Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Deletion of *IFNγ* enhances hepatocarcinogenesis in *FXR* knockout mice

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Background & Aims: Liver tumor, especially hepatocellular carcinoma (HCC), is closely associated with chronic inflammation. We previously showed that farnesoid X receptor knockout ($FXR^{-/-}$) mice displayed chronic inflammation and developed spontaneous liver tumors when they aged. However, the mechanism by which inflammation leads to HCC in the absence of FXR is unclear. Because IFNγ is one of the most upregulated pro-inflammatory cytokines in $FXR^{-/-}$ livers, we generated $IFN\gamma^{-/-}FXR^{-/-}$ double knockout mice to determine IFNγ's roles in hepatocarcinogenesis.

Methods: $IFN\gamma^{-/-}$ mice were crossed with an $FXR^{-/-}$ C57BL/6 background or injected i.p. with the hepatocarcinogen diethylnitrosamine (DEN). Hepatocarcinogenesis was analyzed with biochemical and histological methods.

Results: *IFNγ* deletion accelerated spontaneous hepatocarcinogenesis in $FXR^{-/-}$ mice and increased the susceptibility to DEN-induced hepatocarcinogenesis. *IFNγ* deletion enhanced activation of HCC promoters STAT3 and JNK/c-Jun, but abolished induction of p53 in $IFN\gamma^{-/-}$ livers after acute DEN-induced injury. Furthermore, hepatic p53 expression increased in aged wild type mice but not in aged $IFN\gamma^{-/-}$ and $IFN\gamma^{-/-}FXR^{-/-}$ mice, while activation of STAT3 and JNK/c-Jun was enhanced in aged $IFN\gamma^{-/-}$ and $IFN\gamma^{-/-}FXR^{-/-}$ mice. In addition, IFNγ inhibited liver cancer xenograft growth and impaired IL-6-induced STAT3 phosphorylation by inducing SOCS1/3 expression.

Conclusions: Increased IFNγ expression in $FXR^{-/-}$ livers represents a protective response of the liver against chronic injury and tumorigenesis. IFNγ suppresses hepatocarcinogenesis by inducing p53 expression and preventing STAT3 activation.

Cancer

Introduction

HCC is the fifth most prevalent cancer and the third leading cause of cancer death in the world [1]. HCC commonly develops in a setting of liver damage, and the major risk factor for liver damage and HCC is infection with hepatitis B or C viruses [2]. A common pathological feature of HCC development regardless of etiology is represented by chronic inflammation triggered by hepatocyte death, which leads to continuous compensatory hepatocyte proliferation. HCC is also highly connected to liver metabolic disorders. One particular example is spontaneous HCC development in $FXR^{-/-}$ mice [3].

FXR is a key metabolic regulator of bile acid, lipid, and glucose homeostasis. FXR also regulates host immunity in some contexts. For example, FXR prevents bacterial infection in intestine, modulates concanavalin A-induced T cell hepatitis, and antagonizes LPS-induced hepatic inflammation [4–6]. $FXR^{-/-}$ mice display a low grade chronic inflammation as early as they are 8-weeks-old and spontaneously develop liver cancer when they are over 1 year of age [3]. Furthermore, FXR expression is strongly downregulated in human HCC, and hepatocarcinogenesis in $FXR^{-/-}$ mice mimics human HCC progression [7]. Therefore, $FXR^{-/-}$ mice provide a unique model of HCC in a background of chronic inflammation induced by metabolic disorders. However, the mechanism by which chronic inflammation leads to HCC in the absence of FXR is still unclear.

Keywords: FXR; HCC; STAT3; JNK; NF-κB; p53.
Received 26 January 2012; received in revised form 7 June 2012; accepted 12 June 2012; available online 21 June 2012
☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2012.07.040.
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Abbreviations: HCC, hepatocellular carcinoma; FXR, farnesoid X receptor; DEN, diethylnitrosamine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ROS, reactive oxygen species.

A variety of signaling molecules, particularly cytokines and their downstream mediators, divert inflammation to liver carcinogenesis. These include TNFα, IL-6, IKK/NF-κB, JAK/STAT3, JNK/c-Jun [8–11]. IFNγ is one of the most upregulated cytokines in $FXR^{-/-}$ mouse livers [3], but the exact roles of IFNγ during HCC development in $FXR^{-/-}$ mice are unclear. In this study, we show that *IFNγ* deletion enhanced hepatocarcinogenesis in $FXR^{-/-}$ mice and sensitized mice to DEN-induced tumorigenesis. We also identified a novel role of IFNγ in maintaining aging-induced activation of p53 and NF-κB and preventing hyperphosphorylation of STAT3 and JNK in livers. Our results underscore an important role of IFNγ in suppressing hepatocarcinogenesis.

Materials and methods

Animals

$IFN\gamma^{-/-}$ mice were purchased from Jackson Laboratory. To generate $IFN\gamma^{-/-}FXR^{-/-}$ mice, $IFN\gamma^{-/-}$ mice were crossed with $FXR^{-/-}$ mice in C57BL/6 background. DEN-induced HCC rodent models were generated according to a previous report [12]. Briefly, 100 mg/kg DEN (Sigma, Santa Louis, MO) was i.p. injected into 4-week-old mice, and after 2 weeks, 3 mg/kg TCPOBOP (Sigma) was administered to the mice, once every two weeks for 8 times. Six months after DEN treatment, mice were euthanized and samples collected. Further details on xenograft studies with HuH7 cells and IFNγ are provided in Supplementary data. Mice were maintained in a pathogen-free animal facility under standard 12:12-h light/dark cycle, and were fed standard rodent chow and water *ad libitum*. All procedures followed the NIH guidelines for the care and use of laboratory animals.

Liver histology, TUNEL, and PCNA staining

Livers were fixed in 4% PBS-buffered formalin, dehydrated and embedded in paraffin, sectioned and processed for H&E and immunostaining. Liver specimens were analyzed by pathologists at City of Hope Research Core Lab. Necrosis and leukocyte infiltration (inflammation) were graded as described [13]. TUNEL and PCNA stainings were used to quantify liver cell apoptosis and proliferation with kits from Roche (San Diego, CA) and Invitrogen (San Diego, CA), respectively. The specimens for sectioning were made with approximately the same size and all the positive cells were counted on the specimens. The methods for the other immunostainings are provided in Supplementary data.

Quantitative real-time PCR

RNAs were isolated with TRI reagents (Molecular Research Center, Cincinnati, OH). RNAs were reverse transcribed to cDNA using SuperScript First-Strand Synthesis System (Invitrogen) and quantified by Applied Biosystems 7500 Real-Time PCR System (Forest City, CA). Primers are listed in Supplementary Table 1.

Western blotting

Western blotting was performed as previously described [14]. Anti-β-actin antibody was from Sigma.

All the other antibodies, including phospho-Y701-STAT1 and phospho-Y705-STAT3 antibodies, were purchased from Cell Signaling Technology (Danvers, MA).

Lipid peroxide, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) analysis

Liver lipid peroxides were measured with a kit from Cayman Chemicals (Ann Arbor, MI). Serum was obtained by centrifuging mouse blood at 3500 rpm at 4 °C for 10 min. Serum AST and ALT levels were measured at the City of Hope Helford Research Hospital.

Statistical analysis

All the data were reported as mean ± SEM. Two-tailed Student's-*t* test or one way ANOVA test was used to determine the significance of differences between data groups.

Results

IFNγ deletion enhances spontaneous liver tumorigenesis in $FXR^{-/-}$ mice

The $FXR^{-/-}$ background provided a context of spontaneous liver injury and chronic inflammation [7]. *IFNγ* deletion in $FXR^{-/-}$ mice led to liver tumorigenesis as early as mice were 8-months-old, while no tumor incidence was observed in $FXR^{-/-}$ mice at that time (Table 1). Although livers of 3-month-old $IFN\gamma^{-/-}$ mice did not display morphological differences from wild type mouse livers (Supplementary Fig. 1), sparse HCC incidence was observed in aged $IFN\gamma^{-/-}$ mice but not their wild type littermates over 15-months-old (Table 1). $FXR^{-/-}$ mice had a low tumor incidence rate at 10 months of age (Table 2). In contrast, *IFNγ* deletion in $FXR^{-/-}$ mice resulted in more than 80% incidence and much larger tumors (Table 2, Fig. 1A). Immunohistochemistry analysis of hepatic expression of CD34, CK19, and CK20 revealed that tumors were hepatocellular carcinomas and not derived from bile ducts or intestinal tissues [15] (Fig. 1B).

Deletion of *IFNγ* elevated levels of ALT and AST in 10-month-old wild type and $FXR^{-/-}$ mice (Table 2). These results suggested that *IFNγ* deletion promoted spontaneous liver injury during the aging process, which was supported by hepatocyte degeneration and focal necrosis in the livers (Fig. 1C, Supplementary Fig. 2). Furthermore, *IFNγ* deletion significantly enhanced apoptosis and inflammatory cell infiltration in $FXR^{-/-}$ mice (Fig. 1C, Supplementary Fig. 3A), and in turn led to increased compensatory hepatocyte proliferation in $IFN\gamma^{-/-}FXR^{-/-}$ mice (Fig. 1C, Supplementary Fig. 3B), which is believed to be a major driving force of tumor initiation and expansion. In addition, collagen deposition and fibrosis-related gene expression were enhanced by *IFNγ* and/or *FXR* deletion (Supplementary Fig. 4A and B), which is consistent with the role of IFNγ against fibrosis [16].

IFNγ deletion enhances chemical-induced liver tumorigenesis

We used DEN-induced HCC models to further determine IFNγ's roles in hepatocarcinogenesis and followed a protocol of HCC induction described previously [12]. This method led to ~70% HCC incidence in 7-month-old wild type mice (Table 3). In contrast, all the $IFN\gamma^{-/-}$, $FXR^{-/-}$, and $IFN\gamma^{-/-}FXR^{-/-}$ mice developed liver tumors at this age. Moreover, $IFN\gamma^{-/-}$ mice developed more and larger hepatocellular carcinomas than wild type mice, and $IFN\gamma^{-/-}FXR^{-/-}$ mice displayed enhanced hepatocarcinogenesis compared with $FXR^{-/-}$ mice (Table 3, Fig. 2A, Supplementary Fig. 5).

Serum AST and ALT levels were higher in $IFN\gamma^{-/-}$ mice than in wild type mice, confirming the protective role of IFNγ against liver injury (Table 3). This role was further supported by the more severe necrosis and apoptosis in the non-tumor liver tissue of $IFN\gamma^{-/-}$ mice after DEN treatment compared with wild type controls (Fig. 2B). Histological studies revealed more inflammatory cell infiltration (Supplementary Fig. 6, Fig. 2B) and fibrogenesis in $IFN\gamma^{-/-}$ mice than in wild type mice (Supplementary Fig. 7A and B). In addition, oval cell-like cells appeared more frequently in mice with *IFNγ* deletion, indicating activation of liver progenitor cells was enhanced in these mice (Supplementary Fig. 8), which was confirmed by the immunostaining for the oval cell marker A6 (Supplementary Fig. 9) [17]. Consistent with the spontaneous liver tumorigenesis model, *IFNγ* deletion led to enhanced compensatory hepatocyte proliferation in DEN-induced HCC

Table 1. Spontaneous hepatocarcinogenesis.

Tumor incidence/ mice	WT	*IFNγ*$^{-/-}$	*FXR*$^{-/-}$	*IFNγ*$^{-/-}$ *FXR*$^{-/-}$
6 mo	0/8	0/8	0/8	0/12
8 mo	0/8	0/6	0/12	5/13
10 mo	0/8	0/12	2/14	24/29
15 mo	0/12	3/21	12/12	17/17

(Fig. 2B). These results highlight a key role of IFNγ in suppressing the development of both spontaneous and chemical-induced HCC.

IFNγ deletion enhances cell deaths and compensatory proliferation after DEN treatment

Injury-induced inflammation and compensatory proliferation following exposure to carcinogen play essential roles in cancer initiation. To investigate IFNγ's roles in HCC initiation, the acute phase of DEN-induced liver injury was evaluated in 4-week-old *IFNγ*$^{-/-}$ mice. *IFNγ*$^{-/-}$ mice showed more than 2.5-fold body weight loss and much more robust ALT increase (Fig. 2C) than wild type mice, 2 days after a single DEN injection (100 mg/kg). Consistently, *IFNγ*$^{-/-}$ mice carried more severe focal necrosis, or even submassive necrosis and more extensive inflammatory cell infiltration (Fig. 2D, Supplementary Fig. 10A). In addition, TUNEL staining revealed more apoptotic cells in *IFNγ*$^{-/-}$ livers than in wild type controls (Fig. 2D, Supplementary Fig. 10B). In response to cell deaths and inflammation, compensatory hepatocyte proliferation following acute injury was enhanced in *IFNγ*$^{-/-}$ mice (Fig. 2D, Supplementary Fig. 10B). Consistently, activation of inflammation mediators STAT3 and JNK/c-Jun was augmented (Fig. 2E). Furthermore, both basal and DEN-induced p53 expression was absent in *IFNγ*$^{-/-}$ mice, though no difference in NF-κB activation was observed. Even without DEN treatment, 4-week-old *IFNγ*$^{-/-}$ mice already exhibited higher levels of phosphorylated STAT3 and c-Jun than wild type mice. Overall, the altered responses of STAT3, JNK, and p53 may contribute to enhanced cell deaths, inflammation, and compensatory proliferation in *IFNγ*$^{-/-}$ livers.

IFNγ is required to maintain p53 and NF-κB activation in aging livers

Many types of cancers, including liver cancer, have a strong correlation with aging. The production of IFNγ is altered in aged human individuals [18,19], which prevents proliferation of aged hepatocyte and may help protect against tumorigenesis [20]. Indeed, hepatic expression of IFNγ was also higher in 10-month-old mice than in 3-month-old mice (Supplementary Fig. 11A). Therefore, we asked whether the enhanced hepatocarcinogenesis in *IFNγ*$^{-/-}$ mice was associated with aging–related stresses. We compared hepatic activation of proto-oncogenes and tumor-suppressor genes between 3- and 10-month-old wild type mice. Among many signal pathways we examined, STAT1 and STAT3 activation did not clearly show a tendency of increase, but p53 expression and NF-κB activation were significantly upregulated (Fig. 3A). p53 acts as a checkpoint protein in the cell cycle and suppresses the uncontrolled cancer cell duplication, while hepatocyte NF-κB inhibits hepatocarcinogenesis by repressing reactive oxygen species (ROS) accumulation and preventing necrosis [9]. The activation of these pathways can protect aging livers from hepatocarcinogenesis.

More surprisingly, absence of IFNγ or FXR greatly reduced STAT1 phosphorylation in precancerous livers (Supplementary Fig. 11B, and Fig. 3B). Furthermore, induction of p53 and phosphorylation of hepatic IκB and p65 were reduced or abolished in knockout mice, indicating that the age-related activation of p53 and NF-κB required the presence of IFNγ and FXR, consistent with the reported interaction and synergistic activation of IFNγ/STAT1 and NF-κB pathways [21,22]. ROS, which is capable of inducing DNA damage, genomic instability, and activating STAT3 and JNK, is antagonized by one of the NF-κB target genes, MnSOD. MnSOD catalyzes the dismutation of two molecules of superoxide anion into water and hydrogen peroxides, and thus reduces ROS and protects the liver from oxidative stresses [9]. Indeed, levels of lipid peroxides, the ROS products, were upregulated in all the precancerous tissues of knockout mice (Fig. 3C), probably due to a decreased MnSOD expression resulting from absence of hepatic NF-κB activation (Fig. 3D).

Table 2. Spontaneous HCC in 10-month-old mice.

	WT	*IFNγ*$^{-/-}$	*FXR*$^{-/-}$	*IFNγ*$^{-/-}$*FXR*$^{-/-}$
Tumor incidence x100%	0.0	0.0	14.0	82.7
Tumor number per liver	0.0	0.0	0.6 ± 0.5	7.4 ± 1.3**
No. of tumor diameter >0.2 cm	0.0	0.0	0.0 ± 0.0	2.2 ± 0.4**
Maximum tumor diameter/cm	0.0	0.0	0.1 ± 0.1	0.5 ± 0.1**
Liver/body weight ratio x100%	5.0 ± 0.1	4.8 ± 0.1	6.3 ± 0.2	6.4 ± 0.1
ALT (U/L)				
3 mo	129.3 ± 7.4	120 ± 6.1	267.0 ± 29.7	288.0 ± 14.0
10 mo	131.2 ± 13.2	473.1 ± 50.4††	572.0 ± 78.2	805.7 ± 52.5*
AST (U/L)				
3 mo	44.0 ± 6.1	74.7 ± 29.7	214.0 ± 33.2	255.0 ± 61.8
10 mo	37.6 ± 4.7	228.4 ± 36.8††	261.7 ± 71.1	368.0 ± 92.3

††p <0.01. *IFNγ*$^{-/-}$ *vs.* wild type.
*p <0.05; **p <0.01. *IFNγ*$^{-/-}$*FXR*$^{-/-}$ *vs. FXR*$^{-/-}$.

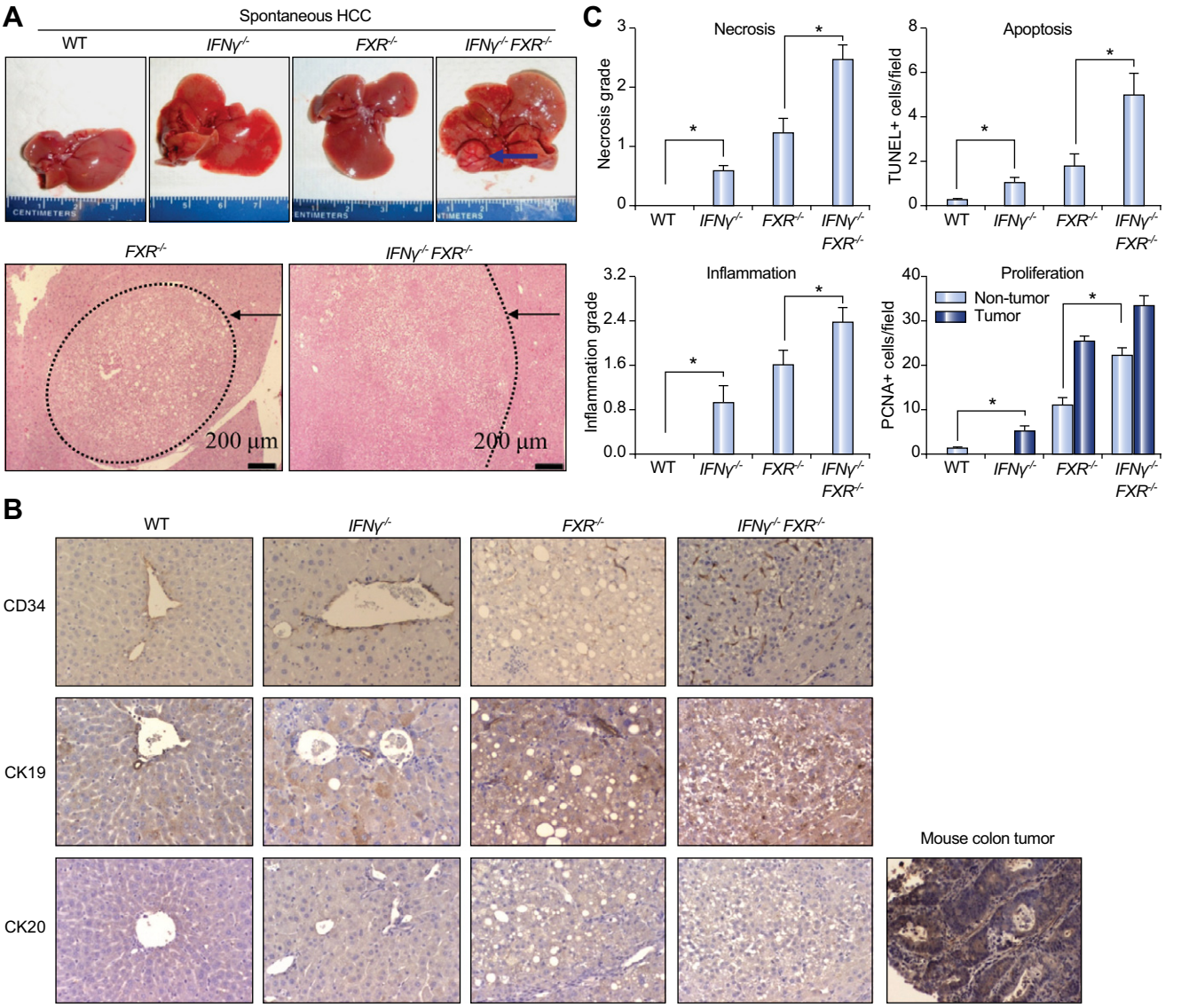

Fig. 1. *IFNγ* deletion promotes spontaneous liver tumorigenesis in *FXR*$^{-/-}$ mice. (A) Representative images of liver tumorigenesis and H&E staining in 10-month-old mice. Arrows indicate tumors. (B) Immunohistochemical analysis of spontaneous liver tumors in 10-month- old mice, with CD34, CK19, and CK20 antibodies. The right bottom panel shows a mouse colon tumor provided by the City of Hope Research Pathology Core, which served as a positive control for CK20 immunostaining. Magnification, 200×. (C) Grading of necrotic hepatocytes and inflammatory cell infiltration by H&E staining, and quantification of apoptotic and proliferating cells by TUNEL and PCNA staining. Student *t* test was applied for statistical analysis. $^{*}p$ <0.05; n = 5 or more. [This figure appears in colour on the web.]

Enhanced STAT3 and JNK1/2 activation and decreased p53 expression in IFNγ$^{-/-}$ mice

STAT1 plays a tumor-suppressor role by antagonizing STAT3 [23–25], and hepatocyte NF-κB suppresses hepatocarcinogenesis by attenuating both STAT3 and JNK activation, in part by controlling ROS [9,26]. Therefore, we speculated that in *IFNγ*$^{-/-}$, *FXR*$^{-/-}$, and *IFNγ*$^{-/-}$*FXR*$^{-/-}$ mouse livers the exaggerated activation of STAT3 and JNK can be observed due to decreased STAT1 and NF-κB activation. Indeed, STAT3 and JNK1/2 were hyperphosphorylated, especially in *IFNγ*$^{-/-}$*FXR*$^{-/-}$ tumors (Fig. 3E). STAT3 can target many anti-apoptotic genes, including *Bcl-2*, *Bcl-xl*, and *Mcl-1*. Expression of these 3 genes was increased in knockout animals (Fig. 3F). The substrate of JNK1/2, c-Jun, is a strong tumor-promoter in the liver, since *c-Jun*$^{-/-}$ mice are more resistant to hepatocarcinogenesis due to loss of c-Jun suppression on p53 [11]. c-Jun phosphorylation was dramatically increased in aged *IFNγ*$^{-/-}$, *FXR*$^{-/-}$, and *IFNγ*$^{-/-}$*FXR*$^{-/-}$ mouse livers. c-Jun has a positive auto-feedback loop of its transcription by binding to its own promoter after being phosphorylated. Consistently, we observed that a robust increase in c-Jun expression in aged knockout animals, which indicated that loss of IFNγ in the aging liver greatly enhanced some oncogenic signaling such as c-Jun. In addition, upregulation of Cyclin D1 and c-Myc and downregulation of p53 in knockout livers (Fig. 3E and F and Supplementary Fig. 11C) appeared to be critical. In fact, c-Myc is suppressed by IFNγ [27], and Cyclin D1 and c-Myc are also activated by STAT3 in HCC. Besides, the hyperactivation of STAT3 and JNK1/2, which

Table 3. DEN-induced HCC in 7-month-old mice.

	WT	*IFNγ*$^{-/-}$	*FXR*$^{-/-}$	*IFNγ*$^{-/-}$*FXR*$^{-/-}$
Tumor incidence x100%	70.0	100.0	100.0	100.0
Tumor number per liver	4.4 ± 0.9	24.7 ± 4.3††	38.2 ± 4.6	64.1 ± 2.6**
No. tumor diameter >0.2 cm	1.7 ± 0.6	4.0 ± 0.4††	7.8 ± 0.7	8.7 ± 0.7
No. tumor diameter >0.5 cm	0.2 ± 0.1	0.8 ± 0.3	0.8 ± 0.4	2.1 ± 0.3*
Maximum tumor size/cm	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	1.0 ± 0.1*
Liver/body weight ratio x100%	5.6 ± 0.2	7.1 ± 0.3	8.3 ± 0.6	8.6 ± 0.4
ALT (U/L)	202.0 ± 24.1	803.2 ± 31.2††	670.0 ± 151.3	1056.0 ± 96.5*
AST (U/L)	74.0 ± 15.9	272.7 ± 30.9††	196.0 ± 37.9	423.6 ± 31.7**

††p <0.01. *IFNγ*$^{-/-}$ *vs.* wild type.
*p <0.05; **p <0.01. *IFNγ*$^{-/-}$*FXR*$^{-/-}$ *vs. FXR*$^{-/-}$.

could also reflect increased hepatic inflammation and immune cell infiltration, could be attributed to the increased expression of hepatic TNFα and IL-6, two cytokines well known for promoting hepatocarcinogenesis (Supplementary Fig. 11C).

STAT3 and JNK are persistently hyperactive in IFNγ$^{-/-}$ livers after DEN treatment

We further confirmed the activation of STAT3 and JNK1/2 in the cancer progression stage in DEN-induced HCC models. In response to decreased phosphorylation of STAT1, STAT3 and JNK/c-Jun were hyperactivated in *IFNγ*$^{-/-}$ precancerous tissues (Fig. 4A). Consistently, STAT3 activator lipid peroxide/ROS was upregulated in *IFNγ*$^{-/-}$ mice, and expression of STAT3 target genes *Bcl2*, *Cyclin D1*, and *c-Myc* was significantly increased (Fig. 4B). However, deregulation of STAT3 and JNK was independent of NF-κB pathways since IκB phosphorylation was higher in *IFNγ*$^{-/-}$ livers than in wild type controls, probably due to a higher expression of IKKα/β (Fig. 4A). Unlike short-term post-DEN stimulation and spontaneous HCC model, p53 expression at this stage was not reduced in *IFNγ*$^{-/-}$ livers. Differences in NF-κB and p53 activation might be due to different progression stages and different mechanisms of tumorigenesis. In accord with the spontaneous HCC model, hepatic expression of certain tumor-promoting cytokines, for instance TNFα, IL-6, and TGFβ, was upregulated (Supplementary Fig. 12).

IFNγ blunts IL-6-induced STAT3 phosphorylation in liver cells and inhibits liver cancer xenograft

To investigate the direct suppressive effects of IFNγ on STAT3 in liver cells, we pretreated HepG2 and Huh7 cells with IFNγ and then added IL-6 to the cells. IFNγ greatly reduced IL-6-induced STAT3 phosphorylation in both cell lines (Fig. 4C). Moreover, the STAT1 inhibitor fludarabine slightly increased IL-6-induced STAT3 phosphorylation (Supplementary Fig. 13), though the inhibitor did not directly alter IκBα and JNK activation by TNFα. The suppressive effects of IFNγ might be due to the induction of SOCS1 and SOCS3 in liver cells since SOCS proteins were specific inhibitors of STAT3 phosphorylation (Fig. 4D). Furthermore, we found that treatment of the HuH7 xenograft with IFNγ decreased tumor growth (Fig. 4E and F), which is consistent with reports on applications of IFNγ on liver cancer models [16].

Discussion

Liver cancer is one of the most common cancers worldwide. Recent studies have focused on the associations of HCC with metabolic diseases. The possible causal link between metabolic diseases and HCC, independent of other well-recognized risk factors, such as viral infections and alcohol, suggests that metabolic dysfunction of the liver may be an important etiology of hepatocarcinogenesis. Metabolic dysfunction may act synergistically with other etiological agents, such as viruses, to promote HCC. Previously, we have observed that both male and female *FXR*$^{-/-}$ mice spontaneously develop liver tumors as they age [3]. Before tumors emerged, liver injury, inflammation, and irregular liver regeneration were observed in *FXR*$^{-/-}$ mice, but not in wild type mice of the same age [3,7,28]. Therefore, *FXR*$^{-/-}$ mice provide a unique animal model for studying metabolic deregulation-related HCC. A key feature of these mice is the chronic inflammation and upregulation of several inflammatory cytokines such as IFNγ in their livers. Interestingly, IFNγ modulates several aspects of metabolism, for instance cytochrome P450 enzyme expression, insulin signaling, and lipid storage [29–31]. In addition, IFNγ plays critical roles in many liver diseases [16]. IFNγ helps recover from infections of hepatitis viruses by activating cytotoxic T lymphocytes, prevents fibrosis induced by viral infection or chemical carcinogen exposure, and decelerates hepatocellular carcinoma progression, whereas inhibition of IFNγ might be required for liver regeneration and to prevent graft rejection after liver transplantation. Therefore, IFNγ might be suitable for therapeutic applications. Elucidation of IFNγ's roles in hepatocarcinogenesis in *FXR*$^{-/-}$ mice will provide insights into the relationship between metabolic disorders and HCC.

Inflammation could be either pro-tumorigenic or tumor-suppressive, depending on causes, timing, persistence, and intensity [32]. It is the balance among different immune mediators and regulators that determines the outcomes of inflammation. IFNγ, secreted by innate and adaptive immune cells, is a critical inflammatory and modulatory cytokine against infection of bacteria and viruses, including hepatitis B or C viruses [33]. It is also involved in both anti-tumor and tumor-promoting inflammation. On the one hand, IFNγ helps reject transplanted tumors by mechanisms such as cytotoxicity enhancement to cancer cells [34], angiogenesis inhibition [35], and regulation of cancer cell immunogenicity and immunosurveillance [36]. On the other hand, IFNγ promotes development of cancers such as melanoma and colorectal carcinoma by inducing chronic inflammation [37,38]. In fact,

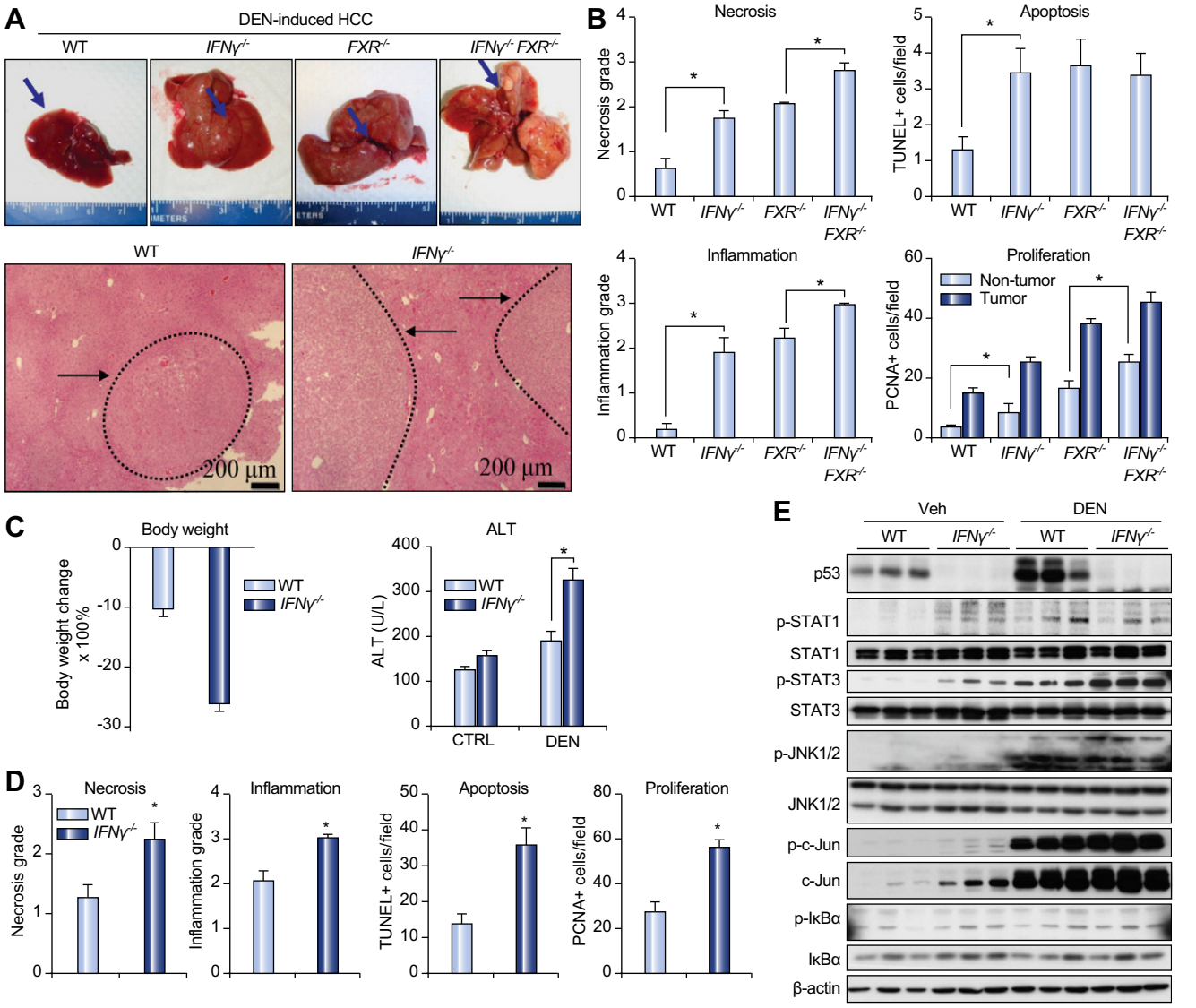

Fig. 2. *IFNγ* deletion enhances DEN-induced HCC and potentiates acute DEN-induced liver injury. (A) Representative images of liver tumorigenesis and H&E staining in DEN-treated mice. Arrows indicate tumors. (B) Grading of necrosis and inflammation, and quantification of apoptotic and proliferating cells. Student *t* test was applied for statistical analysis. **p* <0.05; n = 5 or more. (C) Body weight loss and serum ALT levels in wild type and *IFNγ*$^{-/-}$ mice 2 days after DEN treatment. (D) Grading of necrosis and inflammation, and quantification of apoptotic and proliferating cells in the acute model. Eight fields were randomly chosen (under 100× magnification) for quantification of TUNEL and PCNA staining. Student *t* test was applied. **p* <0.05; n = 3–4. (E) Western blotting for responses of tumor-suppressive or pro-inflammatory genes, 4 h after DEN treatment. PBS was used as vehicle control. [This figure appears in colour on the web.]

endogenous IFNγ is potentially a liver proto-oncogene, because *IFNγR*$^{-/-}$ mice display slightly decreased tumorigenesis with chronic DEN treatment in drinking water [39], and loss of IFNγ/STAT1 suppressor SOCS1 promotes liver fibrosis and carcinogenesis [24]. Nevertheless, the tumor-suppressor role of IFNγ is supported by the evidence that HCC patients with low IFNγ receptor expression have significantly poorer prognosis [40] and exogenous IFNγ inhibits HCC in both tissue culture and carcinogen-challenged rodents by inducing apoptosis [34,41,42]. Taking advantage of our unique HCC model in *FXR*$^{-/-}$ mice, we demonstrate that IFNγ indeed plays a tumor-suppressor role in hepatocarcinogenesis. In *FXR*$^{-/-}$ mice, accumulation of toxic bile acids in the liver induces chronic inflammation and IFNγ is highly upregulated. In contrast to the pro-tumorigenic effect of pro-inflammatory cytokines TNFα and IL-6, our results suggest that IFNγ is induced to protect the liver from injury and suppress signals for cell proliferation. Therefore, the interaction between both pro-tumorigenic and tumor-suppressive cytokines may determine the final outcome of hepatocarcinogenesis.

Our studies identified a novel role of IFNγ in suppressing HCC by maintaining the activities of aging-related responses. IFNγ signaling was elevated in aged rodent livers in order to tightly control cell cycling. In primates, the production of IFNγ is altered in aged individuals [18,19]. This implies that IFNγ might be essential for the liver to adapt to metabolism and microenvironment alternations during the aging process and to prevent tumor initiation or expansion of transformed hepatocytes. We found that the aging process in mouse livers induces NF-κB activation and

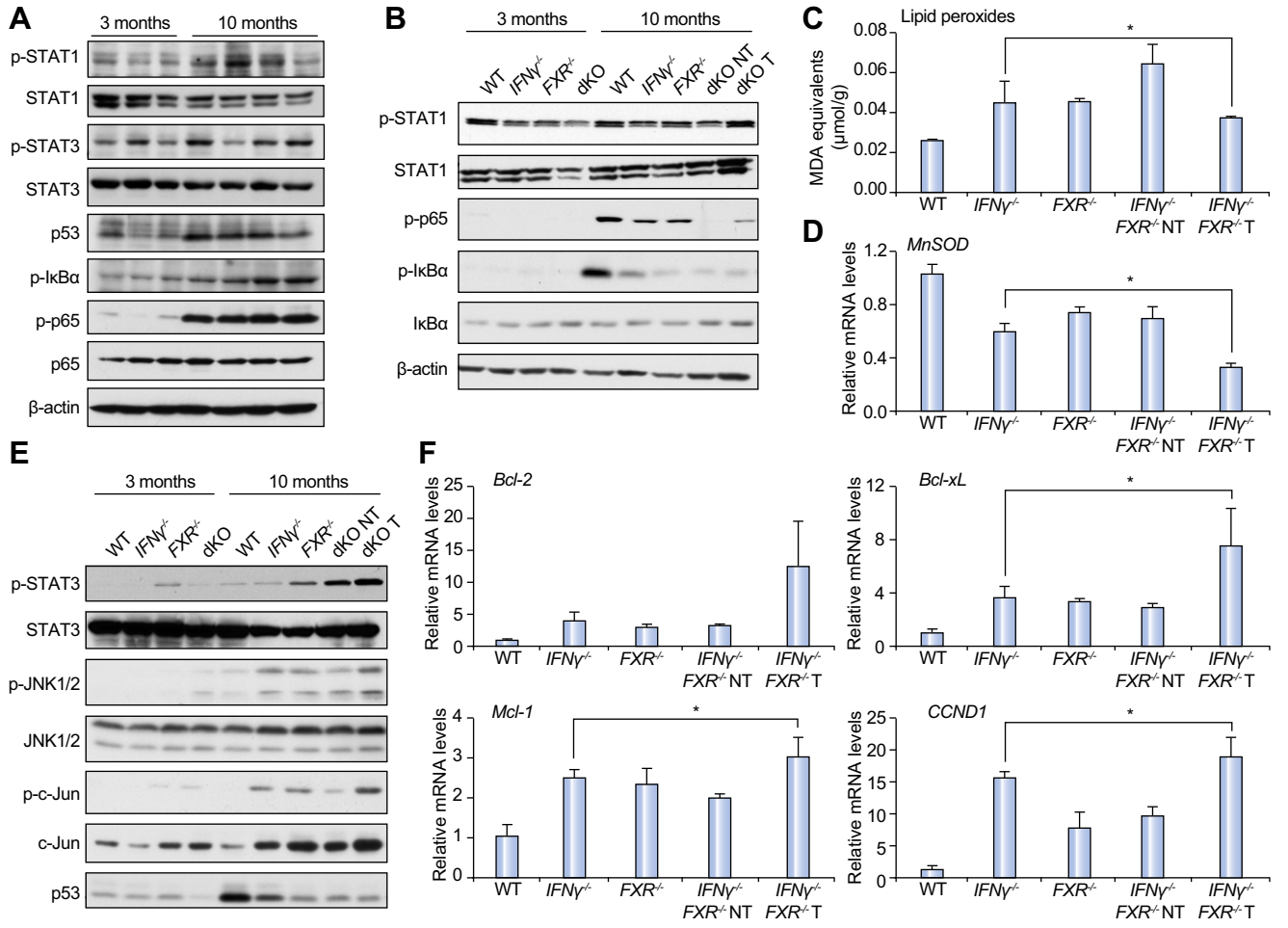

Fig. 3. *IFNγ* deletion alters age-related expression of tumor-suppressive and pro-inflammatory genes. (A) Western blot of liver lysates from 3-month-old (3 replicates) and 10-month-old (4 replicates) wild type mice. (B) Western blot of liver lysates from 3-month-old (pool of 3 replicates) and 10-month-old (pool of 4 replicates) mice. NT, non-tumor; T, tumor. (C and D). Quantification of (C) lipid peroxides and (D) *MnSOD* mRNA levels in livers of 10-month-old mice. (E) Western blot of liver lysates from 3- and 10-month-old mice. (F) Quantitative real-time PCR analysis of STAT3 target genes *Bcl-2*, *Bcl-xL*, *Mcl-1*, and Cyclin D1 (*CCND1*) in livers of 10-month-old mice. One way ANOVA test was applied for statistical analysis. $^{*}p$ <0.05; n = 4.

p53 expression, which has not been reported so far. In *IFNγ*$^{-/-}$ livers, these inductions were absent. Since NF-κB and p53 are key tumor-suppressors in the liver [8,9,43], increased activation of these two signaling pathways should help reduce the liver tumor burden. In fact, IFNγ is involved in NF-κB activation and IFNγ inhibits cell cycle progression of both primary hepatocytes and hepatocyte-derived cell lines via p53-and/or STAT1-dependent manners [16]. Here, we show that *IFNγ* deletion in mice leads to deficient p53 and NF-κB signaling in cancer initiation and progression. Our results highlight the significance of interaction between IFNγ, p53, and NF-κB during hepatocarcinogenesis.

In hepatocytes, IFNγ/STAT1 negatively regulates STAT3 by inducing SOCS1/3 [16,25]. The reduced hepatic STAT1 phosphorylation in *IFNγ*$^{-/-}$ mice can thus exaggerate STAT3 activities during hepatocarcinogenesis. This notion is consistent with studies in human HCC indicating that STAT1 phosphorylation was extensive in non-HCC tissues compared with HCC regions while STAT3 was hyperphosphorylated in HCC regions compared with non-HCC regions [24]. Furthermore, deficient p53 and NF-κB signaling in *IFNγ*$^{-/-}$ livers also contribute to aberrant STAT3 activation by a variety of mechanisms [23,24,26,44]. Similarly, JNK/c-Jun is also hyperphosphorylated in *IFNγ*$^{-/-}$ mice due to accumulated ROS and/or reduced NF-κB activities [26]. These results indicate that endogenous IFNγ is essential for preventing hyperphosphorylation of STAT3 and JNK/c-Jun in part by maintaining the activities of NF-κB, p53, and STAT1 in aging livers. The aberrant activation of STAT3 and JNK/c-Jun has been repeatedly documented in human HCC and animal models [26,43]. STAT3 and JNK/c-Jun appear to play central roles in HCC initiation and progression [45]. Deletion of hepatocyte STAT3 or JNK1 in mice induces resistance to DEN-induced hepatocarcinogenesis [45,46], and pharmacological inhibition of JAK/STAT3 or JNK/c-Jun suppresses liver cancer progression [46,47]. Future studies should dissect the activation of JNK and STAT3 in hepatocytes and non-parenchymal cells in *IFNγ*$^{-/-}$ mice, which would provide more information on the roles of IFNγ in inflammatory signaling cross-talk and hepatocarcinogenesis. Nonetheless, the identification of IFNγ as an endogenous modulator of JAK/STAT3 and JNK/c-Jun will provide more insights into the future therapeutics for HCC. In this study, the therapeutic strategy of peri-tumor subcutaneous injection, which extended elimination half-life of IFNγ by slower release and could simultaneously provide a similar effect

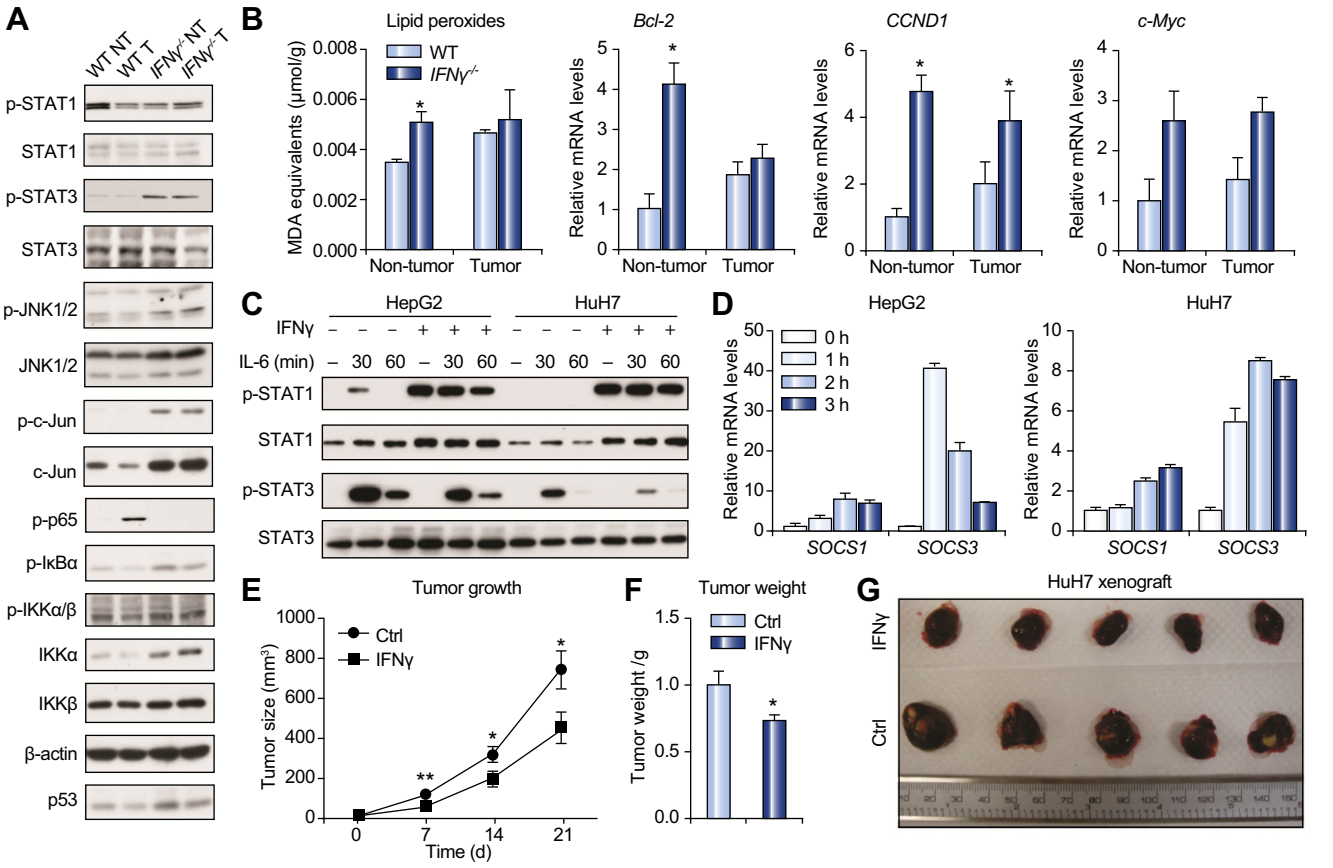

Fig. 4. IFNγ prevents hyperphosphorylation of STAT3 in DEN-induced HCC. (A) Western blot of liver lysates from DEN-treated wild type and *IFNγ*$^{-/-}$ mice. NT, non-tumor; T, tumor. (B) Quantification of liver lipid peroxides and quantitative real-time PCR analysis of STAT3 target genes *Bcl-2*, Cyclin D1 (*CCND1*), and *c-Myc*. $^{*}p$ <0.05; n = 3. Student *t* test was applied. (C) Western blot of samples from liver cell lines HepG2 and HuH7 pretreated with IFNγ and then treated with IL-6. Cells were harvested at the indicated time points. (D) Real-time PCR analysis of *SOCS1/3* induction by IFNγ. (E) Tumor volume and weight of HuH7 xenograft after IFNγ treatment. $^{**}p$ <0.01; $^{*}p$ <0.05; n = 10. (F) Representative images of xenograft tumors on day 21.

with localized deliveries such as intratumoral injection by shortening the delivery distance to tumor sites, effectively inhibited liver tumor xenograft growth.

In summary, *IFNγ* deletion increases the susceptibility to spontaneous hepatocarcinogenesis in *FXR*$^{-/-}$ mice and chemical-induced HCC. Our studies also demonstrate a key role of IFNγ in maintaining activation of p53 and NF-κB and preventing hyperphosphorylation of STAT3 and JNK in aging livers.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Financial support

W.H. is supported by NCI R01-139158. R.X. is supported by National Natural Science Foundation of China (No. 81070420). G.L. is supported by a grant from National Natural Science Foundation of China (No. 30600299).

Authors' contribution

Z.M. and X.W. designed and performed most of the experiments, analyzed the results, and wrote the manuscript. Y.G., Y.Z., H.Z., and J.W. provided technical support. G.L. and C.V. assisted in the genotyping of the mice. H.Y. C.H. discussed the project and made intelligent contribution. R.X. and W.H. conceived the project, supervised the project, and revised the manuscript.

Acknowledgements

We thank Dr. Valentina Factor for providing A6 antibodies. We also thank the Research Pathology Core for pathological analysis, and Dr. Richard Ermel and the Animal Resource center for the technique support in animal experiments.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.06.016.

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