

# Hepatitis C Virus Infection Triggers a Tumor-Like Glutamine Metabolism

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Chronic infection with hepatitis C virus (HCV) is one of the main causes of hepatocellular carcinoma. However, the molecular mechanisms linking the infection to cancer development remain poorly understood. Here we used HCV-infected cells and liver biopsies to study how HCV modulates the glutaminolysis pathway, which is known to play an important role in cellular energetics, stress defense, and neoplastic transformation. Transcript levels of glutaminolytic factors were quantified in Huh7.5 cells or primary human hepatocytes infected with the Japanese fulminant hepatitis 1 HCV strain as well as in biopsies of chronic HCV patients. Nutrient deprivation, biochemical analysis, and metabolite quantification were performed with HCV-infected Huh7.5 cells. Furthermore, short hairpin RNA vectors and small molecule inhibitors were used to investigate the dependence of HCV replication on metabolic changes. We show that HCV modulates the transcript levels of key enzymes of glutamine metabolism *in vitro* and in liver biopsies of chronic HCV patients. Consistently, HCV infection increases glutamine use and dependence. We finally show that inhibiting glutamine metabolism attenuates HCV infection and the oxidative stress associated with HCV infection. **Conclusion:** Our data suggest that HCV establishes glutamine dependence, which is required for viral replication, and, importantly, that glutamine addiction is a hallmark of tumor cells. While HCV induces glutaminolysis to create an environment favorable for viral replication, it predisposes the cell to transformation. Glutaminolytic enzymes may be interesting therapeutic targets for prevention of hepatocarcinogenesis in chronic hepatitis C. (HEPATOLOGY 2016; 00:000-000).

Over 170 million people are chronically infected with hepatitis C virus (HCV) worldwide, placing them at risk to develop chronic liver inflammation and fibrosis and to progress to cirrhosis and hepatocellular carcinoma (HCC) in the long term. Incidences of HCV-induced liver disease and cancer will remain high in the coming decade despite recent progress in the development of direct-acting antiviral agents.<sup>(1)</sup> With new treatments, sustained virological response (SVR) is predicted to become attainable for most patients.<sup>(2)</sup> A therapeutically obtained SVR improves inflammation and fibrosis and decreases the risk for HCC. However, in up to 12% of

SVR patients, fibrosis levels do not reverse and even progress to cirrhosis.<sup>(3)</sup> Furthermore, HCC can still occur in SVR patients, even long after treatment, and an advanced pretreatment fibrosis stage is considered a risk factor.<sup>(4)</sup> Among the risk factors for fibrosis progression in chronic hepatitis C are features of the metabolic syndrome including obesity, dyslipidemia, insulin resistance, and type 2 diabetes. Among these, insulin resistance and steatosis are frequent in patients and suspected—at least partially—to be directly induced by the virus.<sup>(5,6)</sup> However, metabolic fluxes in HCV-infected cells have not been analyzed in detail, despite their pivotal role in cellular transformation.

*Abbreviations:* ATP, adenosine triphosphate; dpi, days postinfection; GLS, glutaminase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus;  $\alpha$ KG,  $\alpha$ -ketoglutarate; MOI, multiplicity of infection; NS3/NS5A, nonstructural proteins 3 and 5A; PHH, primary human hepatocyte; qRT-PCR, quantitative reverse-transcription PCR; shRNA, short hairpin RNA; SLC, solute carrier; SVR, sustained virological response; TCA, tricarboxylic acid.

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The predominant nutrient sources for cancer cells are glucose and glutamine. Both provide bioenergetics (adenosine triphosphate, ATP) and intermediates for macromolecular synthesis. So far only a few reports have described changes to glucose uptake and use in HCV infection, and these have presented contradictory results.<sup>(7,8)</sup> Altered glucose use with augmented lactate production and glutamine uptake have been reported in HCV-infected hepatoma cells,<sup>(8,9)</sup> but no underlying details have been published. Glutamine supports many metabolic functions needed for cell survival, growth, and proliferation. In particular it serves as a precursor for other amino acids, nucleotides, and lipids and as a substrate for oxidative phosphorylation. In addition, it is required for glutathione synthesis and redox homeostasis.<sup>(10)</sup> Glutaminolysis is initiated by the conversion of glutamine to glutamate, catalyzed by the rate-limiting enzyme glutaminase (GLS). Glutamate can serve as a precursor for nucleic acid and serine synthesis, drive the uptake of other amino acids, or be further converted by glutamate dehydrogenase or transaminases to  $\alpha$ -ketoglutarate ( $\alpha$ KG) which enters the tricarboxylic acid (TCA) cycle. The phenomenon of glutamine-driven anaplerosis provides biosynthetic precursors and allows many tumor cells to sustain ATP production by providing enough reducing equivalents to the electron transport chain.<sup>(11)</sup> Two human GLS genes exist, *GLS* (*GLS1*) and *GLS2*. *GLS* displays oncogenic activity, while the potential role of *GLS2* in cancer remains less clear.<sup>(12)</sup> Two *GLS* isoforms have been described, KGA and GAC; but their respective functional differences remain uncharacterized.

Given the importance of glutamine in carcinogenesis, we investigated the role of glutaminolysis in HCV infection. We show that HCV increases expression of glutaminolytic enzymes, glutamine uptake, and glutamine use in *in vitro* infected Huh7.5 cell cultures. Transcripts of key glutaminolytic genes were also increased by HCV in

infected primary human hepatocytes (PHHs). Importantly, we show using GLS-specific pharmacological inhibitors that HCV replication is sensitive to glutaminolysis. Furthermore, expression of several glutaminolytic genes increased together with fibrosis stage and inflammation grade in HCV-liver biopsies. Our results suggest that HCV stimulates glutamine metabolism, a metabolic adaptation that boosts viral replication and that may play an important role in the predisposition to HCC in chronically infected patients.

## Materials and Methods

### CELL CULTURE

Huh7.5 cells were maintained in Dulbecco's modified Eagle medium containing 25 mM glucose, 1× GlutaMAX, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Life Technologies) as well as 10% fetal calf serum (Thermo Scientific) in a 5% CO<sub>2</sub>-normoxia incubator at 37°C. For glucose and glutamine titration and deprivation experiments, Huh7.5 cells were cultured in Dulbecco's modified Eagle medium without glucose or glutamine, supplemented or not with 25 mM glucose and 2 mM glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10% dialyzed fetal calf serum (Life Technologies).

### CELL CULTURE-PRODUCED HCV PRODUCTION

The HCV Japanese fulminant hepatitis 1 strain described by Delgrange et al.<sup>(13)</sup> was *in vitro* transcribed and electroporated into Huh7.5 cells. Supernatants were harvested, filtered (0.45  $\mu$ m), and used to infect naive Huh7.5 cells. Pooled supernatants were quantified using the median tissue culture infective dose (TCID<sub>50</sub>) method.

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## GLUCOSE/GLUTAMINE USE AND LACTATE PRODUCTION MEASUREMENTS

Glucose, glutamine, and lactate were quantified by calculating the difference in concentration in the culture medium after 24-hour culture. Glucose titration reagent contained 0.1 M triethanolamine (pH 7.5; Sigma), 2 mM nicotinamide adenine dinucleotide phosphate (Roche), 2 mM ATP (Sigma), 2 U/mL hexokinase (Sigma), and 2 U/mL glucose-6-phosphate dehydrogenase (Roche). Glutamine was quantified using the L-Glutamine/Ammonia Assay Kit (Megazyme), glutamate was quantified with the Glutamate Assay Kit (Abcam), and glutaminase activity was quantified with the Glutaminase Microplate Assay Kit (Cohesion Biosciences). Lactate titration reagent contained hydrazine/glycine (0.4/0.5 M; pH 9; Sigma), 2 mM oxidized reduced nicotinamide adenine dinucleotide (Sigma) and 2 U/mL lactate dehydrogenase (Sigma). Reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate production was quantified by measuring the endpoint optical density of reactions at 340 nm.

## LIVER BIOPSIES

Liver biopsies from control (Hodgkin lymphoma,  $n = 12$ ) and chronic hepatitis C ( $n = 119$ ) patients and paired biopsies from chronic hepatitis C patients before and during  $\alpha$ -interferon/ribavirin treatment were acquired during routine diagnostic work whenever sufficient material was available. Information on HCV genotype was available for 77 patients: 49 were infected with genotype 1 and 28 with genotype 2. Biopsies were used under the French institutional review board Comité de Protection des Personnes Sud-Est 287 IV agreement (no. 11/040 obtained in 2011). Written informed consent was obtained.

## QUANTITATIVE REVERSE-TRANSCRIPTION PCR

RNA was extracted from cells with Trizol reagent (Life Technologies). Total RNA (1  $\mu$ g) was deoxyribonuclease-treated (Roche) and reverse-transcribed (Moloney murine leukemia virus reverse transcriptase; Life Technologies). RNA from liver biopsy samples was purified (Nucleospin RNA/protein kit; Macherey-Nagel). RNA (350 ng) was reverse-transcribed (Superscript Vilo enzyme; Life

Technologies). Transcript levels of target genes were quantified by quantitative reverse-transcription PCR (qRT-PCR) using Sybr Green I Master Mix (Roche) on a LightCycler 480 (Roche). Glucuronidase beta served as a reference gene for the *in vitro* study and phosphomannomutase 1 for liver biopsies. All primer sequences are available upon request. For the Japanese fulminant hepatitis strain 1, specific primers were adapted from the RC1 and RC21 primers.<sup>(14)</sup>

## STATISTICS

Statistical analyses were performed with the Student  $t$  test or the nonparametric Mann-Whitney U, with Wilcoxon signed rank tests and Spearman correlation analysis.

Additional methods are described in the [Supporting Information](#).

## Results

### HCV INFECTION UP-REGULATES SEVERAL KEY FACTORS OF GLUTAMINE METABOLISM

Basal transcript levels of glutaminolytic factors were compared in Huh7.5 cells and PHHs. MYC is a key transcription factor that drives glutamine addiction in cancer cells by inducing the transcription of various glutamine transporters such as solute carrier family 1 member 5 (SLC1A5) and SLC7A5 as well as of GLS, the rate-limiting enzyme of glutaminolysis. Overall, transcript levels of these genes were lower in PHHs compared to Huh7.5 cells, likely due to the transformed background of the hepatoma cell line (Fig. 1A). Although there were elevated baseline levels of transcripts of glutaminolytic factors in Huh7.5 cells, HCV increased mRNA levels of MYC and both glutaminase transporters significantly not only in PHHs but also in Huh7.5 cells, infected at a multiplicity of infection (MOI) of 5 and 1, respectively (Fig. 1B,C). HCV also induced the two GLS isoforms, KGA and GAC, in Huh7.5 cells, while only GAC was increased in PHHs. Both isoforms are associated with carcinogenesis. In contrast, HCV did not induce transcript levels of GLS2, the isoform expressed in the liver under normal physiological conditions, in Huh7.5 cells or in PHHs. Induction of glutaminolytic transcripts was not observed with ultraviolet-irradiated virus (Fig. 1C), and induction of MYC and GLS by naive virus was confirmed on the protein level in Huh7.5 cells (Fig.

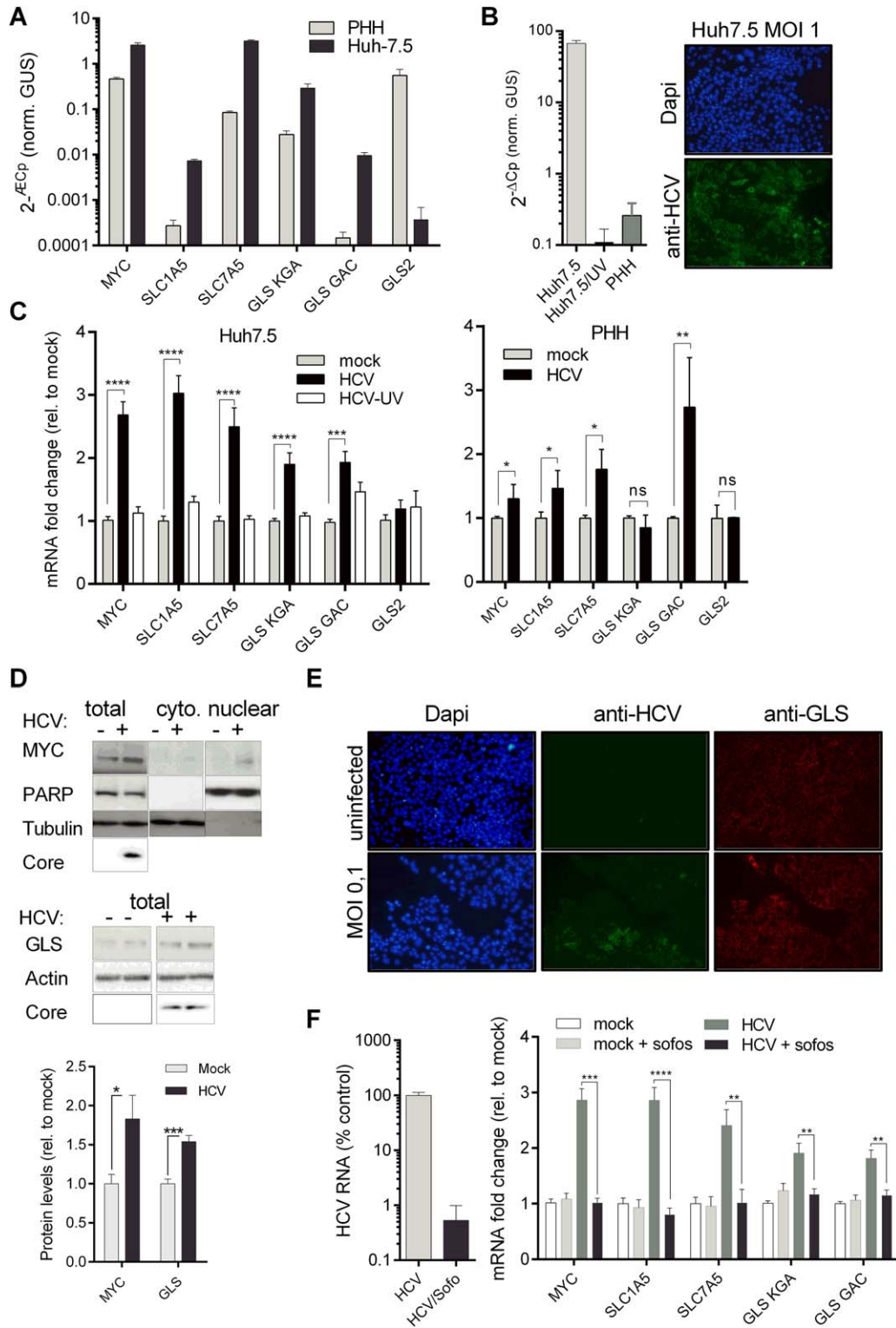


FIG. 1

1D). In infected cells, MYC accumulated particularly in nuclear fractions, indicating activation of its transcriptional activity. In order to test whether induction of glutaminolytic factors by the virus is a direct effect, colocalizations were performed on infected Huh7.5 cells (MOI 0.1) at 3 days postinfection (dpi), using an antibody specific to GLS isoforms KGA and GAC. The virus was present in a large majority of cells, but expression levels were heterogeneous. GLS expression was overall induced by the virus in the culture, but strongly HCV-positive cells did not display the highest GLS staining (Fig. 1E), suggesting that GLS induction may not be directly mediated by HCV but rather by virus-induced oxidative stress or inflammatory processes. GLS induction by HCV was furthermore found to be reversible. Huh7.5 cells were infected at an MOI of 0.1 for 4 days and then treated with the direct-acting antiviral agent sofosbuvir for a further 2 days (Fig. 1F). mRNA levels of *MYC*, *GLS* KGA and GAC, *SLC1A5*, and *SLC7A5* were increased in cultures at 4 dpi but returned to baseline upon successful elimination of HCV by sofosbuvir treatment, demonstrating that the transcriptional induction of these genes is reversible.

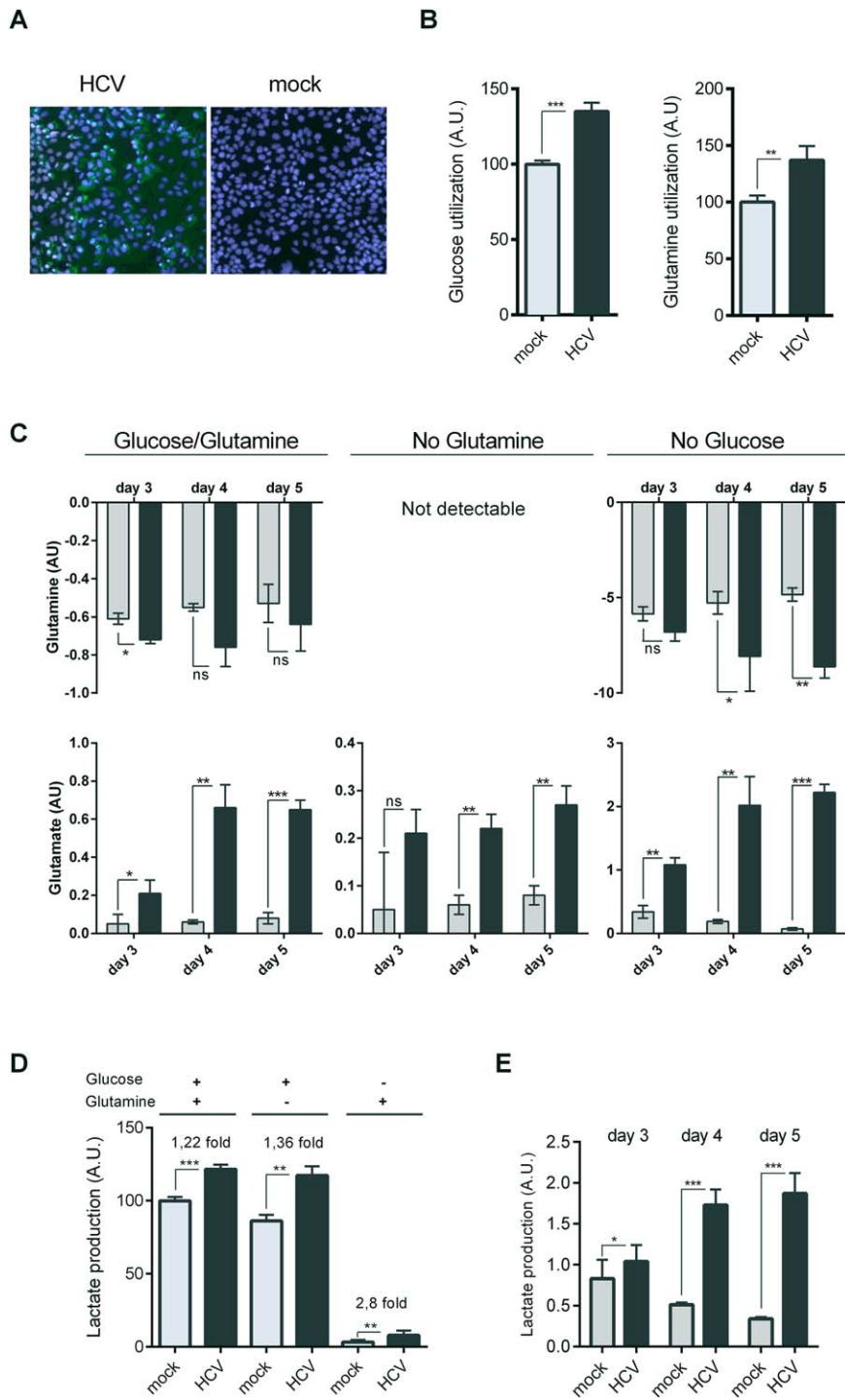
## GLUTAMINE METABOLIZATION IS INCREASED IN HCV-INFECTED CELLS

The effect of HCV on glucose and glutamine use was assessed. At 3 dpi, when the virus had spread throughout the culture (Fig. 2A), culture medium was

changed and harvested 24 hours later to quantitate glucose and glutamine. In infected cells, consumption of both nutrients was increased (Fig. 2B), suggesting that HCV induces glycolysis as well as glutaminolysis. To test the impact of nutrient composition of the culture medium on HCV-induced glutamine uptake, mock and infected cells were cultured from 3 dpi onward in conditioned medium containing glucose or glutamine only or a combination of both. Supernatants were harvested 24 hours later, and the procedure was repeated on days 4 and 5. Supernatants were analyzed by nuclear magnetic resonance. HCV induced significant changes to levels of several amino acids. However, only glutamine and glutamate levels were changed in mock versus infected cells irrespective of the culture medium used (Table 1 and Fig. 2C). Infected cells depleted more glutamine compared to mock cells from the supernatant, while glutamate levels increased, pointing to an active glutamate export from infected cells, possibly in exchange for other amino acids. In order to determine whether glutamine taken up by infected cells feeds into the TCA cycle and in order to assess the respective contributions of glutaminolysis and glycolysis to the TCA cycle, lactate production was quantified. Glucose is the predominant substrate for lactate production. However, lactate can also be produced from glutamine through the TCA cycle intermediate malate. At 3 dpi media were replaced with conditioned media containing both glucose and glutamine, glutamine only, or glucose only and cells were harvested 24 hours later. In the presence of glucose-containing media, HCV increased lactate production by 20%-30% (Fig. 2D).

**FIG. 1.** Glutaminolytic factors are increased in HCV-infected cells and return to basal levels upon viral clearance. (A) Expression levels of MYC, SLC1A5, SLC7A5, and GLS isoforms KGA and GAC or GLS2 transcripts were quantified by qRT-PCR in Huh7.5 cells and PHHs using GUS as a housekeeping gene (mean  $\pm$  standard error of the mean (SEM) values of three or more independent experiments). (B) HCV replication levels at 3 dpi in Huh7.5 cells infected (MOI 1) with native or ultraviolet-irradiated virus analyzed by qRT-PCR and immunofluorescence with an anti-HCV serum (green). PHHs were infected with HCV at an MOI of 5 to 10 and harvested between 3 and 5 dpi (absolute quantification revealed 4,000-9,000 HCV genome copies/ $\mu$ g RNA). (C) MYC, SLC1A5, SLC7A5, GLS KGA /GAC, and GLS2 transcript levels were amplified using GUS as a housekeeping gene in Huh7.5 cells and PHHs. Fold changes standardized to mock infection are shown (mean  $\pm$  SEM, n = 2-10 independent experiments). (D) Total lysates and nuclear and cytoplasmic fractions of HCV-infected Huh7.5 cells were immunoblotted with antibodies specific for MYC, PARP, tubulin, GLS, actin, and core. Protein quantification was performed with ImageJ software. Results were standardized to PARP (MYC) or actin (GLS) expression and are expressed as fold change between infected and uninfected cells (a representative immunoblot is shown; graph, mean  $\pm$  SEM, n = 4). (E) Huh7.5 cells were infected with HCV (MOI 0.1). At 3 dpi, cells were fixed and stained with anti-HCV serum and anti-GLS antibody. Representative images are shown (n = 4). (F) At 4 dpi infected (MOI 0.1) and uninfected Huh7.5 cells were treated with 5  $\mu$ M sofosbuvir or carrier for 2 days. HCV, MYC, SLC1A5, SLC7A5, and GLS mRNA levels were quantified by qRT-PCR at 4 and 6 dpi using GUS as a housekeeping gene. HCV data were standardized to infection at 4 dpi. MYC, SLC1A5, SLC7A5, and GLS were standardized to mock infection (mean  $\pm$  SEM, n  $\leq$  7) (C-F) Student *t* test: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. Abbreviations: Dapi, 4',6-diamidino-2-phenylindole; GUS,  $\beta$ -glucuronidase; ns, not significant; PARP, poly(adenosine diphosphate ribose) polymerase; SEM, standard error of the mean; Sofos, sofosbuvir.





**FIG. 2.** HCV infection increases glutamine use. (A-E) Huh7.5 cells were mock-infected or HCV-infected (MOI 1) for 3 days before cell medium change. At 24 hours after the cell medium change, cells were counted and supernatants harvested for metabolite quantification. Metabolite concentrations were corrected for cell numbers. Gray bars present mock and black bars HCV-infected cultures. (A) HCV infection was controlled at 3 dpi by immunofluorescence with an anti-HCV serum (green). (B) Glucose and glutamine consumption was measured biochemically in normal medium (25 mM glucose, 2 mM glutamine), and data were standardized to mock-infected cells (mean  $\pm$  SEM,  $n = 4$ ) (C) Supernatants of mock-infected and infected cells cultured in normal medium or conditioned medium containing only glucose or only glutamine were harvested 3, 4, and 5 dpi and subjected to nuclear magnetic resonance analysis. Glutamine and glutamate concentrations (arbitrary units) were standardized for cell numbers (mean  $\pm$  standard deviation,  $n = 3$ ). (D) Lactate was quantified biochemically in supernatants of infected/uninfected cells cultured in the indicated conditioned cell media at 3 dpi. Data are standardized to mock-infected cells cultured in normal medium (mean  $\pm$  SEM,  $n = 4$ ). (E) Lactate was quantified by nuclear magnetic resonance in supernatants of mock-infected and infected cells cultured in only glutamine-containing medium at 3, 4, and 5 dpi. Lactate concentrations (arbitrary units) were standardized for cell numbers (mean  $\pm$  SEM,  $n = 3$ ). Abbreviations: A.U., arbitrary units; ns, not significant; SEM, standard error of the mean.

When only glutamine was available overall lactate production was lower, but HCV increased lactate production by close to 300%, indicating that partial glutamine oxidation through the TCA cycle is

significantly activated in HCV-infected cells. These data were confirmed in a 3-day time course in medium containing glutamine only, where lactate production increased over time (Fig. 2E).

TABLE 1. HCV Alters Amino Acid Levels in Supernatants of Infected Cells

Metabolites	Glucose/Glutamine			Glucose Only			Glutamine Only			
	Mock	HCV <sup>+</sup>	<i>P</i>	Mock	HCV <sup>+</sup>	<i>P</i>	Mock	HCV <sup>+</sup>	<i>P</i>	
Arg	-0.096	-0.069	0.2332	-0.208	-0.021	0.0420	-0.437	-1.230	↓	<b>0.0017</b>
Gln	-0.564	-0.709	↓ <b>0.0041</b>	—	—	—	-5.321	-7.832	↓	<b>0.0003</b>
Glt	0.063	0.508	↑ <b>0.0003</b>	0.061	0.234	↑ <b>0.0003</b>	0.202	1.771	↑	<b>0.0003</b>
Gly	—	—	—	—	—	—	0.320	0.862	↑	<b>0.0003</b>
Ile	-0.004	0.049	0.0851	0.187	0.158	0.4263	-0.042	-0.119		0.3311
His	-0.005	-0.024	0.2891	-0.043	0.015	0.0703	0.015	0.034		0.4529
Met	-0.042	0.056	↑ <b>0.0003</b>	0.005	0.024	0.0467	-0.217	-0.245		0.6911
Thr	-0.064	0.073	↑ <b>0.0469</b>	-0.057	0.189	0.0772	-0.041	-0.280	↓	<b>0.0041</b>
Tyr	0.038	0.050	0.3536	0.048	0.088	0.0849	0.130	0.089		0.5660
Val	-0.095	-0.142	0.0468	-0.213	-0.261	0.1451	-0.343	-0.766	↓	<b>0.0023</b>

\*At 3 dpi, supernatant of mock or infected cells was replaced with conditioned growth medium (25 mM glucose, 2 mM glutamine, 25 mM glucose, 2 mM glutamine). 24 hours later media were harvested and replaced. The procedure was repeated on 4 and 5 dpi. Metabolites in supernatants were quantified by nuclear magnetic resonance. Values were corrected for metabolite concentration in the culture media not exposed to cells. Data (in arbitrary units) present the mean concentration measured at 3-5 dpi, corrected for cell density. Tendencies of metabolite concentrations to decrease or increase in infected cells are indicated by arrows. *P* values (Mann-Whitney U) are indicated. Statistically significant changes are marked in bold.

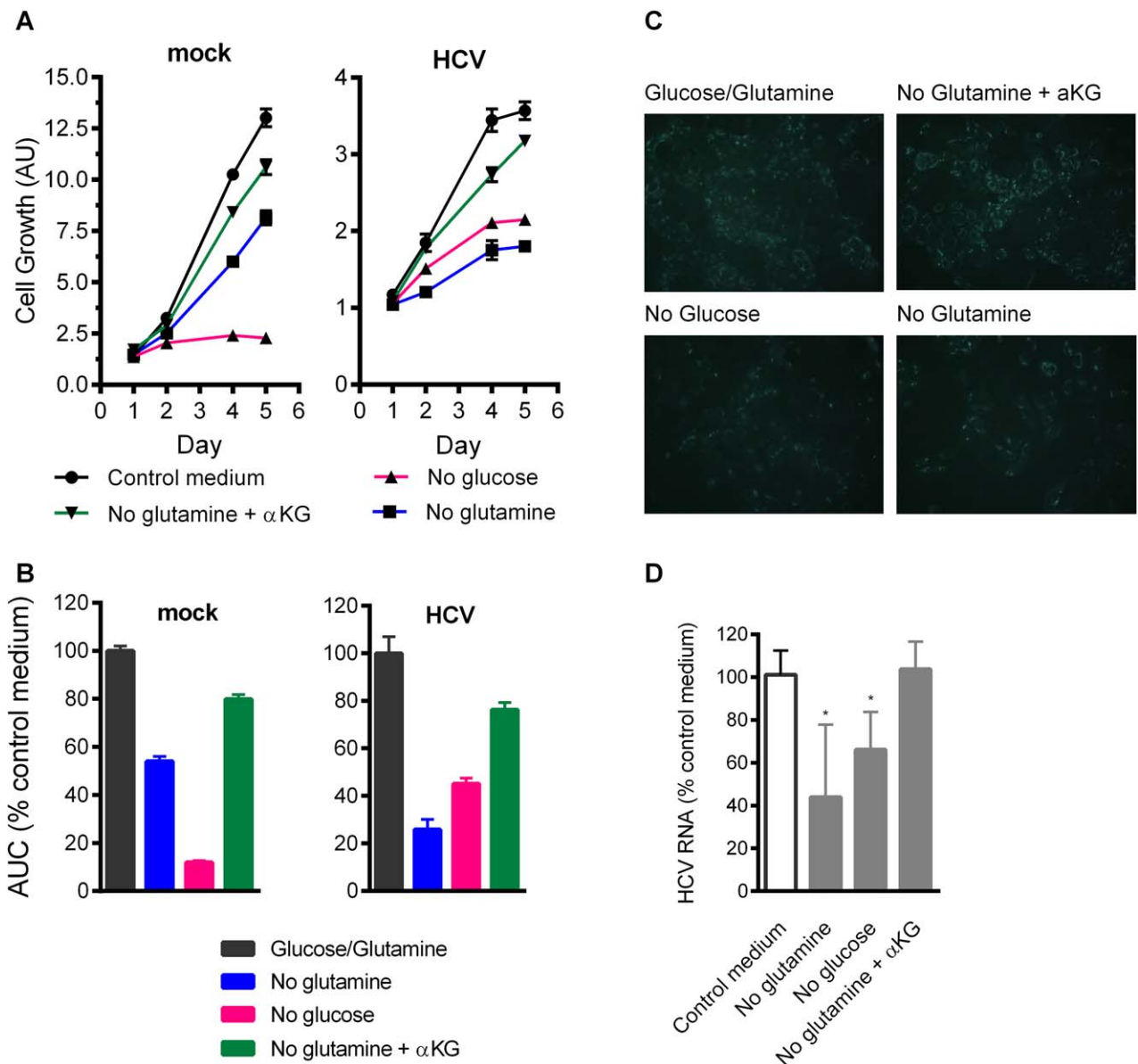
## GLUTAMINE IS MORE ESSENTIAL THAN GLUCOSE FOR THE PROLIFERATION OF HCV-INFECTED CELLS

To estimate the relative importance of glucose and glutamine in HCV infection, proliferation of mock-infected and HCV-infected cells was monitored in conditioned media containing or not glucose and glutamine. HCV-infected Huh7.5 cells were seeded and cultured in the indicated growth media, and cell proliferation was quantified each day. Proliferation of control cells was overall faster than that of HCV-infected cells and more sensitive to glucose than to glutamine deprivation. In contrast, proliferation of HCV-infected cells was more sensitive to glutamine than to glucose withdrawal, underlining the importance of glutamine in HCV-infected cells (Fig. 3A,B). HCV expression was still detectable under all culture conditions 4 days after medium change (Fig. 3C,D), suggesting that the differences in proliferation were predominantly due to metabolic restraints induced by the virus. Furthermore, the effects of glutamine withdrawal on proliferation of both mock-infected and HCV-infected cells could be almost completely rescued by  $\alpha$ KG supplementation (Fig. 3A,B), suggesting that HCV induces glutaminolysis in order to maintain TCA cycle activity.

## GLUTAMINOLYSIS IS REQUIRED FOR HCV REPLICATION

To investigate the dependence of HCV on glutamine metabolism, Huh7.5 cells were infected and

culture media were changed 4 hours later with control and glutamine-deprived media. Viral RNA was quantified by qRT-PCR at 3 dpi and standardized to cellular RNA content in order to compensate for effects on cell growth (Fig. 4A). Glutamine withdrawal from cell culture medium inhibited the establishment of an HCV infection by over 60%. Addition of nonessential amino acids, glutamate, or  $\alpha$ KG to the glutamine-free medium rescued HCV replication (Fig. 4A). This indicates that HCV requires glutamine for production of  $\alpha$ KG, likely for anaplerotic purposes. To verify the importance of glutaminolysis in the HCV life cycle, transcript levels of *MYC* and *GLS* were silenced in Huh7.5 cells with short hairpin RNA (shRNA). Knockdown efficiencies of the shRNAs were verified the same day the cells were seeded for infection. *MYC* and *GLS* transcript levels were found to be reduced by 67% and 71%, respectively in comparison to control shRNA (Fig. 4B). At 3 dpi HCV replication was quantified and found to be inhibited by 53% and 38% in shMYC and shGLS cells, respectively, compared to scramble control. Silencing of *GLS* could be partially rescued by addition of  $\alpha$ KG. To validate MYC and GLS as antiviral targets, the effect of pharmacological small molecule inhibitors on HCV replication was tested. The MYC inhibitor 10058-F4 is known to have a potent anticancer effect *in vitro*.<sup>(15)</sup> The GLS inhibitors bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) and CB-839 are known to block the catalytic center of GLS. CB-839 is currently under evaluation in a phase 1 clinical trial for several types of cancers.<sup>(16,17)</sup> No cell toxicity of these inhibitors was



**FIG. 3.** Proliferation of HCV-infected cells, but not of uninfected cells, is highly sensitive to glutamine withdrawal. Huh7.5 cells were infected (MOI 1) and reseeded 5 dpi using the indicated conditioned cell media. Cell proliferation was monitored from the day of reseeded onward (mean  $\pm$  SEM of one representative experiment of four performed with six biological replicates). (A) Cell proliferation count in the presence of conditioned medium standardized to day 1 of reseeded ( $\alpha$ KG, 2 mM). (B) Cell growth depicted in Fig. 2A represented as area under the curve, taking day 1 as baseline. (C,D) Viral infection at day 4 post-reseeded by immunofluorescence using an anti-HCV serum and by HCV qRT-PCR (standardized to replication in control medium). Abbreviations: A.U., arbitrary units; AUC, area under the curve; SEM, standard error of the mean.

observed on Huh7.5 cells with the exception of CB-839, where cytotoxicity became detectable at the strongest dose tested (Fig. 4C). Huh7.5 cells were infected with MOIs of 0.1 and 1, the inhibitor or carrier was added 4 hours later, and replication was quantitated at 3 dpi. All three molecules inhibited the establishment

of an infection in a dose-dependent fashion, with the strongest effect displayed by CB-839 (Fig. 4C). CB-839 treatment also reduced HCV nonstructural protein 3 (NS3) and NS5A protein levels at 3 dpi (Fig. 4D). To control the pharmacological inhibition of GLS activity, intracellular glutamate levels as well as



glutaminase activity were assessed in the presence of CB-839. HCV increased glutaminase activity in infected cells by approximately 1.5-fold (Fig. 4D). This increase was inhibited in a dose-dependent manner by CB-839. Intracellular glutamate levels were not increased by the virus, possibly due to the important turnover and use of glutamate for amino acid import/exchange and the TCA cycle (as shown in Fig. 2C,D). CB-839, however, decreased intracellular glutamate in a dose-dependent fashion (Fig. 4D). The antiviral effect of CB-839 could be partially reversed by addition of  $\alpha$ KG, suggesting that, besides an increased glutaminolytic flux into the Krebs cycle, glutaminase may fulfill additional functions in the viral life cycle (Fig. 4D). To investigate whether the pharmacological inhibitors of MYC and GLS also inhibit an already established infection, Huh7.5 cells were infected and cultured for a minimum of 10 days. Infected cells were reseeded; 10058-F4, CB-839, or dimethyl sulfoxide carrier was added; and HCV replication was quantified 3 days later. Inhibition of MYC had a reproducible, moderate inhibitory effect. Inhibition of GLS blocked HCV replication in a dose-dependent manner by up to 40% within the 3-day period (Fig. 4E).

HCV has previously been shown to augment production of oxidative stress and in particular superoxide anions.<sup>(18)</sup> In addition, glutaminolysis in cancer cells is required for redox homeostasis. Therefore, the effect of CB-839 on superoxide anion levels in HCV-infected cells was assessed. CB-839 partially blocked the 2-fold increase of superoxide anions induced by HCV (Fig. 4F).

## MYC, SLC7A5, AND GLS LEVELS ARE ELEVATED IN CHRONIC HEPATITIS C PATIENTS

To explore whether glutaminolysis is induced *in vivo*, MYC, SLC1A5, SLC7A5, and GLS (KGA and GAC) transcript levels were quantified by qRT-PCR in liver biopsies from patients with chronic hepatitis C (n = 119) diagnosed with genotype 1 or 2 as well as patients with liver complications from Hodgkin lymphoma as control (n = 12). No difference in the transcript levels of SLC1A5 was detected. However, mRNA levels of MYC, SLC7A5, and both GLS isoforms were significantly elevated in the HCV group (Fig. 5A). Among the HCV patient cohort, activity grades and fibrosis stages were available for 117/119 and 101/119 biopsies, respectively. MYC, SLC1A5, and GLS (KGA and GAC) mRNA levels increased

together with activity grade and fibrosis stage (Fig. 5A). Additional clinical data could be obtained for some of the HCV patients: age (for 90/119 patients), body mass index (65/119), alkaline phosphatase (77/119), aspartate aminotransferase (78/119), alanine aminotransferase (78/119),  $\gamma$ -glutamyltransferase (78/119), and albumin (67/119). Results of association studies are presented in Table 2. Weak but significant positive correlations were detected between MYC, SLC1A5, GLS KGA, GLS GAC,  $\gamma$ -glutamyltransferase, and aspartate aminotransferase levels; further correlations were found between GLS KGA, GLS GAC, and body mass index as well as between GLS GAC and alanine aminotransferase levels. Furthermore, a tendency for a correlation between MYC, SLC1A5, and GLS KGA mRNA and alanine aminotransferase levels (*P* values between 0.0575 and 0.0708) was observed. No difference in the transcript levels of all these genes between patients infected with HCV genotypes 1 and 2 was observed (not shown), suggesting that induction of glutaminolysis is induced in the context of both genotypes. To determine whether increases in MYC, SLC7A5, and GLS levels in patients were virus-induced and reversible upon antiviral treatment, transcripts of HCV, MYC, SLC1A5, SLC7A5, and GLS were quantified in nine paired biopsy couples obtained from patients before and during or after  $\alpha$ -interferon/ribavirin treatment (Fig. 5B). MYC and SLC7A5 were significantly decreased with antiviral treatment alongside HCV levels. GLS levels showed a tendency to decrease (*P* = 0.13), and no difference in transcript levels was observed for SLC1A5.

## Discussion

The molecular mechanisms involved in the progression from chronic HCV infection to HCC are not well established. Here, we report that HCV infection may predispose hepatocytes to liver tumorigenesis through alterations of glutamine metabolism (Fig. 6). In tumor cells glutamine use and the subsequent induction of key target genes involved in glutamine uptake and oxidation are frequently driven by the transcription factor MYC.<sup>(19)</sup> Induction of MYC expression by HCV-induced beta-catenin and Akt signaling has been reported,<sup>(20,21)</sup> but we have extended this finding to MYC-target genes that drive glutamine metabolism: the two glutamine transporters SLC1A5 and SLC7A5 as well as two cancer-associated isoforms of glutaminase—GLS KGA and GAC. Even though basal

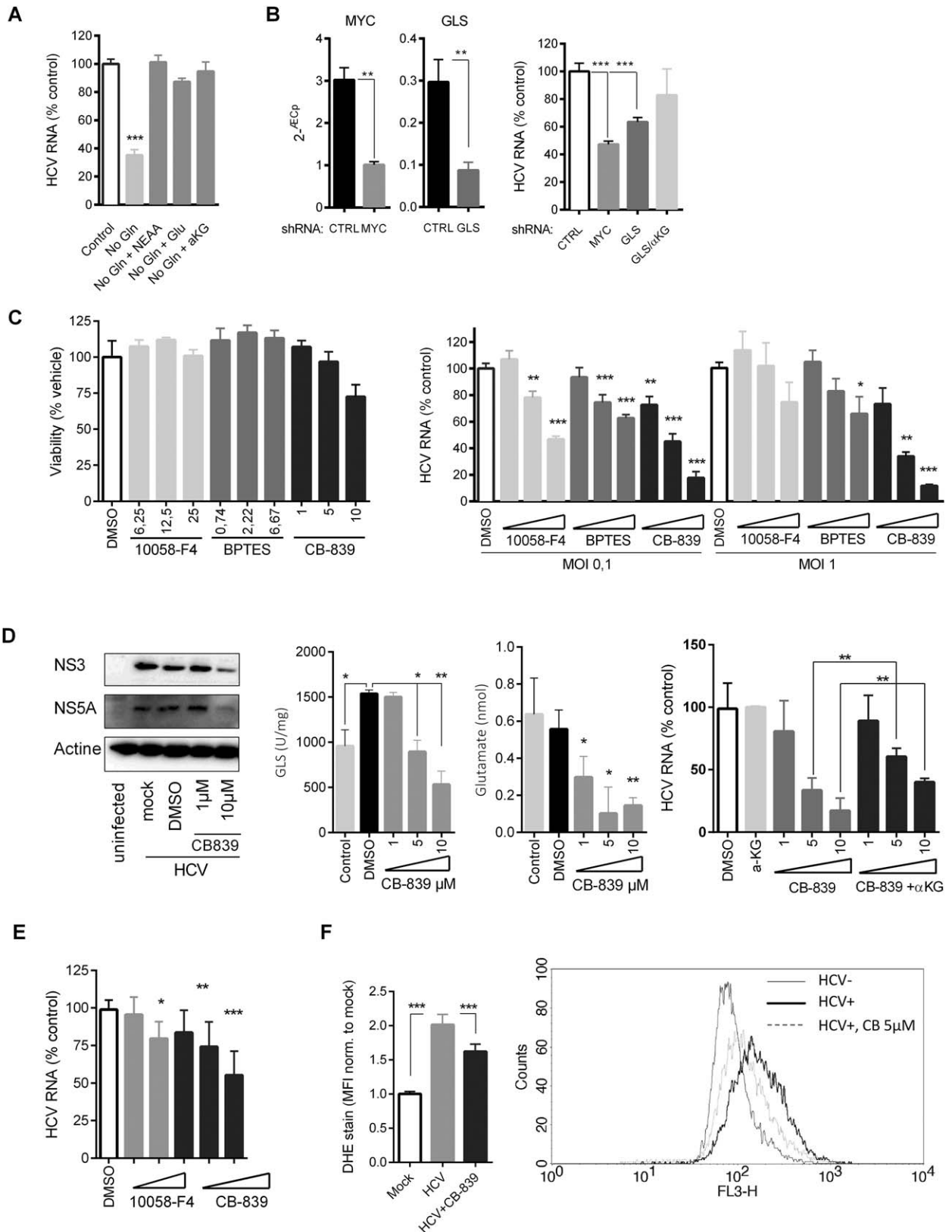


FIG. 4

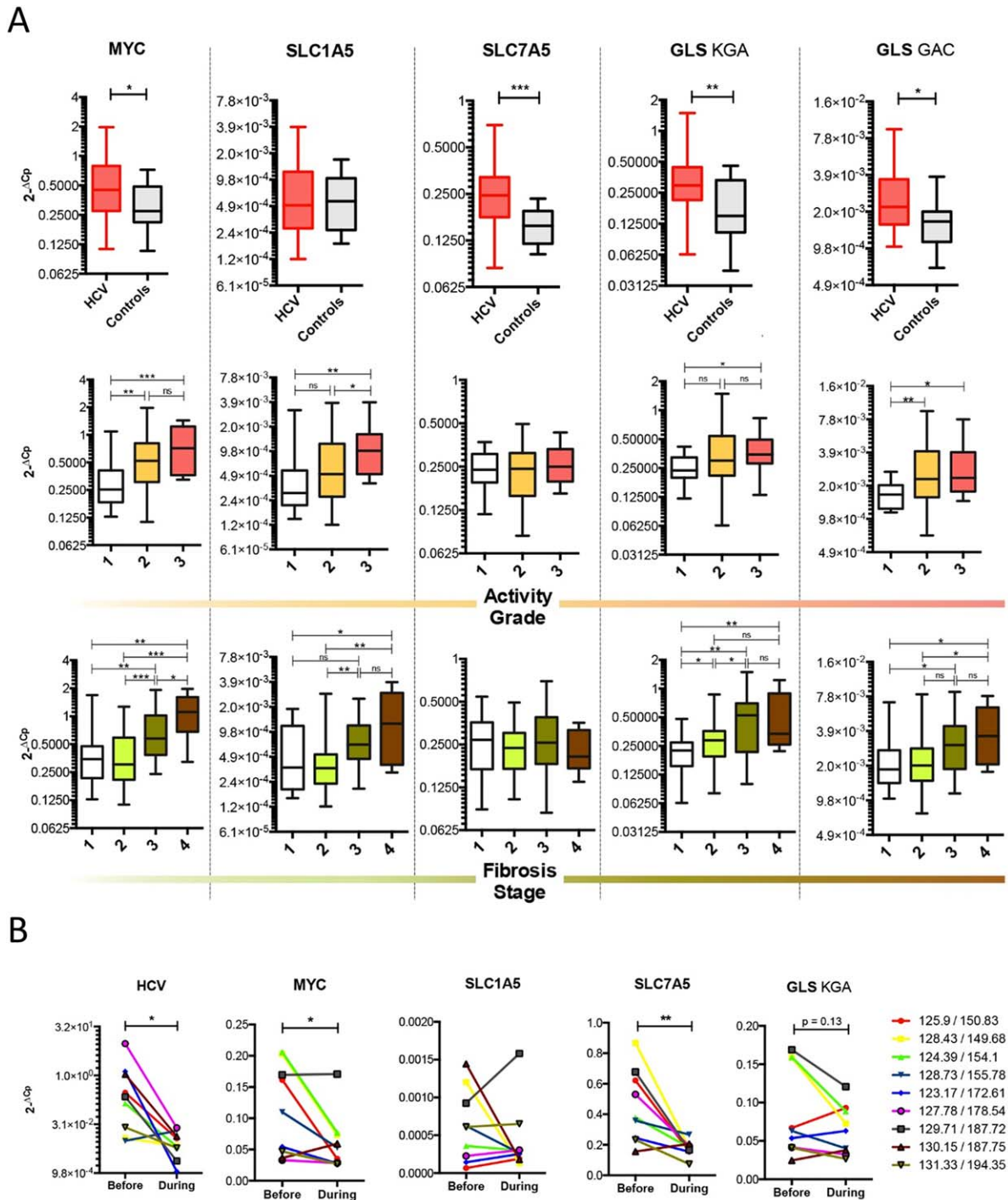
expression of glutaminolytic factors is elevated in Huh7.5 cells in comparison to PHHs, HCV still induces a significant additional increase of these factors in this cell line. We therefore based our experimental approaches on Huh7.5 cells and corroborated these findings in a physiologically appropriate background including experimentally infected PHHs and liver biopsies of HCV-infected patients.

Interestingly, SLC1A5 and SLC7A5 have been described in the literature to be up-regulated in a broad range of human cancers, including HCC.<sup>(22)</sup> Likewise, high expression and activity of GLS *KGA* and *GAC* in cancer cells have been extensively reported.<sup>(10,23)</sup> GLS is known to be important for liver cancer. Indeed, GLS activity is known to correlate with the volume doubling time of rat hepatomas.<sup>(24)</sup> Here, we observed that HCV infection increased the transcripts of both *KGA* and *GAC* isoforms to a similar extent in Huh7.5 cells; however, only the *KGA* isoform was found induced by HCV in PHHs, while both *KGA* and *GAC* isoforms were induced in patient biopsies. The reasons for this discrepant finding are unclear at this stage and warrant further investigation. Transcript levels of *GLS2*, the glutaminase endogenously expressed in the liver, were at the limit of detection in Huh7.5 cells and not increased by the virus in Huh7.5 cells or in PHHs. In addition, we observed increased glutamine consumption in HCV-infected Huh7.5 cells together with an elevation of glucose use and lactate production. These results are consistent with the recently reported stabilization of hypoxia inducible factor 1 $\alpha$ , increase of

hexokinase 2 activity, and stimulation of aerobic glycolysis in infected cells.<sup>(8,25)</sup> Furthermore, the cancer-specific form of pyruvate kinase, PKM2, which mediates the final step of glycolysis, has been shown to interact with HCV polymerase NS5B and to be required for HCV replication.<sup>(26,27)</sup> These data, as well as a recent proteomic analysis of HCV-infected cells,<sup>(9)</sup> suggest that glycolysis plays a major role in the HCV life cycle.

A frequent consequence of aerobic glycolysis is the inhibition of glucose-derived pyruvate entry in the mitochondria, where the TCA cycle produces precursors for biosynthetic processes such as lipid, nucleotide, and amino acid synthesis. Tumor cells are known to compensate for the loss of glucose-derived TCA cycle intermediates by activating glutaminolysis.<sup>(10)</sup> Our results strongly suggest that HCV reprograms metabolism by increasing use of glutamine for anaplerotic purposes. However, a part of the absorbed glutamine is resecreted in the form of glutamate, probably in exchange for other amino acids. This finding has already been reported in various cancer cells and confirms an important role for amino acid metabolism in HCV infection.<sup>(28)</sup> The deleterious effects of glutamine withdrawal on the growth of HCV-infected cells could be fully rescued by  $\alpha$ KG. Increased glutaminolysis and TCA cycle activity have been associated with increased respiratory activity and production of reactive oxygen species in cancer cells. The glutaminase inhibitor CB-839 partially reduced the HCV-induced increase of superoxide anion production in Huh7.5

**FIG. 4.** Glutaminolysis is required for HCV replication. (A) Huh7.5 cells were infected with HCV (MOI 0.1). After 4 hours, culture media were replaced with conditioned medium (control, no glutamine, no glutamine + nonessential amino acids, no glutamine + 2 mM glutamate, no glutamine + 2 mM  $\alpha$ KG). Intracellular HCV RNA levels were quantitated 3 dpi and standardized to infection in control medium. (B) shCTRL, shMYC, and shGLS Huh7.5 cell lines<sup>(37,38)</sup> were controlled for MYC and GLS silencing efficiencies and infected with HCV (MOI 0.1).  $\alpha$ KG (2 mM) was added with the virus as indicated. Intracellular HCV RNA levels were measured by qRT-PCR 3 dpi. (C) Left: Viability was assessed in Huh7.5 cells by neutral red incorporation after 3 days of treatment with the MYC inhibitor 10058-F4 and GLS inhibitors BPTES and CB-839 at the indicated concentrations (micromoles). Middle/right: Huh7.5 cells were infected with HCV at the indicated MOIs. After 4 hours, cells were treated with vehicle (dimethyl sulfoxide) or increasing concentrations of 10058-F4 (6.25, 12.5, and 25  $\mu$ M), BPTES (1, 3, and 6  $\mu$ M), and CB-839 (1, 5, and 10  $\mu$ M). Intracellular HCV RNA levels were measured by qRT-PCR 3 dpi. Dimethyl sulfoxide volumes were the same for each drug condition. (D) Glutaminase inhibitor CB-839 was added at the indicated concentrations 4 hours after infection (MOI 1) of Huh7.5 cells. The impact on HCV protein levels (representative western with anti-NS3 and NS5A antibodies), intracellular glutaminase activity (units per milligram), and intracellular glutamate levels was assessed 3 dpi. In the rightmost graph, 2 mM  $\alpha$ KG was added together with CB-839 to Huh7.5 cells 4 hours after infection (MOI 1), and HCV replication levels were quantitated by qRT-PCR at 3 dpi. (E) Persistently infected Huh7.5 cells (>10 days) were treated for 3 days with dimethyl sulfoxide, 10058-F4 (12.5 and 25  $\mu$ M), and CB-839 (1, 5, and 10  $\mu$ M) and HCV replication quantitated by qRT-PCR and standardized to replication levels at the moment of addition of drugs. (F) Huh7.5 cells were infected with HCV (MOI 0.1) and treated with dimethyl sulfoxide or 5  $\mu$ M CB-839. At 3 dpi superoxide anion levels were quantified by fluorescence-activated cell sorting using dihydroethidium. A representative fluorescence-activated cell sorting histogram is depicted. (A-F) Mean  $\pm$  SEM, n = minimum 3 experiments performed in triplicate. Abbreviations: BPTES, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; CTRL, control; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; Gln, glutamine; Glu, glutamate; MFI, mean fluorescence intensity; NEAA, nonessential amino acid.



**FIG. 5.** Glutaminolytic factors are increased in liver biopsies of chronic HCV patients and MYC and SLC7A5 levels decrease with antiviral treatment. (A) Top panel: Transcript levels of MYC, SLC1A5, SLC7A5, and GLS were measured by qRT-PCR in liver biopsies of 119 HCV and 12 control patients. Minimum to maximum box plots of  $2^{-\Delta C_p}$  values are shown. Mann-Whitney U test. Middle and bottom panels: MYC, SLC1A5, SLC7A5, and GLS (KGA and GAC) transcript levels were plotted against activity grade (A1-A3) and fibrosis stage (F1-F4) of chronic hepatitis C liver biopsies. Group sizes: Activity, 1, n = 18; 2, n = 87; 3, n = 12; Fibrosis, 1, n = 22; 2, n = 37; 3, n = 32; 4, n = 10. Mann-Whitney U test. (B) Transcript levels of HCV, MYC, SLC1A5, SLC7A5, and GLS were measured in nine paired biopsies from chronic HCV patients before and during or after interferon- $\alpha$ /ribavirin therapy. Results are presented as  $2^{-\Delta C_p}$  values. Wilcoxon signed rank test. Abbreviation: ns, not significant.

**TABLE 2. Spearman Correlation Analysis Between MYC, SLC1A5, SLC7A5, and GLS (KGA and GAC) Transcript Levels and Body Mass Index Score, Alkaline Phosphatase, Gamma-Glutamyl Transferase, Aspartate and Alanine Aminotransferase, and Albumin Levels and Patients' Age Within the Chronic HCV Cohort**

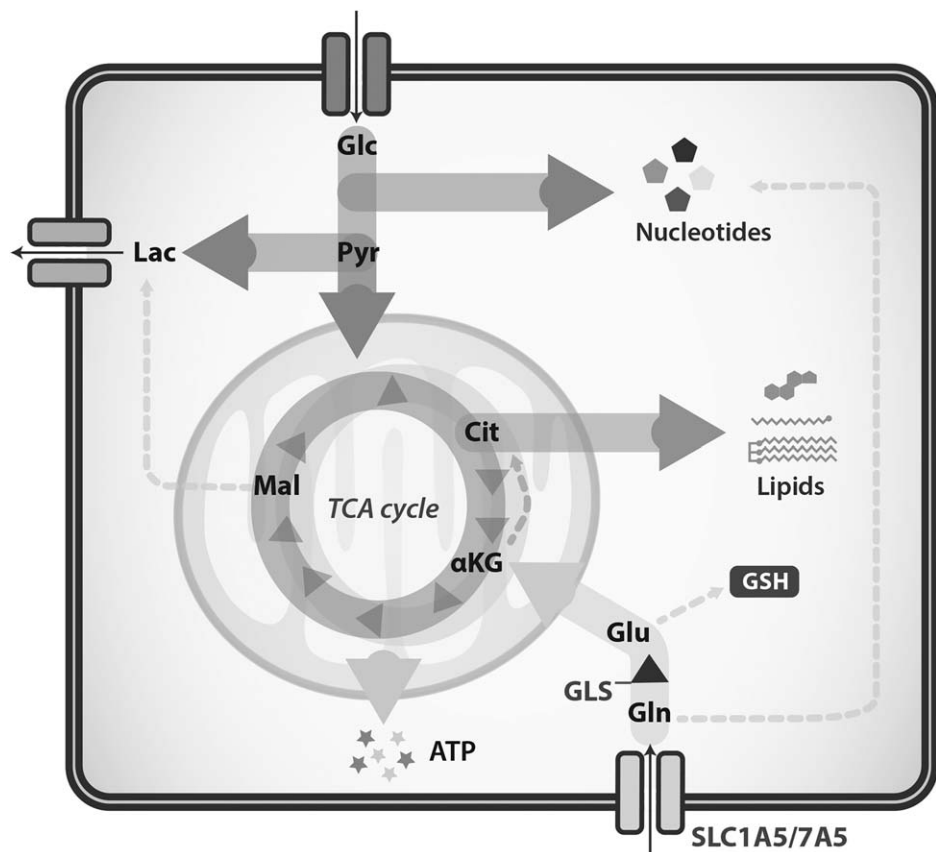
		MYC	SLC1A5	SLC7A5	GLS KGA	GLA GAC
BMI	<i>r</i>	0.0512	0.1774	-0.0632	<b>0.3113</b>	<b>0.2553</b>
n = 65	<i>P</i>	0.6905	0.1789	0.6201	<b>0.0138</b>	<b>0.0452</b>
AP	<i>r</i>	0.0951	0.0657	-0.1106	-0.0720	-0.1392
n = 77	<i>P</i>	0.4171	0.5860	0.3450	0.5419	0.2368
GGT	<i>r</i>	0.0591	<b>0.2379</b>	-0.0541	<b>0.2294</b>	<b>0.2824</b>
n = 78	<i>P</i>	0.6096	<b>0.0427</b>	0.6406	<b>0.0462</b>	<b>0.0141</b>
ASAT	<i>r</i>	<b>0.2692</b>	<b>0.2514</b>	0.1606	<b>0.3884</b>	<b>0.3951</b>
n = 78	<i>P</i>	<b>0.0179</b>	<b>0.0319</b>	0.1630	<b>0.0005</b>	<b>0.0005</b>
ALAT	<i>r</i>	0.2174	0.2127	0.0290	0.2154	<b>0.2705</b>
n = 78	<i>P</i>	0.0575	0.0708	0.8025	0.0616	<b>0.0189</b>
Albumin	<i>r</i>	-0.2137	-0.0564	-0.1008	-0.1696	-0.0270
n = 67	<i>P</i>	0.0825	0.6608	0.4206	0.1700	0.8312
Age	<i>r</i>	<b>0.2760</b>	<b>0.2970</b>	<b>0.2095</b>	<b>0.4830</b>	<b>0.3585</b>
n = 90	<i>P</i>	<b>0.0092</b>	<b>0.0061</b>	<b>0.0487</b>	<0.0001	<b>0.0007</b>

Bold indicates significance.

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; AP, alkaline phosphatase; BMI, body mass index; GGT, gamma-glutamyl transferase.

cells, suggesting that at least in part the increase in oxidative stress in infected cells is due to mitochondrial/respiratory activity. Therefore, it will be interesting to

investigate whether glutaminase inhibitors reduce the oxidative and inflammatory environment that contributes to fibrosis progression in the infected liver.



**FIG. 6.** Glutamine is essential in many aspects of cell metabolism. After entering the cell through specific transporters, glutamine can be partially oxidized through glutaminolysis. The first and rate-limiting step is the conversion of glutamine to glutamate by the enzyme GLS. Glu can then enter the TCA cycle after being converted to  $\alpha$ KG. Gln is also a major precursor for nucleotide as well as glutathione synthesis. Abbreviations: Cit, citrate; Glc, glucose; Gln, glutamine; Glu, glutamate; GSH, glutathione; Lac, lactate; Mal, malate; Pyr, pyruvate.



Furthermore, we found that early glutamine withdrawal after HCV infection inhibited viral replication. Again, the reason is likely to be a drop in TCA cycle intermediates as the effect could be rescued by  $\alpha$ KG supplementation; however, the rescue was less efficient than rescue in the context of Gln withdrawal or GLS silencing for reasons that warrant further investigation.

It was recently shown that reductive carboxylation of glutamine-derived  $\alpha$ KG was the predominant source of *de novo* lipid synthesis in many tumor cells.<sup>(29-31)</sup> Considering the importance of lipids in the HCV life cycle,<sup>(6)</sup> reductive carboxylation from glutamine-derived carbons may be activated in HCV-infected cells to provide lipids in favor of the HCV life cycle. Further experiments are required to verify this hypothesis. In the present study, targeting MYC or GLS, through shRNA or pharmacological inhibitors, significantly attenuated HCV replication. Considering the major functions of MYC in the promotion of cell growth, many pathways are likely to be deregulated by its inhibition. However, targeting GLS more specifically suppresses glutaminolysis, which strengthens the important role of this pathway in HCV replication. Interestingly, glutaminase inhibitors have recently also been shown to display antiviral activity against adenovirus, herpes simplex virus 1, and influenza A,<sup>(32)</sup> suggesting that glutaminase inhibitors may be interesting to develop as general antivirals because they block the metabolic requirements for progeny virion production.

The importance of glutaminolysis in HCV replication *in vitro* was validated *in vivo* in HCV liver biopsies. MYC, SLC7A5, and both forms of GLS were increased in biopsies of HCV-infected patients compared to control liver biopsies derived from Hodgkin lymphoma, which are characterized by hepatic infiltration. Transcripts of glutaminolytic factors tended to decrease upon antiviral treatment, suggesting that glutaminolysis is at least partially reversible *in vivo*. Moreover, transcript levels of MYC, SLC1A5, and both forms of GLS progressively increased together with fibrosis stage, activity grade, and serum levels of several liver enzymes. These data suggest that although some direct effects of HCV are likely to participate, oxidative stress, inflammation, and liver disease may also be linked to and amplify the increase of glutaminolysis. The possibility of an indirect induction of glutaminolysis by HCV is furthermore supported by our finding that the levels of GLS expression did not always correlate with HCV protein levels in *in vitro* infected Huh7.5 cells. Surprisingly little is known on the links between glutaminolysis, oxidative stress, and immune

reactions; however, an increase in hepatic glutamine transport has been associated with inflammation in a model of endotoxemic rats.<sup>(33)</sup> In contrast to the other glutaminolytic factors, SLC7A5 levels were already higher in HCV patients with low or no liver damage and did not increase further with activity grade or with fibrosis stage. Interestingly, SLC7A5 is necessary for mammalian target of rapamycin activation, a factor that is induced in up to 50% of HCCs<sup>(34)</sup> and that has been shown to be required for HCV replication.<sup>(28,35,36)</sup>

Collectively, our data revealed a link between HCV infection and glutaminolysis. We show that viral replication depends on glutaminolysis using genetic and pharmacological approaches. Importantly, increased levels of glutaminolysis are likely to provide a metabolic environment in infected cells that predisposes to and favors the development of HCC. Thus, glutaminolysis inhibitors, currently in clinical trials for cancer treatment, may prove useful for prevention of fibrosis progression and cellular transformation particularly in patients at risk of disease progression toward HCC despite viral elimination with the newly available direct-acting antiviral agents.

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