

June 2002

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Kladney, Raleigh D.; Cui, Xiaoyen; Bulla, Gary A.; Brunt, Elizabeth M.; and Fimmel, Claus J., "Expression of GP73, A Resident Golgi Membrane Protein, in Viral and Nonviral Liver Disease" (2002). *Faculty Research & Creative Activity*. 130.
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Expression of GP73, A Resident Golgi Membrane Protein, in Viral and Nonviral Liver Disease

Raleigh D. Kladney,¹ Xiaoyen Cui,² Gary A. Bulla,² Elizabeth M. Brunt,³ and Claus J. Fimmel¹

GP73 is a novel type II Golgi membrane protein of unknown function that is expressed in the hepatocytes of patients with adult giant-cell hepatitis (Gene 2000;249:53-65). Its expression pattern in human liver disease and the regulation of its expression in hepatocytes have not been systematically studied. The aims of the present study were to compare GP73 protein levels in viral and nonviral human liver disease and in normal livers, to identify its cellular sources, and to study the regulation of its expression in hepatoma cells *in vitro*. GP73 protein levels were quantitated in explant livers of patients with well-defined disease etiologies and compared with the levels in normal donor livers. GP73-expressing cells were identified immunohistochemically. GP73 expression *in vitro* was studied by Western blotting and immunofluorescence microscopy in HepG2 and SK-Hep-1 cells and in the HepG2-derived, hepatitis B virus (HBV)-transfected HepG2215 and HepG2T14.1 cell lines. Whole organ levels of GP73 were low in normal livers. Significant increases were found in liver disease due to viral causes (HBV, HCV) or nonviral causes (alcohol-induced liver disease, autoimmune hepatitis). In normal livers, GP73 was constitutively expressed by biliary epithelial cells but not by hepatocytes. Hepatocyte expression of GP73 was dramatically up-regulated in diseased livers, regardless of the etiology, whereas biliary epithelial cell expression did not change appreciably. GP73 was present at high levels in HepG2215 cells (a cell line that supports active HBV replication), but was absent in HepG2T14.1 cells (an HBV-transfected cell line that does not support HBV replication) and in HBV-free HepG2 cells. In SK-Hep-1 cells, GP73 expression was increased in response to interferon gamma (IFN- γ), and inhibited by tumor necrosis factor α (TNF- α). In conclusion, increased expression of GP73 in hepatocytes appears to be a general feature of advanced liver disease, and may be regulated via distinct pathways that involve hepatotropic viruses or cytokines. (HEPATOLOGY 2002;35:1431-1440.)

In search of novel, disease-induced hepatocyte proteins we recently identified GP73, a 73-kd resident Golgi membrane protein of unknown function. GP73 is a type II transmembrane protein with a single,

N-terminal transmembrane domain and an extensive, C-terminal coiled-coil domain located on the luminal surface of the Golgi apparatus.¹ A number of structurally similar Golgi membrane proteins have been identified in recent years, including p63,² GPP130/GIMPC,³ and Golgin-84.⁴ Based on their structural characteristics, several of these proteins have been implicated in Golgi trafficking. However, their exact biological roles have not been defined and the regulation of their expression has not been studied in any detail. Interestingly, the porcine homologue of one of these proteins, p63, was found to be induced in hepatocytes during circulatory shock, suggesting a potential role in the hepatocyte stress response.⁵

Our initial studies showed that GP73 was expressed at high levels in hepatocyte-derived, multinucleated giant cells in patients with adult giant-cell hepatitis.¹ Furthermore, *in situ* hybridization studies on cirrhotic explant livers suggested that GP73 messenger RNA levels were increased in patients with viral hepatitis,⁶ suggesting a potential role of the protein in the host response to viral infection. On the other hand, we found that GP73 was

Abbreviations: GP73, 73-kd Golgi protein (GenBank # AF236056); HBV, hepatitis B virus; HCV, hepatitis C virus; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; CK19, cytokeratin 19; DAB, 3,3'-diamino-benzidine; AEC, 3-amino-9-ethylcarbazole; DMEM-F12, Dulbecco's modified Eagle medium and nutrient mixture F-12; FBS, fetal bovine serum; TNF- α , tumor necrosis factor α ; IFN- γ , interferon gamma; DAPI, 4,6-diamidino-2-phenylindole; ANOVA, analysis of variance.

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Received August 21, 2001; accepted January 23, 2002.

Supported by a VA Merit Review to C.J.F.

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doi:10.1053/jhep.2002.32525

normally expressed by epithelial cells in multiple tissues, including the intestine, trachea, and prostate gland. These data implied a housekeeping role for GP73 in cells of the epithelial lineage and provided evidence that multiple pathways might be involved in the regulation of its expression.

The aims of the present study were 3-fold: (1) to study the disease-specific expression of GP73 in viral and nonviral liver disease, (2) to identify the hepatic cell types responsible for GP73 expression in normal and diseased liver, and (3) to develop suitable *in vitro* models for the regulation of GP73 in hepatocyte-derived cell lines.

Our data show that GP73 expression is specifically up-regulated in hepatocytes in viral and nonviral chronic liver disease, suggesting that the protein may be involved in the cellular disease response of hepatocytes. Our *in vitro* data point to the existence of separate regulatory pathways that are activated by infection with hepatotropic viruses or in response to cytokines.

Materials and Methods

Frozen Liver Tissues. Samples of cryopreserved (-80°C) human explant livers ($n = 70$) were obtained from the Minnesota Tissue Repository (LTPADS, director: Harvey Sharp) and from collaborating investigators. The samples were obtained from patients with well-characterized end-stage liver disease. Supplemental medical histories and laboratory data were reviewed to ascertain the cause of liver disease. Particular attention was paid to exclude samples in which multiple viral and nonviral causes of liver disease were present. The following disease groups were included: chronic hepatitis B virus (HBV) infection with cirrhosis ($n = 14$), chronic hepatitis C virus (HCV) infection with cirrhosis ($n = 23$), alcohol-induced cirrhosis ($n = 9$), and cirrhosis caused by autoimmune hepatitis ($n = 7$). Control samples were derived from normal donor livers ($n = 17$).

GP73 Protein Measurements. GP73 protein levels were measured by Western blotting in lysates of human liver samples and cultured cells. Aliquots of cryopreserved liver samples (approximately 100 mg per sample) were homogenized for 1 minute in 20 volumes of lysis buffer (50 mmol/L Tris-Cl pH 6.8, 5% β -mercaptoethanol, 1% sodium dodecyl sulfate [SDS]), using an SDT Tissumizer (Tekmar Company, Cincinnati, OH). Lysates were boiled for 5 minutes, sonicated (3×10 -second bursts), and centrifuged at 14,000 rpm at 4°C for 10 minutes. The supernatants were stored at -80°C , and subsequently analyzed in batch fashion.

For measurements of GP73 protein in cultured cells, cells were washed 3 times with phosphate-buffered saline

(PBS), followed by the addition of 300 μL lysis buffer (62.5 mmol/L Tris-Cl pH 6.8, 20 mmol/L dithiothreitol, 10% glycerol, 1% SDS) per well (6-well plates, surface area: 962 mm^2/well). Adherent cells were dislodged with plastic scrapers, and the lysates were transferred to microcentrifuge tubes. Samples were boiled for 3 minutes, sonicated for 30 seconds, and centrifuged at 14,000 rpm at 4°C for 20 minutes. The cleared lysates were stored at -80°C , and analyzed in batch fashion.

Protein concentrations in the lysates were determined using a modification of the micro-Lowry method,⁷ using bovine serum albumin as a standard (Sigma Protein Assay Kit; Sigma, St. Louis, MO). Aliquots corresponding to 15 μg of total cellular protein were subjected to SDS-polyacrylamide gel electrophoresis, by using precast 10% Tris-HCl gels (Ready Gel; Bio-Rad, Hercules, CA) in a running buffer containing 25 mmol/L Tris-Cl, 192 mmol/L glycine, and 0.1% SDS. Gels were run at 100 V for 8 minutes, then 200 V for 30 minutes. A control sample consisting of 7.5 μg of total cellular protein from 293 cells was included in each gel run to allow quantitative comparisons between gels. Separated proteins were transferred to polyvinylidene difluoride membranes (PVDF-Plus; Micron Separations, Westborough, MA) with a semidry transfer cell (Trans-Blot SD; Bio-Rad), by using an optimized buffer (48 mmol/L Tris-Cl, 39 mmol/L glycine, 0.005% SDS, 20% methanol) and standardized transfer conditions (10 V, 30 minutes). Membranes were kept overnight in blocking buffer (10% nonfat dry milk in TBST) at 4°C . They were then washed 3 times in wash buffer (0.5% nonfat dry milk in TBST) and incubated for 1 hour at room temperature in 15 mL wash buffer containing rabbit polyclonal GP73 antibody (1:5,000 vol/vol). Several washes with high-salt buffer (0.5% nonfat dry milk in TBST containing 0.5 mol/L NaCl) and wash buffer were then performed. This was followed by an incubation in washing buffer with 1:1,000 vol/vol preadsorbed goat anti-rabbit IgG-HRP conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes. After repeat washes with high-salt and wash buffer, GP73 immunoreactivity was visualized by enhanced chemiluminescence and autoradiography, using a commercial kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, England). Target band intensities were quantitated by densitometry of the autoradiograms, and normalized relative to the control signal obtained with lysates from 293 cells, a cell line that we had previously found to constitutively express GP73.¹

Formalin-Fixed Liver Tissues. Samples of normal donor livers and from cirrhotic explants were obtained from the Department of Pathology at Saint Louis University Medical School, from the Pathology Section at the St.

Louis VA Medical Center, and from collaborating investigators at various institutions throughout the United States. Samples were fixed overnight in formalin and embedded in paraffin. Sections of approximately 5- μ m thickness were then prepared for immunohistochemical staining.

GP73 Immunohistochemistry. Staining of liver sections was performed by a 2-stage, indirect immunoenzymatic/immunohistochemical technique, using a commercial kit (DAKO EnVision; Dako Corporation, Carpinteria, CA). Endogenous peroxidase activity was blocked by a 5-minute incubation in the presence of 0.03% hydrogen peroxide. Sections were incubated for 30 minutes in the presence of rabbit polyclonal antisera directed against recombinant GP73 at a dilution of 1:1,000 (vol/vol) as previously described.¹ After washes, the sections were incubated with a peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulin for 30 minutes. GP73 immunoreactivity was visualized with the chromogen 3-amino-9-ethylcarbazole (AEC). Rabbit preimmune sera was used for control experiments. In some cases, parallel stains were performed to identify hepatocytes, by using a mouse monoclonal antibody directed against the hepatocyte protein HepPar1 at a dilution of 1:25 (clone OCH1E5; Dako Corporation),⁸ or to identify biliary epithelial cells, by using a mouse monoclonal anti-human cytokeratin 19 (CK19) antibody at a dilution of 1:50 (clone RCK108; Dako Corporation).⁹ In these cases, immunoreactivity was visualized using the chromogen 3,3'-diamino-benzidine (DAB). All samples were counterstained with Mayer's hematoxylin.

Cell Lines and Culture Conditions. HepG2 and SK-Hep-1 cells were obtained from the American Tissue Type Collection (ATCC, Rockville, MD). The cells were cultured in 6-well plates in the presence of a 1:1 mixture of Dulbecco's modified Eagle medium and nutrient mixture F-12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Tumor necrosis factor α (TNF- α) was obtained from Life Technologies (Life Technologies, Gaithersburg, MD). Recombinant human interferon gamma (IFN- γ) was obtained from Biosource International (Camarillo, CA). Agonists or the corresponding vehicle solutions in DMEM-F12 containing 1% FBS were added to subconfluent cells for time periods ranging from 24 to 48 hours. All experiments involving cultured cells were performed in triplicate.

HepG2215 and HepG2T14.1 cells were obtained from Daniela Simon (MCP Hahnemann University, Philadelphia, PA). HepG2215 cells are HBV-transfected variants of HepG2 cells that have the ability to replicate HBV.¹⁰ HepG2T14.1 cells were independently derived

from HepG2 cells by stable transfection with the HBV genome.¹¹ However, these cells subsequently lost the ability support HBV replication or particle formation *in vitro*.¹² The cell lines were carried in RPMI 1640 supplemented with 10% FBS, penicillin, streptomycin, and 200 μ g/mL G418.

GP73 Immunofluorescence. Cultured cells were washed once with PBS prior to fixation, and fixed for 10 minutes in ice-cold methanol containing 4,6-diamidino-2-phenylindole (DAPI). After an additional PBS wash, the slides were incubated for 20 minutes with a blocking solution containing 4% (vol/vol) goat serum in PBS. This was followed by a 1-hour incubation with polyclonal rabbit GP73 antibody (1:400 vol/vol in PBS) at room temperature. After 3 PBS washes, slides were washed again and incubated in PBS containing 4% goat serum and goat anti-rabbit IgG (1:400 vol/vol) conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR). Slides were mounted in elvanol,¹³ and viewed using a Nikon Optiphot-2 microscope equipped with epifluorescence. Fluorescent images were photographed using Kodak Elite CHROME 400 film, and the final images were processed using Photoshop.

Statistics. GP73 protein levels in the human liver samples were compared by Kruskal-Wallis one-way analysis of variance (ANOVA).¹⁴ Dunn's method was used to compare differences between the control and disease groups.¹⁵ A *P* value of <.05 was considered statistically significant.

Human Subject Committee Review. All studies were approved by the Institutional Review Board of the St. Louis VA Medical Center, the Saint Louis University Human Studies Committee, and the University of Minnesota Human Studies Committee. Patient anonymity was maintained by labeling of the clinical samples with code numbers.

Results

GP73 Expression Is Up-regulated in Viral and Nonviral Liver Disease. GP73 expression was minimal or nondetectable in most control livers (Fig. 1). GP73 protein levels, as measured by densitometric quantitation of Western blots, were 0.25 ± 0.14 in normal liver, 17.70 ± 9.20 in HBV, 9.00 ± 4.68 in HCV, 18.10 ± 8.15 in alcohol-induced liver disease, and 14.50 ± 4.73 in autoimmune hepatitis (median \pm 25% percentile), corresponding to an increase of up to 70-fold in the disease groups. Statistical comparisons between the groups of samples were performed using nonparametric statistical testing because the data were not normally distributed. Using Kruskal-Wallis ANOVA on ranks, significant dif-

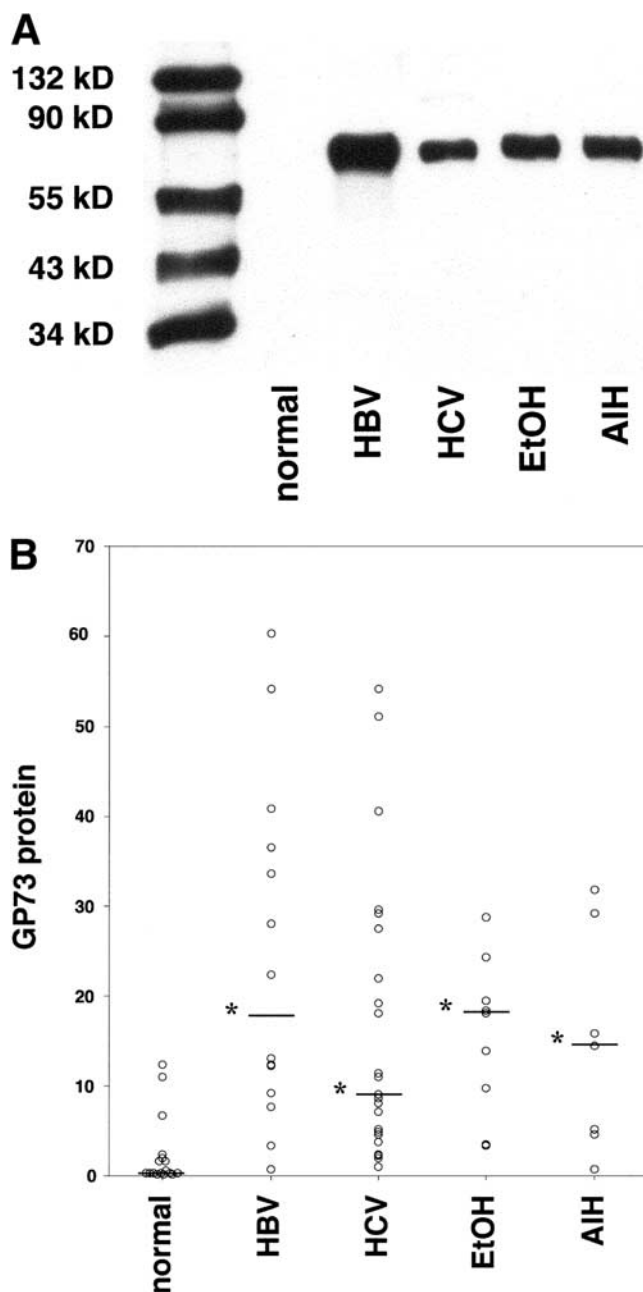


Fig. 1. Expression of GP73 in viral and nonviral liver disease. GP73 protein levels were measured by Western blotting in normal donor livers, and in livers with cirrhosis caused by HBV, HCV, alcohol-induced liver disease (EtOH), and autoimmune hepatitis (AIH). (A) Western blot of representative samples. Individual samples from the control and from each disease group were chosen to closely approximate the median data shown in B. GP73 protein is detected as a specific band with a molecular size of approximately 73 kd. (B) Quantitation of GP73 signals in normal livers ($n = 17$), and livers with HBV ($n = 14$), HCV ($n = 23$), EtOH ($n = 9$), and AIH ($n = 7$). Each data point represents an individual patient sample. Median values for each group are represented by **solid lines** (normal, 0.25; HBV, 17.70; HCV, 9.00; EtOH, 18.10; AIH, 14.50). The differences between the 4 disease groups and the normal controls was statistically significant ($P < .05$, Kruskal-Wallis ANOVA, Dunn's Method [*]). No statistical difference was found between the individual disease groups.

ferences were found between the 5 groups of samples ($P = .0000151$). Further testing using Dunn's method revealed that each disease group differed significantly from the normal controls ($P < .05$). On the other hand, no significant differences were found between the individual disease groups ($P > .1$).

Cellular Sources of GP73 Expression in Normal Liver. GP73-expressing cells in normal donor livers were identified by comparing the staining pattern for GP73 with that of known marker proteins for biliary epithelial cells and hepatocytes (CK19 and HepPar1), respectively (Fig. 2). GP73 immunoreactivity was consistently detected in biliary epithelial cells in the portal tracts and throughout the lobules (Fig. 2A). The identity of GP73-expressing biliary epithelial cells in portal tracts was confirmed by the costaining with CK19 (2A, 2C). In this cell type, GP73 immunoreactivity was localized to an area between the nucleus and apical membrane, typical for a Golgi distribution in polarized epithelial cells.¹⁶ GP73 signals were occasionally detected in hepatocytes located in zone 1, as shown by the colocalization of GP73 and HepPar1 (Fig. 2A and D). The majority of hepatocytes, however, did not express GP73. In hepatocytes, GP73 immunoreactivity was present as discrete lobules, with a preferential subcellular localization to an area adjacent to the canalicular membrane. In contrast, the cytoplasm adjacent to the sinusoidal hepatocyte membrane was typically devoid of GP73 signal. This pattern is in agreement with previous studies on the intracytoplasmic localization of the Golgi apparatus in normal hepatocytes.¹⁶

Cellular Sources of Increased Hepatic GP73 Expression in Advanced Liver Disease. Prominent GP73 signals were detected in hepatocytes in cirrhosis caused by HBV or HCV infection, alcohol-induced hepatitis, or autoimmune hepatitis (Fig. 3). In contrast to its sporadic expression in normal hepatocytes, GP73 was expressed in virtually every hepatocyte, regardless of the disease etiology. The staining intensity was particularly strong in hepatocytes that were located adjacent to connective tissue septa and cirrhotic nodules. Similar to the findings in normal hepatocytes, the subcellular localization of GP73 immunoreactivity appeared to be preferentially pericanalicular. These data suggest that the main change in the GP73 signal was due to an increase in immunoreactivity within the same Golgi compartments. However, we cannot rule out subtle changes in GP73 immunolocalization or in the Golgi morphology in the disease conditions. Bile duct epithelial reactivity was present as in nondiseased, normal donor controls. No GP73 immunoreactivity was found in nonparenchymal liver cells. These data suggest that the increased GP73 at the whole organ level is due to

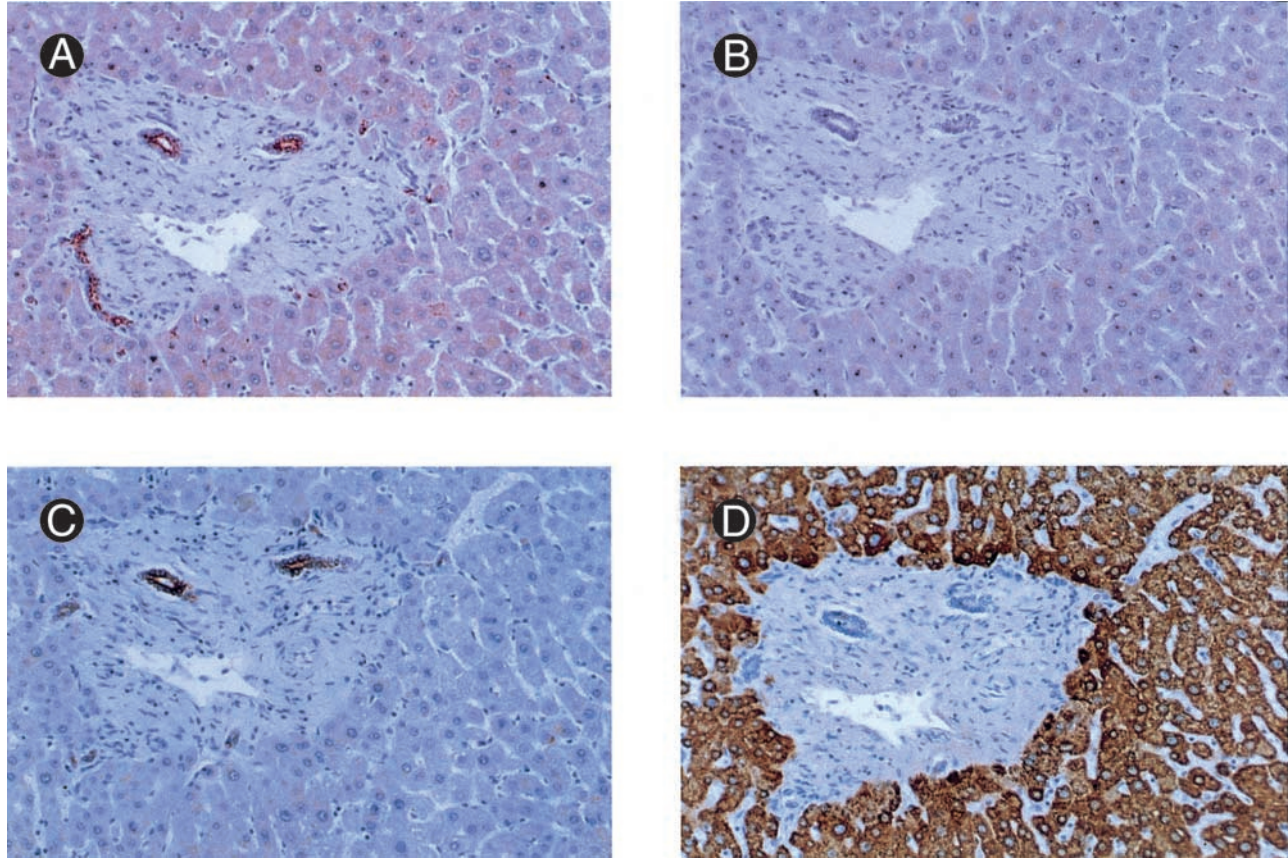


Fig. 2. Cellular sources of GP73 expression in normal liver. GP73 expressing cells in normal liver were identified immunohistochemically, using GP73-specific rabbit polyclonal antisera. (A) GP73 immunoreactivity was visualized by a two-step method, using the chromogen AEC (red signal). (B) A control experiment was performed using rabbit preimmune sera. Serial sections were also stained with monoclonal antibodies directed against (C) CK19, and (D) HepPar1, which were used as markers of biliary epithelial cells and hepatocytes, respectively. DAB was used as a chromogen in C and D (brown signal). All sections were counterstained with Mayer's hematoxylin. (A) A prominent GP73 signal was present in biliary epithelial cells within the portal tracts and throughout the lobules. GP73 immunoreactivity was localized to the perinuclear region of the cells, a pattern that is characteristic of the Golgi apparatus in polarized epithelial cells. A less prominent signal was detected in occasional hepatocytes in zone 1. (B) No specific immunoreactivity was obtained with the control sera. (C) CK19 immunoreactivity in biliary epithelial cells. Note the colocalization of the CK19 signal with the GP73 signal in the same cells shown in A. (D) HepPar1 immunoreactivity in hepatocytes. Note the colocalization of the HepPar1 signal with the GP73 signal in occasional, periportal hepatocytes shown in A. Original magnification $\times 200$.

the up-regulation of expression in hepatocytes, and that this increase is a general feature of advanced liver disease, regardless of the disease etiology.

GP73 Expression Is Present in HBV-Replicating Hepatoma Cells. Our *in vivo* data suggested that viral infection might represent one mechanism for the increased expression of GP73 in hepatocytes. We addressed this issue in a cell culture model of HBV infection (Fig. 4). GP73 expression was measured in highly differentiated HepG2 hepatoma cells, and in HepG2215 and HepG2T14.1 cells, two cell lines which were independently derived from HepG2 cells by transfection with the HBV genome. Previous studies by others have shown that HepG2T14.1 cells—in contrast to HepG2215 cells—do not support HBV replication and the formation of infectious HBV particles *in vitro*. Immunofluorescence staining for GP73 in HepG2 cells was negative, reminiscent of

the data obtained in normal hepatocytes *in vivo*. Similarly, HepG2T14.1 cells did not express GP73. In contrast, a prominent signal was observed in HepG2215 cells. The immunofluorescence data were confirmed by Western blot analysis, which showed the absence of GP73 expression in HepG2 and HepG2T14.1 cells and a substantial GP73 signal in HepG2215 cells (Fig. 4B). Our data suggest that active HBV replication may be one of the pathways that can trigger the expression of GP73 in hepatocytes.

GP73 Expression In Vitro Is Modulated by Cytokines. The finding of an increased expression of GP73 in alcohol-induced and autoimmune liver disease suggested that GP73 expression can be regulated in a virus-independent manner. We tested this possibility by studying GP73 expression in SK-Hep-1 cells, a cell line that spontaneously expresses low levels of GP73. As shown in Fig. 5,

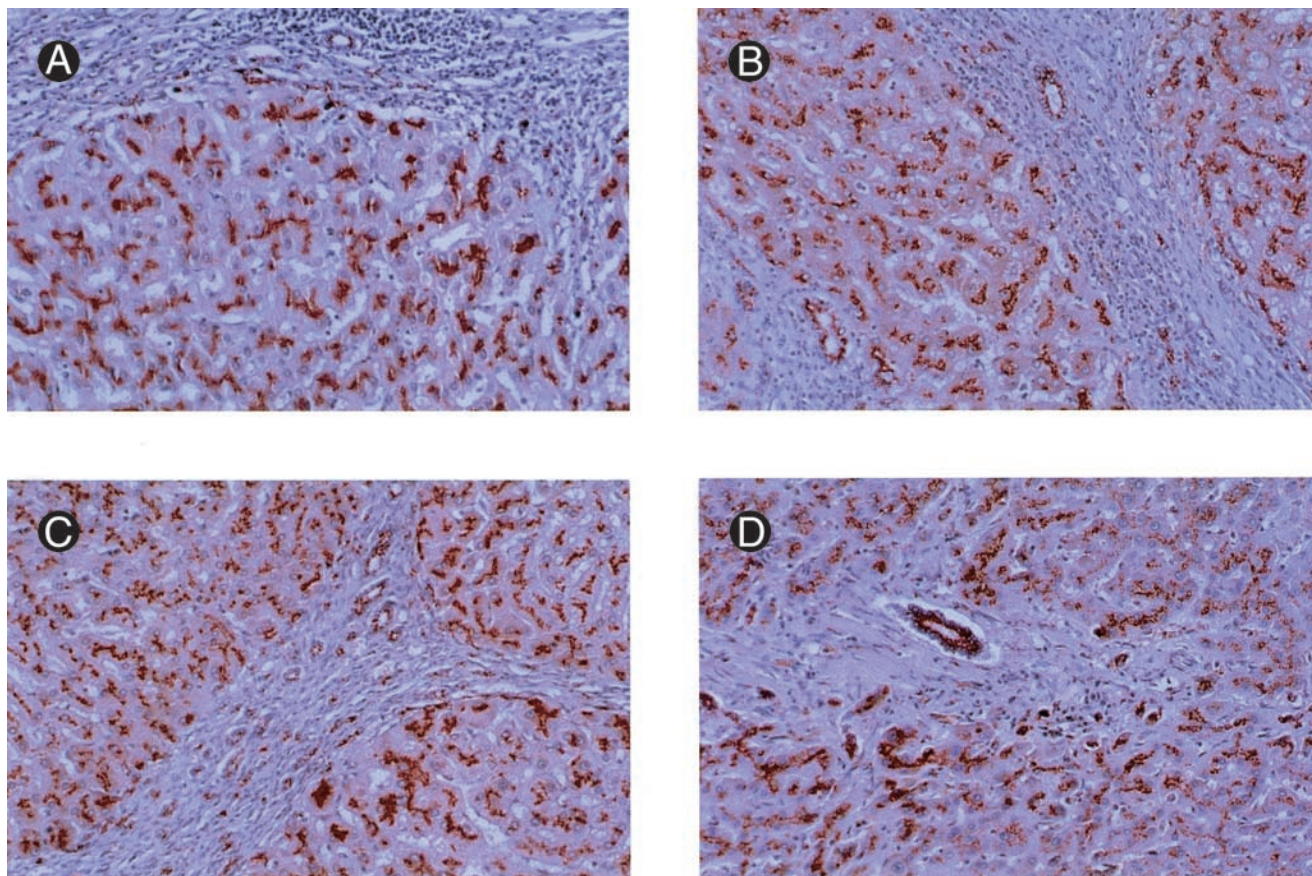


Fig. 3. Cellular sources of GP73 in advanced liver disease. GP73 expression was studied in representative livers of patients with cirrhosis caused by (A and B) HBV or HCV infection, (C) alcohol-induced cirrhosis, or (D) cirrhosis caused by autoimmune hepatitis. GP73 immunoreactivity was visualized using the chromogen AEC (**red signal**). Background staining: Mayer's hematoxylin. (A-D) Hepatocytes were strongly reactive for GP73 immunoreactivity, regardless of the cause of the liver disease. Biliary epithelial cells consistently stained positive, with a signal intensity similar to that observed in normal liver (compare to Fig. 2A). No GP73 staining was detected in inflammatory cells or within the fibrotic septa. Original magnification $\times 200$.

exposure of SK-Hep-1 cells to IFN- γ resulted in significantly increased levels of GP73, whereas treatment with TNF- α was associated with a reduction in GP73 expression. These data suggest that GP73 expression in hepatocytes may be regulated by cytokines, using pathways that do not require the presence of viral infection. Together with the findings in HBV-transfected cells, these results suggest that the expression of GP73 in hepatocytes may be controlled by distinct virus-dependent and -independent mechanisms.

Discussion

GP73 was first identified in a genetic screen for proteins with differential expression in adult giant-cell hepatitis. The screen had originally been designed to isolate unique complementary DNAs derived from a putative viral agent thought to be involved in the disease.¹⁷ GP73 protein levels were found to be dramatically increased in the hepatocyte syncytia of patients with giant-cell hepatitis.

Further experiments showed GP73 to be a novel type II integral membrane protein that was localized to the Golgi apparatus.¹ In preliminary studies, we found that GP73 messenger RNA levels were increased in HCV-related human liver disease.⁶ These data suggested that GP73 might function in the hepatocyte response to viral injury.

The present study was designed to systematically examine the disease- and cell-specific expression patterns of GP73 in human liver disease. Using explant livers of patients transplanted for HBV, HCV, alcohol- or autoimmune disease-induced cirrhosis, we found striking increases in GP73 protein expression at the whole organ level. Similar changes were observed in other forms of chronic liver disease, including primary biliary cirrhosis, and primary sclerosing cholangitis.¹⁸ Our data clearly establish increased GP73 expression as a general feature of cirrhotic liver disease, irrespective of the underlying viral or nonviral disease etiology.

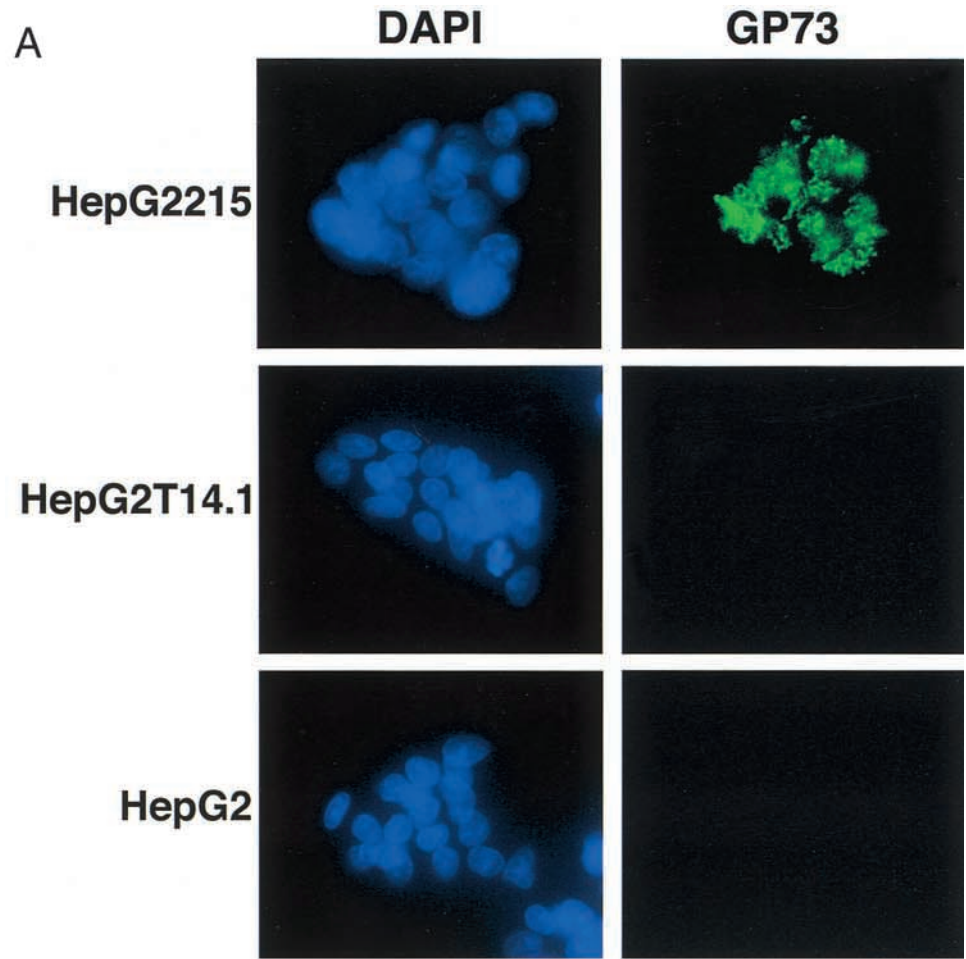
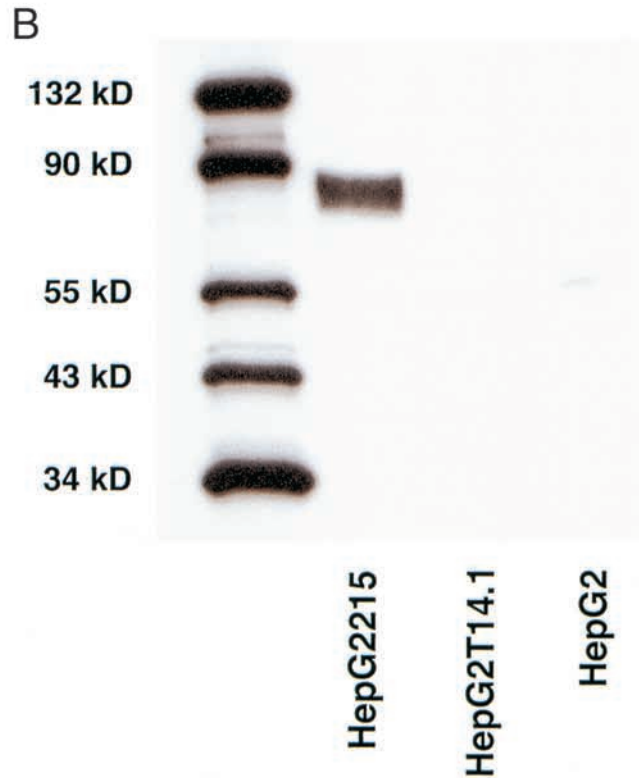


Fig. 4. GP73 expression in HBV-transfected hepatoma cell lines. GP73 expression was studied in monolayers of HBV-transfected HepG2215 and HepG2T14.1 cells, and in HepG2 cells. (A) Immunofluorescence studies. Subconfluent cells were stained using GP73-specific rabbit polyclonal antisera. GP73 immunoreactivity was visualized by using an Alexa Fluor 488 secondary antibody (**green fluorescence**). Cellular DNA was stained with DAPI (**blue fluorescence**). No GP73 signal was detected in HepG2 and HepG2T14.1 cells. In contrast, a prominent GP73 signal was present in HepG2215 cells. The GP73 signal was present in distinct, punctate or tubulovesicular perinuclear structures, typical of a Golgi distribution in this cell type (original magnification $\times 100$). (B) GP73 protein levels were quantitated in cellular lysates by Western blot analysis. Equal amounts of protein ($15 \mu\text{g}$) were loaded in each lane. GP73 protein was present in lysate of HepG2215 cells as a specific band of the expected molecular size. This band was undetectable in HepG2T14.1 and HepG2 cells.



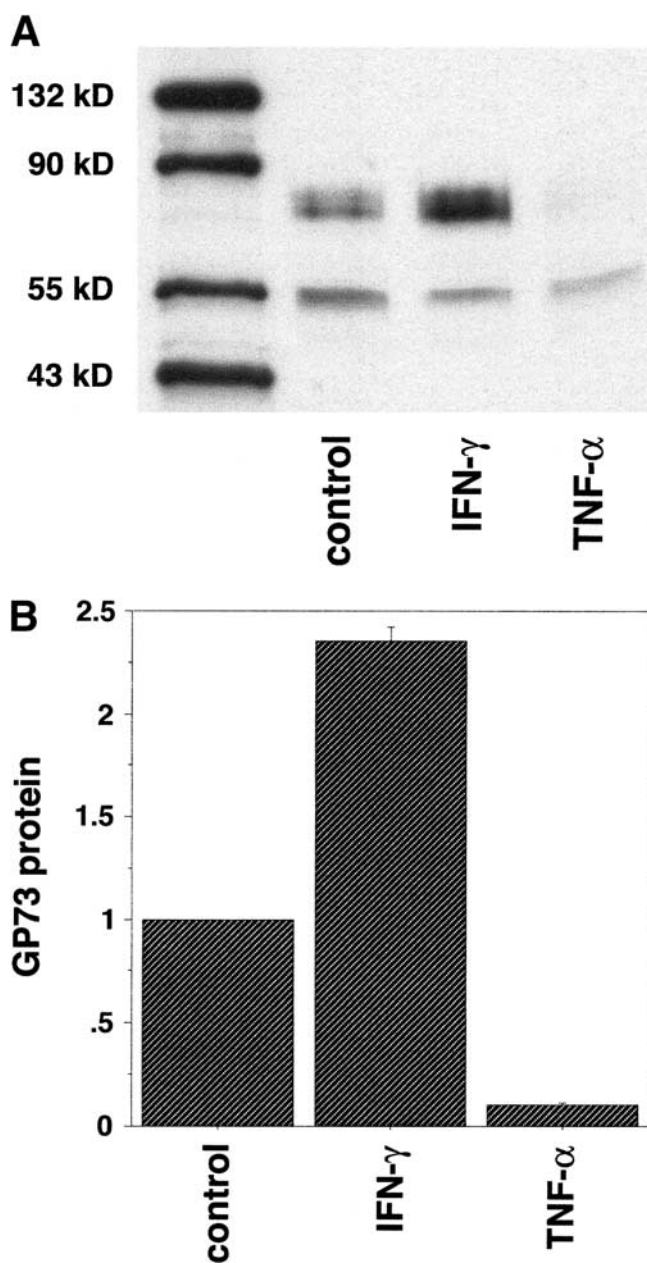


Fig. 5. Modulation of GP73 expression by cytokines in SK-Hep-1 cells. SK-Hep-1 cells were grown for 32 hours in the absence (control) or presence of IFN- γ (1,000 U/mL) or TNF- α (50 ng/mL). Whole cell lysates were subjected to Western blot analysis, using affinity-purified rabbit polyclonal antisera directed against recombinant GP73. (A) Data from a representative experiment. Each lane corresponds to 15 μ g of cellular protein. A modest GP73 signal of the anticipated molecular size (approximately 73 kD) was detected in control cells. IFN- γ treatment resulted in an increase of the GP73 signal, whereas incubation in the presence of TNF- α was associated with a substantial decrease in the GP73 band. The lower molecular weight band (approximately 55 kD) is nonspecific and represents a cross-reacting protein. (B) Quantitation of three independent experiments. GP73 levels were expressed as arbitrary densitometric units, and the control signal was assigned a relative value of 1. GP73 levels were increased to 2.35 ± 0.07 (mean \pm SD) after IFN- γ treatment and decreased to 0.10 ± 0.01 after TNF- α treatment.

Our immunohistochemical studies show that the GP73 signal in normal livers was predominantly derived from biliary epithelial cells, whereas hepatocytes contributed little to the overall GP73 expression. In contrast, high-level expression of GP73 was present in diseased hepatocytes, regardless of the cause of liver disease. GP73 was not expressed in inflammatory cells or in cells within the cirrhotic septa, and its expression levels in biliary epithelial cells did not appear to be altered. Based on the decreased percentage of hepatocytes as compared to non-parenchymal cells in cirrhotic liver disease, the degree of up-regulation of GP73 expression in hepatocytes likely exceeds the 36- to 72-fold increase measured at the whole tissue level. This striking increase suggests that GP73 may function in an important cellular pathway of liver disease.

Our data raise the question as to whether GP73 expression is limited to cirrhotic liver disease, or whether it occurs during the earlier stages of the disease process. One way to address this question would be to study liver biopsy samples of patients with acute hepatitis. However, such samples are difficult to obtain and typically yield insufficient amounts of material for quantitative protein measurements. An alternative approach would be to study GP73 expression in explant livers of patients with fulminant hepatic failure without preexisting, chronic liver disease. We have performed preliminary studies on such tissues and have found increased GP73 expression in surviving hepatocytes (Fimmel, unpublished observations, March 2002). These data suggest that GP73 up-regulation may be a feature of the early stages of liver disease.

The function of GP73 remains unknown, and multiple search algorithms for protein motifs did not reveal any obvious catalytic or enzymatic properties. All resident Golgi proteins are peripheral or integral membrane proteins, and the majority of them are involved in the intracellular modification of secretory proteins. GP73 has no homologies to the known glycosyltransferases and is unlikely to have catalytic functions in this regard.¹⁹ Furthermore, GP73 has no significant sequence homologies or structural similarities to any of the known nucleotide, sugar, or ATP transporters of the Golgi apparatus.²⁰ In recent years, a number of Golgi membrane proteins with N- or C-terminal membrane anchors similar to GP73 have been described. They include the recently described Golgin-84,⁴ p63,² and GPP130.³ A common feature of these proteins is the presence of coiled-coil domains in the N- or C-terminus of the molecule. The biochemical functions of these proteins are unknown, and there are few studies addressing the regulation of their expression. In one study, the porcine homologue of p63 was found to be up-regulated in hepatocytes during cardiogenic shock, a finding that raises the possibility that the protein might be

involved in a cellular stress response.⁵ It has been suggested that their coiled-coil domains function as homotypic or heterotypic protein interaction sites that are essential for the binding, docking, and trafficking of transport vesicles to the cisternal membranes. Experiments to test whether GP73 functions in Golgi membrane trafficking are currently ongoing in our laboratory.

In the absence of obvious structural clues for the function of GP73, we utilized *in vitro* cell culture models to study its regulation. Our data provide evidence for virus-dependent as well as virus-independent modulation of GP73 expression. In earlier work, we observed that adenovirus infection induced the expression of GP73 in HepG2 and Hep3B hepatoma cells. GP73 expression in this model was rapid in onset (within 8 hours of infection), and dependent on the presence of the adenoviral E1A protein, suggesting regulation at the level of transcription.¹ These data prompted us to examine GP73 in a cell culture model of HBV infection. HepG2215 cells were originally developed by stable transfection of the entire HBV genome into HepG2 cells.¹⁰ They express the HBV polyprotein, and support the formation of infectious HBV particles *in vitro*. As shown in this report, HepG2215 cells showed robust expression of GP73, whereas HepG2 cells and HepG2T14.1 cells, an HBV-transfected cell line incapable of supporting productive HBV infection, did not express GP73.¹² These data suggest that GP73 may be expressed in response to infection with hepatotropic viruses. We hypothesize that GP73 may function in the processing of viral proteins in the Golgi apparatus or in the formation of infective virions, and might be beneficial to the invading virus. Alternatively, GP73 might be part of the host cell's antiviral response, and might thus be expected to limit viral toxicity or replication. These possibilities can now be elucidated in our *in vitro* models, for example, by the use of antisense oligonucleotide technology.

To elucidate potential nonviral mechanisms of GP73 regulation, we hypothesized that GP73 expression might be modulated by cytokines that play a role in the development or perpetuation of liver disease. We focused our attention on IFN- γ and TNF- α , two pleiotropic cytokines that are involved in many aspects of the initiation and perpetuation of the hepatic injury response.^{21,22} Using SK-Hep-1 cells, a cell line that spontaneously expresses moderate levels of GP73, we found that IFN- γ treatment stimulated GP73 expression approximately 2-fold. The response to IFN- γ occurred with a lag time of approximately 14 hours, and peaked at 24 hours, suggesting that the effect occurred as a downstream event in the intracellular signaling cascade. The IFN- γ signaling pathway has been extensively studied in recent years. It is

activated by binding of IFN- γ to a specific receptor, which is expressed on nearly all cell types, followed by signaling through JAK1, JAK2, and STAT1. Nuclear translocation of STAT1 results in the initiation of transcription for a group of approximately 100 genes, which together play vital roles in the cellular response to viral infection, and in the regulation of cellular growth and apoptosis.²³ Our data suggest that GP73 represents a downstream target of IFN- γ signaling.

In a second set of experiments, we observed that GP73 was down-regulated by TNF- α . TNF- α signaling involves a specific cellular receptor, and a signaling cascade that requires the activation of the Jun N-terminal kinase. The pathway involves phosphorylation of the c-Jun proto-oncogene, which results in the regulation of cellular genes that play important roles in the regulation of apoptosis, necrosis, and proliferation.^{21,22,24} Taken together, these experiments establish that GP73 expression in hepatocytes can be regulated by relevant cytokines, and suggest that the protein may be an important intracellular target in the hepatocyte injury response.

Interestingly, IFN- γ and TNF- α had no consistent effects on GP73 expression in HepG2 and Hep3B cells, even though both cell lines expressed GP73 in response to adenovirus infection, and HepG2-derived HepG2215 cells expressed large amounts of the protein. HepG2, Hep3B, and SK-Hep-1 cells are members of a cluster of hepatocyte-derived tumor cell lines that are genetically related but distinct.²⁵ These data suggest that the cytokine response of GP73 varies in response to the cellular genetic background.

In addition to its regulated expression via viral and nonviral pathways, our previous studies suggest that GP73 is constitutively expressed at high levels in extrahepatic epithelial cells, including intestinal epithelial cells, pancreatic acinar cells, trachea epithelial cells, and prostate epithelial cells.¹ This pattern is reminiscent of the consistent GP73 expression in normal biliary epithelial cells and suggests GP73 has housekeeping functions in cells of the epithelial lineage.

To date, few studies have been performed on the role of the Golgi apparatus and its associated proteins in disease states.²⁶ Our study provides a suitable model to address the involvement of this organelle in human liver disease. Further experiments are needed to elucidate the function of GP73, using protein overexpression, antisense oligonucleotide inhibition, and the identification of potential interacting protein in normal and diseased cells.

Acknowledgment: The authors thank Bruce A. Luxon for help with the statistical analysis. Daniela Simon pro-

vided the HBV-transfected hepatoma cell lines. Human liver tissues were obtained from the Minnesota Tissue Repository (director: Harvey Sharp) and from Harvey Solomon. Adrian DiBisceglie, Bruce Bacon, and John Edwards provided helpful comments on the manuscript.

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