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Deletion of IFN γ enhances hepatocarcinogenesis in FXR knockout mice

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Abstract

Background and 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 IFN γ is one of the most up-regulated pro-inflammatory cytokines in FXR^{−/−} livers, we generated IFN γ ^{−/−} FXR^{−/−} double knockout mice to determine IFN γ 's roles in hepatocarcinogenesis.

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The authors have no conflict of interest for disclosure.

Author 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., J.W. provided technique 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.

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Methods—IFN γ ^{-/-} mice were crossed to a FXR^{-/-} C57BL/6 background or injected i.p with hepatocarcinogen diethylnitrosamine (DEN). Hepatocarcinogenesis was analyzed with biochemical and histological methods.

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

Conclusion—Increased IFN γ expression in FXR^{-/-} livers represents a protective response of liver against chronic injury and tumorigenesis. IFN γ suppresses hepatocarcinogenesis by inducing p53 expression and preventing STAT3 activation.

Keywords

FXR; HCC; STAT3; JNK; NF- κ B; p53

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 the infection with hepatitis B or C viruses [2]. A common pathological feature of HCC development regardless of etiology is that hepatocyte death triggers chronic inflammation, which leads to continuous compensatory hepatocyte proliferation. HCC is also highly connected to liver metabolic disorders. One particular example is the 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-week-old and spontaneously develop liver cancer when they are over 1-year-old [3]. Furthermore, FXR expression is strongly down-regulated 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.

A variety of signaling molecules, particularly cytokines and their downstream mediators, divert inflammation to liver carcinogenesis. These include TNF α , IL6, IKK/NF- κ B, JAK/STAT3, JNK/c-Jun [8–11]. IFN γ is one of the most up-regulated cytokines in FXR^{-/-} mouse livers [3], but the exact roles of IFN γ during HCC development in FXR^{-/-} mice are unclear. In this study, we show that IFN γ deletion enhanced the hepatocarcinogenesis in FXR^{-/-} mice and sensitized mice to DEN-induced tumorigenesis. We also identified a novel role of IFN γ in maintaining aging-induced activation of p53 and NF- κ B and preventing hyperphosphorylation of STAT3 and JNK in livers. Our results underscore an important role of IFN γ in suppressing hepatocarcinogenesis.

Materials and Methods

Animals

IFN γ ^{-/-} mice were purchased from Jackson Laboratory. To generate IFN γ ^{-/-} FXR^{-/-} mice, IFN γ ^{-/-} mice were crossed to FXR^{-/-} mice in the C57BL/6 background. The 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 treated to the mice once every two weeks for 8 times. 6 months after DEN treatment, mice were euthanized and samples were collected. The details of xenograft studies with Huh7 cells and IFN γ were provided in the supporting documents. The 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 staining 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 immunostaining are provided in the supplemental documents.

Quantitative Real-time PCR

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

Western blot

Western blot 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 by 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 were measured at the City of Hope Helford Research Hospital.

Statistical Analysis

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

Results

IFN γ deletion enhances spontaneous liver tumorigenesis in FXR^{-/-} mice

The FXR^{-/-} background provided a context of spontaneous liver injury and chronic inflammation [7]. IFN γ deletion in FXR^{-/-} mice led to liver tumorigenesis as early as the mice were 8-month-old, while no tumor incidence was observed in FXR^{-/-} mice at this

time (Table 1). Although livers of 3-month-old IFN γ ^{-/-} mice did not display morphological differences from wild-type mouse livers (SFigure 1), sparse HCC incidence was observed in aged IFN γ ^{-/-} mice but not their wild-type littermates over 15-month-old (Table 1). FXR^{-/-} mice had a low tumor incidence rate at 10-month-old (Table 2). In contrast, IFN γ deletion in FXR^{-/-} mice resulted in more than 80% incidence and much larger tumors (Table 2, Figure 1A). Immunohistochemistry analysis of hepatic expression of CD34, CK19 and CK20 revealed that the tumors were hepatocellular carcinomas and not derived from bile ducts or intestinal tissues [15] (Figure 1B).

Deletion of IFN γ elevated levels of ALT and AST in 10-month-old wild-type and FXR^{-/-} mice (Table 2). These results suggested that IFN γ deletion promoted spontaneous liver injury during aging process, which was supported by the hepatocyte degeneration and focal necrosis in the livers (Figure 1C, SFigure 2). Furthermore, IFN γ deletion significantly enhanced apoptosis and inflammatory cell infiltration in FXR^{-/-} mice (Figure 1C, SFigure 3A), and in turn led to increased compensatory hepatocyte proliferation in IFN γ ^{-/-}FXR^{-/-} mice (Figure 1C, SFigure 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 IFN γ and/or FXR deletion (SFigure 4A–B), which is consistent with the role of IFN γ against fibrosis [16].

IFN γ deletion enhances chemical-induced liver tumorigenesis

We used DEN-induced HCC models to further determine IFN γ '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 γ ^{-/-}, FXR^{-/-}, and IFN γ ^{-/-}FXR^{-/-} mice developed liver tumors at this age. Moreover, IFN γ ^{-/-} mice developed more and larger hepatocellular carcinomas than wild-type mice, and IFN γ ^{-/-}FXR^{-/-} mice displayed enhanced hepatocarcinogenesis compared with FXR^{-/-} mice (Table 3, Figure 2A, SFigure 5).

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

IFN γ 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 IFN γ 's roles in HCC initiation, acute phase of DEN-induced liver injury was evaluated in 4-week-old IFN γ ^{-/-} mice. IFN γ ^{-/-} mice showed more than 2.5 times body weight loss and much more robust ALT increase (Figure 2C) than wild-type mice 2 days after a single DEN injection (100 mg/kg). Consistently, IFN γ ^{-/-} mice carried more severe focal necrosis, or even submassive necrosis and more extensive inflammatory cell infiltration (Figure 2D, SFigure 10A). In addition, TUNEL staining revealed more apoptotic cells in IFN γ ^{-/-} livers than in wild-type

controls (Figure 2D, SFigure 10B). In response to cell deaths and inflammation, compensatory hepatocyte proliferation following acute injury was enhanced in IFN γ ^{-/-} mice (Figure 2D, SFigure 10B). Consistently, activation of inflammation mediators STAT3 and JNK/c-Jun was augmented (Figure 2E). Furthermore, both basal and DEN-induced p53 expression was absent in IFN γ ^{-/-} mice, though no difference in NF- κ B activation was observed. Even without DEN treatment, 4-week-old IFN γ ^{-/-} 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 the enhanced cell deaths, inflammation, and compensatory proliferation in IFN γ ^{-/-} livers.

IFN γ is required to maintain p53 and NF- κ B activation in aging livers

Many types of cancers, including liver cancer, have a strong correlation with aging. The production of IFN γ 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 IFN γ is also higher in 10-month-old mice than in 3-month-old mice (SFigure 11A). Therefore, we ask whether the enhanced hepatocarcinogenesis in IFN γ ^{-/-} mice is 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- κ B activation were significantly up-regulated (Figure 3A). p53 acts as a checkpoint protein in the cell cycle and suppresses the uncontrolled cancer cell duplication, while hepatocyte NF- κ B 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 IFN γ or FXR greatly reduced STAT1 phosphorylation in precancerous livers (SFigure 11B, Figure 3B). Furthermore, induction of p53 and phosphorylation of hepatic I κ B and p65 were reduced or abolished in the knockout mice, indicating that the age-related activation of p53 and NF- κ B required the presence of IFN γ and FXR, consistent with the reported interaction and synergistic activation of IFN γ /STAT1 and NF- κ B 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- κ B target gene, MnSOD. MnSOD catalyzes the dismutation of two molecules of superoxide anion into water and hydrogen peroxides, and thus reduces ROS and protects liver from oxidative stresses [9]. Indeed, the levels of lipid peroxides, the ROS products, were up-regulated in all the precancerous tissue of the knockout mice (Figure 3C), probably due to the decreased MnSOD expression resulting from absence of hepatic NF- κ B activation (Figure 3D).

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

STAT1 plays a tumor-suppressor role by antagonizing STAT3 [23–25], and hepatocyte NF- κ B suppresses hepatocarcinogenesis by attenuating both STAT3 and JNK activation in part by controlling ROS [9, 26]. Therefore, we speculate that in the IFN γ ^{-/-}, FXR^{-/-}, and IFN γ ^{-/-}FXR^{-/-} mouse livers the exaggerated activation of STAT3 and JNK can be observed due to the decreased STAT1 and NF- κ B activation. Indeed, STAT3 and JNK1/2 are hyperphosphorylated, especially in the IFN γ ^{-/-}FXR^{-/-} tumors (Figure 3E). STAT3 can target many anti-apoptotic genes, including Bcl-2, Bcl-xl, and Mcl-1. Expression of all these 3 genes is increased in the knockout animals (Figure 3F). The substrate of JNK1/2, c-Jun, is a strong tumor-promoter in 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 IFN γ ^{-/-}, FXR^{-/-} and IFN γ ^{-/-}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 the c-Jun expression in the aged knockout animals, which indicated that loss of IFN γ in the aging liver greatly enhanced some oncogenic signaling such as c-Jun. In addition, the up-regulation of Cyclin D1 and c-Myc and the down-regulation of p53 in the knockout livers (Figure 3E&F & SFigure 11C) appear to be critical. In fact, c-Myc is suppressed by IFN γ [27], and Cyclin D1 and c-Myc are also activated by STAT3 in HCC. Besides, the hyperactivation of STAT3 and JNK1/2, which could also reflect increased hepatic inflammation and immune cell infiltration, can be attributed to the increased expression of hepatic TNF α and IL-6, the two cytokines well known for promoting hepatocarcinogenesis (SFigure 11C).

STAT3 and JNK are persistently hyperactive in the IFN γ -/- 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 are hyperactivated in IFN γ -/- precancerous tissues (Figure 4A). Consistently, the STAT3 activator lipid peroxide/ROS is up-regulated in IFN γ -/- mice, and the expression of STAT3 target genes Bcl2, Cyclin D1, and c-Myc are significantly elevated (Figure 4B). However, the deregulation of STAT3 and JNK were independent of NF- κ B pathways since I κ B phosphorylation is higher in IFN γ -/- livers than in wild-type controls, probably due to higher expression of IKK β (Figure 4A). Unlike the short-term post-DEN stimulation and spontaneous HCC model, p53 expression at this stage was not reduced in IFN γ -/- livers. The differences in NF- κ B and p53 activation might be due to the different progression stages and the different mechanisms of tumorigenesis. In accord with the spontaneous HCC model, hepatic expression of certain tumor-promoting cytokines, for instance TNF α , IL-6, and TGF β , is up-regulated (SFigure 12).

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

To investigate the direct suppressive effects of IFN γ on STAT3 in liver cells, we pre-treated IFN γ to HepG2 and Huh7 cells and then applied IL-6 to the cells. IFN γ greatly reduced IL-6 induced STAT3 phosphorylation in both cell lines (Figure 4C). Moreover, the STAT1 inhibitor Fludarabine slightly increased IL-6-induced STAT3 phosphorylation (SFigure 13), though the inhibitor did not directly alter I κ B α and JNK activation by TNF α . The suppressive effects of IFN γ might be due to its induction of SOCS1 and SOCS3 in the liver cells since SOCS proteins are specific inhibitors of STAT3 phosphorylation (Figure 4D). Furthermore, we found that IFN γ treatment to the Huh7 xenograft decreased the tumor growth (Figure 4E-F), which is consistent with the reports on applications of IFN γ 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 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 up-regulation of several inflammatory

cytokines such as IFN γ in their livers. Interestingly, IFN γ modulates several aspects of metabolisms, for instance cytochrome P450 enzyme expression, insulin signaling, and lipid storage [29–31]. In addition, IFN γ plays critical roles in many liver diseases [16]. IFN γ helps recovery 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 IFN γ might be required for liver regeneration and avoiding graft rejection after liver transplantation. Therefore, IFN γ might be suitable for therapeutic applications. Elucidation of IFN γ 's roles in hepatocarcinogenesis in FXR $^{-/-}$ mice will provide insight 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. IFN γ , 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 one hand, IFN γ helps reject transplanted tumors by mechanisms such as enhancing cytotoxicity to cancer cells [34], inhibiting angiogenesis [35], and regulating cancer cell immunogenicity and immunosurveillance [36]. On the other hand, IFN γ promotes development of cancers such as melanoma and colorectal carcinoma by inducing chronic inflammation [37, 38]. In fact, the endogenous IFN γ is potentially a liver proto-oncogene, because IFN γ R $^{-/-}$ mice display slightly decreased tumorigenesis with chronic DEN treatment in drinking water [39] and loss of IFN γ /STAT1 suppressor SOCS1 promotes liver fibrosis and carcinogenesis [24]. Nevertheless, the tumor-suppressor role of IFN γ is supported by the evidence that HCC patients with low IFN γ receptor expression have significantly poorer prognosis [40] and exogenous IFN γ 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 IFN γ indeed plays a tumor-suppressor role in hepatocarcinogenesis. In FXR $^{-/-}$ mice, accumulation of toxic bile acids in liver induces chronic inflammation and IFN γ is highly up-regulated. In contrast to pro-tumorigenic effect of pro-inflammatory cytokines TNF α and IL6, our results suggest that IFN γ is induced to protect liver from the 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 IFN γ in suppressing HCC by maintaining the activities of aging-related responses. IFN γ signaling was elevated in aged rodent livers in order to tightly control cell cycling. In primates, the production of IFN γ is altered in aged individuals [18, 19]. These imply that IFN γ might be essential for the liver to adapt to metabolism and microenvironment alternations during aging process and to prevent tumor initiation or expansion of transformed hepatocytes. We found that the aging process in the mouse livers induces NF- κ B activation and p53 expression, which has not been reported before. In IFN γ $^{-/-}$ livers, these inductions were absent. Since NF- κ B and p53 are key tumor-suppressor genes in the livers [8, 9, 43], increased activation of these two signaling pathways should help reduce tumor burden for the livers. In fact, IFN γ is involved in NF- κ B activation and IFN γ 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 IFN γ deletion in mice leads to deficient p53 and NF- κ B signaling in cancer initiation and progression. Our results highlight the significance of interaction between IFN γ , p53 and NF- κ B during hepatocarcinogenesis.

In hepatocytes, IFN γ /STAT1 negatively regulates STAT3 by inducing SOCS1/3 [16, 25]. The reduced hepatic STAT1 phosphorylation in IFN γ $^{-/-}$ mice can thus exaggerate STAT3

activities during hepatocarcinogenesis. This notion is consistent with the reports in human HCC that STAT1 phosphorylation was extensive in non-HCC tissues compared with HCC region whereas STAT3 is hyperphosphorylated in HCC regions compared with non-HCC regions [24]. Furthermore, deficient p53 and NF- κ B signaling in IFN γ -/- livers also contribute to aberrant STAT3 activation by a variety of mechanisms [23, 24, 26, 44]. Similarly, JNK/c-Jun is also hyperphosphorylated in IFN γ -/- mice due to accumulated ROS and/or reduced NF- κ B activities [26]. These results indicate that endogenous IFN γ is essential for preventing hyperphosphorylation of STAT3 and JNK/c-Jun in part by maintaining the activities of NF- κ B, 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]. One of future studies would be to dissect the activation of JNK and STAT3 in hepatocytes and non-parenchymal cells in IFN γ -/- mice, which would provide more information for the roles of IFN γ in inflammatory signaling crosstalk and hepatocarcinogenesis. Nonetheless, the identification of IFN γ 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 peritumor subcutaneous injection, which extended elimination half-life of IFN γ by slower release and could simultaneously also provide a similar effect with localized deliveries such as intratumoral injection by shortening the delivery distance to tumor sites, effectively inhibited liver tumor xenograft growth.

In summary, IFN γ deletion increases the susceptibility to spontaneous hepatocarcinogenesis in FXR-/- mice and chemical-induced HCC. Our studies also demonstrate a key role of IFN γ in maintaining the activation of p53 and NF- κ B and preventing hyperphosphorylation of STAT3 and JNK in aging livers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

HCC	hepatocellular carcinoma
FXR	farnesoid X receptor
DEN	diethylnitrosamine
AST	Aspartate Aminotransferase
ALT	Alanine Aminotransferase
ROS	reactive oxygen species

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Figure 1. IFN γ deletion promotes spontaneous liver tumorigenesis of 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 months 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 serves as a positive control for CK20 immunostaining. Magnification, 200 X. 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.

Figure 2. IFN γ 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 IFN γ ^{-/-} mice 2 days after DEN treatment. D. Grading of necrosis and inflammation, and quantification of apoptotic and proliferating cells in the acute model. 8 fields were randomly chosen under the magnification X 100 for quantification of TUNEL and PCNA staining. Student *t* test was applied. *, *p* < 0.05; *n*=3–4. E. Western blotting for the responses of tumor-suppressive or pro-inflammatory genes 4 hours after DEN treatment. PBS was used as the vehicle control.

Figure 3. IFN γ deletion alters age-related expression of tumor-suppressive and pro-inflammatory genes

A. Western blotting with liver lysates from 3-month-old (3 replicates) and 10-month-old (4 replicates) wild-type mice. B. Western blotting with 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&D. Quantification of lipid peroxides (C) and MnSOD mRNA levels (D) in the livers of 10-month-old mice. E. Western blotting with 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 in the livers of the 10-month-old mice. One way ANOVA test was applied for statistical analysis. *, $p < 0.05$; $n=4$.

Figure 4. IFN γ prevents hyperphosphorylation of STAT3 in DEN-induced HCC

A. Western blotting of liver lysates from DEN-treated wild-type and IFN γ ^{-/-} 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, and c-Myc. **, $p < 0.01$; *, $p < 0.05$; $n=3$. Student t test was applied. C. Western blotting of liver cell lines HepG2 and Huh7 pretreated with IFN γ and then treated with IL-6. The cells are harvested at the indicated time points. D. Real-time PCR analysis of SOCS1/3 induction by IFN γ . E. Tumor volume and weight of Huh7 xenograft after IFN γ treatment. **, $p < 0.01$; *, $p < 0.05$; $n=10$. F. Representative images of the xenograft tumors on Day 21.

Table 1

Spontaneous Hepatocarcinogenesis

Tumor incidence/mice	WT	IFN γ ^{-/-}	FXR ^{-/-}	IFN γ ^{-/-} FXR ^{-/-}
6 months	0/8	0/8	0/8	0/12
8 months	0/8	0/6	0/12	5/13
10 months	0/8	0/12	2/14	24/29
15 months	0/12	3/21	12/12	17/17

Table 2

Spontaneous HCC in 10 months old mice

	WT	IFN γ ^{-/-}	FXR ^{-/-}	IFN γ ^{-/-} FXR ^{-/-}
Tumor incidence $\times$ 100%	0.0	0.0	14.0	82.7
Tumor number per liver	0.0	0.0	0.6 $\pm$ 0.5	7.4 $\pm$ 1.3 **
No. of tumor diameter >0.2cm	0.0	0.0	0.0 $\pm$ 0.0	2.2 $\pm$ 0.4 **
Maximum tumor diameter/cm	0.0	0.0	0.1 $\pm$ 0.1	0.5 $\pm$ 0.1 **
Liver/body weight ratio $\times$ 100%	5.0 $\pm$ 0.1 %	4.8 $\pm$ 0.1 %	6.3 $\pm$ 0.2 %	6.4 $\pm$ 0.1 %
ALT (U/L)	3 month	129.3 $\pm$ 7.4	120 $\pm$ 6.1	288.0 $\pm$ 14.0
	10 months	131.2 $\pm$ 13.2	473.1 $\pm$ 50.4 **	805.7 $\pm$ 52.5 *
AST (U/L)	3 month	44.0 $\pm$ 6.1	74.7 $\pm$ 29.7	255.0 $\pm$ 61.8
	10 months	37.6 $\pm$ 4.7	228.4 $\pm$ 36.8 **	368.0 $\pm$ 92.3

** , p < 0.01. IFN γ ^{-/-} vs. WT.

* , p < 0.05;

** , p < 0.01. IFN γ ^{-/-} FXR^{-/-} vs. FXR^{-/-}.

Table 3

DEN-induced HCC in 7 months old mice

	WT	IFN γ ^{-/-}	FXR ^{-/-}	IFN γ ^{-/-} FXR ^{-/-}
Tumor incidence $\times$ 100%	70.0	100.0	100.0	100.0
Tumor number per liver	4.4 $\pm$ 0.9	24.7 $\pm$ 4.3 ^{**}	38.2 $\pm$ 4.6	64.1 $\pm$ 2.6 ^{**}
No. tumor diameter >0.2cm	1.7 $\pm$ 0.6	4.0 $\pm$ 0.4 ^{**}	7.8 $\pm$ 0.7	8.7 $\pm$ 0.7
No. tumor diameter >0.5cm	0.2 $\pm$ 0.1	0.8 $\pm$ 0.3	0.8 $\pm$ 0.4	2.1 $\pm$ 0.3 [*]
Maximum tumor size/cm	0.3 $\pm$ 0.1	0.6 $\pm$ 0.2	0.5 $\pm$ 0.2	1.0 $\pm$ 0.1 [*]
Liver/body weight ratio $\times$ 100%	5.6 $\pm$ 0.2%	7.1 $\pm$ 0.3%	8.3 $\pm$ 0.6%	8.6 $\pm$ 0.4%
ALT (U/L)	202.0 $\pm$ 24.1	803.2 $\pm$ 31.2 ^{**}	670.0 $\pm$ 151.3	1056.0 $\pm$ 96.5 [*]
AST (U/L)	74.0 $\pm$ 15.9	272.7 $\pm$ 30.9 ^{**}	196.0 $\pm$ 37.9	423.6 $\pm$ 31.7 ^{**}

^{**}, p < 0.01. IFN γ ^{-/-} vs. WT.

^{*}, p < 0.05;

^{**}, p < 0.01. IFN γ ^{-/-} FXR^{-/-} vs. FXR^{-/-}.