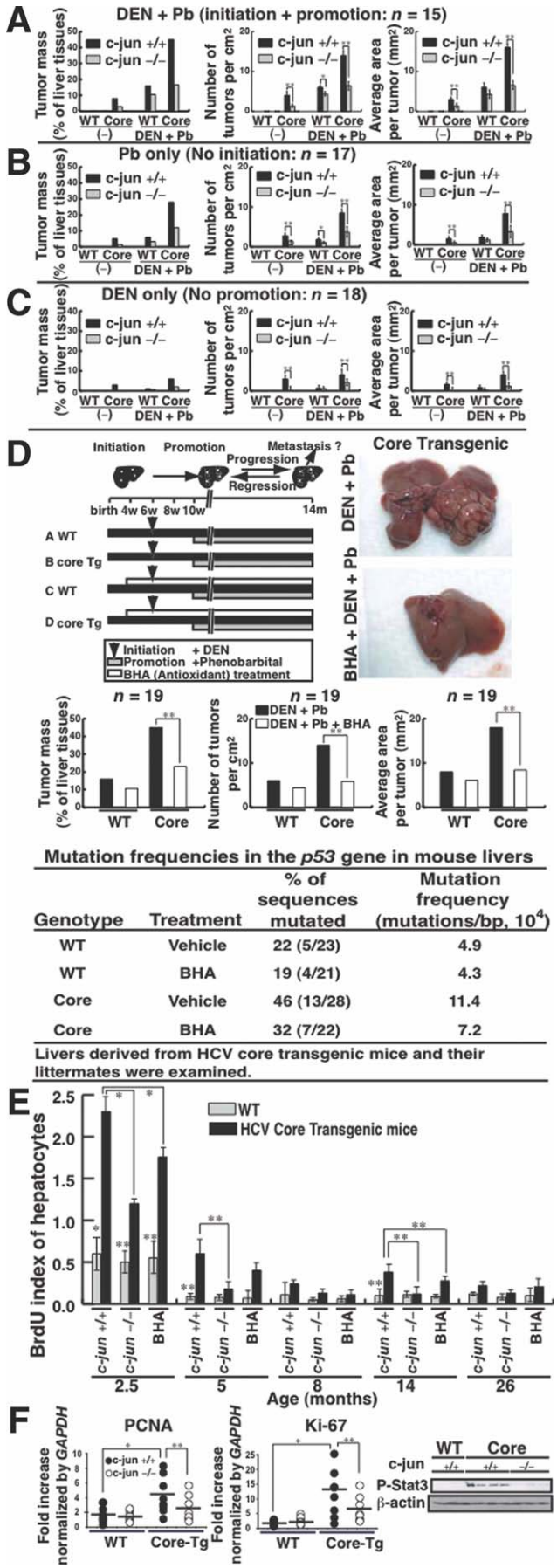



Fig. 2. c-Jun mediates HCV core–induced hepatocarcinogenesis. (A) Experimental conditions to define the role of c-Jun in HCV core-induced liver tumors. Development of tumors was initiated at 6 weeks of age (arrow) by a single injection of DEN, and tumor promotion was started at 10 weeks with food containing the tumor promoter Pb. The c-Jun proteins were detected by immunoblot. $\beta$-actin blot serves as a loading control. The *c-jun* and *gapdh* mRNA levels were quantified by qRT-PCR and normalized by *gapdh* control. (B) Mortality of *c-jun* $-/-$ or *c-jun* $+/+$ HCV core Tg or wild-type mice with the DEN + Pb treatment. (C) Tumor incidence of *c-jun* $+/+$ or *c-jun* $-/-$ HCV core Tg mice. $*P < 0.05$, $**P < 0.001$. Disruption of *c-jun* reduces tumor incidence in HCV core Tg mice. (D) HCC formation in HCV *core* Tg mice, but not in WT mice, less in *c-jun*$-/-$ *core* Tg mice. Shown are (E) hematoxylin & eosin histology and (F) cancer stem cell marker ($CD133^+$ and $CD49f^+$) images in liver sections.

sequencing from genomic DNA using p53 gene as a marker of HCV core transgenic mice in the presence or absence of antioxidant treatment (BHA). The data showed that core transgenic mice have a significantly higher frequency of mutation, which is abrogated by BHA treatment (Fig. 3D, table; $P < 0.01$). These results indicate that HCV core-induced ROS/RNS enhances DNA mutation frequency of major tumor suppressor gene *p53*, which is abrogated by blocking ROS/RNS, in livers of HCV core transgenic mice.




***c-Jun Enhances Tumor Cell Proliferation.*** Next, we tested whether suppressed liver tumor formation with BHA is associated with inhibition of hepatocellular proliferation. For this analysis, we examined 5-bromo-2′deoxyuridine (BrdU) incorporation in the livers at various time points (2.5∼26 months) of DEN/Pb treatment in WT and core Tg mice, with or without BHA treatment (Fig. 3E). In parallel, we also analyzed the effect of c-Jun deficiency. At the young age of 2.5 months, the proliferative activity is high, particularly in core Tg mice treated with DEN/Pb, and this is reduced 50%∼60% by c-Jun deficiency and 30% by BHA treatment. BrdU index was lower in all groups at the older ages, but the effects of c-Jun deficiency and BHA were still evident ($P < 0.05$; Fig. 3E). Suppressed proliferation in c-Jun–deficient core Tg mice also corroborated reduced PCNA and Ki-67 messenger RNA (mRNA) levels detected by qRT-PCR (Fig. 3F). These results demonstrate that the contribution of core-enhanced cellular proliferation takes place during the early stage of DEN/Pb-induced carcinogenesis, consistent with the notion that core serves as a tumor initiator. The BHA effect indicates a role of oxidative stress in hepatocellular proliferation (Fig. 3E). Interestingly, levels of the phosphorylated STAT3 (pSTAT3) were also reduced by *c-jun* disruption in *core* Tg mice, suggesting that core-induced STAT3 activation is dependent on c-Jun (Fig. 3F). These data demonstrate that HCV core promotes hepatocellular proliferation via oxidative stress and c-Jun. Because pSTAT3 has known mitogenic effects,[19] c-Jun–dependent STAT3 phosphorylation suggests the contribution of this mitogenic factor as a downstream effector of c-Jun for core-induced hepatocyte proliferation.

Fig. 3. Antioxidant reduces HCV core-initiated tumor development through core-induced proliferation. (A) Mean number of foci per cubic centimeter in 14-month-old core Tg ($n = 15\sim18$) and wild-type (WT) ($n = 15\sim18$) animals (A) in tumor initiation plus promotion, (B) in tumor promotion only, and (C) in tumor initiation only, and for tumor progression. Values were obtained by measuring foci area in three liver tissue sections (chosen at random) per animals, as described in Materials and Methods. The standard deviation is indicated by a vertical error bar. $*P < 0.05$; $**P < 0.01$ compared with controls. (D) Antioxidant reduces HCV core-initiated tumor development. Values were obtained by measuring foci area in three liver tissue sections (chosen at random) per animals. (Table) Enhanced mutation frequency of tumor suppressor *p53* gene in livers of core Tg mice is reduced by BHA treatment. (E) BrdU incorporation of *c-jun* −/− or BHA-treated mice in comparison to those of DEN/Pb-treated mice. $*P < 0.05$; $**P < 0.01$ compared with controls. (F) PCNA and Ki-67 mRNA levels were quantified by qRT-PCR. Phosphorylated STAT3 proteins were detected by phosphospecific antibody. β-actin blot serves as a loading control.

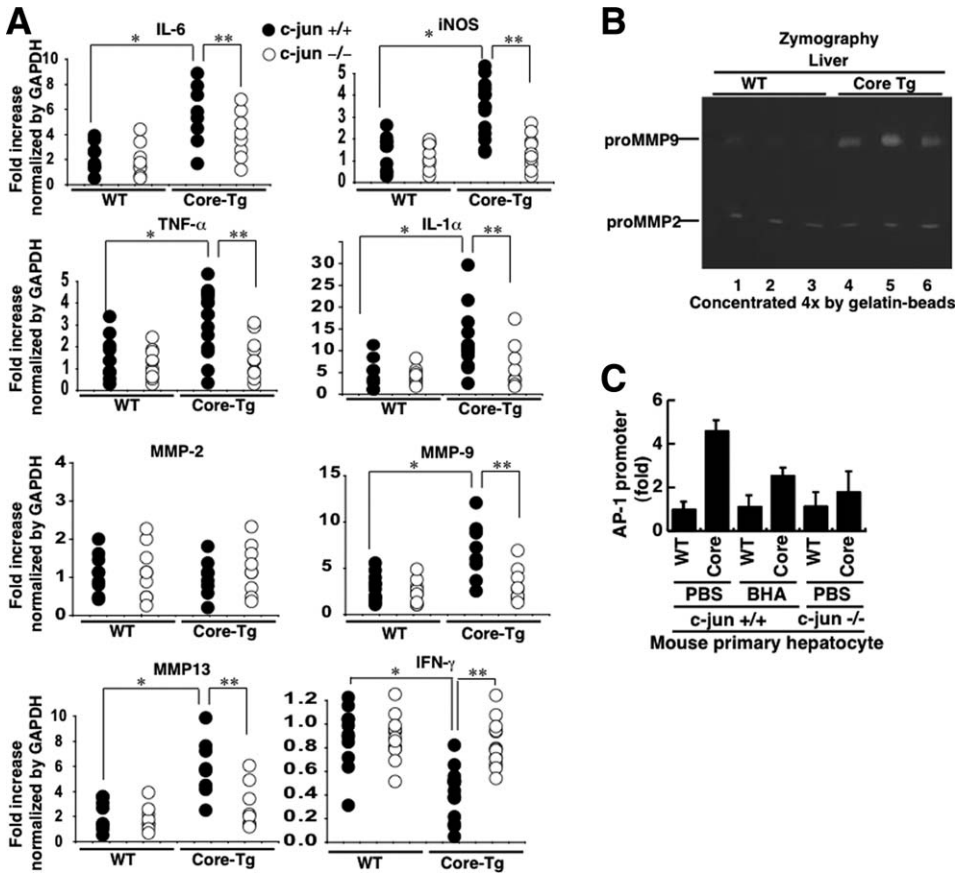



Fig. 4. Downstream significance of c-Jun. (A) Inflammatory cytokines, MMP, and iNOS mRNA expression using qRT-PCR in liver of *core* Tg mice. (B) Zymography of liver homogenates of HCV core transgenic mice. Zymography showing pro-MMP-9 and pro-MMP-2 in liver extracts of three different HCV core or nontransgenic animals. (C) AP-1 promoter activity quantified by luciferase assays in mouse primary hepatocytes.

***iNOS, MMP-9, and IL-6 as Potential c-Jun Target Genes in Core Tg Mice.*** Our results demonstrate the importance of c-Jun in the HCV core protein's ability to synergistically enhance DEN/Pb-induced hepatocarcinogenesis as a tumor initiator. We asked next which c-Jun/AP-1 target genes are up-regulated and implicated in our synergism model. c-Jun/AP-1 activates the promoter of matrix metalloproteinases (MMPs),[20] which play a critical role in acute, fulminant hepatitis by degrading the extracellular matrix and allowing massive leukocyte influx in the liver[21] and is involved in cancer migration, growth, and vasculogenesis.[22] Indeed, our qRT-PCR analysis revealed increased expression of MMP-9 and MMP-13, but not MMP-2 in the livers of core Tg mice given DEN/Pb, as compared to carcinogen-treated WT mice, and abrogation of this induction by c-Jun deficiency (Fig. 4A). Induction of MMP-9 in core Tg mice is also confirmed by zymography (Fig. 4B). Concomitantly, proinflammatory cytokines known to induce MMPs, such as interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α),[23] are up-regulated in core Tg mice and similarly repressed by c-Jun deficiency (Fig. 4A). IL-6 which is a known agonist for STAT3 activation and implicated in carcinogenesis,[19,24,25] is also induced in core Tg mice in a c-Jun–dependent manner (Fig. 4A). HCV core induces inducible NO synthase (iNOS), RNS/ROS generation, and DNA hypermutation *in vitro*,[18] and these changes are implicated in enhanced double-strand DNA breaks and increased levels of oxidatively damaged DNA (8-oxodG) in the livers of core Tg mice.[13] Indeed, our analysis shows up-regulation of iNOS in core Tg mice and its abrogation by c-Jun deficiency, suggesting that the core protein is upstream of c-Jun, which contributes to DNA damage via iNOS induction.

To test direct activation of AP-1 by the core protein, we next performed a transient transfection experiment using an AP-1 reporter construct and primary hepatocytes from WT (*c-jun*+/+) and c-Jun–deficient (*c-jun*−/−) mice. Core expression increased the AP-1 promoter activity in *c-jun*+/+ but not in *c-jun*−/− hepatocytes (Fig. 4C). In addition, BHA significantly reduced the core-induced AP-1 promoter activity (Fig. 4C), suggesting that ROS and RNS play a role in the activation of the AP-1 promoter induced by the core protein.

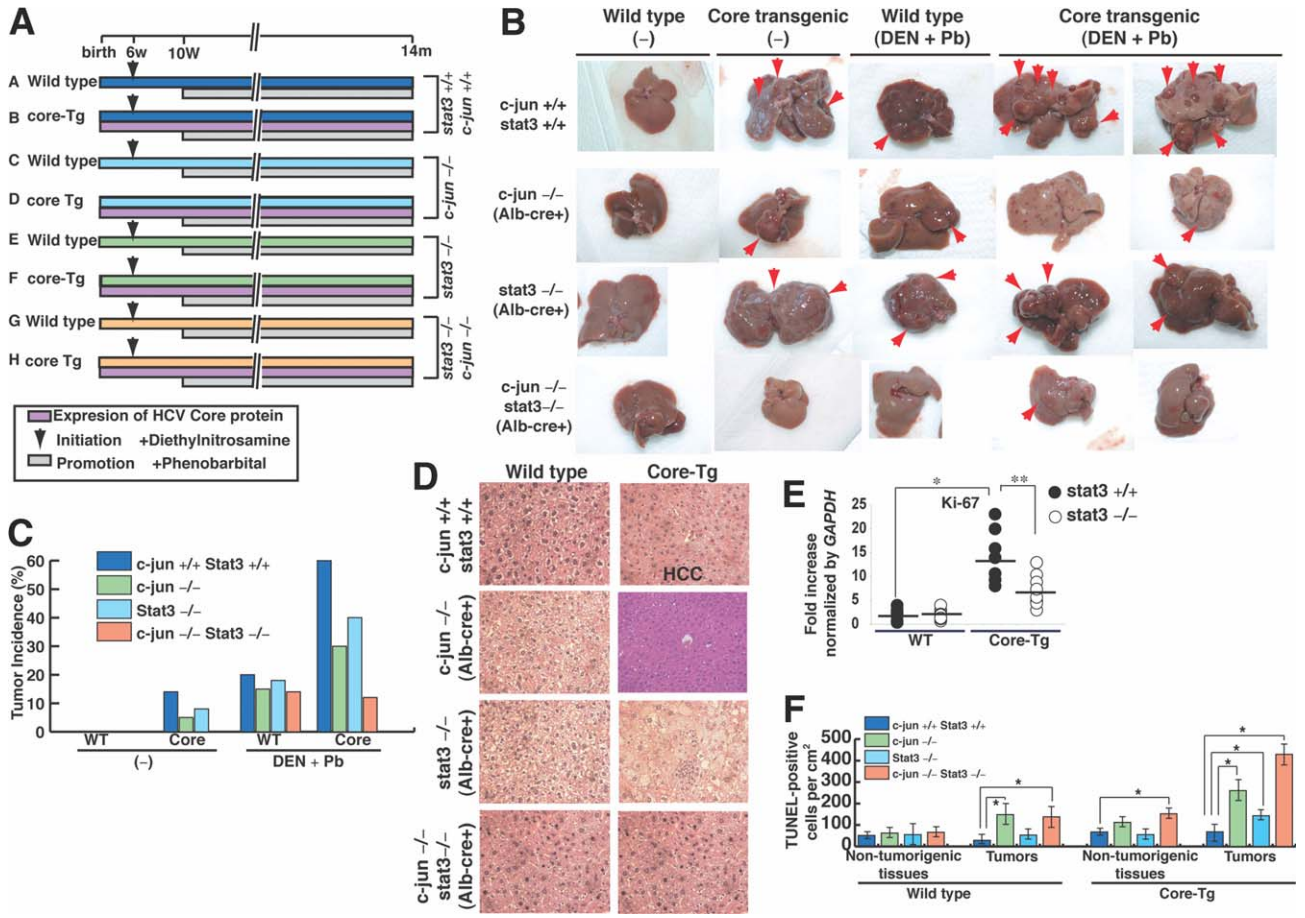



Fig. 5. Cooperation between c-Jun and STAT3 mediates HCV hepatocarcinogenesis. (A) Experimental design to test the role of c-Jun and STAT3 in HCC development in HCV core transgenic mice. HCV core Tg mice or their control non-Tg littermates were injected with the carcinogen DEN at 4 weeks of age and treated with the tumor promoter Pb after 8 weeks of age. The disruption of *c-jun* and/or *stat3* was induced by crossing mice with albumin-promoter-driven *cre* recombinase. The Cre recombinase expressed in hepatocytes, which induces the recombination of lox sites that flank the *c-jun* gene ("floxed"). Mice were sacrificed after 14 months of age for analysis of tumor incidence. (B) Disruption of *c-jun* and *stat3* reduces HCC incidence. (C) Liver tumor incidence of *c-jun*−/− and/or *stat3*−/− HCV core Tg in the presence or absence of DEN/Pb. (D) Sections stained with hematoxylin & eosin from eight groups of mice. (E) Ki-67 mRNA levels in *stat3*+/+ or −/− mice. (F) Apoptotic cell numbers were quantified by TUNEL staining of liver sections from eight different groups.

***Hepatocyte-Specific Knockout of c-Jun and Stat3 Additively Prevent HCV Hepatocarcinogenesis.*** - STAT3 plays an important role in DEN-induced hepatocarcinogenesis.[26] HCV core protein induces generation of ROS[13] and the expression of IL-6 (Fig. 4A), both of which are known agonists for STAT3 activation.[13,14] Indeed, our results demonstrate enhanced activation of STAT in core Tg mice (Fig. 1F) and the potential role of pSTAT3 in c-Jun–dependent pro-oncogenic effects of the core (Fig. 5F). To test the importance of STAT3 in core-induced or core-promoted hepatocarcinogenesis, we examined the effects of hepatocyte-specific deletion of *stat3* (*stat3*$^{flox/flox}$ mice crossed with mice expressing albumin promoter-Cre) on liver oncogenesis induced by DEN/Pb treatment (Fig. 5A). Our results in *c-jun*$^{flox/flox}$ mice injected with the adenoviral vector expressing Cre supported the role of c-Jun in core-mediated and core-enhanced liver tumor formation. However, this technique inevitably deletes *c-jun* in both parenchymal and nonparenchymal liver cells. To further test hepatocyte-specific deletion of *c-jun*, the compound mice harboring a *cre* gene under albumin promoter, *c-jun*$^{flox/flox}$, and a *core* transgene were generated and also tested for DEN/Pb-induced hepatocarcinogenesis. The mice were divided into eight groups ($n$ = 35-48 in each group) based on the presence or absence of *c-jun*, *stat3*, and the viral core protein, and the use of DEN and Pb (Fig. 5A). Conditional knockout of *c-jun* or *stat3* reduced both spontaneous and DEN-induced tumor incidence (Fig. 5B). Furthermore, dual knockout of *c-jun* and *stat3* showed an additive effect, resulting in a remarkable 80% reduction in the incidence (Fig. 5A-C). To determine the role of STAT3 in core-enhanced hepatocellular proliferation, Ki-67 mRNA levels were measured in WT and *Stat3*−/− mice treated with DEN/Pb. Core-

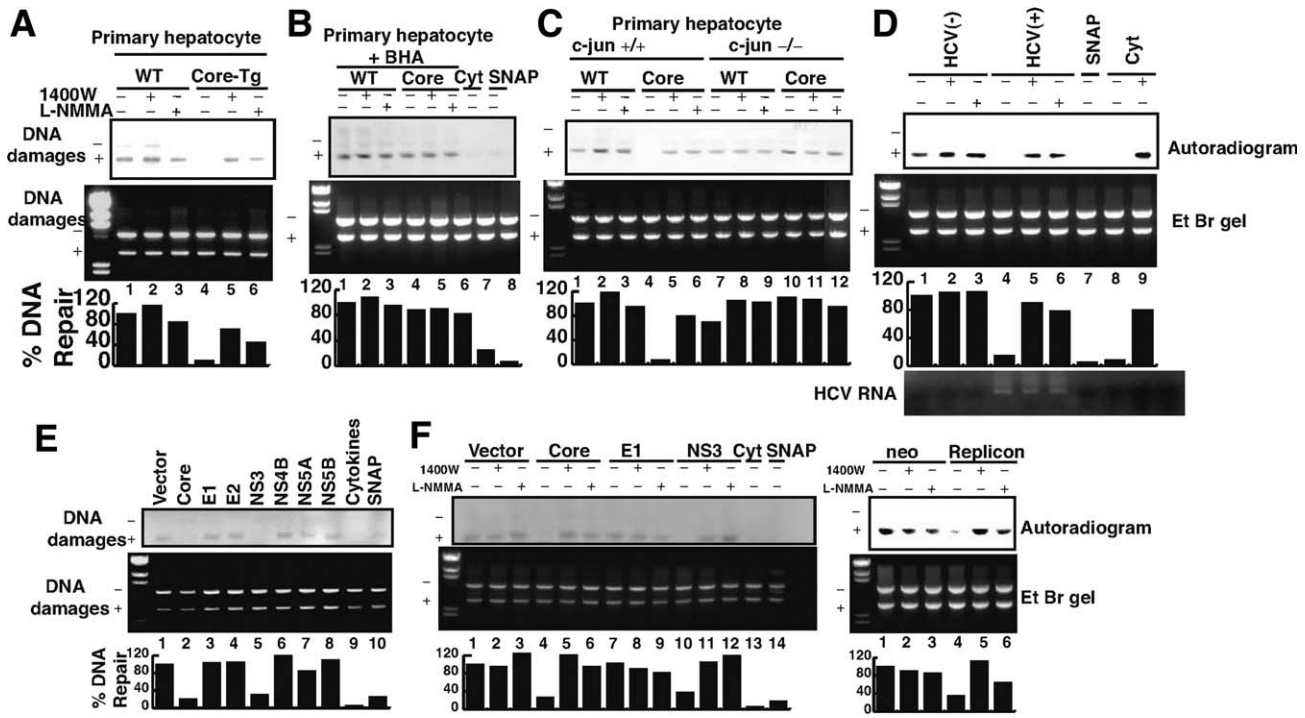



Fig. 6. HCV inhibits DNA repair in a NO-dependent manner through HCV core and NS3 proteins. (A-D) Restoration of the DNA repair activity by NOS inhibitors in mouse primary hepatocytes expressing neomycin resistant gene or core protein in the (A) absence or (B) presence of BHA, in (C) c-jun −/− or c-jun +/+ and (D) HCV-infected Huh7.5.1 cells as determined by *in vitro* dGTP incorporation assays. The photoactivated, methylene blue–damaged plasmid and intact plasmid DNA were incubated with the whole-cell extracts from HCV (+) or HCV (−) cells treated with or without 1400W or L-NMMA in the presence of [$^{32}$P]dGTP. Plasmid DNAs were stained with ethidium bromide. The incorporation of [$^{32}$P]dGTP into the damaged plasmid DNA was determined by autoradiography. Note that only the damaged DNA (lower band) incorporated [$^{32}$P]dGTP. For positive controls, cells were treated with a mixture of cytokines (Cyt) (IL-1$\beta$, interferon-$\gamma$, and TNF-$\alpha$) or SNAP (S-nitrosoacetyl penicillamine), releasing endogenous and exogenous NO, respectively.[28] HCV RNA in the infected cells was detected with RT-PCR. (E) DNA repair assay using extracts from Huh7 cells expressing individual HCV proteins. (F) Restoration of the DNA repair activity by NOS inhibitors in cells expressing individual HCV proteins. (F) Repair incorporation assay using extracts from HCV replicon cells or control cells (neo) containing a neomycin-resistance gene.

induced Ki-67 expression was significantly reduced in STAT3 deficient mice (Fig. 5E). This result and the c-Jun-dependent mitogenic effect (Fig. 3F) suggest that both c-Jun and STAT3 mediate core-induced hepatocellular proliferation. Furthermore, the number of apoptotic cells was significantly increased by c-Jun or STAT3 deficiency in tumor-bearing liver tissues of core Tg mice (Fig. 5F). Interestingly, double knockout of *c-jun* and *stat3* had a synergistic effect on the frequency of apoptotic cells in core Tg mice (Fig. 5F). In tumor-free tissue of core Tg mice or tumor-bearing tissues of WT mice, c-Jun deficiency, but not deficiency in STAT3, significantly increased apoptosis (Fig. 5F). HCV infection is associated with Fas-dependent apoptosis of infected hepatocytes via cytotoxic T lymphocytes.[22] For this reason, we tested the effects of agonistic anti-Fas antibody (Jo2) on hepatocytes isolated from WT and core Tg mice treated with DEN/Pb. Hepatocytes from WT mice released significantly higher levels of AST into the medium and showed frequent TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling) staining in response to Jo2. In contrast, the cells from core Tg mice had significantly reduced release of AST and almost complete absence of TUNEL staining (Supporting Fig. 1A,B,D). Furthermore, hepatocytes from c-Jun–deficient core Tg mice restored Jo2-induced cell death response (Supporting Fig. 1). These differential apoptotic effects between core and WT hepatocytes were closely associated with c-Jun–dependent reduction of Fas expression in core hepatocytes (Supporting Fig. 1C).

***Core-Induced NO Impairs Repair of Oxidative DNA Damage.*** HCV core serves as a tumor initiator (Fig. 3B) through genetic damage caused by core-stimulated generation of ROS or RNS.[18] Furthermore, DNA repair mechanisms may be inhibited by core-generated NO.[27-29] Because the antioxidant BHA inhibits nitrite release[30] and HCV core-induced oncogenesis (Fig. 3D), we hypothesized that core-stimulated generation of NO inhibits DNA damage repair, especially oxidative DNA damage repair. To test this notion, cell lysates from WT and core Tg mouse hepatocytes with or without a prior treatment with NOS

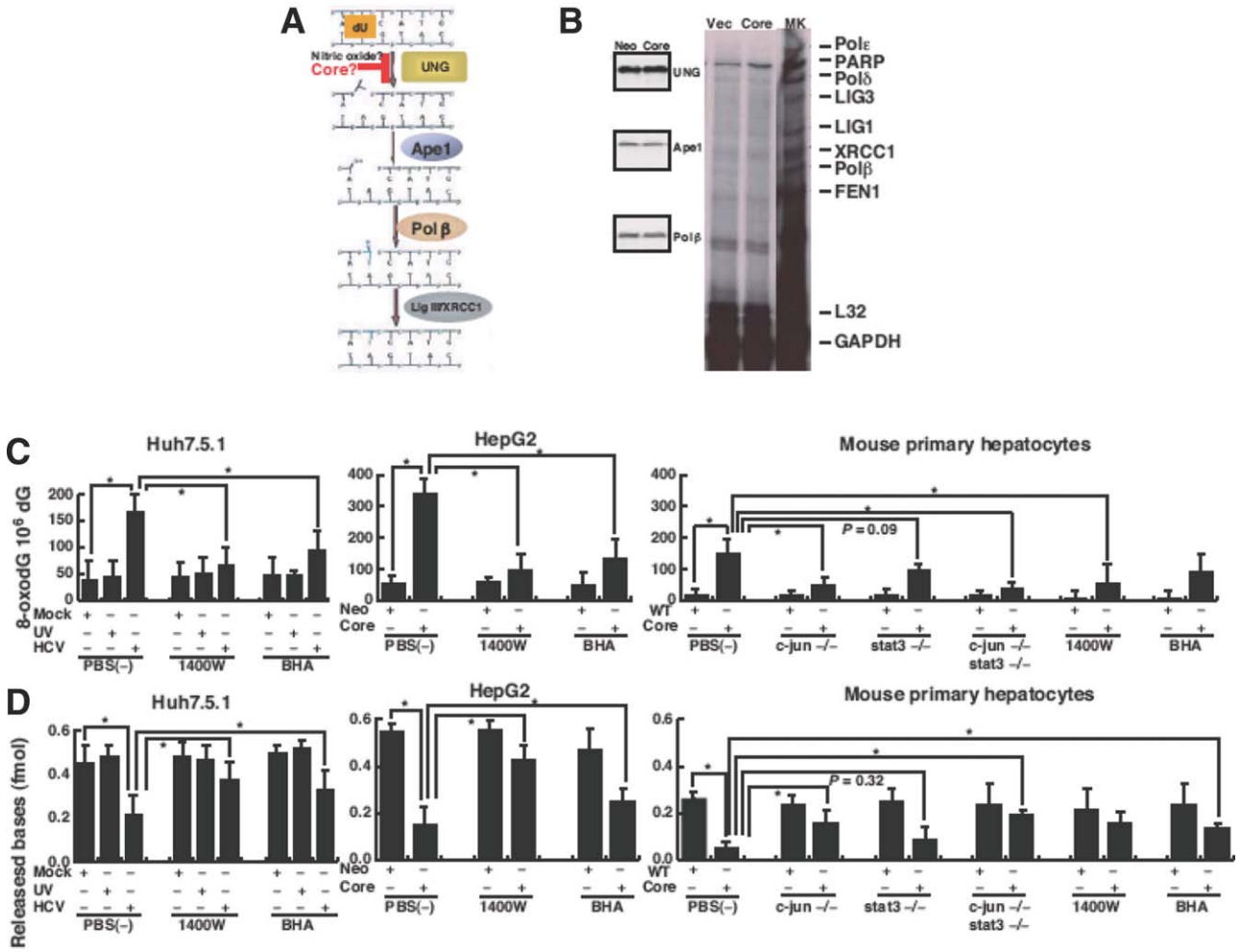



Fig. 7. HCV inhibits DNA repair in an NO-dependent manner through HCV core and NS3 proteins. (A) Postulated mechanism of base excision repair (BER). Immunoblots of UNG, Ape1, and Pol$\beta$. (Right inset). (B) Ribonuclease protection assay of NHEJ components in core-expressing cells. (C) Oxidative DNA damage in HCV-infected Huh7.5.1 and HepG2 cells expressing HCV core protein. DNA sequences were isolated from cells, and their 8-oxodG levels were measured. (D) DNA glycosylase activity in HCV-infected Huh7.5.1, HepG2 cells, and mouse primary hepatocytes. *$P < 0.05$; ** $P < 0.01$, compared with controls.

inhibitors were examined for their ability to promote *in vitro* incorporation of the radiolabeled nucleotide [$^{32}$P]deoxyguanosine triphosphate ([$^{32}$P]dGTP) into a damaged DNA substrate. If dGTP is efficiently incorporated into the substrate with a lysate, this means that the lysate contained fully functional repair mechanisms to excise damaged bases and to incorporate new dGTP. Our results showed that dGTP was incorporated into the damaged DNA when the lysate from WT hepatocytes was used, whereas no dGTP incorporation was evident using the core Tg hepatocyte lysate (Fig. 6A, lanes 1 versus 4). Pretreatment with a specific iNOS inhibitor (1400W) or a general NOS inhibitor (N $\omega$-nitro-L-arginine methyl ester [L-NMMA]) nearly normalized the dGTP incorporation activity with the lysate from Tg hepatocytes (Fig. 6A, lanes 5 and 6). Similarly, the lysate from core Tg hepatocytes treated with BHA also had normal dGTP incorporation as seen in the WT lyaste (Fig. 6B, lane 4). Furthermore, the treatment of WT hepatocytes with a mixture of NO-inducing cytokines (interferon-$\gamma$, TNF-$\alpha$, IL-1$\beta$) or a NO donor (S-nitrosoacetyl penicillamine [SNAP]), caused a complete failure in dGTP incorporation (Fig. 6B, lanes 7 and 8)

Next, we tested the role of c-Jun in core-induced inhibition of dGTP incorporation. The lysate from core Tg mouse hepatocytes deficient in c-Jun (*albumin-cre*:*c-jun*$^{flox/flox}$: *c-jun*$-/-$) showed the normal level of dGTP incorporation as opposed to severely impaired activity with the lysate from core Tg/*c-jun*+/+ mice (Fig. 6C, lane 4 versus 10). These results support the obligatory role of c-Jun in mediating core-induced inhibition of DNA repair via NO. To extend this conclusion to natural HCV infection, we tested the lysate from

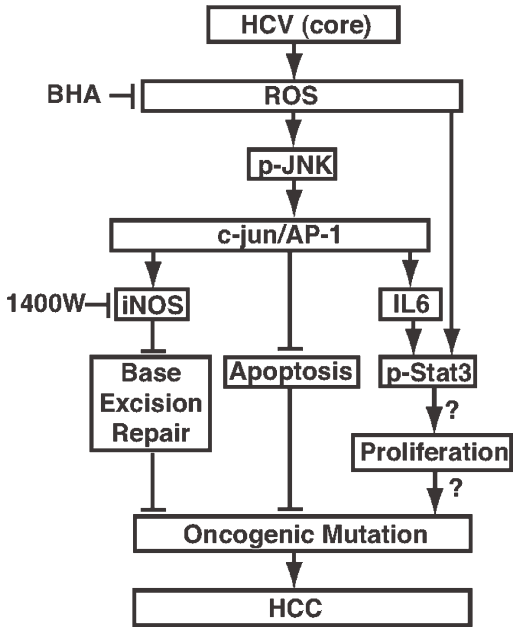



Fig. 8. The postulated mechanism of core-induced HCC induction. Schematic model depicting the proliferative function of c-Jun through STAT3, and c-Jun antagonizing base excision repair (BER) activity by down-regulation, via overexpression of iNOS. Following hepatocyte transformation, c-Jun regulates inflammatory cytokine transcription thereby leading to STAT3 up-regulation and inhibition of oxidatively damaged DNA-repair triggered by the c-Jun target gene *inos*.

Huh7.5.1 cells infected or uninfected with HCV for the dGTP incorporation analysis. Indeed, HCV-infected cell lysate (HCV+) showed impaired incorporation activity, which was again normalized by treatment with 1400W or L-NMMA (Fig. 6D). In addition, the treatment of cells not infected with HCV (HCV−) with the NO donor SNAP or the NO-inducing cytokine mixture obliterated the incorporation activity, and the latter effect was prevented with 1400W (Fig. 6D). Thus, these results confirm HCV-mediated inhibition of oxidative DNA damage repair via NO generation in the setting of HCV infection. HCV expresses several other structural and nonstructural proteins besides core. Thus, we next tested these viral proteins for their effects on DNA repair. For this analysis, the [$^{32}$P]dGTP incorporation assay was performed on Huh7 cells expressing individual viral proteins (Fig. 6E). Among seven viral proteins examined, core and NS3 (nonstructural protein 3) proteins equally impaired the incorporation activity (Fig. 6E), which was restored by treatment with NO inhibitors (Fig. 6F). Similar results were obtained using the lysate from Huh7 cells containing an HCV replicon, which included NS3 (Fig. 6F). The control cell line containing a neomycin-resistant gene exhibited normal dGTP incorporation activity, which was not affected by the NO inhibitors. These results indicate that NO induced by core and NS3 proteins is responsible for inhibition of DNA repair associated with HCV infection (Fig. 6A-F).

***Effect of the HCV Core Protein on Base Excision Repair.*** HCV infection or core protein inhibits dGTP-incorporation activity in a c-Jun and NO-dependent manner, which is mainly facilitated by base excision repair (BER). BER removes a variety of DNA lesions such as spontaneous hydrolytic depurination, deamination of cytosine and 5-methylcytosine, products of reactions with hydroxyl radical, and covalent DNA adducts.[26] The BER components include Pol$\beta$, pol$\delta$, pol$\epsilon$, APE1 (AP-endonuclease), and Ogg1 (8-oxoguanine DNA glycosylase).[31] To determine whether HCV core protein affects the BER, we performed immunoblot analysis to determine the expression of the components of the BER in HepG2 cells with and without stable core protein expression. We also performed coimmunoprecipitation analysis to assess the interactions between the BER components and the HCV core protein. Neither alteration of protein or mRNA levels of the BER components (Fig. 7A,B), nor the interaction of the core protein with the components (the data not shown), was observed.

***DNA Glycosylase Activity in Hepatocytes During HCV Infection.*** We next analyzed whether the accumulation of 8-oxodG in HCV-infected Huh7.5.1 cells, core-transduced HepG2 cells, and primary hepatocytes from core Tg mice, is accompanied by alterations in DNA glycosylase activity for the repair of oxidative damage. For this assessment, we measured the activity which specifically removes 8-oxodG using a duplex oligonucleotide containing a radiolabeled 8-oxodG residue.[32] As predicted, HCV infection or core expression either by stable transduction or as a transgene, increased the content of 8-oxodG in a manner dependent on iNOS or oxidant stress (Fig. 7C). Accumulation of this oxidative DNA modification was also shown to be dependent on c-Jun in primary hepatocytes from core Tg versus Tg:*c-jun*−/− mice (Fig. 7E, the last panel). These increases in the 8-oxodG content are closely associated with concomitant reductions in the release of 8-oxodG by DNA glycosylase activity of the respective cell lysate (Fig. 7D). Furthermore, the protective effects of the iNOS inhibitor (1400W), antioxidant (BHA), or c-Jun deficiency (*c-jun*−/−) tightly correlated with enhanced DNA glycosylase activity (Fig. 7D).

## Discussion

We demonstrated that dual ablation of *c-jun* and *stat3* results in an additive and nearly complete

prevention of both spontaneous and DEN-induced HCC in HCV core Tg mice, highlighting the critical role of both c-Jun and STAT3 in HCV hepatocarcinogenesis. The core-induced proliferative effects on hepatocytes required activation of c-Jun/AP-1 and STAT3, particularly during tumor initiation and early progression (Fig. 8). Furthermore, our data suggest that c-Jun is upstream of STAT3 activation (Fig. 3F), probably via c-Jun–mediated IL-6 induction (Fig. 4A). The antioxidant effect of BHA is most likely upstream, scavenging ROS, which in turn suppresses c-Jun activation[33] and oxidative DNA damage.

These results demonstrate that HCV core protein induces specific signaling via c-Jun and STAT3 that culminate in the multiple levels of mutagenic and prooncogenic effects as a tumor initiator to induce spontaneous HCC and to enhance carcinogen/promoter-induced hepatic carcinogenesis. Based on this conclusion, c-Jun and STAT3 inhibitors[34] may be particularly useful during precancerous stages such as cirrhosis or chronic viral infection, as chemopreventive agents.

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