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Malnutrition Impairs Interferon Signaling Through mTOR and FoxO Pathways in Patients With Chronic Hepatitis C

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BACKGROUND & AIMS: Patients with advanced chronic hepatitis C (CH-C) often are malnourished, but the effects of malnutrition on interferon (IFN) signaling and response to treatment have not been determined. We assessed the importance of the nutritional state of the liver on IFN signaling and treatment response. **METHODS:** We studied data from 168 patients with CH-C who were treated with the combination of pegylated-IFN and ribavirin. Plasma concentrations of amino acids were measured by mass spectrometry. Liver gene expression profiles were obtained from 91 patients. Huh-7 cells were used to evaluate the IFN signaling pathway, mammalian target of rapamycin complex 1 (mTORC1), and forkhead box O (FoxO). Antiviral signaling induced by branched-chain amino acids (BCAAs) was determined using the in vitro hepatitis C virus replication system. **RESULTS:** Multivariate logistic regression analysis showed that Fischer's ratio was associated significantly with nonresponders, independent of interleukin-28B polymorphisms or the histologic stage of the liver. Fischer's ratio was correlated inversely with the expression of BCAA transaminase 1, and was affected by hepatic mTORC1 signaling. IFN stimulation was impaired substantially in Huh-7 cells grown in medium that was low in amino acid concentration, through repressed mTORC1 signaling, and increased Socs3 expression, which was regulated by Foxo3a. BCAA could restore impaired IFN signaling and inhibit hepatitis C virus replication under conditions of malnutrition. **CONCLUSIONS: Malnutrition impaired IFN signaling by inhibiting mTORC1 and activating Socs3 signaling through Foxo3a. Increasing BCAAs to up-regulate IFN signaling might be used as a new therapeutic approach for patients with advanced CH-C.**

Keywords: HCV; Liver Disease; Therapy; Diet.

Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C), but approximately 50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.¹

Recent landmark studies of genome-wide associations identified genomic loci associated with treatment responses to pegylated (Peg)-IFN and RBV combination therapy,^{2,3} and a polymorphism in the interleukin (IL)-28B gene was found to predict hepatitis C treatment-induced viral clearance. Moreover, we previously showed that expression of hepatic IFN-stimulated genes (ISGs) was associated with the IL-28B polymorphism and might contribute to the treatment response.⁴ In addition to the IL-28B polymorphism, host factors such as fibrosis stage and metabolic status of the liver might be associated with the treatment outcome^{4,5}; however, the significance of these factors in conjunction with the IL-28B polymorphism has not been evaluated fully.

In CH-C livers, prolonged liver cell damage, fibrosis development, and microcirculation failure can lead to a state of malnutrition in hepatocytes, resulting in the impairment of multiple metabolic pathways. In patients with advanced stage CH-C, hypoalbuminemia and decreased plasma values for the Fischer's ratio of branched-amino acids (BCAA; leucine, isoleucine, and valine) to aromatic amino acids (tyrosine and phenylalanine) commonly are observed. BCAA are the essential amino acids necessary for ammonium metabolism in muscle when the liver is unable to perform this function. Recent reports have shown that BCAA activates albumin synthesis in rat

Abbreviations used in this paper: BCAA, branched-chain amino acid; BCAT1, branched chain amino-acid transaminase 1; CH-C, chronic hepatitis C; CHIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FBE, Foxo binding element; FBEmut, Foxo binding element mutant; FoxO, forkhead box, subgroup O; GLuc, Gaussia luciferase; IFN, interferon; IL, interleukin; ISG, interferon-stimulated genes; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NR, no response; PCR, polymerase chain reaction; Peg, pegylated; p-mTOR, phosphorylated form of mammalian target of rapamycin; pS6K, phosphorylated form of p70 S6 protein kinase; pSTAT1, phosphorylated form of signal transducer and activator of transcription 1; Raptor, regulatory associated protein of mTOR; RBV, ribavirin; S6K, p70 S6 protein kinase; siRNA, small interfering RNA; SVR, sustained viral response; TR, transient response.

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primary hepatocytes⁶ and cirrhotic rat liver⁷ through mammalian target of rapamycin (mTOR) signaling, a central regulator of protein synthesis, by sensing nutrient conditions.⁸ Thus, peripheral amino acid composition is closely related to signaling pathways in the liver.

In addition to metabolic aspects, recent reports have elucidated new functional roles for mTOR in the IFN signaling pathway. Targeted disruptions of tuberous sclerosis 2 and eukaryotic translation initiation factor 4E binding protein 1, which both inhibit mTOR complex 1 (mTORC1) signaling, substantially enhanced IFN- α -dependent antiviral responses.^{9,10} Therefore, mTORC1 signaling might be involved in the antiviral response as well as in metabolic processes. However, these issues have not yet been addressed in terms of IFN treatment for CH-C. In the present study, therefore, we evaluated the clinical relevance of the nutritional state of the liver, as estimated by the plasma Fischer's ratio, on Peg-IFN and RBV combination therapy. We also evaluated antiviral signaling induced by BCAA using an in vitro HCV replication system.

Materials and Methods

Patients

A total of 168 patients with CH-C at the Graduate School of Medicine at Kanazawa University Hospital (Kanazawa, Japan) and its related hospitals in Japan (Table 1, Supplementary Table 1) were evaluated in the present study. The clinical characteristics of these patients have been described previously.⁴ All patients were administered Peg-IFN- α 2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks. The definition of the treatment response was as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapse during the follow-up period; and no response (NR). Genetic variation of the IL-28B polymorphism at rs8099917 was evaluated in all patients using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) as described previously.⁴ Gene expression profiling in the liver was performed in 91 patients using the Affymetrix Human 133 Plus 2.0 microarray chip (Affymetrix, Santa Clara, CA) as described previously (Supplementary Table 1).⁴

Plasma Amino Acid Analysis

Amino acid concentrations in plasma samples were measured by high-performance liquid chromatography-electrospray ionization-mass spectrometry, followed by derivatization.¹¹ Detailed experimental procedures are described in the Supplementary Materials and Methods section.

Culture Medium

Huh-7 and Huh-7.5 cells (kindly provided by Professor C. M. Rice, Rockefeller University, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10%

fetal bovine serum and 1% penicillin/streptomycin (normal medium). Amino acid-free medium (ZERO medium) was prepared by mixing 5.81 g nutrition-free DMEM (Nacalai Tesque, Kyoto, Japan), 1.85 g NaHCO₃, 1 g glucose, and 0.5 mL 1M (mol/L) sodium pyruvate in 500 mL Milli-Q water, then sterilizing with a 0.22- μ m filter (Millipore, Billerica, MA). Low amino acid media ($\times 1/5$, $\times 1/10$, $\times 1/30$, and $\times 1/100$ DMEM) were prepared by diluting $\times 1$ DMEM with ZERO medium. Powdered BCAA (leucine-isoleucine-valine, 2:1:1.2) (Ajinomoto Pharma, Tokyo, Japan) was freshly dissolved with distilled water at 100 mmol/L, then applied to cultured medium at 2 mmol/L, 4 mmol/L, or 8 mmol/L.

Western Blotting and Immunofluorescence Staining

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium and maintained for up to 24 hours. Western blotting was performed as previously described.¹² Cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer containing complete Protease Inhibitor Cocktail and PhosSTOP (Roche Applied Science, Indianapolis, IN). The membranes were blocked in Blocking One-P (Nacalai Tesque). The antibodies used for Western blotting are summarized in the Supplementary Materials and Methods section.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS, then permeabilized with 0.1% Triton-X 100 in PBS. The primary anti-forkhead box O (Foxo)3a antibody (Abcam, Cambridge, MA) was used at a final concentration of 2 μ g/mL in PBS containing 2% fetal bovine serum at 4°C for 16 hours. Incubation with the Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA) at a 500-fold dilution in PBS containing 3% fetal bovine serum antibody was performed for 4 hours, and cells were stained with Hoechst 33258 to visualize nuclear DNA (Vector Laboratories, Burlingame, CA).

Quantitative Real-Time Detection Polymerase Chain Reaction

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium, to which IFN- α and/or BCAA was added, and maintained for 24 hours. Rapamycin treatment (100 nmol/L) was performed for 30 minutes in normal medium before a medium change. RNA was isolated using TriPure isolation reagent (Roche Applied Science), and complementary DNA (cDNA) was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real-time detection polymerase chain reaction (PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) containing specific primers according to the manufacturer's

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR	NR	Univariate P value	Multivariate odds (95% CI)	Multivariate P value
Patients, n	125	43		—	
Age and sex					
Age, y	57 (30–72)	56 (30–73)	.927	—	
Sex, male vs female	68 vs 57	24 vs 19	.872	—	
Liver histology					
F stage (F1–2 vs F3–4)	95 vs 30	20 vs 23	.001	6.35 (2.02–23.7)	.001
A grade (A0–1 vs A2–3)	68 vs 57	19 vs 24	.248	—	
Host gene factors					
IL-28B (TT vs TG/GG) ^a	109 vs 12	12 vs 31	<.001	19.7 (5.74–82.7)	<.001
ISGs (Mx, IFI44, IFIT1), (<3.5 vs ≥3.5)	103 vs 22	12 vs 31	<.001	5.26 (1.65–17.6)	.005
Metabolic factors					
BMI, kg/m ²	23.2 (16.3–34.7)	23.4 (19.5–40.6)	.439	—	
TG, mg/dL	98 (30–323)	116 (45–276)	.058	—	
T-Chol, mg/dL	167 (90–237)	160 (81–214)	.680	—	
LDL-Chol, mg/dL	82 (36–134)	73 (29–123)	.019	—	
HDL-Chol, mg/dL	42 (20–71)	47 (18–82)	.098	—	
FBS, mg/dL	94 (60–291)	96 (67–196)	.139	—	
Insulin, μU/mL	6.6 (0.7–23.7)	6.8 (2–23.7)	.039	—	
HOMA-IR	1.2 (0.3–11.7)	1.2 (0.4–7.2)	.697	—	
Fischer ratio	2.3 (1.5–3.3)	2.1 (1.5–2.8)	.005	8.91 (1.62–55.6)	.011
Other laboratory parameters					
AST level, IU/L	46 (18–258)	64 (21–283)	.017	—	
ALT level, IU/L	60 (16–376)	82 (18–345)	.052	—	
γ-GTP level, IU/L	36 (4–367)	75 (26–392)	<.001	—	
WBC, /mm ³	4800 (2100–11100)	4800 (2500–8200)	.551	—	
Hb level, g/dL	14 (9.3–16.6)	14.4 (11.2–17.2)	.099	—	
PLT, ×10 ⁴ /mm ³	15.7 (7–39.4)	15.2 (7.6–27.8)	.378	—	
Viral factors					
ISDR mutations ≤1 vs ≥2	80 vs 44	34 vs 9	.070	4.12 (1.25–15.9)	.019
HCV-RNA, KIU/mL	2300 (126–5000)	1930 (140–5000)	.725	—	
Treatment factors					
Total dose administered					
Peg-IFN, μg	3840 (960–7200)	3840 (1920–2880)	.916	—	
RBV, g	202 (134–336)	202 (36–336)	.531	—	
Achieved administration rate					
Peg-IFN, %					
≥80%	84	28	.975	—	
<80%	42	14			
RBV (%)					
≥80%	76	24	.745	—	
<80%	50	18			
Achievement of EVR	101/125 (81%)	0/43 (0%)	<.001	—	

BMI, body mass index; CI, confidence interval; FBS, fasting blood sugar; γ-GTP, gamma-glutamyl transpeptidase; Hb, hemoglobin; HDL-chol, high density lipoprotein cholesterol; LDL-chol, low density lipoprotein cholesterol; PLT, platelets; T-chol, total cholesterol; TG, triglycerides; WBC, leukocytes.

^aIL-28B SNP at rs8099917.

instructions. The primer sequence for real-time detection PCR is given in the Supplementary Materials and Methods section. HCV RNA was detected as described previously¹² and expression was standardized to that of glyceraldehyde-3-phosphate dehydrogenase.

Reporter Assay

Construction of the interferon stimulated response element (ISRE)-luc reporter plasmid and Socs3-luc or Socs3 (FoxO binding element mutant [FBEmut])-luc reporter plasmids is described in the Supplementary Materials and Methods section.

Huh-7 cells were transfected with the ISRE-luc reporter plasmid 24 hours before IFN-alfa treatment. Cells were

treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid media. After 24 hours, luciferase activities were measured using the Dual Luciferase assay system (Promega, Madison, WI). For Socs3 promoter activities, Huh-7 cells were transfected with Socs3-luc or Socs3 (FBEmut)-luc reporter plasmids together with the Foxo3a expression plasmid, and luciferase activities were measured after 24 hours. Values were normalized to the luciferase activity of the co-transfected pGL4.75 Renilla luciferase-expressing plasmid (Promega).

Knockdown Experiments

Huh-7 cells were transfected with Ctrl (Stealth RNAi Negative Control Low GC Duplex #2; Invitrogen) or

targets (regulatory associated protein of mTOR [Raptor] and Foxo3a) (Supplementary Materials and Methods) small interfering RNA (siRNA) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 hours, cells were cultured in normal or low-amino-acid media for a further 24 hours. The knockdown effect was confirmed by Western blotting.

Chromatin Immunoprecipitation Assay

Detailed experimental procedures are described in the Supplementary Materials and Methods section.

HCV Replication Analysis

pH77S3 is an improved version of pH77S, a plasmid containing the full-length sequence of the genotype 1a H77 strain of HCV with 5 cell culture-adaptive mutations that promote its replication in Huh-7 hepatoma cells.¹³ pH77S.3/Gaussia luciferase (GLuc)2A is a related construct in which the GLuc sequence, fused to the 2A autocatalytic protease of foot-and-mouth virus RNA, was inserted in-frame between p7 and NS2¹⁴ (Supplementary Materials and Methods). A signal sequence in GLuc directs its secretion into cell culture media, allowing real-time, dynamic measurements of GLuc expression to be performed without the need for cell lysis.

A 10- μ g aliquot of synthetic RNA transcribed from pH77S.3/GLuc2A was used for electroporation. Cells were pulsed at 260 V and 950 μ F using the Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA) and plated in fresh normal medium for 12 hours to recover. Cell medium was changed to \times 1 DMEM without serum for 8 hours, then changed to low-amino-acid medium containing 0–8 mmol/L BCAA for a further 24 hours. Cells and culture medium were collected and used for GLuc assays, real-time detection PCR, and Western blotting. The number of viable cells was determined by a (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) assay (Promega).

Continuously JFH-1-infecting Huh-7 cells were obtained by the infection of Huh-7 cells with JFH-1 cell culture-derived HCV at a multiplicity of infection of 0.01. Cells were maintained in normal medium by passaging every 3–4 days for approximately 6 months. About 20%–30% of the cells consistently were positive for HCV core protein (Supplementary Figure 4). Culture medium of JFH-1-infecting Huh-7 cells was changed to the low-amino-acid medium containing 0–8 mmol/L BCAA for 24 hours. Cells then were collected and used for assays.

Statistical Analysis

Results are expressed as mean \pm standard deviation. Significance was tested by 1-way analysis of variance with the Bonferroni method, and differences were considered statistically significant at a *P* value of less than .05.

Results

Fischer's Ratio as a Predictive Factor for Treatment Response

The clinical characteristics of patients who received Peg-IFN and RBV combination therapy are shown in Table 1 and Supplementary Table 1, and explanations of these characteristics have been described previously.⁴ All patients were infected with HCV genotype 1b and had a high viral load (>100 IU/mL). We compared patients with SVR + TR against those with NR, as assessed by the overall plausibility of treatment response groups using Fisher's C statistic as previously described.⁴ We included data on the IL-28B polymorphism and plasma amino acid composition (aminogram).

Univariate regression analysis showed that no single amino acid was associated significantly with treatment response; however, using Fischer's ratio, the BCAA (Ile+Leu+Val)/aromatic amino acids (Phe+Tyr) ratio was associated significantly with treatment response (*P* = .005) (Table 1). Of the 121 patients with IL-28B major type, SVR, TR, and NR were observed in 53%, 37%, and 10%, respectively, and among 33 patients with IL-28B minor type, SVR, TR, and NR were observed in 15%, 17%, and 68%, respectively (*P* < .001) (data not shown). Fischer's ratio of SVR, TR, and NR was 2.35 ± 0.38 , 2.30 ± 0.29 , and 2.10 ± 0.31 , respectively (*P* < .015) (data not shown).

We selected IL-28B polymorphism, hepatic ISG expression, fibrosis stage, HCV RNA, interferon sensitivity determining region mutation, and Fischer's ratio as factors for multivariate analysis. Multivariate analysis revealed that the minor type of IL-28B polymorphism (TG or GG at rs8099917) (odds ratio, 19.7; *P* < .001), advanced fibrosis stage of the liver (F3–4) (odds ratio, 6.35; *P* = .001), high hepatic ISGs (≥ 3.5) (odds ratio, 5.26; *P* = .005), low Fischer's ratio (continuous range, 1.5–3.3) (unit odds, 8.91; *P* = .011), and presence of ISDR mutation (≤ 1) (odds ratio, 4.12; *P* = .019) independently contributed to NR (Table 1).

The distribution of the Fischer's ratio according to fibrosis stage is shown in Supplementary Figure 1. The ratio decreased significantly in advanced fibrosis stage (F3–4) compared with early fibrosis stage (F1). No significant association between major or minor type of IL-28B polymorphism and different fibrosis stages of the liver was observed (Supplementary Figure 1A). In early fibrosis (F1–2) (Supplementary Figure 1B), 90% (80 of 89) of SVR+TR cases had the major type of IL-28B polymorphism, and 94% (16 of 17) of NR cases had the minor type. However, in the advanced fibrosis stage of the liver (F3–4) (Supplementary Figure 1C), 85% (23 of 27) of SVR+TR cases had the major type of IL-28B polymorphism and 50% (10 of 20) of NR cases had the minor type. Thus, in advanced fibrosis stages, factors other than the IL-28B polymorphism appear to contribute to NR. Interestingly, the Fischer's ratio was significantly lower in NR patients than SVR+TR pa-

tients in the advanced fibrosis stage of the liver. Therefore, Fischer's ratio could be an important predictor for NR that is independent of IL-28B polymorphism and histologic stage of the liver.

Fischer's Ratio and mTORC1 Signaling in CH-C Livers

Hepatic gene expression in 91 of 168 patients (Supplementary Table 1) was obtained using Affymetrix genechip analysis as described previously.⁴ To examine the relationship between the plasma Fischer's ratio and mTORC1 signaling in the liver we evaluated the expression of key regulatory genes related to mTORC1 signaling. We found that expression of branched chain amino acid transaminase 1 (BCAT1), an important catalytic enzyme of BCAA, was significantly negatively correlated with Fischer's ratio (Figure 1A). This indicates that the plasma Fischer's ratio is regulated in the liver as well as in peripheral muscle. Interestingly, the expression of c-myc, a positive regulator of BCAT1 (Figure 1C),¹⁵ was correlated negatively with the Fischer's ratio (Figure 1B). The expression of PDCD4, a negative transcriptional target of ribosomal p70 S6 protein kinase (S6K), downstream of mTORC1, was correlated significantly with BCAT1 (Figure 1D and E). Thus, in CH-C livers, BCAT1 is induced with progressive liver disease and mTORC1 signaling is repressed, a process that might involve c-myc. Fischer's ratio of the plasma therefore can be seen to reflect mTORC1 signaling in the liver.

Impaired IFN Signaling in Huh-7 Cells Grown in Low-Amino-Acid Medium

Recent reports have shown the functional relevance of mTOR on IFN signaling and antiviral responses.^{9,10} To evaluate IFN- α signaling and the mTOR pathway, we used Huh-7 cells grown in different amino acid conditions ($\times 1$ DMEM, $\times 1/5$ DMEM, $\times 1/30$ DMEM, and $\times 1/100$ DMEM). The phosphorylated forms of mTOR (p-mTOR) and S6K (pS6K), an important downstream regulator of mTORC1 signaling, were decreased substantially in $\times 1/30$ DMEM and $\times 1/100$ DMEM (Figure 2A). Interestingly, the expression of the phosphorylated form of signal transducer and activator of transcription 1 (pSTAT1), an essential transducer of type 1 IFN signaling, also was decreased in these conditions (Figure 2A). Similarly, the expression of p-mTOR and pSTAT1 was repressed significantly in CH-C livers with a low Fischer's ratio compared with those with a high Fischer's ratio (Supplementary Figure 2, Supplementary Table 2).

To examine whether decreased pSTAT1 expression might be owing to repressed mTORC1 signaling, we knocked down the expression of Raptor, a specific subunit of mTORC1. We achieved more than 50% knockdown of Raptor by specific siRNA (Figure 2B). Under these conditions, the expression of p-mTOR and pS6K were repressed, which is consistent with previous reports.¹⁶ The expression of pSTAT1 also was repressed after Raptor knockdown (Figure 2B).

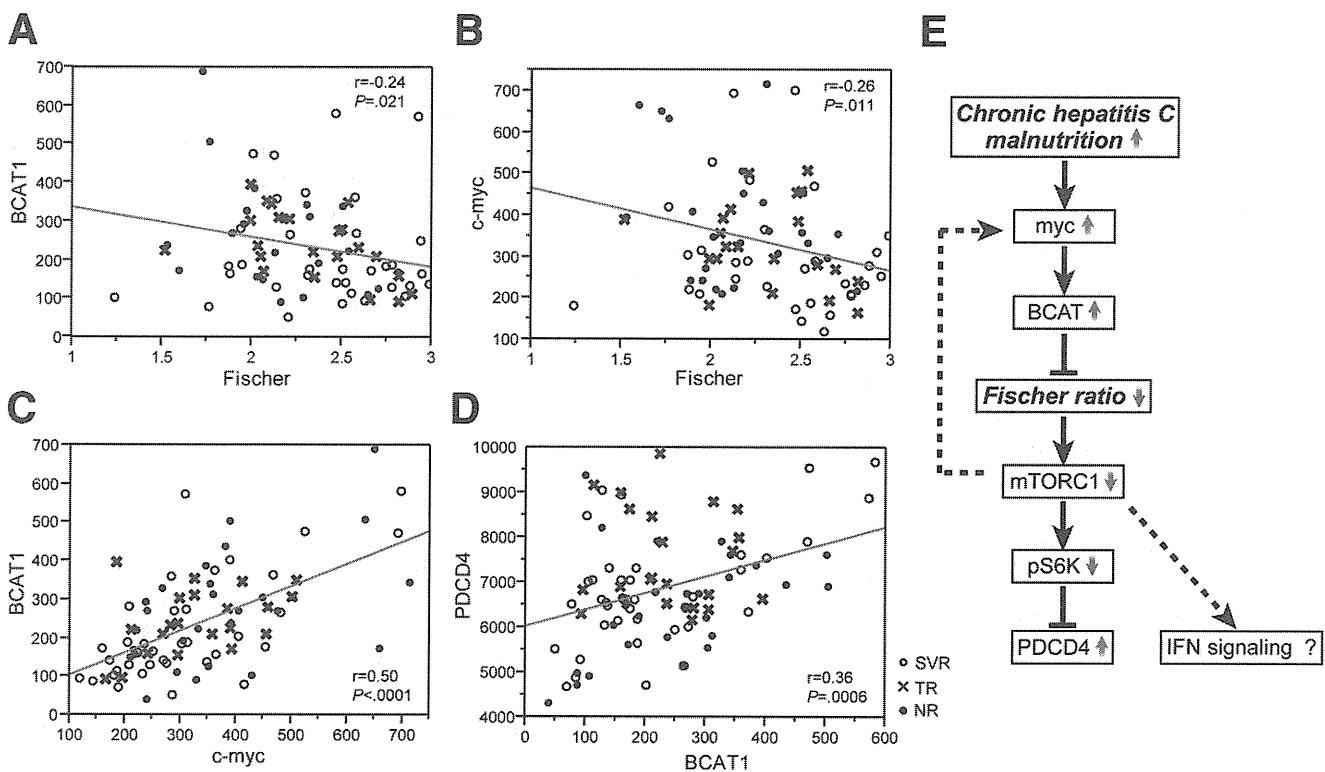


Figure 1. Regression analysis of mTORC1-related gene expression in liver. Gene expression values were determined by probe intensities. (A) BCAT1 and Fischer's ratio. (B) c-myc and Fischer's ratio. (C) BCAT1 and c-myc. (D) PDCD4 and BCAT1. (E) Putative signaling of mTORC1-related genes in CH-C.

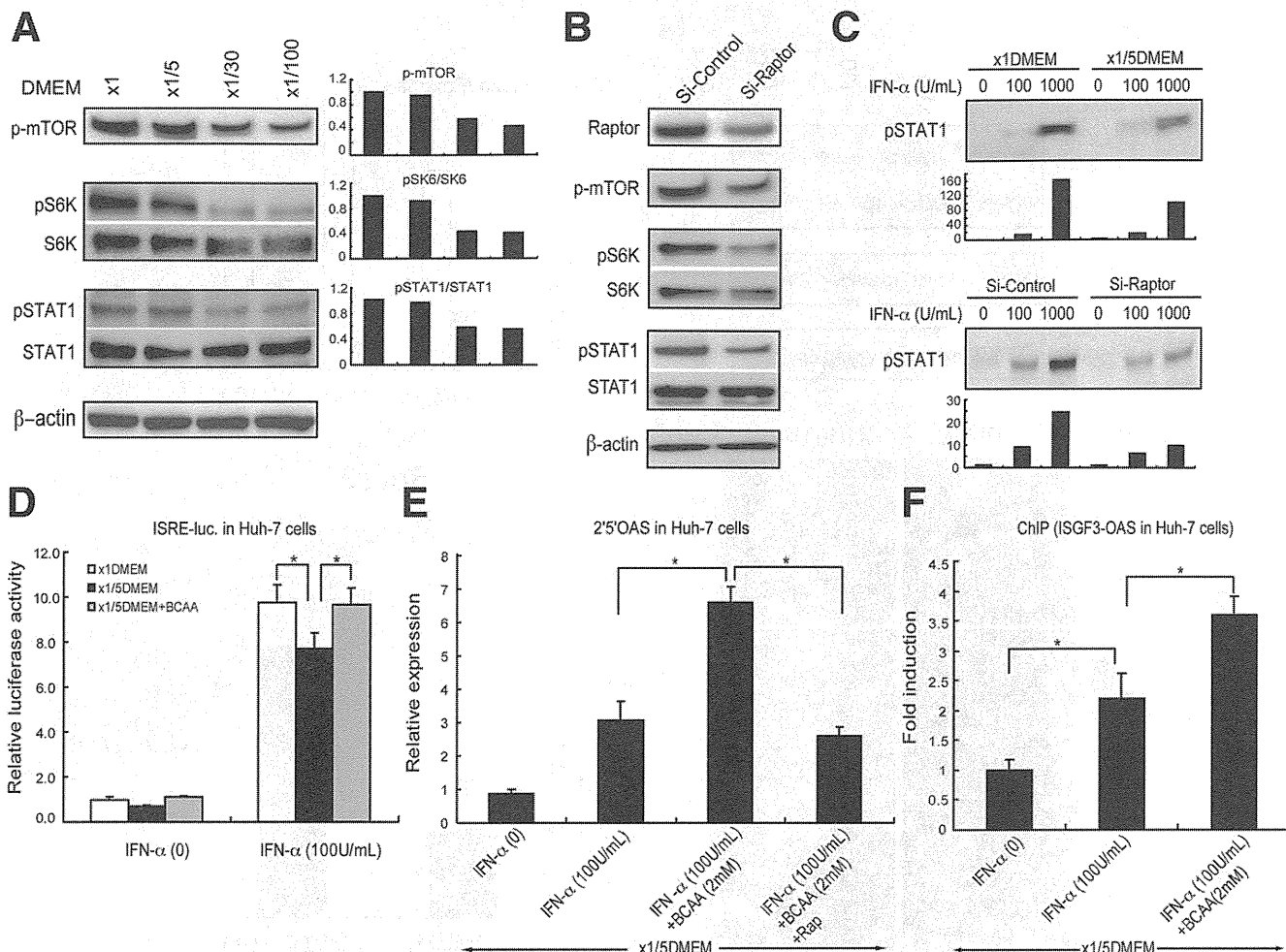


Figure 2. mTORC1 and IFN signaling in Huh-7 cells in low-amino-acid medium. (A) p-mTOR, pS6K, and pSTAT1 expression in different amino acid media. (B) p-mTOR, pS6K, and pSTAT1 expression under Raptor knock-down conditions. (C) IFN-alfa stimulation and pSTAT1 expression in low-amino-acid media or under Raptor knock-down conditions. (D) IFN-alfa stimulation and ISRE reporter activities in normal and low-amino-acid media. (E) IFN-alfa stimulation and 2'5'OAS expression in low-amino-acid medium supplemented with BCAA or rapamycin. (F) Chromatin immunoprecipitation of 2'5'OAS promoter region by ISGF3 γ .

The induction of pSTAT1 by IFN-alfa (1000 U/mL) stimulation was impaired in $\times 1/5$ DMEM or in Raptor knocked-down condition, compared with the control (Figure 2C). Consistent with these results, IFN-alfa-induced ISRE-dependent transcriptional activity, as measured using an ISRE-luciferase reporter assay, was impaired significantly in $\times 1/5$ DMEM compared with $\times 1$ DMEM (Figure 2D). However, this activity could be rescued by the addition of 2 mmol/L BCAA (Figure 2D). These results were confirmed by determining the expression of the endogenous IFN-alfa responsive gene, 2'5'OAS, using quantitative reverse-transcription PCR. Figure 2E shows that BCAA treatment augmented 2'5'OAS expression in low levels of amino acids, and that this could be reversed by the addition of rapamycin, an inhibitor of mTORC1 (Figure 2E). Furthermore, chromatin immunoprecipitation (ChIP) experiments revealed that transcriptional augmentation by BCAA was mediated by the binding of the IFN-alfa-inducible transcription factor, ISGF3 γ , to the promoter region of 2'5'OAS (Figure 2F). These results indicate that

amino acids in culture media play an essential role in IFN-alfa signaling through mTORC1 signaling, and that the addition of BCAA can overcome impaired IFN-alfa signaling in Huh-7 cells.

Induction of Socs3 in Low-Amino-Acid Medium in Huh-7 Cells

Besides being involved in mTOR signaling, Foxo transcriptional factors mediate another important branch of nutrition-sensing signaling pathway.¹⁷ Therefore, we evaluated forkhead box O3A (Foxo3a) expression in low-amino-acid conditions in Huh-7 cells. After 6 hours culture in $\times 1/5$, $\times 1/30$, and $\times 1/100$ DMEM, expression of the phosphorylated form of Foxo3a (pFoxo3a) decreased, whereas that of total Foxo3a increased in $\times 1/5$ and $\times 1/30$ DMEM, and the ratio of pFoxo3a to Foxo3a (pFoxo3a/Foxo3a) substantially decreased (Figure 3A and B). It has been reported that dephosphorylated Foxo3a is translocated to the nucleus before activation of its target genes.¹⁸ In the present study, immunofluorescent staining

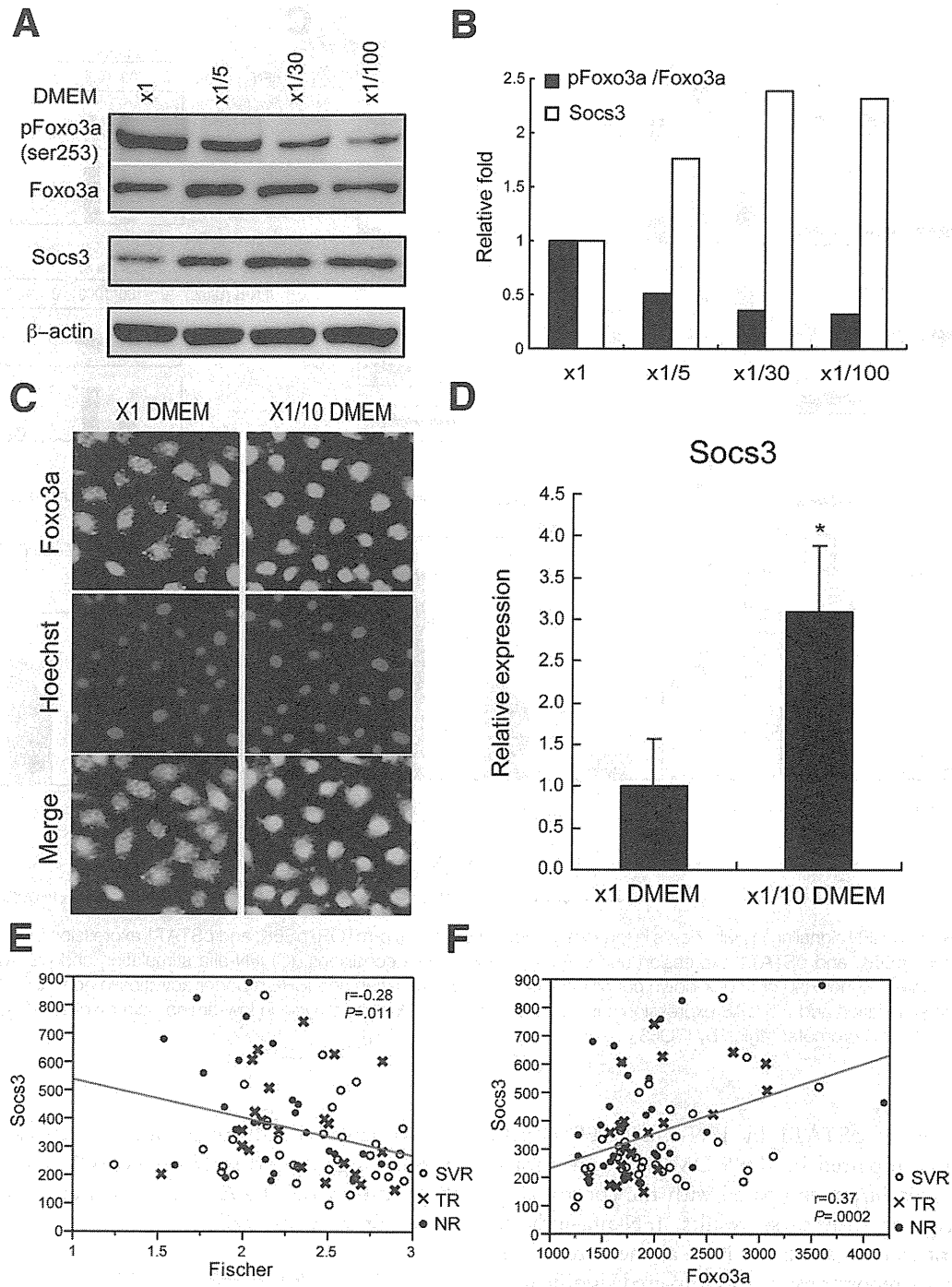


Figure 3. Foxo3a and Socs3 signaling in Huh-7 cells in low-amino-acid medium. (A) Foxo3a and Socs3 expression in different amino acid media. (B) Relative change of pFoxo3a/Foxo3a and Socs3 expression in different amino acid media. (C) Immunofluorescence staining of Foxo3a in Huh-7 cells in normal and low-amino-acid media. (D) Relative change of Socs3 messenger RNA in Huh-7 cells in normal and low-amino-acid media. (E) Regression analysis of Socs3 in liver and Fischer's ratio. (F) Regression analysis of Socs3 and Foxo3a in liver.

with an anti-Foxo3a antibody showed that Foxo3a diffused in both the cytoplasm and nucleus in normal amino acid medium, but localized in the nucleus in low-amino-acid medium ($\times 1/10$ DMEM) (Figure 3C).

Interestingly, in low-amino-acid medium, transcription and protein expression of Socs3 increased significantly (Figure 3A, B, and D). The induction of Socs3 in a state of malnutrition also was confirmed in clinical samples. In CH-C livers there was a significant negative correlation

between the plasma Fischer's ratio and Socs3 expression, implying that Socs3 expression increases during the malnutrition state induced by CH-C. There was also a significant correlation between Foxo3a and the transcriptional level of Socs3 in CH-C livers (Figure 3E and F), suggesting an *in vitro* and *in vivo* biological role for Foxo3a in the activation of Socs3 expression. These findings also were confirmed by Western blotting of CH-C livers (Supplementary Figure 2, Supplementary Table 2).

Socs3 Is a Transcriptional Target of Foxo3a

The significant correlation between *Socs3* and *Foxo3a* in CH-C livers prompted us to analyze the *Socs3* promoter sequence and, in doing so, we identified a putative *Foxo* binding element (FBE) (Figure 4A). To investigate the functional relevance of *Foxo3a* in the transcriptional regulation of *Socs3*, we constructed reporter plasmids containing a luciferase coding region fused to the *Socs3* promoter region (*Socs3-luc*). *Socs3-luc* promoter activity was increased substantially by the overexpression of *Foxo3a* (Figure 4B). The mutations introduced in the putative FBE (FBEmut) in the *Socs3* promoter significantly reduced *Foxo3a*-induced *Socs3* promoter activation (Figure 4B).

Foxo3a then was knocked down by siRNA and *Socs3* induction was evaluated. After suppression of *Foxo3a* (Supplementary Figure 3), *Socs3* promoter activity was repressed significantly in low-amino-acid medium ($\times 1/10$ DMEM) (Figure 4C). Thus, *Foxo3a* appears to be indispensable for activating the *Socs3* promoter under low-amino-acid conditions. Correlating with these results, ChIP assays using an anti-*Foxo3a* antibody showed a significant increase in the association between *Foxo3a* and the FBE of the *Socs3* promoter in low-amino-acid conditions ($\times 1/10$ DMEM) (Figure 4D). Taken together, these results suggest that, besides mTORC1 signaling, the *Foxo3a*-mediated *Socs3* signaling pathway might contribute to impaired IFN signaling in a state of malnutrition in CH-C. BCAA potentially restores this signaling (Figure 4E).

Effect of BCAA on HCV Replication in Huh-7 or Huh-7.5 Cells

Based on the earlier-described results, we used 2 HCV in vitro replication systems to examine whether BCAA affects HCV replication in Huh-7 or Huh-7.5 cells. The first system used a recombinant infectious genotype 1a clone, H77S.3/GLuc2A (Supplementary Materials and Methods, Supplementary Figure 4), including reporter genes, whereas the second used continuously JFH-1-infecting Huh-7 cells (Supplementary Materials and Methods).

The synthetic RNA transcribed from pH77S.3/GLuc2A was introduced into Huh-7.5 cells and replication of H77S.3/GLuc2A was evaluated in normal or low-amino-acid medium supplemented with BCAA. H77S.3/GLuc2A increased significantly by 2.6-fold in Huh-7.5 cells grown in low-amino-acid medium ($\times 1/5$ DMEM) compared with normal amino acid medium ($\times 1$ DMEM). Interestingly, BCAA repressed H77S.3/GLuc2A replication in a dose-dependent manner (Figure 5A). In agreement with these results, the expression of *Mx-1* was increased significantly by the addition of BCAA (Figure 5B). Similar findings were observed in JFH-1-infecting Huh-7 cells (Materials and Methods, Supplementary Figure 4). Although no obvious increase in HCV replication was observed in low-amino-acid medium ($\times 1/5$ DMEM) com-

pared with normal amino acid medium ($\times 1$ DMEM), JFH-1 replication was repressed significantly by the addition of BCAA in a dose-dependent manner (Figure 5D). The expression of *Mx-1* was increased substantially by the addition of BCAA (Figure 5E), suggesting that BCAA significantly repressed HCV replication in cells with either naive or persistent HCV infection. Importantly, there were no significant differences in cell viability between the conditions (Figure 5C and F).

To validate these findings, signaling pathways in HCV replicating cells were examined (Figure 6A and B). BCAA increased pS6K in a dose-dependent manner, implying its involvement in the activation of mTORC1 signaling. Related to this, expression of pSTAT1 was shown to be increased and the ratio of pSTAT1 to total STAT1 (pSTAT1/STAT1) increased 2.5- to 3-fold after the addition of BCAA. Thus, BCAA activated mTORC1 and the JAK-STAT signaling pathway in HCV-infected cells. In addition, the expression ratio of pFoxo3a to total Foxo3a (pFoxo3a/Foxo3a) increased 3- to 4-fold, indicating an increase in the cytoplasmic form of *Foxo3a* that is exposed to proteasome degradation. Concordant with these findings, we observed a decrease in the expression of *Socs3*. In addition, expression of the HCV core protein decreased as shown in Figure 6A and B. Thus, these results clearly show that BCAA repressed HCV replication through activation of IFN signaling and repression of *Socs3*-mediated IFN inhibitory signaling, as proposed in Figure 4E.

Discussion

Thompson et al⁵ showed that the IL-28B polymorphism, HCV RNA, nationality (Caucasian/Hispanic vs African American), hepatic fibrosis stage, and fasting blood sugar level are all significant variables for achieving SVR in patients infected with genotype 1 HCV. However, the significance of variable factors for treatment response in conjunction with the IL-28B polymorphism has not been evaluated fully. In the present study, in addition to previously examined variables,⁴ we included the plasma Fischer's ratio as a nutritional parameter. Multivariate analysis showed that the minor type of IL-28B polymorphism, advanced fibrosis stage, high hepatic ISGs, low Fischer's ratio, and ISDR mutation (≤ 1) independently contributed to NR (Table 1). Interestingly, among patients of similar fibrosis stage (F3-4), the Fischer's ratio was significantly lower in NR than SVR+TR cases. Therefore, the plasma value of Fischer's ratio was associated with the treatment response that was independent of the IL-28B polymorphism and histologic stage of the liver, although patients with advanced hepatic fibrosis are likely to be nutritionally affected.

As a nutrient sensor signaling pathway, the protein kinase mTOR plays an essential role in maintaining homeostasis and regulates protein synthesis in response to nutrient conditions. mTOR is the catalytic subunit of 2 distinct complexes, mTORC1 and mTORC2. In addition

A

Socs3 promoter

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Human  --CGCCCTCG GCGCCCGCGG CCCCTCCCTC ACCCTCCGCG CTCAGCCTTT CTCTGCTGCG
      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
Mouse  TCCAAGCCCG CCCTCCGCGG CCCCTCCCTC GCCCTCCGCG CACAGCCTTT CAGTGC--AG

      FBE                               GAS
AGTAGTGACT AAACATTACA AGAAGGCCGG CCGCGCAGTT CCAGGAATCG GGGGGCGGGG
      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
AGTAGTGACT AAACATTACA AGAAGACC GG CCGCGCAGTT CCAGGAATCG GGGGGCGGGG

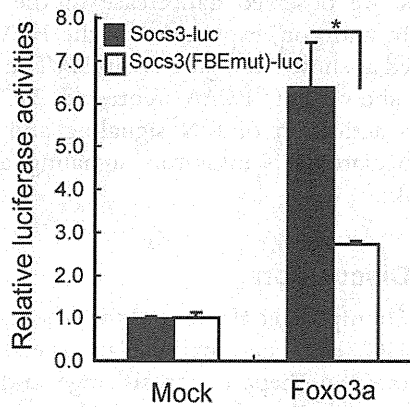
      TATA                               Transcription start site
CGCGGCGGCC GCCTATATAC CCGCGAGCGC GGCCTCCGCG GCGGCTC
      |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
CGTACTGGCC GGGTAAATAC CCGCGCGCGC GGCCTCCGAG GCGGCTC
    
```

FBE of Socs3 promoter

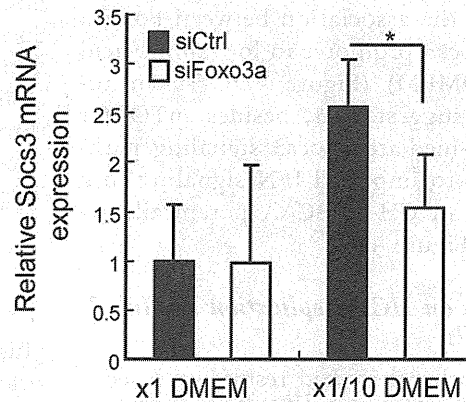
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Wild seq.      TGACTAAACATTACA
Mutated seq.   TGACTCACCATTACA
Consensus seq. (G/A)TAAA(T/C)A
    
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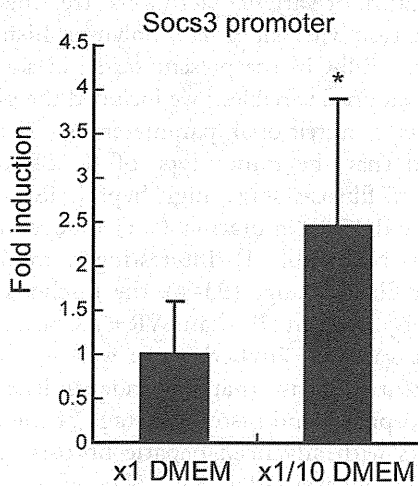
B



C



D



E

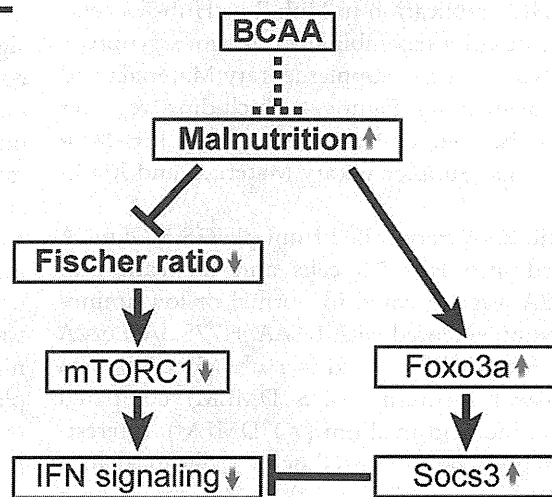


Figure 4. Socs3 promoter assay. (A) Primary structure of putative Foxo binding element in Socs3 promoter region. (B) Socs3-luc and Socs3 (FBEmut)-luc activities after overexpression of Foxo3a in Huh-7 cells. (C) Relative Socs3 messenger RNA (mRNA) expression after knockdown of Foxo3a in normal and low-amino-acid media. (D) Chromatin immunoprecipitation of Socs3 promoter region by Foxo3a in normal and low-amino-acid media. (E) Model of impaired IFN signaling by repressed mTORC1 signaling and increased Socs3 signaling under CH-C state of malnutrition.

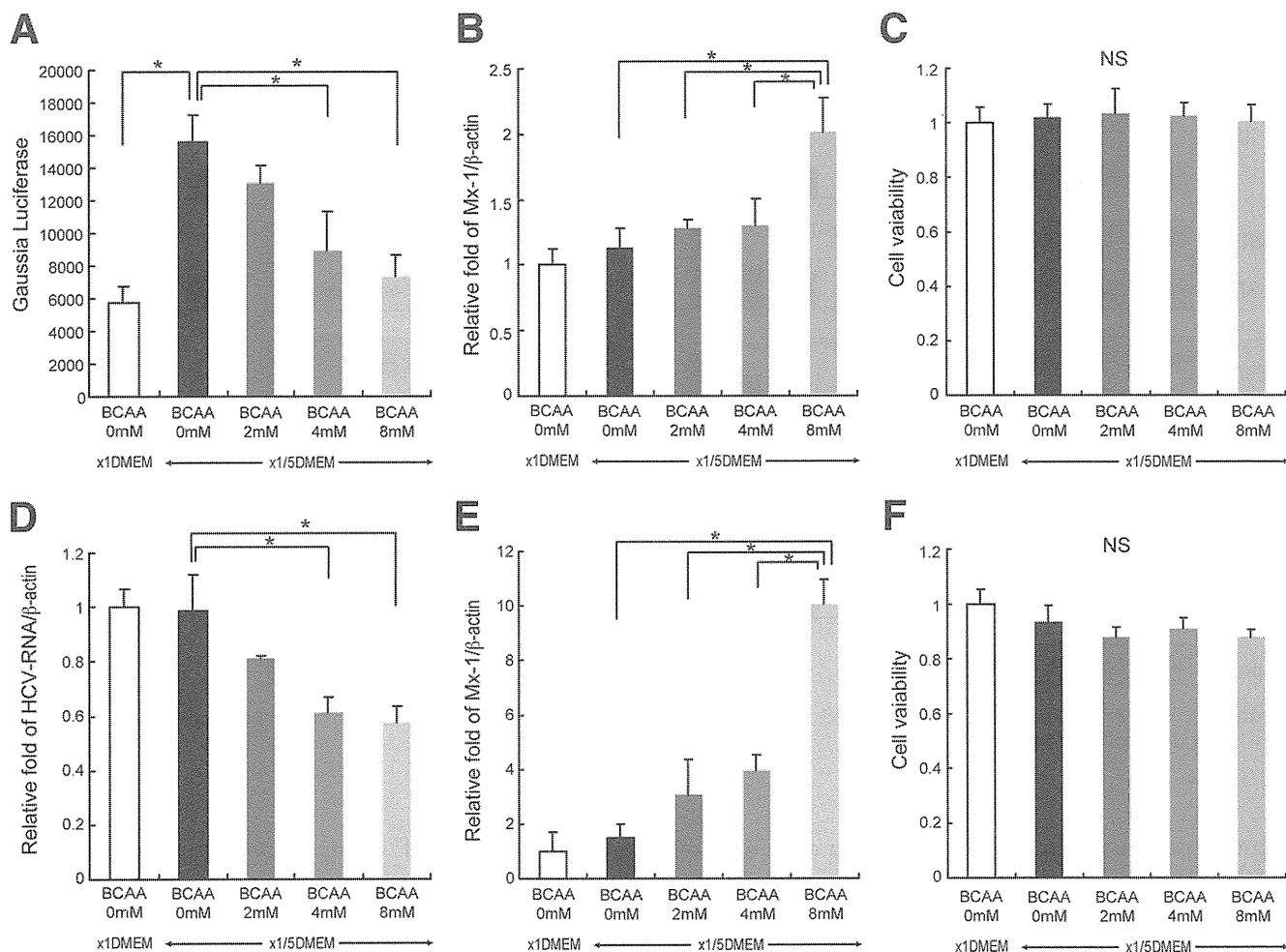


Figure 5. Effect of BCAA on HCV replication in cells in low-amino-acid medium. (A) Effect of BCAA on H77S.3/GLuc2A replication in Huh-7.5 cells. (B) Mx-1 expression in H77S.3/GLuc2A-transfected Huh-7.5 cells supplemented with BCAA. (C) Viability of Huh-7.5 cells. (D) Effect of BCAA on JFH-1 replication continuously infecting Huh-7 cells. (E) Mx-1 expression in continuously JFH-1-infecting Huh-7 cells supplemented with BCAA. (F) Viability of Huh-7 cells.

to these metabolic aspects, recent reports have shown that mTORC1 participates in IFN signaling and antiviral defense responses,^{9,10} although the precise signaling pathway has not yet been clarified. In the present study, we evaluated mTORC1 signaling in CH-C livers using gene expression profiling of 91 patients (Figure 1, Supplementary Table 1). We observed a significant negative correlation between plasma Fischer’s ratio and hepatic expression of BCAT1, an important catalytic enzyme of BCAA (Figure 1A). Moreover, BCAT1 expression was correlated positively with PDCD4 expression, which in turn is regulated negatively by pS6K at the transcriptional level (Figure 1D).¹⁶ Thus, the expression of BCAT1 appears to be a negative indicator of mTORC1 signaling in the liver, and the plasma Fischer’s ratio is partially reflected by mTORC1 signaling in the liver and muscle.

Interestingly, the expression of c-myc was correlated significantly with BCAT1 (Figure 1C) as reported previously.¹⁵ Several studies observed up-regulated c-myc expression in advanced stages of CH-C¹⁹ but, on the other hand, c-myc recently was shown to be a target of

mTORC1 in hepatic cells.¹⁷ The existence of a feedback mechanism between c-myc and mTORC1 signaling to maintain liver homeostasis (Figure 1E) is plausible, although the precise mechanisms need to be confirmed.

Impaired mTORC1 signaling is suggested to affect the IFN- α -induced signaling pathway. To address this, the relationship between mTORC1 and IFN signaling was assessed using a cell culture system. In low-amino-acid medium ($\times 1/5$, $\times 1/30$, and $\times 1/100$ DMEM), expression of pSTAT1 was decreased substantially, correlating with the impaired mTORC1 signaling represented by decreased p-mTOR and pS6K expression in Huh-7 cells (Figure 2A).

The relationship between mTORC1 and IFN signaling was confirmed further by the knock-down experiment of Raptor, a specific subunit of mTORC1 (Figure 2B), although a more precise analysis should be performed to confirm this relationship. Importantly, when Huh-7 cells were stimulated by IFN- α , pSTAT1 induction was repressed significantly in low-amino-acid medium ($\times 1/5$ DMEM) or in Raptor knocked-down conditions (Figure 2C). It therefore could be speculated that IFN treat-

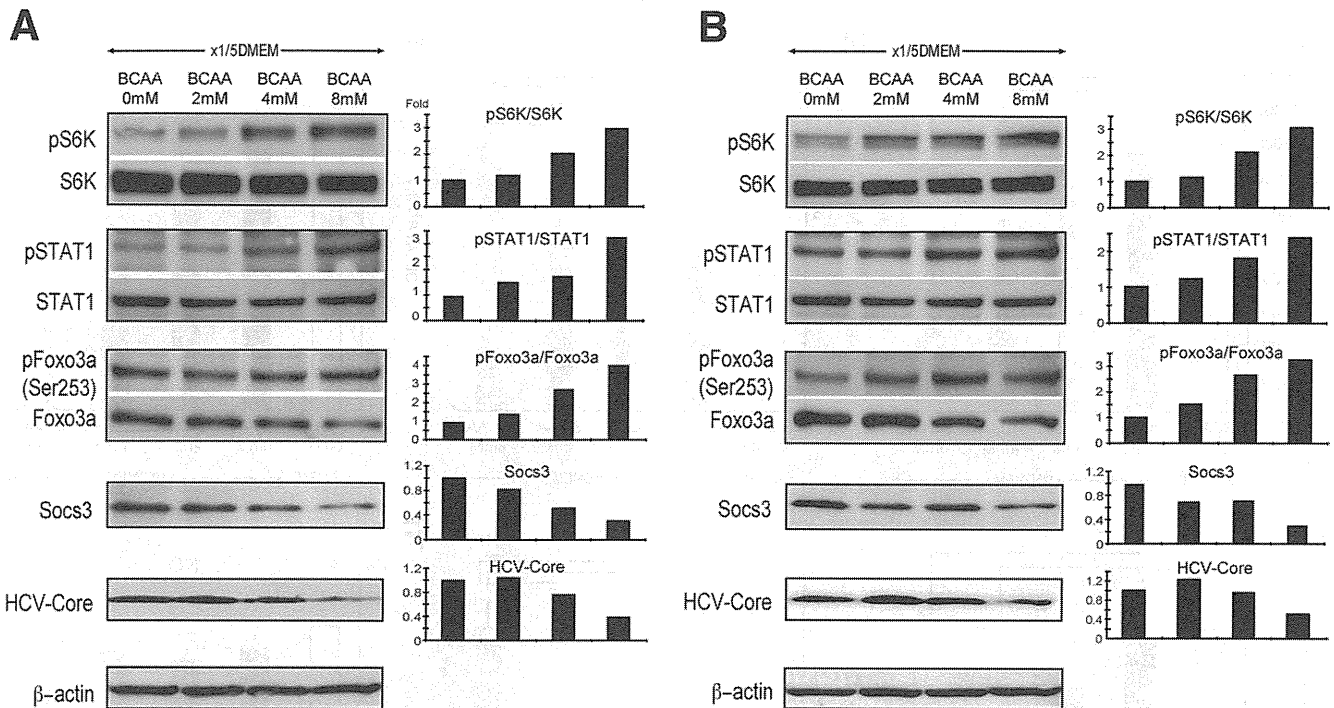


Figure 6. Expression of S6K, STAT1, Foxo3a, Socs3, and HCV core in H77S.3/GLuc2A-transfected Huh-7.5 cells or continuously JFH-1-infected Huh-7 cells supplemented with BCAA.

ment of patients with liver malnutrition and impaired mTORC1 signaling would lead to reduced induction of ISGs. Importantly, BCAA was able to restore impaired IFN signaling through increased binding of ISGF3 γ to its targets (Figure 2D–F).

Besides cross-talk of mTORC1 and IFN signaling, we revealed that Foxo3a also is involved in the IFN inhibitory pathway. In low-amino-acid medium, expression of pFoxo3a (ser253) was decreased substantially whereas that of Socs3 was increased. A decreased pFoxo3a/Foxo3a ratio indicates nuclear accumulation of Foxo3a before activation of its target genes, and this was confirmed by immunofluorescent staining (Figure 3C). The expression of Foxo3a was significantly positively correlated with that of Socs3 in CH-C liver (Figure 3F). These findings prompted us to identify a putative FBE in the Socs3 promoter region (Figure 4A). In fact, Socs3 promoter reporter activity was activated by overexpression of Foxo3a, and mutation of FBE impaired Foxo3a-dependent Socs3 promoter activation. Conversely, induction of Socs3 was not observed when expression of Foxo3a was knocked down by siRNA in low-amino-acid medium. Socs3 induction in low-amino-acid medium was owing to increased binding of Foxo3a to the FBE, which was confirmed by ChIP (Figure 4D). Therefore, in addition to impaired mTORC1 signaling, the Foxo3a-mediated Socs3 IFN inhibitory pathway might be involved in impaired IFN signaling in patients with liver malnutrition (Figure 4E).

Finally, we examined whether BCAA could restore impaired IFN signaling and inhibit HCV replication in cells

under conditions of malnutrition. Importantly, BCAA could repress replication of the recombinant genotype 1a-derived HCV, H77S.3/GLuc2A, in a dose-dependent manner (Figure 5A). H77S.3/GLuc2A RNA produces infectious virus¹⁴ and, therefore, the results indicate that BCAA might act on a naive HCV infection. Moreover, BCAA inhibited JFH-1-infected Huh-7 cells in which JFH-1 continuously was infecting in a dose-dependent manner. These results indicate that BCAA had an inhibitory effect on either naive or persistent HCV infection irrespective of genotypes (1a and 2a). Consistent with these results, BCAA induced the expression of pSTAT1 and Mx protein in a dose-dependent manner, and repressed Socs3 expression through increasing the ratio of pFoxo3a (ser243) to Foxo3a in a dose-dependent manner (Figures 5 and 6). Therefore, BCAA potentially could restore impaired IFN signaling and inhibit HCV replication in a CH-C state of malnutrition.

In conclusion, we addressed the clinical significance of the nutritional state of the liver on the treatment response of Peg-IFN and RBV combination therapy for CH-C. Although further studies are required to fully define the precise mechanisms underlying mTOR and IFN signaling, we showed that plasma values of Fischer's ratio are a useful nutritional parameter associated with treatment response. Fischer's ratio reflects mTORC1 signaling in the liver, which is correlated with IFN signaling and related to Socs3 IFN inhibitory signaling through Foxo3a. The potential usefulness of BCAA for the augmentation of IFN signaling could suggest a new therapeutic application for advanced-stage CH-C.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.03.051.

Appendix A

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Acknowledgments

Participating investigators from the Hokuriku Liver Study Group are listed in Appendix A.

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Conflicts of interest

The authors disclose no conflicts.

Supplementary Materials and Methods

Plasma Amino Acid Analysis

Plasma sample amino acid concentrations were measured by high-performance liquid chromatography–electrospray ionization–mass spectrometry followed by derivatization.¹ An MSQ Plus LC/MS system (Thermo Fischer Scientific, Waltham, MA) equipped with an electrospray ionization source was used in positive ionization mode for selected ion monitoring. Xcalibur version 1.4 SR1 software (Thermo Fischer Scientific, Yokohama, Japan) was used for data collection and processing. The high-performance liquid chromatography separation system consisted of an L-2100 pump, L-2200 autosampler, and L-2300 column oven (Hitachi High-Technologies Corporation, Tokyo, Japan). A Wakosil-II 3C8-100HG column (100, 2.1, 3 mm; Wako Pure Chemical Industries, Osaka, Japan) was used for the separation, and the mobile phase consisted of eluent A (25-mmol/L ammonium formate in water, pH 6.0) and eluent B (water:acetonitrile = 40:60).

Western Blotting

The expression of HCV core protein, Socs3, Foxo3a, phospho-Foxo3a (Ser253) (pFoxo3a), STAT1, pSTAT1 (Tyr701), S6K, pS6K, p-mTOR (Ser2448), Raptor, and β -actin were evaluated with mouse anti-core (Affinity BioReagents, Golden, CO), mouse anti-Socs3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Foxo3a, rabbit anti- β -actin (Sigma-Aldrich, St Louis, MO), rabbit anti-phospho-Foxo3a (Ser253), rabbit anti-STAT1, rabbit anti-p-STAT1 (Tyr701), rabbit anti-p70 S6K, rabbit anti-pS6K, rabbit anti-p-mTOR (Ser2448), and rabbit anti-Raptor (Cell Signaling Technology, Beverly, MA), respectively. Densitometric analysis was conducted directly on the blotted membrane using a charge coupled device camera system (LAS-3000 Mini; Fujifilm, Tokyo, Japan) and Scion Image software (Frederick, MD).

Primer Sequences for PCR and siRNA

Primer sequences for PCR and siRNA were as follows: 2'5'OAS: forward 5'- CTC AGA AAT ACC CCA GCC AAA TC-3', reverse 5'-GTG GTG AGA GGA CTG AGG AA-3'; Socs3: forward 5'-TAC CAC CTG AGT CTC CAG CTT CTC-3', reverse 5'-CCT GGC AGT TCT CAT TAG TTC AGC ATT C-3'; Foxo3a: forward 5'-TGC TGT ATG CAA GAA CTT TCC AGT AGC AG-3', reverse 5'-ACT CTA GCC CCC ATG CTA CTA GTG-3'; glyceraldehyde-3-phosphate dehydrogenase: forward 5'-GAA GGT GAA GGT CGG AGT-3', reverse 5'-GAA GAT GGT GAT GGG ATT TC-3', siFoxo3a (SASL_Hs01_00119127; Sigma) sense: 5'-GAA UGA UGG GCU GAC UGA AdTdT-3', antisense: 5'-UUC AGU CAG CCC AUC AUU CdTdT-3'. Small interfering Raptor was purchased as

part of KIAA1303 siGENOME SMART pool siRNA reagents from Dharmacon, Inc (Lafayette, CO).

Construction of ISRE-Luc Reporter and FBEmut-luc Reporter Plasmids

Oligonucleotides containing the ISRE tandem repeat sequence (sense 5'-TCG AGA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA A-3', antisense 5'-AGC TTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT C-3', consensus 5'-GAA Ann GAA ACT-3') were annealed, and integrated into Xho I and Hind III sites of the pGL4.23 luciferase vector (Promega). The human Socs3 promoter region (-109/+217) was amplified by genomic PCR using specific primers (forward, 5'-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG-3' and reverse, 5'-CCG TGA AGT CCA CAA AGG AGC CTT C-3') and cloned into the EcoR V site of the pGL4.10-luc2 reporter vector (Promega). The Socs3 FBE mutant reporter vector was created by substituting 2 adenines in the putative FBE with guanines (wild-type sequence 5'-CTAAACA-3', mutated sequence 5'-CTGAGCA-3').

ChIP Assay

For the ChIP assay using the anti-ISGF3 γ antibody, 1×10^6 Huh-7 cells were treated with IFN- α (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid medium for 6 hours. For ChIP using the anti-Foxo3a antibody, 1×10^6 Huh-7 cells were cultured in low-amino-acid medium for 24 hours.

Cells were cross-linked with 1% formaldehyde in PBS for 10 minutes at 37°C, and the reaction was stopped with 250 mmol/L glycine for 10 minutes. Cells were suspended in sodium dodecyl sulfate–lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L ethylenediaminetetraacetic acid [EDTA], 50 mmol/L Tris-HCl [pH 8.1]), complete protease inhibitor cocktail (Roche Applied Science), and incubated for 30 minutes at 10°C. Cell lysate was sonicated with Bioruptor (Cosmo Bio, Tokyo, Japan) to obtain chromatin fragments and diluted 10-fold in ChIP dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton-X 100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl, complete protease inhibitor cocktail). Chromatin fragments were incubated with 2 μ g ISGF3 γ antibody (Santa Cruz Biotechnology), 2 μ g Foxo3a antibody (H-144; Santa Cruz Biotechnology), or normal rabbit immunoglobulin G for 18 hours at 4°C. Dynabeads (30 μ L) protein G (Invitrogen) was added and incubated for 1 hour at 4°C. The beads were washed with low-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl), high-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 500 mmol/L NaCl), LiCl wash buffer (250 mmol/L LiCl, 1% NP-40, 1% de-

oxycholate, 1.0 mmol/L EDTA, 1.0 mmol/L Tris-HCl [pH 8.1]) and Tris-EDTA buffer. Immunoprecipitated chromatin fragments were eluted with elution buffer (1% sodium dodecyl sulfate, 100 mmol/L NaHCO₃, 10 mmol/L dithiothreitol), and reverse cross-linked by incubating for 6 hours at 65°C in elution buffer containing 200 mmol/L NaCl. DNA fragments were purified and quantified by real-time detection PCR with primers for putative ISRE in the 2'5'OAS promoter region (forward, 5'-AAA TGC ATT TCC AGA GCA GAG TTC AGA G-3', reverse, 5'-GGG TAT TTC TGA GAT CCA TCA TTG ACA GG-3') or putative FBE in the Socs3 promoter region (forward, 5'-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG -3', reverse, 5'-AGC GGA GCA GGG AGT CCA AGT C -3'). Values were normalized by the measurement of input DNA.

pH77S.3/GLuc2A

pH77S.2 is a modification of pH77S² containing an additional mutation within the E2 protein (N476D in the polyprotein) that promotes infectious virus yields from RNA-transfected cells (Yi et al, unpublished data). To monitor replication, the GLuc sequence, fused at its C terminus to the foot-and-mouth disease virus 2A autoprotease, was inserted between p7 and NS2 of pH77S.2 (Supplementary Figure 4). To insert the GLuc-coding sequence between p7 and NS2 in pH77S.2, followed by the foot-and-mouth disease virus 2A protein-coding sequence, Mlu I, EcoR V, and Spe I restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the Mlu I and EcoR V sites of the modified plasmid after PCR amplification using the primers: 5'-ATA ATA TTA CGC GTA TGG GAG TCA AAG TTC TGT TTG CC-3' (sequence corresponding to the N-terminal GLuc is italicized and that corresponding to Mlu I is underlined) and 5'-ATA AAT AGAT ATC GTC ACC ACC GGC CCC CTT GAT CTT-3' (C terminal GLuc is italicized and EcoR V is underlined). A DNA fragment encoding the 17 amino acids of the foot-and-mouth disease virus 2A protein was generated by annealing the following complementary oligonucleotides: 5'-ATA TGA TAT CAA CTT TGA CCT TCT CAA GTT GGC CGG CGA CGT

CGA GTC CAA CCC AGG GCC CAC TAG CAT AT-3' and 5'-ATA TGC TAG TGG GCC CTG GGT TGG ACT CGA CGT CGC CGG CCA ACT TGA GAA GGT CAA AGT TGA TAT CAT AT-3' (underlined sequences indicate EcoR V and Spe I sites). The annealed oligonucleotides were digested by both restriction enzymes and the product inserted into the corresponding sites of pH77S.2 containing GLuc to generate pH77S.2/GLuc2A. Q41R is a cell-culture adaptive mutation within the NS3 protease domain of pH77S. Because it is not essential for production of infectious virus (Yi et al, unpublished data), pH77S.2 and pH77S.2/GLuc2A constructs underwent this mutation by site-directed mutagenesis of a PCR fragment spanning the Afe I and BsrG I sites to replace Gln₄₁ with wild-type Arg. The resulting plasmids (pH77S.2/R41Q and pH77S.2/GLuc2A/R41Q) were redesignated pH77S.3 and pH77S.3/GLuc2A, respectively.^{3,4} GLuc has several advantages over other luciferase reporter enzymes in that it is smaller and allows more sensitive detection than either firefly or Renilla luciferase.^{3,4} In addition, a signal sequence directs its secretion into cell-culture media, allowing real-time dynamic measurements of GLuc expression without the need for cell lysis. H77S.3/GLuc2A RNA produces infectious virus, although with lower efficiency than H77S.3 RNA (10-fold less).

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) and chronic liver disease worldwide. Recent developments and advances in HCV replication systems *in vitro* and *in vivo*, transgenic animal models, and gene expression profiling approaches have provided novel insights into the mechanisms of HCV replication. They have also helped elucidate host cellular responses, including activated/inactivated signaling pathways, and the relationship between innate immune responses by HCV infection and host genetic traits. However, the mechanisms of hepatocyte malignant transformation induced by HCV infection are still largely unclear, most likely due to the heterogeneity of molecular paths leading to HCC development in each individual. In this review, we summarize recent advances in knowledge about the mechanisms of hepatocarcinogenesis induced by HCV infection.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide.¹ The majority of HCCs arise from a background of chronic liver diseases caused by infection with hepatitis B virus (HBV) or hepatitis C virus (HCV).² Although both viruses are hepatotropic and regarded as causative agents of HCC, the underlying mechanisms of hepatocarcinogenesis are considered to be largely different, partly due to differences in the nature of DNA viruses (with an integration capacity for the host genome) and RNA viruses (with no genome integration capacity).

Hepatitis C virus is an RNA virus that is unable to integrate into the host genome but, instead, its proteins interact with various host proteins and induce host responses that potentially contribute to the malignant transformation of cells. In addition, HCC usually develops in the setting of liver cirrhosis after long-term continuous inflammation/regeneration processes; these accelerate the turnover of hepatocytes with increased risk of replication errors and DNA damage. Furthermore, recent genome-wide association studies have suggested that the natural course of HCV infection might be modified by the genetic background of the host.^{3,4} Thus, both host and virus factors are considered to affect the process of hepatocarcinogenesis in a complex manner.

In this review, we summarize the current knowledge of the mechanisms of hepatocarcinogenesis induced by HCV infection. We also focus on recent findings of transcriptomic characteristics of HCV-related HCC and summarize the potential signaling pathways that are altered in this condition.

Epidemiology

Chronic HCV infection is a major risk factor for the development of HCC worldwide. According to the World Health Organization (WHO), approximately 170 million people are chronically infected with HCV. Although epidemiological evidence has suggested a clear, close relationship between HCV infection and HCC,^{5,6} the prevalence of HCV infection in HCC patients differs noticeably between geographical regions. Thus, HCV infection is found in 70–80% of HCC patients in Japan, 70% in Egypt, 40–50% in Italy and Spain, about 20% in the United States (US), and less than 10% in China.^{7–9} In industrialized countries including the US, a recent increase in HCC incidence and mortality has been observed, potentially due to the rising incidence of HCV infection transmitted through contaminated blood.¹⁰

Hepatitis C virus increases the risk of HCC by promoting inflammation and fibrosis of the infected liver that eventually results in liver cirrhosis. Once HCV-related cirrhosis is established, HCC develops at an annual rate of about 4–7%.¹¹ Other factors including alcohol intake, diabetes, and obesity have also been reported to increase the risk of HCC development by about two- to fourfold, indicating a strong life-style effect on the process of hepatocarcinogenesis.^{12,13} Age and male gender are also contributing risk factors for HCV-related HCC, although the detailed mechanisms are still debatable.

Virus proteins and host responses

Hepatitis C virus belongs to the Flaviviridae family. It has a positive-stranded linear RNA genome of about 9.6-kb containing a

single large open reading frame encoding three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins.¹⁴ The structural proteins form the HCV virions, whereas non-structural proteins are involved in the processes of viral replication, assembly, and maturation. HCV proteins are known to be processed by host and viral proteases. Both structural and non-structural proteins can interact with various host cellular proteins to potentially promote the malignant transformation of hepatocytes (see recent reviews^{7,15,16}). In this review, because of space limitations, we focus on the findings of core and NS5A proteins in terms of host responses potentially evoked during the process of HCV-related hepatocarcinogenesis.

Core protein

Hepatitis C virus core is a 21-kDa nucleocapsid protein with an RNA-binding capacity. In addition to its function in regulating HCV-RNA translation and HCV particle assembly, core protein is known to be involved in mediating the alteration of various host cell signaling pathways, transcriptional activation, modulation of immune responses, apoptosis, oxidative stress, and lipid metabolism.⁷ Several recent studies have indicated the statistically significant high frequency of mutations in the *core* gene in HCV-infected patients who developed HCC.^{17,18} However, the functional relevance of mutant core proteins on the malignant transformation of hepatocytes or the HCV life cycle has yet to be clarified.

Evidence of core protein as a causative agent of HCC was initially obtained from the transgenic mice model in which *core* gene overexpression, under the regulation of the HBV regulatory element used as a promoter, resulted in steatosis of mouse livers in early life, with subsequent development of adenoma and HCC.¹⁹ However, another mouse model using a different promoter and of a different strain background resulted only in steatosis or different phenotypes without HCC development.^{20,21} Similar controversial findings were reported in transgenic mice expressing HCV polyprotein or structural protein with regards to the development of HCC.^{22,23} Thus, the role of core protein alone in the development of HCC remains unclear in transgenic mouse models.

Although the direct role of core protein in the malignant transformation of hepatocytes is still under investigation, it seems to be related to the development of hepatic steatosis.^{19,24} Indeed, steatosis is one of the risk factors for the development of HCV-related HCC,^{25,26} and activation of the lipogenic pathway has been reported in a subset of HCC cases.²⁷ Core protein is associated with the surface of lipid droplets in infected cells and might be directly related to steatosis through several factors responsible for lipid biogenesis and degradation, including peroxisome proliferator-activated receptor alpha and sterol-regulatory element binding protein-1.^{21,28–30} Furthermore, core protein is reported to interact with endoplasmic reticulum (ER) or mitochondrial outer membranes and induce ER stress by perturbation of protein folding or by the accumulation of reactive oxygen species (ROS) through mitochondrial dysfunction.^{31,32} ROS produced in this way might result in DNA damage to the host genome and accelerate the process of hepatocarcinogenesis. Increased hepatic iron deposition may also induce oxidative stress and lipid peroxidation, thus increasing the risk of HCC development in HCV polyprotein transgenic mice.³³

Since the discovery of HCV, various studies have investigated the role of core on host cells. Its effects have been demonstrated on signaling pathways responsible for the cell cycle, and apoptosis through interaction with several tumor suppressors including p53, p73, and p21^{34–39} as well as apoptosis regulators such as tumor necrosis factor- α (TNF- α) signaling or Bcl-2 members.^{40–42} However, the data obtained from these studies are relatively inconsistent with each other and have varied across experimental models. Core protein may influence the growth and proliferation of host cells through activation of signaling pathways such as Raf/mitogen activated protein kinase (MAPK),⁴³ Wnt/beta catenin,¹⁶ and transforming growth factor- β (TGF- β).^{15,44} These pathways are known to be activated in HCC.⁴⁵ The findings therefore indicate a potential role for core in cell proliferation or suppression of apoptosis during malignant transformation of hepatocytes in the liver of chronic hepatitis C, where chronic inflammation and regeneration of hepatocytes continuously occurs.

NS5A protein

NS5A is a 56–58-kDa protein phosphorylated at serine residues by serine-threonine kinase⁴⁶ and is essential for replication of the HCV genome. NS5A protein forms part of the viral replicase complex and is localized mainly in the cytoplasm of infected cells in association with the ER. NS5A can become a lower molecular weight protein through post-translational modification, after which it can undergo translocation to the nucleus where it acts as a transcriptional activator. High frequencies of wild-type NS5A genes were reported to be dominant in liver cirrhosis patients who finally developed HCC compared with those who did not,⁴⁷ but the mechanistic significance of the NS5A wild/mutant genotypes in the process of HCV-related hepatocarcinogenesis remains uncertain.

NS5A protein has been suggested to interact with various signaling pathways including cell cycle/apoptosis⁴⁸ and lipid metabolism^{28,49,50} in host cells and shares some signaling targets with core protein. NS5A is recognized as a transcriptional activator for many target genes⁵¹ including p53 and its binding protein, TATA binding protein (TBP). Transcription factor IID activities were reported to be modified by NS5A in the suppression of p53-dependent transcriptional transactivation and apoptosis.^{52,53} NS5A may also interact with pathways such as Bcl2,⁵⁴ phosphatidylinositol 3-kinase (PI3-K),⁵⁵ Wnt/beta catenin signaling,⁵⁶ and mTOR⁵⁷ to activate cell proliferation signaling and inhibit apoptosis.

Taken together, intriguing data concerning the function of core and NS5A proteins on host cell signaling pathways, transcriptional activation, apoptosis, oxidative stress, and lipid metabolism described above suggest a diverse role for HCV proteins in the pathophysiology of chronic hepatitis C that leads to malignant transformation in infected hepatocytes. Key findings and present concepts are summarized in Figure 1.

Transcriptomic characteristics of HCV-related HCC

As described above, HCV proteins can evoke various host responses in infected cells at transcriptional/translational/post-translational levels. Furthermore, enhanced cell death/regeneration processes are considered to induce DNA damage and accelerate replication errors that cause frequent mutations and genomic alter-

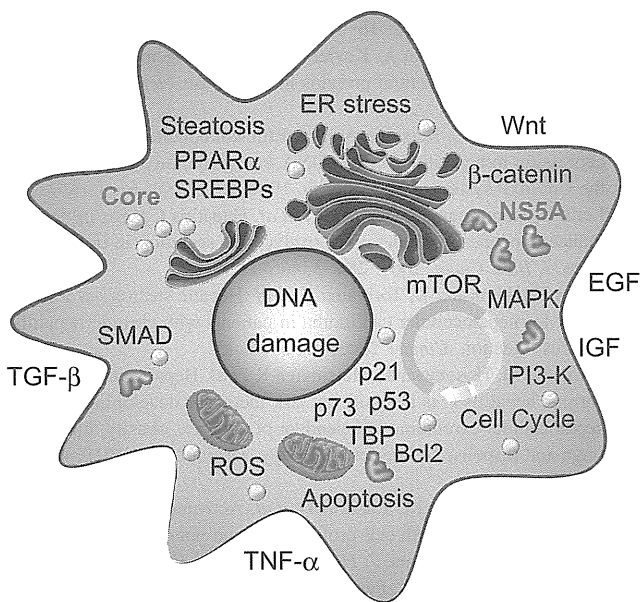


Figure 1 Signaling pathways potentially affected by hepatitis C virus (HCV) proteins. EGF, epidermal growth factor; ER, endoplasmic reticulum; IGF, insulin-like growth factor; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SREBP, sterol-regulatory element binding protein; TBP, TATA binding protein.

ation in the host genome. The central dogma is defined as the flow of genetic information from DNA to mRNA and then to protein, so genetic/genomic alterations and transcriptional/translational modifications are ultimately considered to affect the cellular signaling pathway at the transcriptional level.

Over the past decade, several methods (including differential display, serial analysis of gene expression [SAGE], and microarray) have been developed to allow comparative studies of gene expression between normal and cancer cells on a genome-wide scale,⁵⁸ and the analysis of a set of all RNA molecules (mainly indicating mRNAs) is termed as whole transcriptome analysis. Extensive transcriptome analysis of HCC and corresponding non-cancerous livers has been performed, and the results have greatly increased our knowledge about the transcriptome characteristics of HCV-related HCC.

Early microarray and SAGE studies investigating the gene expression patterns of chronic hepatitis B and C tissues indicated that these two chronic hepatitis tissues had distinct gene expression profiles; the genes activated in chronic hepatitis C were correlated with signaling pathways associated with apoptosis, oxidative stress responses, and Th1 cytokine signaling.^{59,60} An early study comparing genes activated in HCV-related and HBV-related HCCs showed that the genes associated with xenobiotic metabolism were more abundantly expressed in HCV-related HCC,⁶¹ suggesting a detoxification role, which is potentially induced by chronic inflammation and generation of ROS resulting from HCV infection. In contrast, HBV-related HCC might closely correlate with the activation of imprint genes, including insulin-like growth factor-II (IGF-II) as investigated by oligo-DNA

microarray,⁶² suggesting a role of de-differentiation or epigenetic alteration of the host genome in HBV-related HCC. Activation of genes associated with interferon, oxidative stress, apoptosis, and lipid metabolism signaling was detected in HCV-related HCC and chronic hepatitis C specimens,^{27,60,63} consistent with numerous functional studies that have investigated the host response evoked by HCV structural and non-structural proteins.⁴⁸

Transcriptome analysis has also recently shed new light on the transcriptional alteration events occurring in early stages of HCV-related hepatocarcinogenesis. *GPC3* (encoding Glypican 3) was identified as one of the most activated transcripts in the early stage of hepatocarcinogenesis,^{60,64} while several recent studies showed that gene signatures including *GPC3* can successfully discriminate HCCs from pre-malignant dysplastic nodules and cirrhosis nodules.^{65,66} Close examination of genes differentially expressed among cirrhotic nodules, dysplastic nodules, and early and advanced HCV-related HCC tissues has also suggested roles for Toll-like receptor signaling, Wnt signaling, bone morphogenetic protein (BMP)/TGF- β signaling, JAK-STAT signaling, and DNA repair/cell cycle responses in each step of the malignant transformation processes.⁶⁷ These processes might therefore provide candidate molecular targets for the chemoprevention of HCV-related HCC.

Recent advances in transcriptome analysis have also provided detailed information on the status of small noncoding RNAs, microRNAs, that can regulate the expression of target genes and viral replication in normal and cancer tissues. Expression of microRNAs including miR-122 and -199a has been reported to modulate HCV replication,⁶⁸⁻⁷⁰ and miR-122 expression can be regulated by host interferon signaling and responses.⁷¹ HCV protein expression in turn could induce miRNAs and might affect the tumor suppressor DLC1 and the chemosensitivity of malignantly transformed cells.^{72,73} Several microRNAs were also differentially expressed between HCV-related and HBV-related HCCs as well as their corresponding non-cancerous liver tissues. The candidate signaling pathways potentially altered by microRNAs in HCV-related tissues were those associated with antigen presentation, cell cycle, and lipid metabolism,⁷⁴ consistent with the mRNA microarray data described above. MicroRNAs have also recently been reported to successfully discriminate between HCC and cirrhotic liver tissues,⁷⁵ implicating their role in the early stages of malignant transformation. These data suggest that microRNAs may be good targets for the eradication of HCC as well as hepatocytes infected with HCV.

Conclusion

The heterogeneity of genetic/transcriptomic/proteomic events observed in hepatocytes or cell lines expressing HCV proteins and HCV-related HCCs reported thus far has suggested that complex mechanisms underlie malignant transformation induced by HCV infection. These potentially act through convoluted virus-host interactions including HCV replication with host cell cycles, apoptosis, proliferation, quality control of protein synthesis, lipid metabolism, and DNA damage responses. Indeed, HCC is a heterogeneous disease in terms of drug sensitivity, metastatic capacity, and clinical outcome. The heterogeneity of HCV-related HCC may closely correlate with the origin of malignantly transformed cells where multifaceted cellular reactions including apoptosis and

cell proliferation are induced by HCV infection. An in-depth understanding of these molecular complexities associated with HCV-related HCC may provide the opportunity for effective chemoprevention of HCC among those with HCV-cirrhosis, and to design tailor-made treatment options for HCV-related HCC patients in the future.

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