decreased replication and increased infectious virus production. However, the extent of enhanced virus production was substantially lower in JFH-1/C than in JFH-1/S2, which might have led to the earlier elimination of infection in the JFH-1cc–infected chimpanzee. In other words, the potency of infectious virus production and spread seems to correspond to the duration of infection in infected animals.

The association between a lower replication efficiency and persistent infection is still unclear. It has been reported that an escape mutant with an amino acid substitution at the cytotoxic T lymphocyte (CTL) epitope in the NS3 region exhibits lower NS3/4 protease activity and replication capacity *in vitro*.[17,18] The JFH-1/S2 strain contains the T1077A mutation in the NS3 region (Supporting Table 1), and this mutation is located close to mutations reported to be associated with immune evasion and lower replication.[17] Thus, the lower replication efficiency of the JFH-1/S2 strain may be a result of an immune escape mutation at the expense of viral fitness. Meanwhile, we cannot deny the advantage of lower replication in establishing persistent infection. Lower replication may contribute to the avoidance of major histocompatibility class I–mediated antigen presentation and to escape from the host immune system. Either way, by acquiring the ability to produce more viral particles, the JFH-1/S2 strain could rapidly spread to surrounding cells, irrespective of its lower replication efficiency. Importantly, these emerged mutations did not attenuate *in vivo* infectivity, unlike cell culture adaptive mutations reported to cause attenuated infection *in vivo*.[19] Upon inoculation into human hepatocyte–transplanted mice, JFH-1/S1, JFH-1/S2, and JFH-1/C strains could establish infection without any mutations, produced levels of viremia similar to JFH-1/wt, and persisted for a similar observed period of infection (Fig. 2). This observation is different from that in chimpanzees, where JFH-1/wt and JFH-1/C strains were eliminated earlier than JFH-1/S2. In contrast to chimpanzees, human hepatocyte–transplanted mice lack a CTL and natural killer (NK) cell–mediated immune system, which could be responsible for this difference.[6] Taken together, our results suggest that along with efficient infectious virus production, the JFH-1/S2 strain might have acquired an advantage that helps it evade the CTL and NK cell–mediated immune system.

Apoptosis of virus-infected cells by the immune system is crucial as a general mechanism of clearing infections.[20,21] The J6/JFH-1 chimeric virus has been reported to exhibit proapoptotic characteristics in cell culture.[22] However, because HCV needs to escape the host immune system in order to establish chronic infection, immune cell–mediated apoptosis may be inhibited in infected hepatocytes. In the liver, HCV-infected hepatocytes are eliminated by targeted apoptosis induced by NK cells, macrophages, and CTLs with ligand-mediated and receptor-mediated signals such as TNF-α, FasL, and TNF-related apoptosis–inducing ligand.[23-26] Thus, we used TNF-α to mimic natural immunomediated apoptosis and found that the JFH-1/S2–replicating cells have lower susceptibility to the apoptosis induced by these cytokines. In JFH-1/S2–transfected cells, TNF-α–induced apoptosis detected by TUNEL assay was substantially lower than that of JFH-1/wt–transfected cells (Fig. 4). We confirmed it by staining with anticleaved PARP. In complete agreement with the results produced by way of TUNEL assay, the number of anticleaved PARP stained cells among JFH-1/S2–infected cells was significantly lower than that among JFH-1/wt–infected cells (Fig. 5). In our previous study, we reported that HCV-specific immune responses with T cell proliferation and interferon-γ production were maintained until the disappearance of viremia in the patient serum–infected chimpanzee.[11] This finding indicates that continuous selection pressure in the infected chimpanzee might have contributed to the emergence of a clone with an ability to escape the cytokine-induced apoptosis. We are not sure whether this phenotype of JFH-1/S2 is due to its lower replication efficiency and thus lower production of HCV proteins. The accumulation of viral proteins might predispose cells to the apoptosis induced by TNF-α. To answer this question, it will be necessary to investigate the genomic regions of JFH-1/S2 and cellular host factors responsible for the ability of this strain to escape the apoptosis.

By way of mapping analysis for JFH-1/S2, we could determine responsible regions; NS5B was for lower replication efficiency (Supporting Fig. 1B), and P7 and NS2 were for enhanced viral particle assembly (Supporting Table 2). For the evasion of apoptosis, we could not specify the responsible region, because both chimeric constructs, JFH-1/S2-wt and JFH-1/wt-S2, showed less susceptibility to cytokine-induced apoptosis to a certain extent. These data indicate that both structural and nonstructural regions might have contributed to the acquisition of this phenotype. Previously, a potent antiapoptotic effect of the HCV NS5A protein was described.[27] NS5A interacts with Bin1, which is a nucleocytoplasmic c-Myc–interacting protein with tumor suppressor and apoptotic properties, thus inhibiting Bin1-

associated apoptosis. Because JFH-1/S2 contains several mutations in the NS5A region (Supporting Table 1), one or more mutations in this protein may be associated with antiapoptotic effects.

In conclusion, we demonstrated that the JHF-1/S2 strain acquired phenotypes of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis. These phenotypes were associated with mutations that emerged 23 weeks after infection in a chimpanzee, and might have contributed to long-term infection *in vivo*. Such control of viral functions by specific mutations may be a key viral strategy to establish persistent infection.

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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

primary hepatocytes[6] and cirrhotic rat liver[7] 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-alfa–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-alfa 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-μm 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 μg/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-alfa 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, ×104/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.
[a]IL-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 previously[12] 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 NS2[14] (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-μg aliquot of synthetic RNA transcribed from pH77S.3/GLuc2A was used for electroporation. Cells were pulsed at 260 V and 950 μF 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 ×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 ± 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-alfa signaling and the mTOR pathway, we used Huh-7 cells grown in different amino acid conditions (×1 DMEM, ×1/5 DMEM, ×1/30 DMEM, and ×1/100 DMEM). The phosphorylated forms of mTOR (p-mTOR) and S6K (pS6K), an important downstream regulator of mTORC1 signaling, were decreased substantially in ×1/30 DMEM and ×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 pSK6 were repressed, which is consistent with previous reports.[16] The expression of pSTAT1 also was repressed after Raptor knockdown (Figure 2B).
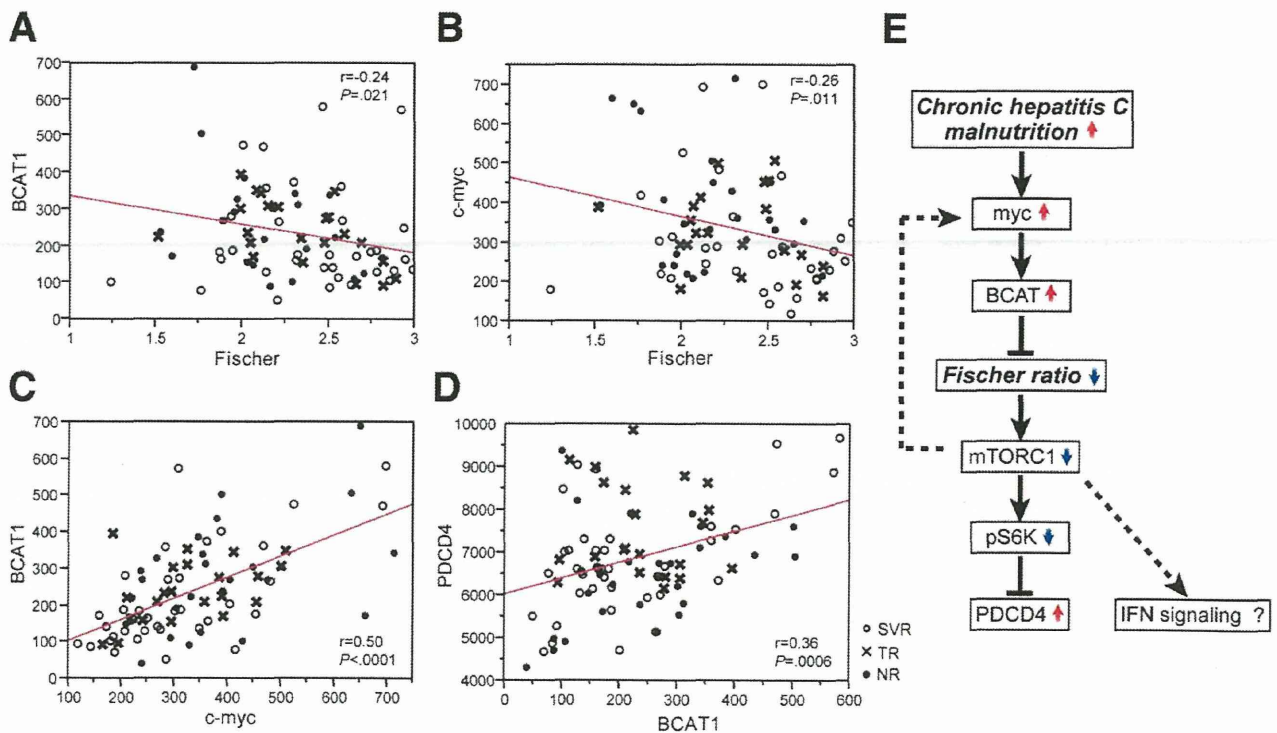

Figure 1. Regression analysis of mTORC1-related gene expression in liver. Gene expression values were determined by probe intensities. (A) BCAT1 and Fischer's ratio. (B) c-myc and Fischer's ratio. (C) BCAT1 and c-myc. (D) PDCD4 and BCAT1. (E) Putative signaling of mTORC1-related genes in CH-C.
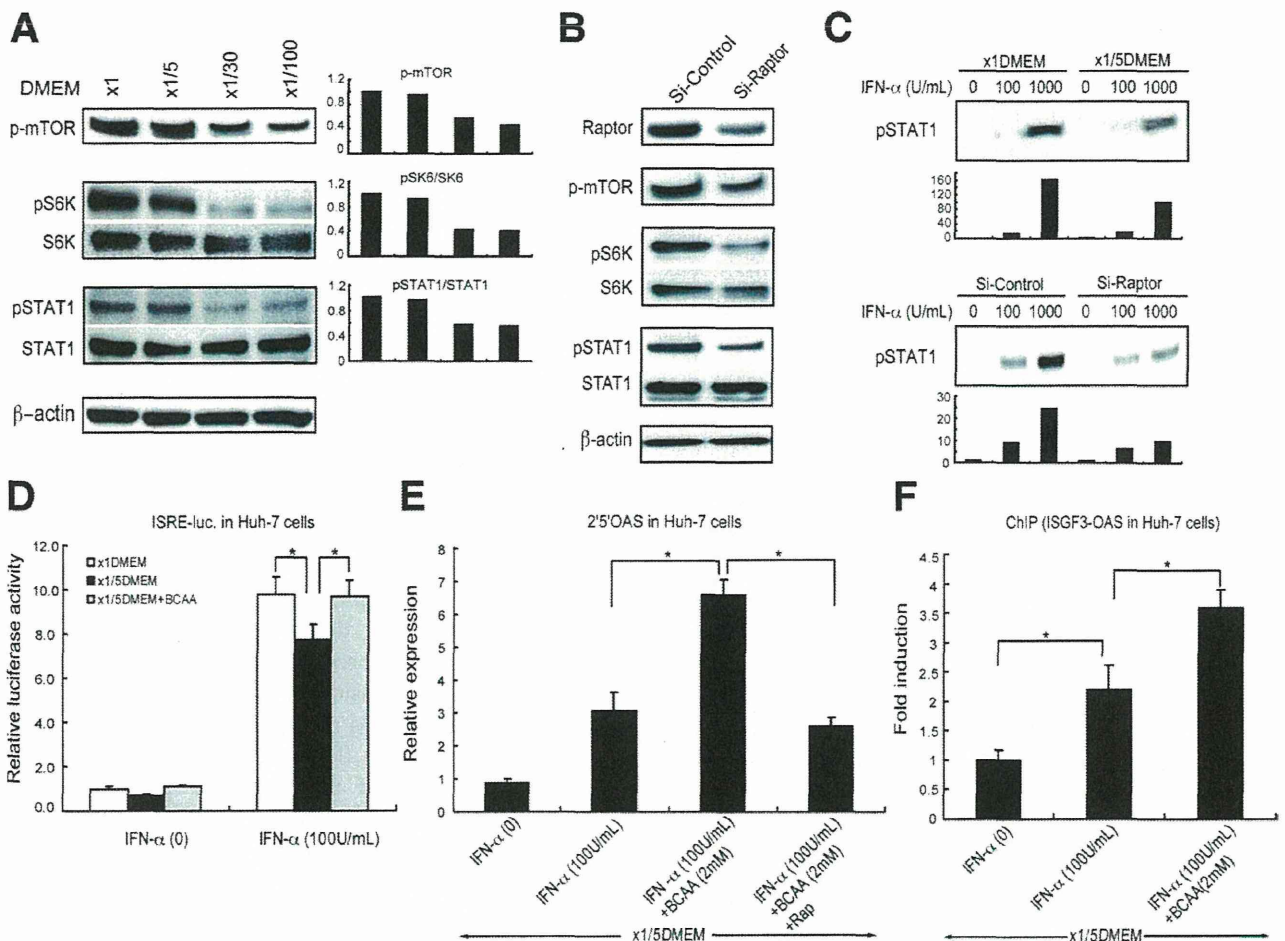
Figure 2. mTORC1 and IFN signaling in Huh-7 cells in low-amino-acid medium. (A) p-mTOR, pS6K, and pSTAT1 expression in different amino acid media. (B) p-mTOR, pS6K, and pSTAT1 expression under Raptor knock-down conditions. (C) IFN-alfa stimulation and pSTAT1 expression in low-amino-acid media or under 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 supplemented with BCAA or rapamycin in low-amino-acid medium. (F) Chromatin immunoprecipitation of 2'5'OAS promoter region by ISGF3γ.

The induction of pSTAT1 by IFN-alfa (1000 U/mL) stimulation was impaired in ×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 ×1/5 DMEM compared with ×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, ISGF3γ, 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 transcription 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 ×1/5, ×1/30, and ×1/100 DMEM, expression of the phosphorylated form of Foxo3a (pFoxo3a) decreased, whereas that of total Foxo3a increased in ×1/5 and ×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
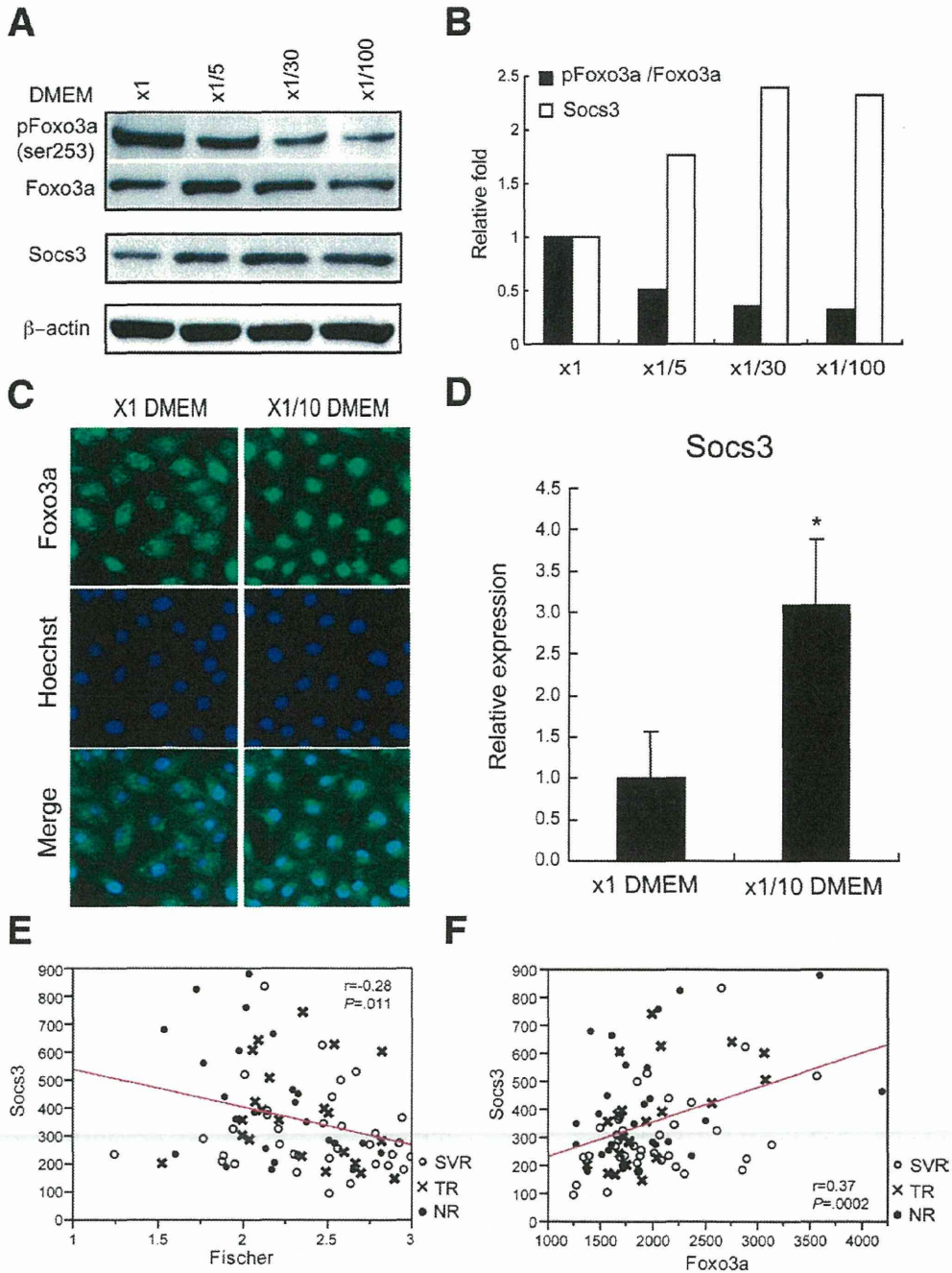
Figure 3. Foxo3a and Socs3 signaling in Huh-7 cells in low-amino-acid medium. (*A*) Foxo3a and Socs3 expression in different amino acid media. (*B*) Relative change of pFoxo3a/Foxo3a and Socs3 expression in different amino acid media. (*C*) Immunofluorescence staining of Foxo3a in Huh-7 cells in normal 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 (×1/10 DMEM) (Figure 3*C*).

Interestingly, in low-amino-acid medium, transcription and protein expression of Socs3 increased significantly (Figure 3*A*, *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 3*E* 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 4*A*). 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 4*B*). The mutations introduced in the putative FBE (FBEmut) in the Socs3 promoter significantly reduced Foxo3a-induced Socs3 promoter activation (Figure 4*B*).

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 ($\times1/10$ DMEM) (Figure 4*C*). 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 ($\times1/10$ DMEM) (Figure 4*D*). 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 4*E*).

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 ($\times1/5$ DMEM) compared with normal amino acid medium ($\times1$ DMEM). Interestingly, BCAA repressed H77S.3/GLuc2A replication in a dose-dependent manner (Figure 5*A*). In agreement with these results, the expression of Mx-1 was increased significantly by the addition of BCAA (Figure 5*B*). 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 ($\times1/5$ DMEM) com-

pared with normal amino acid medium ($\times1$ DMEM), JFH-1 replication was repressed significantly by the addition of BCAA in a dose-dependent manner (Figure 5*D*). The expression of Mx-1 was increased substantially by the addition of BCAA (Figure 5*E*), 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 5*C* and *F*).

To validate these findings, signaling pathways in HCV replicating cells were examined (Figure 6*A* 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 6*A* 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 4*E*.

Discussion

Thompson et al[5] 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