GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Type I Interferon Signaling Accelerates Liver Regeneration by Metabolic Modulation in Noninfectious Conditions

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Type I interferon (IFN-I) has a well-known function in controlling viral infections, but its contribution in hepatocyte proliferation and hepatocellular carcinoma (HCC) formation remains unclear. Mice deficient in IFN- α receptor expression in whole mice or only in hepatocytes (*Ifnar*^{-/-} and *Ifnar*^{liver}) were used to investigate the role of IFN-I signaling in cell proliferation and cancer formation in the liver. *Ifnar*^{-/-} mice were resistant to chemical-induced HCC formation in the absence of infection. The results show that low grade of IFN-I and interferon-stimulated gene were expressed substantially in naïve mouse liver. The low level of IFN-I activation is constantly present in mouse liver after weaning and negatively modulates forkhead box O hepatic expression. The IFN-I signaling can be partially blocked by the clearance of lipopolysaccharide. Mice lacking IFN-I signaling have lower basal proliferation activity and delayed liver regeneration processes after two-thirds partial hepatectomy. The activation of IFN-I signaling on hepatocyte controls glucose homeostasis and lipid metabolism to support proliferation potency and long-term tumorigenesis. Our results reveal a positive role of low-grade IFN-I signaling to hepatocyte proliferation and HCC formation by modulating glucose homeostasis and lipid metabolism. (*Am J Pathol* 2021, 191: 1036–1048; <https://doi.org/10.1016/j.ajpath.2021.03.006>)

The liver has both metabolic and immunoregulatory functions. Various nutrients, xenobiotics, and bacterial components are absorbed in the small intestine and pass through the liver via the portal circulation, undergoing metabolic elimination or conversion.¹ Bacterial translocation, viral infection, and hepatocyte death, caused by xenobiotics, may activate innate immune responses, leading to cytokine and interferon production. After injuries caused by hepatotoxic substances or infection, the liver undergoes compensatory proliferation to maintain homeostasis and tissue mass. Constant inflammation and compensatory regeneration are thought to promote cancer development.² Hepatocellular carcinoma (HCC) is the most common type of liver cancer,³ and persistent infection caused by hepatitis B and C viruses is one of the risk factors for human HCC. Type I interferon (IFN-I) is part of the innate immune response that controls virus infection. Although the role of IFN-I in controlling viral infection is

well recognized, its impact on hepatocyte proliferation and HCC development is poorly understood.

Expression of IFN-I proteins, such as IFN- α and IFN- β , is triggered by pathogen-associated molecular patterns through activation of pattern-recognition receptors, including RIG-I-like receptors and toll-like receptors (TLRs).⁴ Multiple IFN- α subtypes, IFN- β , IFN- ϵ , and IFN- ω , and other IFN-I subtypes bind to the same receptor, which is composed of IFN- α receptor 1 and 2 subunits.⁵ The binding of IFN-I to its receptor activates JAK1 and TYK2 tyrosine kinases, leading to the phosphorylation and dimerization of STAT1 and STAT2, which turn on interferon-stimulated gene (ISG)

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expression to fight viral infections.^{6,7} IFN-I signaling also activates p38 and phosphatidylinositol 3-kinase (PI3K).^{8,9} Along with its well-known antiviral activity, IFN-I has antiproliferative activity and has been used to treat several types of cancer, such as lymphoma and melanoma, in humans.^{7,10} Intriguingly, IFN-I treatment induces dormant hematopoietic stem cells to enter an active cell cycle.¹¹ Besides, IFN-I increases epithelial turnover and wound repair in mice, and apolipoprotein L9 (APOL9) produced by macrophages might be the ISG involved in these processes.¹² The effect of IFN-I signaling on cell fate might be cell type and context dependent.

In the present study, IFN- α receptor knockout (*Ifnar*^{-/-}) mice were used to examine the contribution of IFN-I signaling to HCC development, hepatocyte proliferation, and liver regeneration. A low level of IFN-I activation was found to positively regulate compensatory hepatocyte proliferation by modulating glucose and lipid metabolism, which might contribute to HCC development.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 and *Ifnar*^{-/-} male mice (C57BL/6 background)^{8,13} were maintained at the National Health Research Institutes. *Ifnar*^{F/F} mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and albumin-Cre mice were kindly provided by Dr. Michael Karin. *Ifnar*^{F/F} mice were crossed with albumin-Cre mice to obtain *Ifnar*^{Δliver} mouse line. Unless specified, the mice were weaned at 4 weeks of age and fed a diet of normal mouse chow. All mouse-related experiments were conducted in compliance with the guidelines of the Laboratory Animal Center of the National Health Research Institutes and used approved animal protocols (National Health Research Institutes—Institutional Animal Care and Use Committee 100013, 102126, 103104, 104006, and 106051).

HCC Tumor Model and Two-Thirds PHx

Two-week-old male mice were injected with diethylnitrosamine (DEN; 25 mg/kg; Sigma, St. Louis, MO) intraperitoneally, and HCC tumor development was examined at the age of 8 months.^{14–16} Mice, aged 8 to 12 weeks, were subjected to two-thirds partial hepatectomy (PHx) by following a protocol published previously.¹⁷ The blood collected from mouse tail tip was used to measure glucose level with Accu-Chek Instant glucometer (Roche, Basel, Switzerland).

Antibodies, Chemicals, and Reagents

Antibodies for studying forkhead box O (FoxO) 1, FoxO3, epidermal growth factor receptor, Met, cyclin D1, cyclin A2, cyclin B1, p27, p21, and p15 were purchased from Cell Signaling Technology (Danvers, MA). Cyclin E1 was

obtained from Santa Cruz Biotechnology (Dallas, TX). Tubulin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Adipose differentiation-related protein (ADRP) antibody was purchased from PROGEN Biotechnik GmbH (Heidelberg, Germany). The anti-mouse APOL9 antibody was raised in rabbits (Animal Health Research Institute, Taiwan) immunized with purified His-tagged APOL9 protein. Insulin and intralipid emulsion were purchased from Sigma-Aldrich.

Immunohistochemistry

Paraffin-embedded liver sections were subjected to antigen retrieval with Target Retrieval Solution (S1700; DakoCytomation, Carpinteria, CA) at 95°C for 45 minutes and stained with anti-Ki-67 antibody (GeneTX, Hsinchu, Taiwan) by methods as described previously.¹⁸ The Ki-67 staining images were taken under a microscope and quantified by ImageJ version 1.50i software (NIH, Bethesda, MD; <https://imagej.nih.gov/ij/>, last accessed March 26, 2016). For FOXO1 staining, the antigen retrieval was performed with citrate buffer, pH 6.0, with 0.1% Tween-20 at 80°C for 6 hours and stained with anti-FOXO1 antibody (GeneTX). The hematoxylin and eosin staining for the tissue sections was performed by the pathology core laboratory in National Health Research Institutes.

Quantitative Real-Time PCR Analysis

cDNA synthesis and quantitative real-time PCR were performed as described previously.¹⁸ The primer sequences used in this study were listed in Table 1.

Oil Red O Staining and Lipid Droplet Fractionation

Frozen liver sections were fixed with formalin for 10 minutes and washed in tap water. After being rinsed in 60% isopropanol in water, the liver sections were stained with 1.5% oil red O (Sigma) in isopropanol and water mixture (3:2) for 20 minutes and further counterstained with hematoxylin. Lipid droplet fractionation was performed by the method described previously,¹⁹ with minor modification. In brief, 300 mg mouse livers was lysed in HLM buffer (20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 10 mmol/L sodium fluoride, and 1× Roche protease inhibitor) by grinding with a glass Dounce homogenizer and centrifuged for 10 minutes at 1000 × g, 4°C. The post-nuclear supernatant was mixed with one-third volume of ice-cold HLM containing 60% sucrose and layered on the bottom of the ultracentrifuge tube for SW 41 Ti rotor. A total of 5 mL of HLM containing 5% and 0% sucrose was further layered on top sequentially. Flotation of lipid droplets was performed 30 minutes at 160,000 × g, 4°C. Fractions (1 mL/fraction) were collected from top to bottom and subjected to immunoblotting.

Table 1 Quantitative Real-Time PCR Primer Sequences

Gene	Forward primer sequence	Reverse primer sequence
<i>Cph</i>	5'-ATGGTCAACCCACCGTGT-3'	5'-TTCTTGCTGTCTTTGGAACCTTGTC-3'
<i>Ifna1</i>	5'-ACCCAGCAGATCCTGAACAT-3'	5'-AATGAGTCTAGGAGGGTGTATTCC-3'
<i>Ifna2</i>	5'-GTCCTGGCACAGATGAGGA-3'	5'-ACCTTCTCCAGGGGAATC-3'
<i>Ifna4</i>	5'-TCAAGCCATCCTTGTGCTAA-3'	5'-GTCTTTTGATGTGAAGAGGTCAA-3'
<i>Ifna5</i>	5'-GCCTTAACCTCCTGGTAAAA-3'	5'-TCCTGTGGGAATCCAAAGTC-3'
<i>Ifna6</i>	5'-AGGACTTTGGATTCCCCTTG-3'	5'-CTCAGTCAGGACAGGGATGG-3'
<i>Ifna7</i>	5'-CTGCTGCTTGGGATGGAT-3'	5'-ACAGCCTTGCAGGTCATTG-3'
<i>Ifna8</i>	5'-GGAGGTGGTCAGAGCAGAAG-3'	5'-TTGGTCTCAGGACTCACTCGT-3'
<i>Ifna9</i>	5'-TTCTGATGGTCTTGGTGGT-3'	5'-TGTGCCAGGAGTGTCAAGAT-3'
<i>Ifna11</i>	5'-AGGACTTTGGATTCCCCTTG-3'	5'-AGGGATGGATTGAGCCTTCT-3'
<i>Ifna12</i>	5'-TGACTCTCCTGGCACAAATG-3'	5'-CCTTCTCCTGGGGGAATCTA-3'
<i>Ifna13</i>	5'-GGTGGTCAGAGCAGAAGTCC-3'	5'-TCCTCCTTGCTCAGTCTTGC-3'
<i>Ifna14</i>	5'-GGAGGTGGTCAGAGCAGAAAT-3'	5'-CTTTCAGGCTGGTCAGCAAC-3'
<i>Ifna15</i>	5'-GCCATCCCTGTCTTACAAGA-3'	5'-GCAGCAGATGAGTCTCTTGA-3'
<i>Ifna16</i>	5'-GCCATCCCTGTCTTAAGTGA-3'	5'-ATCCAAGCAGCAGATGAAT-3'
<i>Ifnb</i>	5'-AGCTCCAAGAAAGGACGAACAT-3'	5'-GCCCTGTAGGTGAGGTGTGATCT-3'
<i>Ifng</i>	5'-ATCTGGAGGAAGTGGCAAAA-3'	5'-TTCAAGACTTCAAAGAGTCTGAGGTA-3'
<i>Apol9</i>	5'-AGGCCATCTGGGTCTGT-3'	5'-TTGCCATGTTCTGAGATACCC-3'
<i>Ccnd1</i>	5'-TTTCTTTCCAGAGTCATCAAGTG-3'	5'-TGACTCCAGAAGGGCTTCAA-3'
<i>Ccne1</i>	5'-TTTCTGCAGCGTCATCCTC-3'	5'-TGGAGCTTATAGACTTCGCACA-3'
<i>Ccna2</i>	5'-CTTGGCTGCACCAACAGTAA-3'	5'-CAAACCTCAGTTCTCCAAAAACA-3'
<i>Ccnb1</i>	5'-GCGCTGAAAATTCTTGACAAC-3'	5'-TTCTTAGCCAGGTGCTGCAT-3'
<i>E2f1</i>	5'-GCCCTTGACTATCACTTTGGTCTC-3'	5'-CCTTCCCATTTTGGTCTGCTC-3'
<i>E2f7</i>	5'-GATGCGTTCGTGAATCCCTG-3'	5'-AGAAACTTCTGGCACAGCAGCC-3'
<i>E2f8</i>	5'-GAGAAATCCCAGCCGAGTC-3'	5'-CATAAATCCGCCGACGTT-3'
<i>G6pc</i>	5'-TGGCTGGAGTCTTGTCAAG-3'	5'-GTAGAATCCAAGCGCGAAAC-3'
<i>Fasn</i>	5'-GTAAGTTCTGTGGCTCCAGAG-3'	5'-GCCCTCCCGTACACTCACTC-3'
<i>Acly</i>	5'-CAGCCAAGGCAATTTCAAGAGC-3'	5'-CTCGACGTTTGATTAACCTGGTCT-3'
<i>Acc</i>	5'-ACAGTGAAGGCTTACGTCTG-3'	5'-AGGATCCTTACAACCTCTGC-3'
<i>Apob</i>	5'-GGAACAACCTCTTGTGGTCTCT-3'	5'-ATCCTGAACATCAAGAGGGG-3'
<i>Mtp</i>	5'-CCCAACGTACTTCTAATTTATGGTC-3'	5'-TGCTGGCCAACACGTCTA-3'
<i>Cd36</i>	5'-GATGACGTGGCAAAGAACAG-3'	5'-TCCTCGGGTCTCTGAGTTAT-3'
<i>Ppara</i>	5'-TCGGCGAAGTATTTCGGCTG-3'	5'-GCACTTGTGAAAACGGCAGT-3'

Immunoblotting

A total of 10 to 100 µg cell or liver lysates, prepared with radioimmunoprecipitation assay buffer (10 mmol/L Tris-HCl, pH 8.0, 140 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), was subjected to SDS-PAGE gel electrophoresis and immunoblotting with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The blot was developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). All the images used for quantification were within the linear range.

Results

IFN-I Signaling Enhances Liver Tumor Development

As HCC formation is closely associated with repetitive liver injuries and hepatocyte regeneration processes, whether IFN-I

signaling has an active role in HCC development was examined. Carcinogen DEN can induce HCC development in young male mice with one injection.^{14–16} Acute DEN treatment causes mild liver injury, followed by compensatory regeneration.¹⁴ To test the role of IFN-I in HCC development, two-week-old male WT and *Ifnar*^{−/−} mice were injected with DEN (25 mg/kg) intraperitoneally, and HCC development was examined at the age of 8 months (Figure 1A). *Ifnar*^{−/−} mice are deficient in IFN-α receptor expression. A total of 100% DEN-injected WT male mice developed approximately 20 liver tumors on average (Figure 1B). In contrast, *Ifnar*^{−/−} mice had a slightly lower incidence rate (70%) and lower tumor multiplicity (six tumors on average) with a smaller size (Figure 1, B and C), suggesting that basal IFN-I signaling might have an active role in promoting HCC development in the absence of virus infection. Interestingly, IFN-I activation by the transient expression of hepatitis C virus full-length nonstructural protein 5B expression with adenoviral vector⁸ could further enhance HCC development in a DEN-induced tumor model in 4-week-old mice (Supplemental Figure S1).

Figure 1 Type I interferon signaling enhances chemical-induced hepatocellular carcinoma. Two-week-old wild-type (WT) and interferon- α receptor 1 knockout (*Ifnar*^{-/-}) male mice were subjected to i.p. diethylnitrosamine (DEN) injection (25 mg/kg). **A:** Schematic diagram of the chemical-induced cancer model. **B:** Tumor number and tumor size were evaluated when the mice were 8 months old. **C:** Representative images of mouse livers at the time of tumor examination. **D:** Tissue sections from the tumor-harboring livers were subjected to hematoxylin and eosin staining. The average score of lipid content for each mouse from five selected fields was calculated and plotted. **E:** Tissue sections were subjected to anti-forkhead box O 1 (FOXO1) immunostaining. The FOXO1 expression in tumor nodules was quantified by ImageJ software. $N = 8$ to 10 mice (**B**). * $P < 0.05$, ** $P < 0.01$. Scale bars: 5 mm (**C**); 100 μ m (**D**); 50 μ m (**E**).

To examine microscopic alterations in the tumor-harboring livers, the liver tissue of the DEN-treated mice at 8 months old was subjected to hematoxylin and eosin staining (Figure 1D). In addition to the presence of tumor nodules,

more lipid vacuoles were shown in some of the WT liver sections than in the *Ifnar*^{-/-} liver sections (Figure 1D and Supplemental Figure S2). In addition to STAT1-dependent ISG activation, IFN-I signaling also engages PI3K, leading

Figure 2 Active type I interferon (IFN-I) signaling is present in naïve mouse liver. **A:** The IFN-I (*Ifna6* to *a13*) and IFN-II (*Ifng*) mRNA expression levels in the liver (**top panels**) and spleen (**bottom panels**) from wild-type (WT) and *Ifnar*^{-/-} adult male mice were examined by quantitative real-time PCR. **B:** The *Apol9* mRNA expression levels in 2- and 4-week-old mouse livers were evaluated by quantitative real-time PCR. **C:** The apolipoprotein L9 (APOL9) protein expression level in mouse liver was detected by immunoblotting with anti-APOL9 antiserum. **D:** Forkhead box O (FoxO) 1 and FoxO3 protein expression levels in 4-week-old mouse livers were evaluated by immunoblotting. The image was quantified by ImageJ software. **E:** WT mice were treated with subnanometer gold nanocluster with modified methyl motifs (SAuNC-M) nanoparticles (75 mg/kg, intraperitoneally) in 24-hour intervals, and livers were collected on day 2 or 3. APOL9 expression was examined by immunoblotting and quantified after being normalized with tubulin. *n* = 4 (**A** and **B**); *n* = 3 (**E**). **P* < 0.05, ***P* < 0.01.

Figure 3 Type I interferon signaling enhances hepatocyte proliferation in naïve adult mice. **A:** Liver sections obtained from wild-type (WT) or *Ifnar*^{-/-} mice of different ages were stained with anti-Ki-67 antibody to label proliferating cells. **B:** The number of Ki-67⁺ cells in the ×400 magnification field was quantified. **C:** The nuclear size of the Ki-67⁺ cells was analyzed by ImageJ software. Red dashed line indicates the 2000 arbitrary unit (AU) nuclear size of the differentiated hepatocyte. Red boxed areas indicate the differentiated hepatocytes with nuclear size >2000 AU and their percentage to total Ki-67⁺ cells in the 4-week group. ***P* < 0.01. Scale bar = 20 μm (A).

to AKT activation, which inhibits FOXO transcription factor activity via proteasome-dependent degradation.²⁰ FOXOs regulate the expression of genes related to glucose and lipid metabolism, cell cycle, and apoptosis.²⁰ The liver sections of the DEN-treated mice were therefore stained for FOXO1 by immunohistochemistry. As shown in Figure 1E, the FOXO1 expression was found sporadically in the nontumor region and enhanced in some tumor regions. The FOXO1 expression was significantly stronger in the *Ifnar*^{-/-} tumor nodules than in the WT tumor nodules.

A Basal Level of IFN-I Signaling Is Present in the Liver

To examine whether IFN-I signaling is activated in a normal physiological state in the liver, IFN-I mRNA expression in adult WT and *Ifnar*^{-/-} mice was evaluated by quantitative real-time PCR. As shown in Figure 2A, *Ifna9* was the main IFN-I expressed in adult mouse liver. Most other IFN-Is, including *Ifnb*, *a1*, *a2*, *a4*, *a5*, *a6*, *a7*, *a8*, *a11*, *a12*, *a13*, *a15*, and *a16*, were at a low level or not detected in the liver. *Ifng* (type II IFN) mRNA expression was also low in unchallenged liver tissues. No difference was observed in IFN-I mRNA expression between the WT and *Ifnar*^{-/-} liver. IFN-I mRNA expression in mouse spleen was also evaluated (Figure 1A). The *Ifna6*, *a9*, and *a13* mRNA expression levels were detected in the spleen. As lymphocytes are

abundant in the spleen, *Ifng* mRNA expression in the lymphocytes was much higher than in the liver. The results suggest a high level of *Ifna9* expression in the naïve mouse liver, which may activate IFN-I signaling constantly.

To evaluate whether IFN-I signaling is active in the liver during postnatal liver development, the expression of an interferon-stimulated gene, *Apol9*, was examined using quantitative real-time PCR. *Apol9* mRNA expression was significantly increased in the 4-week-old WT mouse liver but was much lower in the *Ifnar*^{-/-} mouse liver (Figure 2B). The APOL9 protein expression was also detected in the 4-week-old WT liver but was undetectable in *Ifnar*^{-/-} livers (Figure 2C). The results suggest that activation of IFN-I signaling in the WT liver began at a young age. Interferon-γ receptor knockout (*Ifngr*^{-/-}) adult mice expressed a comparable amount of *Apol9* mRNA, as in naïve WT mouse liver (Supplemental Figure S3), suggesting that type II IFN signaling did not contribute to *Apol9* mRNA expression in naïve mouse liver.

To examine whether activation of IFN-I signaling in the liver modulates the PI3K/AKT/FOXO pathway, FOXO protein expression in 4-week-old mouse liver was evaluated by immunoblotting. As shown in Figure 2D, both FOXO1 and FOXO3 expression levels were slightly lower in 4-week-old WT liver relative to *Ifnar*^{-/-} liver, suggesting that IFN-I might fine-tune PI3K/AKT/FOXO pathway activity in naïve liver.

Figure 4 Type I interferon signaling accelerates hepatocyte regeneration induced by two-thirds partial hepatectomy (PHx). **A:** Wild-type (WT) and *Ifnar*^{-/-} mice were subjected to two-thirds PHx. Liver tissues were collected at 48 hours and 7 days after surgery and subjected to Ki-67 staining. **B** and **C:** Cell cycle-associated gene expression was evaluated by quantitative real-time PCR (**B**) and immunoblotting (**C**). **P* < 0.05, ***P* < 0.01. Scale bar = 10 μm (**A**). EGFR, epidermal growth factor receptor.

The negative regulation of IFN-I signaling on FOXO level was also observed in mouse liver with recombinant adenovirus to expressing hepatitis C virus active nonstructural protein 5B mutant, which can activate IFN-I production (Supplemental Figure S4).⁸ Human hepatoma Huh7 cells treated with IFN-β also showed a reduction in FOXO level (Supplemental Figure S5). These results suggest that IFN-I signaling can modulate FOXO level in the liver.

Lipopolysaccharide (LPS), a component of Gram-negative bacterial cell wall, activates TLR4 and induces IFN-I expression.²¹ To examine whether LPS translocated from intestine to liver could be the trigger of IFN-I signaling in naïve mice, the subnanometer gold nanocluster with modified methyl motifs nanoparticles that block LPS and LPS-associated inflammation²² were used. As shown in Figure 2E, WT livers treated with subnanometer gold nanocluster with modified methyl motifs (75 mg/kg) for 2 or 3 days showed a 40% reduction in APOL9 protein level, suggesting that LPS/TLR4-dependent IFN expression participated in IFN-I activation in the liver.

IFN-I Signaling Enhances Basal Hepatocyte Proliferation in Naïve Mice

To evaluate whether IFN-I signaling modulates hepatocyte proliferation during postnatal development, liver tissue was stained to examine the proliferation marker Ki-67 using immunohistochemistry. As shown in Figure 3, A and B, liver tissue from 2-week-old mice contained high levels of proliferating cells, and these cells were significantly reduced in liver tissue obtained from 4-week-old weaning mice. The number of Ki-67⁺ cells in young (aged 2 and 4 weeks) mice

was not significantly different between the WT and *Ifnar*^{-/-} mice, but *Ifnar*^{-/-} mice had less proliferating cells in adult naïve mice (aged 8 or 12 weeks), as quantified in Figure 3B. Hepatocytes are diploid after birth and differentiate into mature hepatocyte cells with multiple chromosome copies (polyploidy) to fulfill the needs for protein synthesis and metabolic functions. Mature hepatocytes, therefore, have a large nuclear size.²³ Quantification of the nuclear size of proliferating cells (Figure 3C) indicated that Ki-67⁺ hepatocytes in 2-week-old mouse liver had an average nuclear size of 1000 arbitrary units based on pixel size. The differentiated hepatocytes in adult mice were a mixed population ranging from 1000 to 3000 arbitrary units in size, with an average size of 2000 arbitrary units. Although the average nuclear size of the proliferating hepatocytes was not significantly different between the WT and *Ifnar*^{-/-} in any age group, in 4-week-old mice, Ki-67⁺ hepatocytes with a nuclear size >2000 arbitrary units were more abundant in *Ifnar*^{-/-} livers than in WT livers (Figure 3C), suggesting that the differentiation program in *Ifnar*^{-/-} liver might start earlier than in WT mice. Assessment of cell cycle-associated cyclins by real-time quantitative PCR in different ages of mice indicated significantly higher levels of *E2f1* and *Ccnd1* mRNA in 4-week-old WT mice relative to *Ifnar*^{-/-} mice (Supplemental Figure S6). Decreased proliferation and early onset of hepatocyte differentiation in *Ifnar*^{-/-} mice were further confirmed in 4-week-old mice challenged with the recombinant adenovirus expressing hepatitis C virus nonstructural protein 5B active mutant (Supplemental Figure S7). Taken together, these data suggest that IFN-I signaling participates in the regulation of the balance of hepatocyte proliferation and differentiation during postnatal liver development.

Figure 5 Transient lipid accumulation during liver regeneration is positively modulated by type I interferon signaling. Wild-type (WT) and *Ifnar*^{-/-} liver tissues, collected at 0 or 6 hours after two-thirds partial hepatectomy (PHx) surgery, were subjected to quantitative real-time PCR to detect gene expression related to gluconeogenesis (A), lipogenesis (B), lipid export (C), and fatty acid oxidation (D). WT and *Ifnar*^{-/-} liver sections collected from naïve mice (E) or mice after two-thirds PHx (F) were subjected to oil red O staining. **P* < 0.05, ***P* < 0.01. Scale bar = 20 μm (E and F). *Acc*, acetyl-CoA carboxylase; *Apob*, apolipoprotein B; *G6pc*, glucose-6-phosphatase, catalytic subunit; *Ppara*, peroxisome proliferator-activated receptor-α.

Ablation of IFN-I Signaling Delays Hepatocyte Regeneration

To evaluate the role of IFN-I signaling in hepatocyte proliferation in the absence of pathogen invasion, the hepatocyte regeneration process was monitored in WT and *Ifnar*^{-/-} liver after two-thirds PHx. Two-thirds PHx is a surgery that removes two-thirds of the mouse liver to evaluate its regeneration ability.¹⁷ The peak of hepatocyte proliferation occurs between 36 and 48 hours after liver resection, and the remaining liver completes regeneration to its original size in a week.¹⁷ As shown in Figure 4A, WT mice had extensive proliferating cells

in the liver at 48 hours after PHx and few proliferating cells on day 7 after surgery. In contrast, the *Ifnar*^{-/-} liver had significantly lower numbers of Ki-67⁺ cells at 48 hours after surgery. The regeneration process was still ongoing on day 7 after liver PHx in the *Ifnar*^{-/-} liver (Figure 4A), which may be because liver mass had not reached homeostasis. To sustain metabolic homeostasis, the liver size is maintained in proportion to the body size.²⁴ The liver weight/body weight ratio was significantly elevated on day 7 in WT mice but not in *Ifnar*^{-/-} mice (Supplemental Figure S8). In line with the slow proliferation rate, *Ifnar*^{-/-} liver expressed lower amounts of *Ccna2* and *Ccnb1* mRNA at 48 hours following PHx (Figure 4B).

Figure 6 Type I interferon signaling enhances lipid droplet formation. A and B: Protein expression of apolipoprotein L9 (APOL9) and adipose differentiation-related protein (ADRP) in total liver lysates (A) or lipid-enriched fractions (fractions 1 to 3; B) collected from a sucrose gradient were detected by immunoblotting. C: Lipid-enriched fractions prepared from the wild-type (WT) and *Ifnar*^{-/-} mouse livers [24 hours after partial hepatectomy (PHx)] were analyzed by immunoblotting.

Figure 7 Delayed liver regeneration in *Ifnar*^{-/-} mice can be restored by insulin treatment. **A:** The blood glucose level in wild-type (WT) and *Ifnar*^{-/-} mice with 10 hours starvation was measured. **B:** WT and *Ifnar*^{-/-} mice were subjected to the two-thirds partial hepatectomy (PHx), and two of the *Ifnar*^{-/-} mice were injected with insulin (intraperitoneally, 0.75 U/kg) at 2 hours after surgery. Blood glucose level was measured at different time points after PHx. **C:** Mouse livers were collected at 48 hours after PHx and subjected to immunohistochemistry with the anti-Ki-67 antibody. **D:** *Ifnar*^{-/-} mice were injected with intralipid (100 mg/kg, intraperitoneally), and blood glucose level was measured. **E:** *Ifnar*^{-/-} mice were subjected to the two-thirds PHx, and the mice were injected with intralipid (100 mg/kg, intraperitoneally) at 24 hours after surgery. Mouse liver sections were stained with the anti-Ki-67 antibody. *n* = 5 (**A**); *n* = 3 WT mice (**B**); *n* = 5 *Ifnar*^{-/-} mice (**B**); *n* = 3 *Ifnar*^{-/-} mice (**D**). **P* < 0.05, ***P* < 0.01. Scale bar = 20 μ m (**C** and **E**).

Reduced cyclin A2 and B1 protein expression at 48 hours after hepatectomy in the *Ifnar*^{-/-} liver was also observed (Figure 4C). To control cell cycle progression, cell cycle kinases are negatively regulated by members of the Kip/Cip family of cell cycle kinase inhibitors, including p27. p27 expression remained at a higher level in *Ifnar*^{-/-} liver at 48 hours following PHx (Figure 4C). Interestingly, fewer proliferating cells and more cell cycle kinase inhibitor p27 and p21 accumulation were also observed in *Ifnar*^{-/-} liver compared with the WT liver during the regeneration time after virus infection (Supplemental Figures S7 and S9). Similarly, compensatory liver regeneration, induced by DEN challenge, was also decreased in *Ifnar*^{-/-} liver (Supplemental Figure S10). Taken together, the data suggest that hepatic IFN-I signaling positively modulates compensatory regeneration processes in the liver.

IFN-I Signaling Enhances Hepatic Lipid Metabolism during Regeneration

FOXOs are transcription factors involved in the regulation of metabolism and cell fate decisions in responding to

various metabolic conditions, growth factor stimulation, and stress.²⁰ FOXOs are translocated to the nucleus to activate target gene expression. FOXO1 expression was found predominantly in the nucleus in naïve WT and *Ifnar*^{-/-} mice (Supplemental Figure S11). Metabolic changes, including hypoglycemia and transient hepatic lipid droplet accumulation, occur before cell proliferation during the liver regeneration process.²⁵ As the activation of IFN signaling modulates the FOXO level in the liver (Figure 2D and Supplemental Figure S4), IFN signaling was suspected to modulate metabolic alterations during the regeneration process. Therefore, the change of metabolic genes involved in gluconeogenesis and lipid metabolism after PHx was examined by real-time PCR (Figure 5, A–D). *G6pc* gene encodes glucose-6-phosphatase, a catalytic subunit that is an enzyme functioning in gluconeogenesis and glycogenolysis. The baseline *G6pc* mRNA expression was slightly higher in *Ifnar*^{-/-} liver compared with the expression level in WT liver. Although both significantly reduced at 6 hours after PHx, the *G6pc* expression level was kept significantly higher in *Ifnar*^{-/-} liver than in WT liver (Figure 5A). As lipid metabolism can be regulated at various levels, such as

Figure 8 Type I interferon signaling in parenchymal cells modulates glucose and lipid metabolism and contributes to liver regeneration. **A:** The *Apol9* mRNA expression levels in wild-type (WT; *Ifnar*^{F/F} × albumin-Cre⁺) and *Ifnar*^{Δliver} (*Ifnar*^{F/F} × albumin-Cre⁺) mouse livers were evaluated by quantitative real-time PCR. **B:** The blood glucose level in naïve mice was measured. **C:** Mice were subjected to two-thirds partial hepatectomy (PHx), and the blood glucose level was measured at different time points after PHx. **D** and **E:** Mouse livers were collected at 24 hours after PHx for oil red O staining (**D**) and 48 hours after PHx for anti-Ki-67 immunohistochemistry (**E**). *n* = 3 (**A**); *n* = 4 to 5 (**B**). **P* < 0.05. Scale bar = 20 μm (**D** and **E**).

lipid uptake, *de novo* lipogenesis, fatty acid oxidation, and lipid export, the change of gene expression related to these processes was examined. Genes involved in *de novo* lipogenesis [acetyl-CoA carboxylase (*Acc*)] and lipid export [apolipoprotein B (*Apob*)] were activated in WT liver at 6 hours after PHx but not in *Ifnar*^{−/−} liver (Figure 5, B and C). The expression level of peroxisome proliferator-activated receptor-α gene (*Ppara*), which is involved in fatty acid oxidation, was significantly down-regulated in WT liver at 6 hours after PHx compared with the expression level in *Ifnar*^{−/−} liver (Figure 5D). The results suggest that the IFN signaling modulates hepatic metabolism during the regeneration process.

Whether IFN-I signaling participates in the transient lipid accumulation during liver regeneration was further

evaluated. A low amount of small lipid droplets were present in the livers of both WT and *Ifnar*^{−/−} naïve mice, as indicated by oil red O staining (Figure 5E). High amounts of large lipid droplets accumulated in the WT liver at 24 hours after PHx (Figure 5F). In contrast, only the smaller sized lipid droplets increased in *Ifnar*^{−/−} liver (Figure 5F). The results suggest a role of IFN-I signaling in lipid accumulation during the liver regeneration process.

ADRP is one of the lipid droplet-associated membrane proteins that are required for stabilization of the lipid droplet structure.²⁶ The protein expression of ADRP was highly elevated at 24 hours after PHx in WT livers but to a lesser extent in *Ifnar*^{−/−} livers (Figure 6A), which is consistent with the oil red O staining result. To examine whether APOL9 functions in lipid accumulation in the liver, lipid

droplets in the liver were purified on a discontinuous 0% to 20% sucrose gradient, in which lipid droplets were enriched in the top three fractions (Supplemental Figure S12).¹⁹ As shown in Figure 6B, both ADRP and APOL9 were present in the lipid-enriched fractions separated from WT mouse liver from 24 hours after PHx, but not in the lipid fractions from naïve WT mouse liver. Lower ADRP amounts were induced in liver lysate and lipid fractions separated from *Ifnar*^{-/-} mice liver from 24 hours after PHx (Figure 6C). Taken together, the results suggest that IFN-I signaling might participate in the metabolic regulation during the regeneration process.

IFN-I Signaling Modulates Glucose Homeostasis

Glucose and lipid metabolic alterations occur after PHx to initiate the liver regeneration process. As lipid metabolism was dysregulated in *Ifnar*^{-/-} liver, whether glucose metabolism is also affected by IFN-I signaling was further evaluated. The average fasting blood glucose level in WT mice was 110 mg/dL and was elevated to 140 mg/dL in *Ifnar*^{-/-} mice (Figure 7A). With the high serum glucose, the insulin level was also slightly elevated in *Ifnar*^{-/-} mice at baseline (Supplemental Figure S13A). The serum insulin level was not significantly different between WT and *Ifnar*^{-/-} mice after PHx (Supplemental Figure S13B). *Ifnar*^{-/-} mice did not show insulin-resistant phenotype in the insulin tolerance test and glucose tolerance test (Supplemental Figure S13C). Transient hypoglycemia occurred in WT mice after PHx surgery, and the blood glucose level returned to normal within 24 hours (Figure 7B). In contrast, the blood glucose level in *Ifnar*^{-/-} mice decreased at PHx and was kept at a low level at 24 hours after PHx. As dextrose supplementation has been known to have an inhibitory effect on liver regeneration,²⁷ the slow regeneration phenotype in *Ifnar*^{-/-} mice might be due to the high blood glucose level and dysregulation of catabolic metabolism in these mice. Whether lowering blood glucose level could accelerate the regeneration process in *Ifnar*^{-/-} mice was examined. In *Ifnar*^{-/-} mice treated with insulin (0.75 U/kg, intraperitoneally) at 2 hours after PHx, the kinetics of blood glucose level in insulin-treated *Ifnar*^{-/-} mice became similar to the WT mice (Figure 7B). The number of Ki-67⁺ hepatocytes in the insulin-treated *Ifnar*^{-/-} mice was also significantly increased (Figure 7C). The transient hepatic lipid droplet accumulation might be triggered by hypoglycemia to maintain glucose homeostasis. Therefore, whether lipid supplement affects blood glucose level and regeneration was examined in *Ifnar*^{-/-} mice. On the i.p. injection of 20% intralipid emulsion (soybean oil, 100 mg/kg), blood glucose was significantly elevated (Figure 7D) and liver regeneration was enhanced (Figure 7E) in *Ifnar*^{-/-} mice. Taken together, these results suggest that IFN-I signaling regulates glucose homeostasis to support a timely regeneration process.

Liver-Specific IFN Signaling Regulates Metabolism to Initiate Regeneration

To address whether parenchymal cells in liver are the main cell type receiving IFN signaling during liver regeneration, the conditional knockout mice (*Ifnar*^{Aliver}; *Ifnar*^{F/F} × *albumin-Cre*⁺) lacking IFN-I receptor in albumin-expressing parenchymal cells, including hepatocytes and cholangiocytes, were established. As shown in Figure 8A, the *Apol9* mRNA expression level in *Ifnar*^{Aliver} liver was significantly lower than in WT (*Ifnar*^{F/F}) liver. *Ifnar*^{Aliver} mice had a slightly higher baseline blood glucose level (Figure 8B). On PHx, the blood glucose level in *Ifnar*^{Aliver} mice decreased at 6 hours after PHx and remained at a low level at 24 hours (Figure 8C). In contrast, the blood glucose level in WT mice decreased at 6 hours after PHx and gradually increased at 24 hours after PHx. Similar to *Ifnar*^{-/-} mice, *Ifnar*^{Aliver} mice had less amount of lipid droplet accumulation at 24 hours after PHx and less hepatocyte proliferation compared with the WT mice (Figure 8, D and E). Taken together, IFN-I signaling modulates metabolic alterations in parenchymal cells and facilitates liver regeneration.

Discussion

In the present study, a basal level of IFN-I signaling was identified in the mouse liver, leading to constant activation of ISG expression and FOXO degradation. The low level of IFN-I activation enhanced baseline proliferation and compensatory liver regeneration, which might be dependent on the regulation of glucose and lipid metabolism in hepatocytes. Moreover, the constant hepatic IFN-I activation enhanced HCC development. The study reveals the possible contribution of IFN-I signaling to the regulation of glucose and lipid metabolism, hepatocyte proliferation, and HCC development in the absence of infection.

IFN-I has been used to treat several types of cancer,²⁸ and its ability to inhibit cell growth at high concentrations can be easily observed in cell culture. The positive role of IFN-I signaling in hepatocyte proliferation identified in this study might be intensity- and context-dependent, providing the hepatocyte with a capacity to tolerate stress and facilitate subsequent proliferation. In the absence of infection, trace amount of LPS translocated from the intestine is one of the potential stimuli to induce a low level of IFN production to modulate hepatocyte physiology, including metabolic homeostasis.

Signals transduced directly from IFN receptor or downstream ISG expression might participate in shaping liver homeostasis. IFN-I signaling activates STAT1, PI3K, and p38 pathways. Lack of these signaling pathways can all potentially be involved in the slow proliferation and regeneration ability of *Ifnar*^{-/-} hepatocytes. Genetic ablation of p38α in hepatocytes enhances chemical-induced injury, compensatory proliferation, and HCC development,¹⁵ therefore, the role of p38 downstream of IFN-I signaling in the regulation of hepatocyte

proliferation might not be dominant. FOXO1, a critical effector of the PI3K/Akt pathway, regulates multiple metabolic pathways, including gluconeogenesis and lipogenesis in the liver. The current findings suggest that the basal IFN-I activation in the liver regulates FOXO1, which might, therefore, modulate blood glucose level. The coordinated metabolic status in hepatocytes may provide a higher capacity to proliferate during the regeneration process. On the other hand, disruption of IFN-I signaling leads to an accumulation of FOXO1 and an unregulated blood glucose level. Dysregulation of FOXO1 and blood glucose level then affect subsequent lipid metabolism, which leads to delayed regeneration. In line with this, insulin treatment to lower the glucose and FOXO1 level could accelerate liver regeneration in the *Ifnar*^{-/-} mice. Insulin binds to the insulin receptor, activating extracellular signal-regulated kinase and PI3K/Akt—mechanistic target of rapamycin pathways, which may also contribute to cell proliferation.²⁹ Therefore, in addition to the modulation of the FOXO1 and glucose level, the insulin treatment might also potentially contribute to hepatocyte proliferation during liver regeneration in *Ifnar*^{-/-} mice.

ISG expression downstream of the IFN-I/STAT1 pathway may also play a role to engage IFN-I signaling to glucose and lipid metabolism during liver regeneration. Although the exact role of the APOL family is not clear yet, APOL9, which is highly induced by IFN-I activation in the liver, may be involved in lipid droplet accumulation during hepatocyte regeneration. APOL9 expression is maintained at a high level in mouse liver after weaning and is diminished in *Ifnar*^{-/-} mice, making APOL9 a good marker for tracing IFN-I signaling activity in the liver. Mice, aged 3 to 4 weeks, begin eating solid food during weaning and are exposed to various food-borne substances, some of which may lead to direct IFN-I induction in the liver or through effects on the microbiota at the mucosal immune system. For instance, LPS reaching the liver through the portal vein activates TLR4 and induces IFN-I expression. Interestingly, *Tlr4*^{-/-} mice also show delayed liver regeneration after partial hepatectomy,³⁰ and both TLR4 and the intestinal microbiota are involved in HCC promotion.³¹ Whether other ISGs downstream of IFN-I signaling modulate various metabolic homeostasis levels in the liver needs further investigation.

Transient hypoglycemia and lipid droplet accumulation in the liver are critical events to initiate liver regeneration after PHx, but the interplay between these physiological events remains unclear. Blood glucose level reduces right after PHx, which may be a trigger for lipid metabolism, including *de novo* lipogenesis. Transient lipid accumulation, induced by metabolic disturbance after PHx, may be used as an energy resource for liver regeneration. Lipid supplementation is also known to enhance liver regeneration. The role of IFN-I signaling in modulating glucose and lipid metabolism during the regeneration process may contribute to HCC development and will be worthy of further exploration.

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Author Contributions

M.-S.W., Y.-P.K., Y.-C.L., and D.-J.T. performed most of the experiments. C.-Y.L. and T.-H.C. conducted apolipoprotein L9 antiserum production. S.-Y.L. provided nanoparticles. W.-T.T. and P.-J.L. participated in animal work. M.-S.W., S.-Y.L., and T.-H.C. participated in experimental design, data interpretation, and manuscript preparation with G.-Y.Y.

Supplemental Data

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