

Antiproliferative Effects of Interferon Alpha on Hepatic Progenitor Cells *In Vitro* and *In Vivo*

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Hepatic progenitor cells (called oval cells in rodents) proliferate during chronic liver injury. They have been suggested as targets of malignant transformation in chronic liver diseases, including chronic hepatitis C. Interferon alpha therapy reduces the risk of hepatocellular carcinoma (HCC) in chronic hepatitis C regardless of viral clearance. The aim of this study was to determine whether interferon alpha could reduce the risk of HCC by modifying preneoplastic events in the hepatic progenitor cell population. Pre- and post-treatment liver biopsies were evaluated for changes in the hepatic progenitor cell population in 16 patients with non-responding chronic hepatitis C. Interferon alpha-based treatment significantly reduced the numbers of c-kit-positive hepatic progenitor cells by 50%. To determine the mechanism of cell number reduction, the effects of interferon alpha on murine hepatic progenitor cells were studied *in vitro*. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay and proliferating cell nuclear antigen staining showed that interferon alpha had a dose-dependent, anti-proliferative effect. Interferon alpha stimulated hepatocytic and biliary differentiation of the oval cell lines reflected by increased expression of albumin and cytokeratin19 accompanied by decreased expression of alphafetoprotein and Thy-1. To validate these results *in vivo*, mice were placed on the choline-deficient, ethionine-supplemented diet to induce liver injury and oval cell proliferation and treated with pegylated interferon alpha 2b for 2 weeks. This resulted in a significant four-fold reduction in the number of oval cells ($P < .05$). **In conclusion**, interferon alpha-based treatment reduced the number of hepatic progenitor cells in chronic liver injury by modulating apoptosis, proliferation, and differentiation. *Supplementary material for this article can be found on the HEPATOLOGY website (<http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>). (HEPATOLOGY 2006;43:1074-1083).*

Abbreviations: HCC, hepatocellular carcinoma; π -GST, π -glutathione-S-transferase; AFP, alphafetoprotein; MPK, M2-pyruvate kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling; PCR, polymerase chain reaction; G6Pase, glucose-6-phosphatase; CDE diet, Choline deficient ethionine supplemented diet.

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Up to 3% of the world's populations may be infected with hepatitis C virus. Ten percent of chronically infected individuals progress to cirrhosis, with 2% to 9% of subjects with cirrhosis developing hepatocellular carcinoma (HCC) each year.¹⁻⁴ Evidence suggests that HCC may arise due to the transformation of adult hepatic progenitor cells, called "intermediate hepatobiliary" cells in humans⁵ and "oval" cells in mice.⁶

In humans, hepatic progenitor cells proliferate after chronic liver injury due to chronic viral hepatitis, alcoholic liver disease, or metabolic liver conditions, and their numbers increase in direct proportion with the disease severity.⁷⁻⁹ In rodents, oval cells proliferate in response to a variety of stimuli, primarily associated with exposure to hepatocarcinogens.¹⁰ We have shown in a mouse model that an attenuated oval cell response correlates with reduced incidence of HCC after prolonged liver injury.¹¹ Oval cells are easily transformed in culture, giving rise to cells capable of forming solid tumors in immune-deficient mice.^{12,13} Thus, despite early skepticism, the concept of progenitor cells as targets of malignant transformation is now widely accepted, and hence differentiation therapy, in addition to therapeutic control of proliferation and

apoptosis of pre-neoplastic cells, is a plausible solution to overcoming their malignant potential.

Currently, the gold standard for treatment of chronic hepatitis C infection is combination treatment with interferon alpha and ribavirin.¹⁴ In addition to its anti-viral capabilities, interferon alpha plays an essential role in controlling cellular proliferation and survival of a variety of diverse cell types, as well as exerting potent immunomodulatory and anti-angiogenic actions.¹⁵ Interferon alpha has been included in the treatment protocols for malignancies such as hairy-cell leukemia, chronic myelogenous leukemia, and Kaposi's sarcoma.¹⁶ Furthermore, recent studies have shown that interferon therapy significantly reduces the risk for development of HCC, regardless of sustained virological response.¹⁷⁻²¹ This effect could be mediated via pre-neoplastic effects on the hepatic progenitor cell compartment. Thus, the aim of this study was to determine the effect of interferon alpha therapy on the numbers of hepatic progenitor cells observed in the livers of patients with chronic hepatitis C and to elucidate the effects of interferon alpha on the behavior of oval cells in experimental murine models, *in vitro* and *in vivo*.

Methods

Human Studies. Liver biopsies collected before and after interferon-based treatment were available from 16 randomly selected individuals with non-responding chronic hepatitis C (predominantly genotype 1). The interval between pre- and post-treatment biopsies was 18 months; patients were treated for 48 weeks and the post-treatment biopsy was performed at 6 months after end of treatment. Initial METAVIR scores ranged from 0 to 4. Informed consent was obtained for the use of these biopsies for research purposes, and the study was approved by the Human Research Ethics Committee, Fremantle Hospital, Fremantle, Western Australia. Standard histological examination was undertaken using hematoxylin-eosin and Masson's trichrome staining for assessment of fibrosis.

Human Liver Immunohistochemistry. All sections were antigen retrieved in citrate buffer (10 mmol/L citric acid, pH 6.8) by boiling for 15 minutes as described by Pileri et al.²² Sections were blocked for endogenous peroxidases and endogenous biotin using a biotin-blocking system (Dako, Carpinteria, CA) according to the manufacturer's instructions. Sections were then blocked for non-specific antigens using serum-free protein block (Dako). Primary antibodies against, c-kit (1:200, Dako), CK19 (1:100, Dako) and π -GST (1:400, Rockland, Gilbertsville, PA) were applied to tissue sections and incu-

bated overnight at 4°C. Detection was performed using the Universal LSAB2 Kit (Dako) and liquid DAB (Dako). Intermediate hepatobiliary cells were identified as small cells with ovoid nuclei and scant cytoplasm that were immunoreactive for cytokeratin 19 (CK19), π -glutathione S-transferase (π -GST), or c-kit.

In Vitro Oval Cell Studies. Well-characterized immortalized murine oval cell lines (PIL-2 and PIL-4) that exhibit phenotypic similarities with oval cells *in vivo* were used for *in vitro* studies.^{12,23,24} PIL cells are immunoreactive for the hepatocyte markers albumin and transferrin and the oval cell markers alpha-fetoprotein (AFP), A6 and M2-pyruvate kinase (MPK). They are capable of differentiating along both the hepatic and biliary lineages²⁴ and exhibit increased proliferation in response to increasing concentrations of serum.²⁵ PIL-2 cells are tumorigenic whereas PIL-4 cells are not.¹² PIL cells were maintained in culture as previously described.¹²

In Vitro Proliferation Studies. PIL-2 and PIL-4 cells were seeded in 96-well microtiter plates (BD Falcon, Franklin Lakes, NJ) at a density of 2,000 cells/well. After adherence, cells were exposed to media containing pegylated interferon alpha 2b (Schering Plough Pty Ltd, Kenilworth, NJ) at various concentrations (0 to 1000 U/mL) and cultured for 24 hours. Numbers of metabolically active cells present at the end of the experiment were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay, which measures mitochondrial activity.²⁵ Staining for proliferating cell nuclear antigen (PCNA) was performed to assess the proliferative status of the cells, using a previously described method.²⁶ In brief, cells were fixed using 4% (v/v) buffered formalin for 8 minutes and 99% ethanol for 2 minutes. Cells were then washed in phosphate-buffered saline (PBS) and blocked for endogenous peroxidases with 3% (v/v) hydrogen peroxide for 1 minute, followed by a biotin blocking step using a biotin blocking system. Cells were also blocked with a serum-free protein block for 10 minutes before incubating with the mouse monoclonal anti-PCNA primary antibody diluted in PBS at 1:200, for 1 hour at 37°C. After this, cells were washed in PBS and incubated with biotinylated anti-mouse secondary antibody and streptavidin-labeled horseradish peroxidase using a Universal LSAB2 Kit for 30 minutes each. Cells positive for PCNA were visualized using liquid DAB. PCNA-positive cells were counted in 10 sequential fields of view and normalized to the total number of cells present as determined by phase-contrast microscopy. The percentage of PCNA-positive cells in each field were expressed as mean \pm SEM.

In Vitro Apoptosis Studies. PIL-2 and PIL-4 cells were seeded in 4-chambered slides (BD Falcon) at a den-

Table 1. Primers for Target Gene Sequences Analyzed by Quantitative Polymerase Chain Reaction

Target Gene	Primer Sequence	Product Size	Reference
Albumin	5' CTT AAA CCG ATG GGC GAT CTC ACT 3' CCC CAC TAG CCT CTG GCA AAA T	130 bp	27
α -fetoprotein	5' TCG TAT TCC AAC AGG AGG 3' AGG CTT TTG CTT CAC CAG	176 bp	27
G6Pase	5' CGC CTT CTA TGT CCT CTT TCC C 3' GTT TCA GCC ACA GCA ATG CC	459 bp	27
CK19	5' CAT GGT TCT TCT TCA GGT AGG C 3' GCT GCA GAT GCA TTC AGA ACC	177 bp	28
Thy-1	5' AAC CAA AAC CTT CGC CTG GAC 3' AAG CTC ACA AAA GTA GTC GCC C	231 bp	27

sity of 200 cells/chamber. After allowing for adherence, cells were serum-starved overnight before 3 days' stimulation with 250 IU/mL pegylated interferon alpha 2b. Apoptotic cells were detected using terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay (Promega, Madison, WI) according to manufacturer's instructions. Cells undergoing apoptosis were also identified using Annexin V-fluorescein isothiocyanate (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

In Vitro Differentiation Studies. PIL-2 and PIL-4 cells were seeded in 6-well plates at a medium density of 2,000 cells/well and cultured for 8 days in the presence of pegylated interferon alpha 2b 250 IU/mL. Cells were then harvested, and total RNA was isolated using TRIzol (Invitrogen, Calsbad, CA) and converted to cDNA using the ThermoScript Reverse Transcription System (Invitrogen) according to the instructions of the manufacturer. Quantitative polymerase chain reaction (PCR) was performed using specific primers directed against murine albumin, CK19, AFP, glucose-6-phosphatase (G6Pase), and Thy-1. Each reaction contained 1 \times reaction buffer (Fisher Biotech, Perth, Australia), 2.5 mmol/L MgCl₂ (Fisher Biotech), 0.25 mmol/L dNTPs (Fisher Biotech), Platinum Taq (Invitrogen, Australia), and 0.25 \times SYBR green (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using the Rotorgene system (Corbett Research, Australia). Platinum Taq polymerase was activated by preincubation at 95°C for 2 minutes, before cycling for 40 cycles of 95°C, 20 seconds; 60°C, 20 seconds; and 72°C, 30 seconds. Fluorescence was measured at the 72°C extension phase. The level of gene expression was determined in arbitrary units by comparison with plasmid DNA standards, specific for the gene of interest as previously described.²⁴ The levels of β -actin mRNA was determined in the same samples, again by comparisons with plasmid DNA standards. Gene expression of each marker was normalized to β -actin levels to account for variation in RNA loading and the

efficiency of reverse transcription. Sequences of primers and product sizes are listed in Table 1.^{27,28}

Animal Studies. To determine the *in vivo* effects of pegylated interferon alpha 2b on the hepatic progenitor cell population, 4-week old C57Bl/6 male mice weighing between 14 and 16g were placed on the choline-deficient ethionine-supplemented (CDE) diet to induce proliferation of the progenitor cells as previously described.¹¹ Animals were administered either 10⁵ units pegylated interferon alpha 2b (Schering Plough) intraperitoneally once every 3 days or saline (placebo). Administration of interferon commenced on the first day of CDE feeding and continued for the duration of the experiment. Mice were sacrificed 2 weeks later. Livers were perfused with saline, and portions of liver were sampled for histological studies. All animal experiments were performed in a pathogen-free animal holding facility in accordance with guidelines of the National Health and Medical Research Council of Australia and approved by the University of Western Australia Animal Ethics Committee.

Histology and Immunohistochemistry. Portions of perfused liver were either fixed in neutral buffered formalin and embedded in paraffin or immersed in cryomatrix (Tissue-Tek OCT, Sakura Finetek, Japan) and snap-frozen in liquid nitrogen. Five-micron sections were cut and stained for the presence of specific antigens using immunohistochemistry.

Sections for MPK and phospho-STAT-3 staining were antigen retrieved in EDTA buffer (1 mmol/L EDTA, pH 8.0) by boiling for 15 minutes as previously described by Pileri et al.²² Sections were blocked for endogenous peroxidases followed by blocking for non-specific antigens using serum-free protein block (DAKO). Primary antibodies against PCNA (1:200; DAKO), MPK (1:500; Rockland, Gilbertsville, PA), A6 (1:30; a gift from Dr Valentina Factor, National Cancer Institute, National Institute of Health, Bethesda) and phospho-STAT-3 (1:20, Cell Signaling Technology, Danvers, MA) were applied to tissue sections and incubated overnight at 4°C and

detected using biotinylated secondary antibodies and liquid DAB (DAKO, Carpinteria, CA).

In Situ TUNEL Staining. Apoptotic cells were detected in paraffin-embedded tissues using the same TUNEL assay (Promega) as described previously. Modifications to allow for detection in tissue sections were made as recommended by the manufacturer.

Cell Counts. Cell counts were performed on an inverted phase microscope using a 400 \times magnification. Counts were performed on 10 adjacent, non-overlapping peri-portal fields. Total cell number was also counted in each field, for purposes of data normalization. Liver progenitor cells were identified as cells approximately 10 μ m in diameter with ovoid nucleus and scant cytoplasm. Hepatocytes were identified as cells 20 μ m or more in diameter with round nucleus and abundant cytoplasm. Where a specific marker was not used, care was taken to exclude other cell types, including inflammatory cells, endothelial cells, Kupffer cells, and stellate cells, from oval cell counts.

Statistical Methods. Each *in vitro* experiment was performed three times and in duplicate. Data are reported as the mean \pm SEM. Comparisons between groups were performed using unpaired *t* test or Fisher's exact test (GraphPad Prism version 4.03 for Windows, San Diego, CA). Means were deemed to be different for $P < .05$. Non-parametric data are reported as median values and compared using the Mann-Whitney *U* test (GraphPad Prism).

Results

Interferon Alpha-Based Therapy Reduced Numbers of c-kit-Positive Intermediate Hepatobiliary Cells in Subjects With Chronic Hepatitis C. Hepatic progenitor cells were stained using three markers: c-kit, CK19, and π -GST. Each of these correlate to a different stage of maturation or lineage. c-kit is a hematopoietic marker specific to primitive progenitor cells and is abruptly switched off as the cell differentiates.²⁹⁻³¹ π -GST is expressed by fetal hepatocytes and duct-like progenitor cells,³²⁻³⁴ whereas CK-19 is expressed by mature and immature biliary epithelium.^{35,36} The number of c-kit-positive intermediate hepatobiliary cells detected in liver biopsies was reduced by over 50% in 14 of 16 subjects (13 non-responders) post-treatment with interferon alpha compared with the paired pre-treatment biopsies (pre-treatment 3.5% \pm 1.0% versus post-treatment 1.7% \pm 0.5%, $P < .05$, Fig. 1A) (for individual subject details see Supplementary Table 1). In contrast, no consistent change in the numbers of intermediate hepatobiliary cells that were immunoreactive for CK19 (Fig. 1B) or π -GST

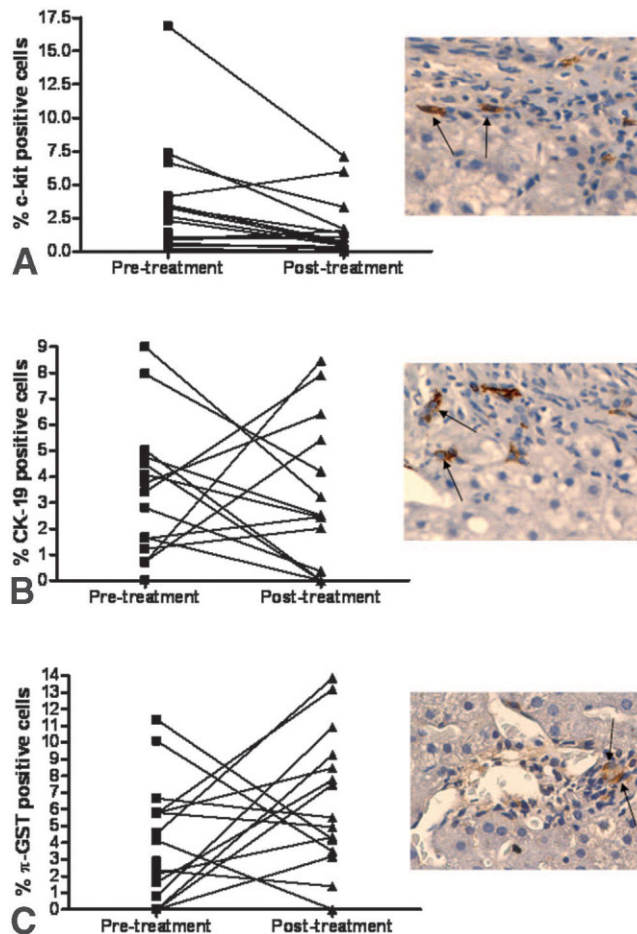


Fig. 1. Changes in the numbers of intermediate hepatobiliary cells in chronic hepatitis C patients after treatment with interferon alpha. There was a significant decrease by at least 50% in the numbers of c-kit-positive intermediate hepatobiliary cells in 14 of 16 patients (A, $P < .05$). The number of CK-19 (B) and π -GST (C)-positive intermediate hepatic progenitor cells did not change.

(Fig. 1C) was observed between pre-and post-interferon treatment biopsies. Fibrosis scores remained unchanged in seven subjects, worsened by at least one level in five subjects, and improved in three subjects (all non-responders). Hepatic inflammatory activity remained unchanged in five subjects, worsened in six subjects, and improved in five subjects (3 responders, 2 non-responders).

Pegylated Interferon Alpha 2b Inhibits Oval Cell Proliferation and Induces Apoptosis In Vitro. To determine whether interferon treatment modulates proliferation or apoptosis of hepatic progenitor cells, PIL-2 and PIL-4 cells were grown in increasing concentrations of pegylated interferon alpha 2b. The results showed a dose-dependent reduction in metabolically active cells in the presence of increasing concentrations of interferon as determined by MTT assay (Fig. 2A). Based on these data, the median lethal dose was determined to be approxi-

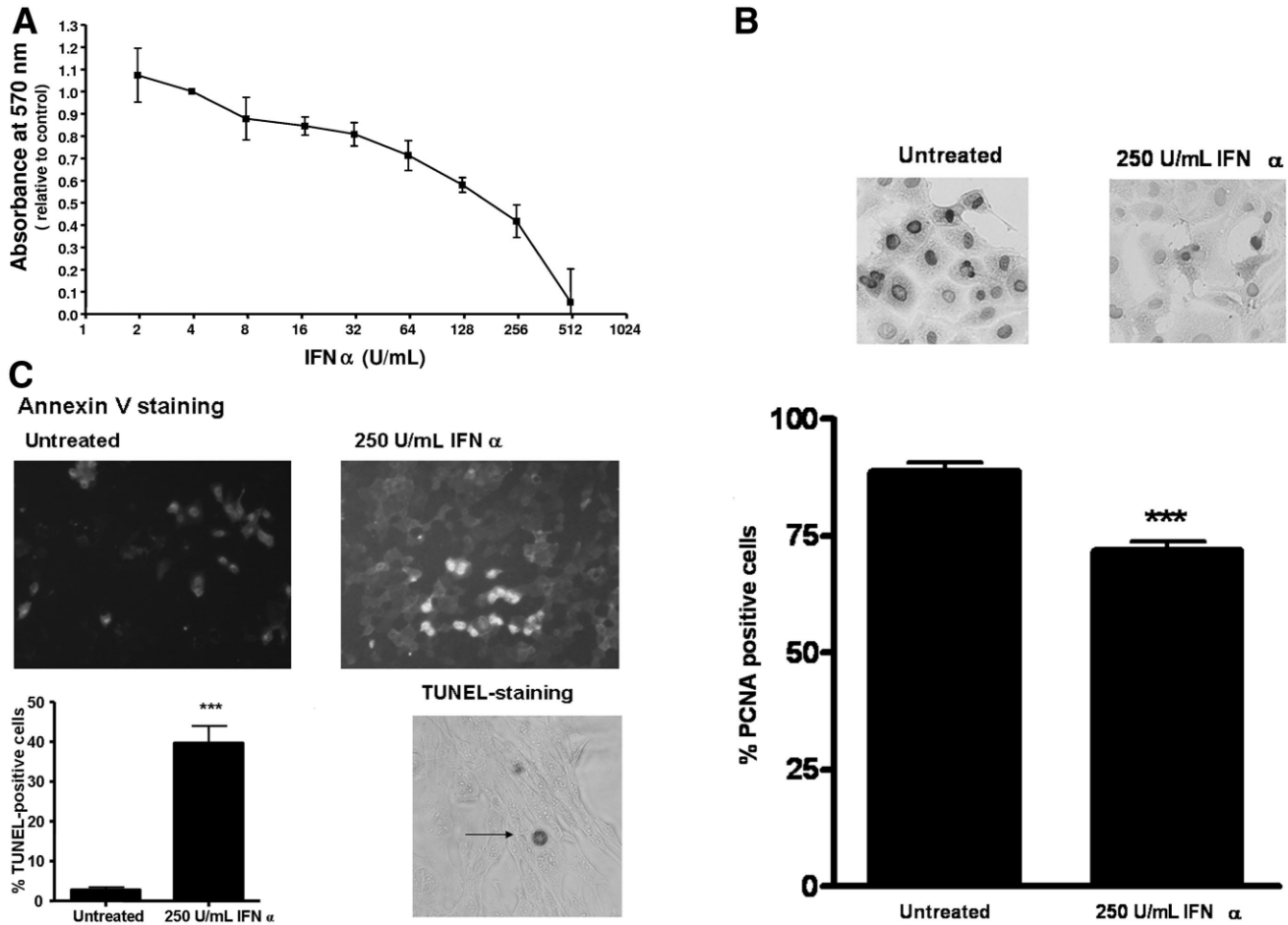


Fig. 2. Effect of pegylated interferon alpha 2b on proliferation of PIL cells. PIL-2 and PIL-4 cells were cultured with interferon at concentrations of 500, 250, 125, 62.5, 16.6, 7.8, and 1.95 IU/mL. As the results for both cells types were similar, only the results for PIL-4 cells are presented. The numbers of PIL-4 cells decreased with increasing concentrations of interferon (A). After 3 days culture with interferon, a 16% decrease in the numbers of PCNA-positive PIL-4 cells was seen (B) together with a ninefold increase in the numbers of apoptotic cells as detected using TUNEL assay (C). Annexin V fluorescein isothiocyanate clearly shows increased expression in cells treated with interferon (C). *** $P < .001$. PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling.

mately 250 IU/mL. The reduction in numbers of metabolically active cells was due to a reduction in proliferation as demonstrated by PCNA staining ($P < .001$, Fig. 2B) and increased apoptosis as demonstrated by TUNEL assay ($P < .001$, Fig. 2C).

Pegylated Interferon Alpha 2b Induces Hepatic Progenitor Cell Differentiation In Vitro. To assess whether interferon alpha treatment of oval cell lines was associated with an alteration in maturation state, the relative expression of key hepatic developmental markers was determined *in vitro* after treatment with either vehicle or interferon. After 8 days' exposure to pegylated interferon alpha 2b, albumin mRNA levels increased by 19-fold in PIL-2 cells ($P < .001$, Fig. 3A), whereas in PIL-4 cells the levels remained unchanged. CK19 mRNA levels increased threefold after treatment in both cell lines ($P < .05$, Fig. 3B, G). PIL-2 and PIL-4 cells exhibited a decrease in AFP gene expression of 1- and 323-fold, respec-

tively (Fig. 3C, H). Thy-1 was only expressed in PIL-2 cells, and its expression was significantly reduced after administration of interferon ($P < .05$, Fig. 3E). G6Pase mRNA levels increased by 414-fold in PIL-4 cells after administration of interferon ($P < .05$, Fig. 3I) but remained unaffected in PIL-2 cells.

Pegylated Interferon Alpha 2b Inhibits Proliferation of Murine Hepatic Progenitor Cells In Vivo. To substantiate the *in vitro* anti-proliferative effects of interferon alpha on murine oval cells, we induced chronic liver injury in mice using a CDE diet, and concurrently administered either a placebo control or interferon. After 2 weeks of CDE diet feeding, treatment with pegylated interferon alpha 2b resulted in a fourfold reduction in the number of A6-positive oval cells present in the liver, in comparison with animals that received placebo ($P < .05$, Fig. 4). This was accompanied by a one-third decrease of MPK-positive oval cells ($P < .05$, Fig. 4).

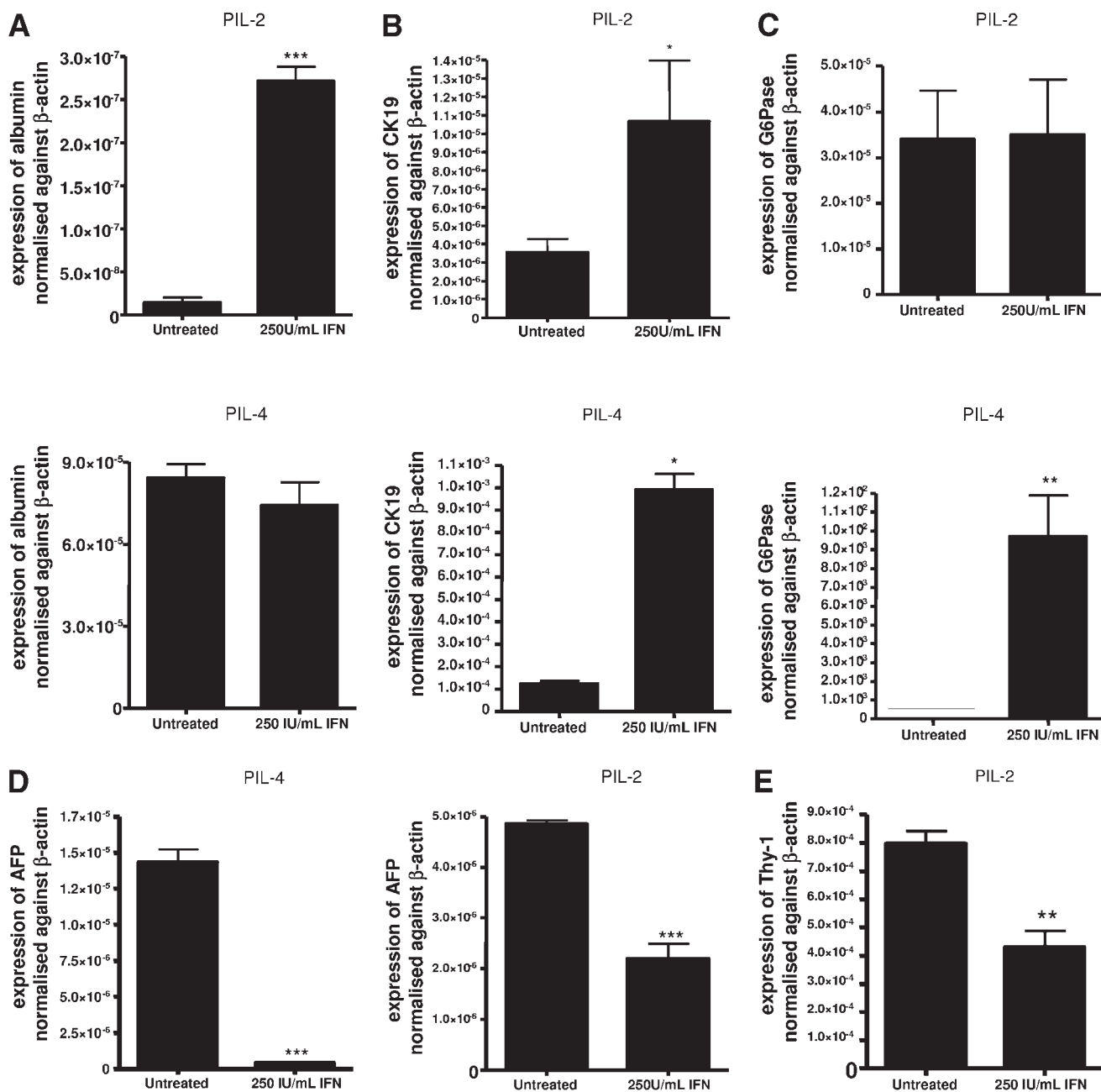


Fig. 3. Change in expression of differentiation markers after treatment with pegylated interferon alpha 2b. After 8 days treatment with 250 IU/mL interferon, quantitative PCR was performed to analyze changes in gene expression. Significant increases in expression of albumin (A) and CK-19 (B) were observed in PIL-2 cells accompanied by a marked decrease in the expression of AFP (D) and Thy-1 (E). PIL-4 cells demonstrated increased CK-19 (B) and G6Pase (C) expression and substantially reduced expression of AFP (D) after co-culture with interferon. * $P < .05$, ** $P < .01$, *** $P < .001$. PCR, polymerase chain reaction; AFP, alpha-fetoprotein.

PCNA staining of liver tissues showed that treatment with interferon alpha 2b resulted in a significant reduction in the numbers of PCNA-positive oval cells, whereas increasing numbers of PCNA-positive hepatocytes ($P < .05$, Fig. 5A). No significant effect on apoptosis of either cell type was evident from TUNEL staining of interferon- or placebo-treated livers ($P < .001$, Fig. 5B).

Pegylated Interferon Alpha 2b Treatment Induces Stat-3 Phosphorylation in Hepatic Cells. STAT-3 is

known to be a major downstream mediator in the interferon alpha signaling pathway and has been implicated in the control of oval cell proliferation and differentiation *in vitro*.^{24,25,37} To determine whether STAT-3 phosphorylation may be involved in the mediation of interferon effects in the CDE model, numbers of phosphorylated STAT-3-positive cells were assessed in the livers of interferon- or placebo-treated animals. Pegylated interferon alpha 2b-treated mice had significantly increased numbers of phos-

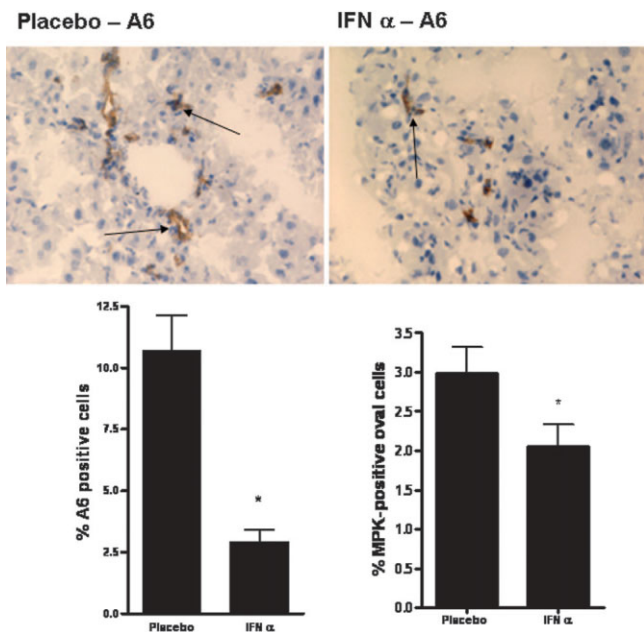


Fig. 4. Pegylated interferon alpha 2b causes a reduction in numbers of hepatic progenitor cells in mice fed the CDE diet. Administration of pegylated interferon alpha 2b to mice fed the CDE diet for 2 weeks resulted in substantial reductions in the percentages of A6- and MPK-positive hepatic progenitor (oval) cells compared with mice treated with placebo. * $P < .05$. CDE diet, Choline deficient ethionine supplemented diet.

phorylated STAT-3-positive cells compared with placebo, confirming the activation of this signaling pathway after interferon treatment ($P < .001$, Fig. 6).

Discussion

Interferon alpha has been suggested to exert anti-tumorigenic effects in the livers of patients with chronic hepatitis C independent of its anti-viral capabilities. Mounting evidence suggests that at least a subset of liver tumors occurring in mice and humans might arise due to transformation of adult liver progenitor cells. As such, we investigated the effects of interferon treatment in human subjects with chronic hepatitis C and in the injury-induced murine liver progenitor cell compartment, to determine whether it modulates the behavior of these cells, *in vitro* and *in vivo*.

Treatment with interferon alpha reduced the numbers of c-kit-positive intermediate hepatobiliary cells in 14 of 16 non-responding subjects with chronic hepatitis C. C-kit is a well-described marker for hepatic progenitor cells,^{26,34,38} and has been previously studied in human liver biopsies from patients with viral hepatitis, both with and without HCC.^{34,39,40} Furthermore, we have recently shown that numbers of c-kit-positive cells in patients with chronic hepatitis B correlate with disease severity (Knight B et al, unpublished observa-

tions). Selective targeting of the c-kit cell population has also been suggested to offer potential benefits in the treatment of HCC.⁴¹ Previous reports have suggested that a subset of c-kit-expressing hepatic progenitor cells also express CK19.^{38,42} Numbers of CK19-positive cells were not affected in our study; this suggests that the anti-tumorigenic effects of interferon therapy in patients with hepatitis C may be mediated by selective reduction of the c-kit-positive, CK19-negative progenitor cell pool. The significance of this subset of hepatic progenitor cells in the pathogenesis of chronic viral hepatitis is not yet known, and warrants further exploration.

To elucidate the mechanism by which interferon alpha treatment reduced the numbers of intermediate hepatobiliary cells present in the chronic hepatitis C patients, we examined the effects of pegylated interferon alpha 2b on the proliferation, apoptosis, and differentiation of two well-characterized murine oval cell lines. The results suggest that interferon may exert direct effects on hepatic progenitor cells, reducing their rate of cell growth as well as stimulating them to undergo apoptosis. This is in accordance with the observed effects of interferon on human HCC-derived

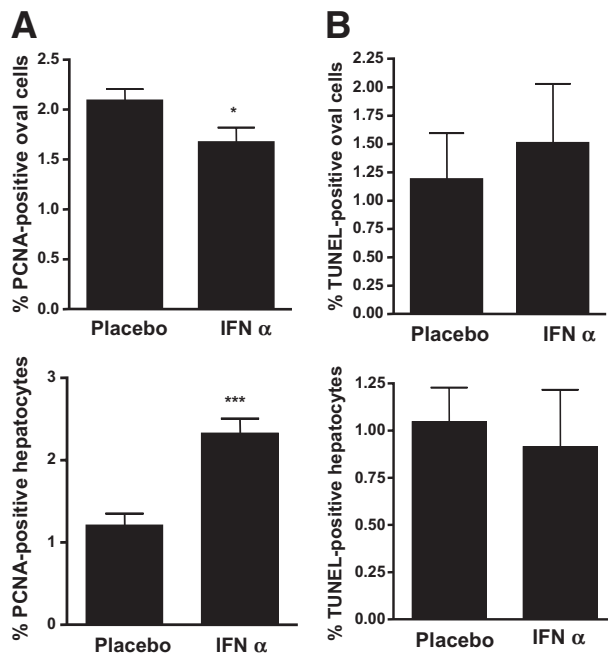


Fig. 5. Pegylated interferon alpha 2b exerts differential effects on proliferation of oval cells versus hepatocytes but does not appear to affect apoptosis in either cell type *in vivo*. Administration of pegylated interferon alpha 2b reduced the proportion of PCNA-positive oval cells but increased the proportion of PCNA-positive hepatocytes compared with placebo (A). Pegylated interferon alpha 2b did not increase apoptosis in hepatocytes or oval cells, as judged by TUNEL staining (B). * $P < .05$, *** $P < .001$. PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling.

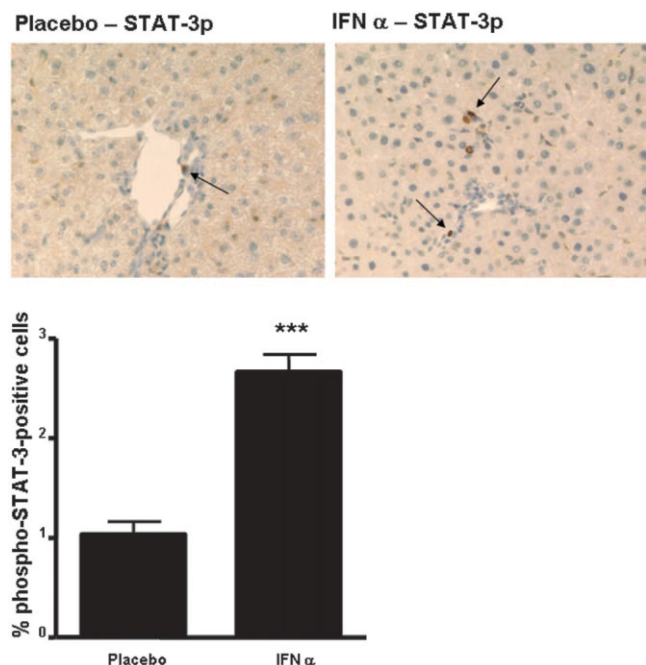


Fig. 6. Pegylated interferon alpha 2b increases the numbers of phosphorylated STAT-3-positive cells in the liver. A significant increase in the percentage of cells positive for phosphorylated STAT-3 was observed in the livers of mice treated with pegylated interferon alpha 2b, compared with placebo. *** $P < .001$.

cell lines, in which interferon alpha caused delayed S-phase progression through inhibition of cyclin-dependent kinases⁴² and induced caspase-dependent apoptosis.⁴³ Interestingly, after treating PIL-2 and PIL-4 cells with interferon alpha for 8 days, significant changes were observed in the expression of various marker genes corresponding to different stages of hepatic progenitor cell maturity and lineage. The expression of the immature cell markers AFP and Thy-1 was dramatically reduced after culture in the presence of interferon alpha, suggesting that their maturation was cytokine stimulated. These findings were corroborated by the increased expression of the mature biliary and hepatocytic markers CK19, albumin, and G6Pase in one or both of the cell lines. This suggests that in addition to its growth-modulatory effects, interferon alpha may promote the maturation of liver progenitor cells.

Previous studies in rodents have shown that interferon treatment inhibits hepatocarcinogenesis via anti-proliferative effects; however, these studies did not address whether this was associated with a reduction in the oval cell response.¹⁸ In the current work we showed that interferon therapy in the CDE model of hepatocarcinogenesis reduced the number of A6- and MPK-positive oval cells compared with placebo. Our findings also suggest that interferon alpha 2b exerts a differen-

tial effect on the proliferation of oval cells compared with hepatocytes, increasing numbers of PCNA-positive hepatocytes but reducing numbers of PCNA-positive oval cells. This implies some degree of specificity involved in the interferon alpha 2b-induced inhibition of proliferation. However, the number of TUNEL-positive oval cells or hepatocytes did not change significantly in interferon alpha-treated livers, suggesting that the reduced numbers of progenitor cells seen *in vivo* was likely to be mediated primarily through reduced growth or increased maturation, rather than by a direct apoptotic mechanism.

The observed discrepancy between the apoptosis-inducing effects of interferon alpha on oval cells *in vitro* and *in vivo* could be due to one or more of several reasons: (1) a dose dependence, whereby *in vivo* oval cells were not exposed to high enough concentrations of interferon to induce significant apoptosis, or (2) a time dependence, whereby most of the apoptotic effects on the oval cell population induced by interferon occurred before or after the 2-week time point, when samples were examined. Alternately, the discrepancy may simply reflect the limitation of cell culture systems for predicting *in vivo* biological effects; cell-cell interactions, as well as circulating co-factors, may significantly modify the effect of direct cytokine stimulation on isolated cells.

In this study we show that interferon alpha treatment increases the numbers of cells expressing phosphorylated STAT-3, a key component and multifaceted member of the Jak-STAT signaling pathway. STAT-3 activation has been shown in previous studies to be associated with differentiation of hepatic progenitor cells.^{44,45} Furthermore, we have shown that phosphorylated STAT-3 inhibits proliferation and promotes apoptosis of the PIL cell lines.²⁴ Thus, we propose that the molecular events leading to the inhibition of proliferation and differentiation of hepatic progenitor cells in response to interferon treatment may be mediated by STAT-3. Future studies using siRNA technology to knockdown STAT-3 expression in our cell lines are planned to test this hypothesis.

In conclusion, interferon-based therapy of human subjects with chronic hepatitis C, or rodents with experimentally induced liver injury, results in a reduction in the numbers of hepatic progenitor cells. Our results suggest that these effects may be mediated by direct effects of interferon on the hepatic progenitor cell compartment, including inhibition of proliferation, induction of apoptosis, and promotion of differentiation. These findings provide further support for the concept that interferon-based therapies reduce the risk of HCC by modulating the response of intermediate hepatobili-

ary cells in chronic hepatitis C. Our results support the suggestion made previously that even non-responders may benefit from continued interferon therapy^{20,46,47} and strongly suggest that the anti-carcinogenic properties of interferon alpha in the liver should be explored further.

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