impaired VEGF-stimulated phosphorylation of VEGF receptor (VEGFR)2 (Tyr1175) and extracellular signal-regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204) in HUVECs (Fig. 3a, b). Co-administration of BSO partially rescued the inhibitory effect of SeP on VEGF signalling (Fig. 3a, b). The mRNA expression of *VEGFR2* (also known as *KDR*) in HUVECs was unaffected by treatment with purified human SeP protein (Fig. 3c). These results indicate that SeP at physiological concentrations impairs VEGF signal transduction in vascular endothelial cells.

SeP suppresses VEGF-induced acute generation of ROS in HUVECs To clarify the mechanism by which the antioxidative protein SeP impairs VEGF signalling, we assessed the action of SeP on the acute generation of ROS stimulated by VEGF. The VEGF-induced ROS burst is reported to be required for the subsequent VEGF signal transduction [27]. Stimulation with 50 ng/ml VEGF for 5 min significantly increased intracellular levels of ROS in HUVECs (Fig. 3d). Pretreatment with SeP suppressed intracellular levels of ROS both with and without VEGF stimulation (Fig. 3d). These results suggest that SeP-induced VEGF resistance is associated with a reduction in the ROS burst stimulated by VEGF.

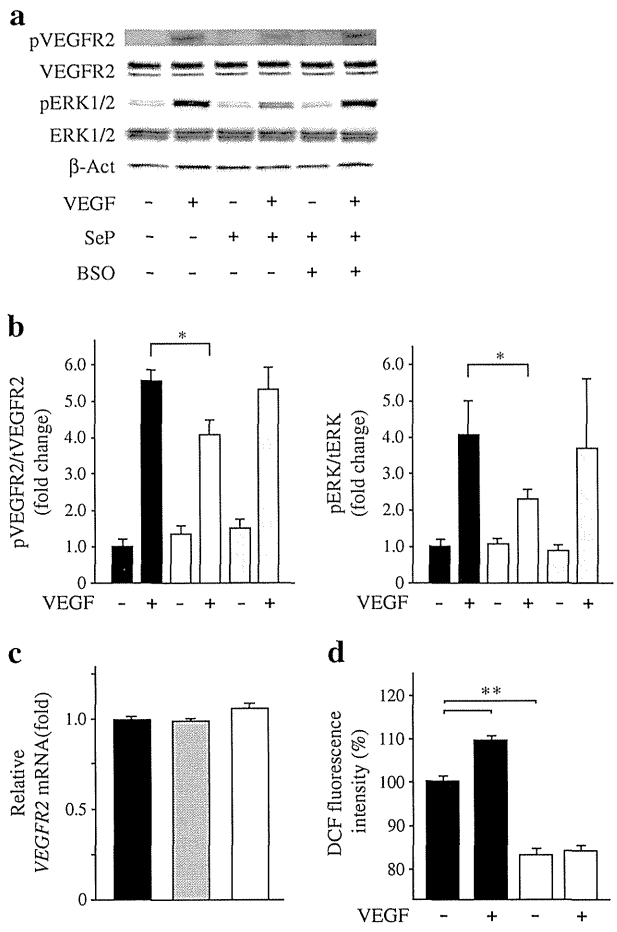

Fig. 3 SeP impairs VEGF signal transduction in endothelial cells. (**a**) VEGF signalling in HUVECs treated with SeP (10 μg/ml). (**b**) Quantification of phosphorylated VEGFR2 and ERK normalised to total VEGFR2 and total ERK in HUVECs (n=6). (**c**) Gene expression levels for *VEGFR2* in HUVECs treated with SeP for 24 h normalised to *GAPDH* (n=6). (**d**) ROS levels in HUVECs stimulated with VEGF for 5 min (n=8). ROS levels were measured as DCF fluorescence intensity. Black bars, control; dark-grey bars, SeP 5 μg/ml; white bars, SeP 10 μg/ml; light-grey bars, SeP 10 μg/ml and BSO 0.2 mmol/l. Data are mean ± SEM. *p<0.05 and **p<0.01. WT, wild-type

SeP delays wound healing of skin in mice To clarify whether hepatic overexpression of SeP affects angiogenesis-related disorder in vivo, we used a hydrodynamic injection method to generate mice that overexpress human *SEPP1* mRNA in the liver. Levels of *SEPP1* gene expression in the liver and SeP protein in the blood were significantly elevated in these mice (Fig. 4a, b), whereas serum levels of total selenium in wild-type and SeP-transgenic mice, which were 322.6 ng/ml and 331.0 ng/ml respectively, were not significantly different (Fig. 4c).

We created excisional wounds (10 mm) in the dorsal skin of the mice and quantified the rate of wound healing. Wound closure was significantly impaired in the mice overexpressing *SEPP1* at 3, 5 and 7 days (Fig. 4d, e). In contrast, *Sepp1*$^{-/-}$mice showed an improvement of the wound closure at 9 days compared with the wild-type animals (Fig. 4f, g). These results indicate that the hepatokine SeP delays the wound healing of the skin in mice.

Sepp1-heterozygous-knockout mice show enhanced angiogenesis after hindlimb ischaemia To determine whether attenuation of SeP expression enhances angiogenesis in vivo, we generated hindlimb ischaemia in *Sepp1*$^{+/-}$mice. We previously reported that *Sepp1*-homozygous-knockout mice exhibit enhancement of insulin signalling in skeletal muscle, whereas *Sepp1*-heterozygous-knockout mice show marginal changes in insulin signalling [15]. Hence, we selected *Sepp1*-heterozygous-knockout mice in the present study to assess the direct actions of SeP on the vascular system, independent of insulin signalling. At 5 days after femoral artery ligation, *Sepp1*$^{+/-}$mice showed a significant increase in blood flow compared with wild-type mice (Fig. 5a). This increase continued for 15 days after artery ligation (Fig. 5b). Consistent with these findings, histological examination showed increased vessel density in the hindlimb musculature as determined by immunostaining with anti-CD31 antibody (Fig. 5c, d).

Discussion

The present study indicates that the liver-derived secretory protein SeP impairs angiogenesis both in vitro and in vivo. SeP directly attenuates VEGF signal transduction in vascular

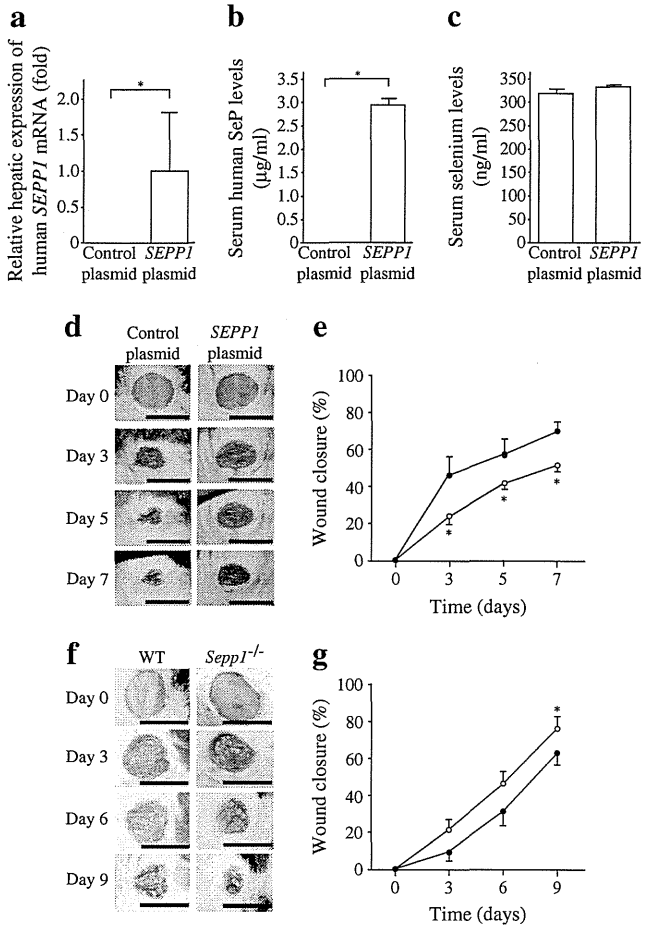

Fig. 4 Hepatic overexpression of SeP impairs wound healing in mice. (**a**) Levels of human *SEPP1* mRNA normalised to 18S rRNA in the livers of mice injected with plasmid DNA via the tail vein (n=9). (**b**) Serum human SeP levels in mice injected with a plasmid encoding *SEPP1* (n=9). (**c**) Serum levels of selenium in mice injected with a plasmid encoding *SEPP1* (n=3). (**d**) Representative images of full-thickness excision rounds on the backs of mice injected with *SEPP1* plasmid. (**e**) Quantification of wound closure in mice injected with *SEPP1* plasmid (white circles) and control (black circles) (n=9). (**f**) Representative images of full-thickness excisional wounds on the backs of *Sepp1*$^{-/-}$mice. (**g**) Quantification of wound closure in *Sepp1*$^{-/-}$mice (white circles) and control (black circles) (n=6–12). Data are mean ± SEM. *p<0.05, scale bars, 10 mm. WT, wild-type

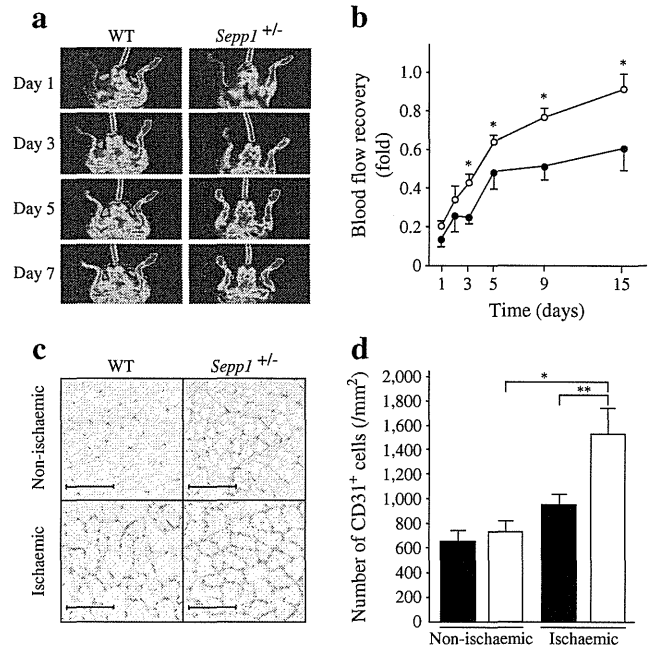

Fig. 5 *Sepp1*$^{+/-}$mice show enhanced angiogenesis during hindlimb ischaemia. (**a**) Representative images of perfusion recovery following hindlimb ischaemia in *Sepp1*$^{+/-}$mice. (**b**) Quantification of blood flow recovery in *Sepp1*$^{+/-}$mice (white circles) and control (black circles) (n=5). Ratios of perfusion from non-ischaemic leg to ischaemic leg are shown. (**c**) Representative images of CD31-stained sections of lower limb tissues of *Sepp1*$^{+/-}$mice at 15 days after ligation. Scale bars 100 μm. (**d**) Quantification of CD31-positive cells in the hindlimb of *Sepp1*$^{+/-}$mice (white bars) and WT (black bars). Data are from 16 fields per section. Data are mean ± SEM. *p<0.05 and **p<0.01

endothelial cells, resulting in suppression of VEGF-induced cell proliferation, migration and tube formation. We reported previously that levels of both hepatic *SEPP1* mRNA and serum SeP protein are elevated in type 2 diabetes [15]. Taken together with our previous report, the present study suggests that hepatic overproduction of SeP may contribute to the onset of impaired angiogenesis in type 2 diabetes (Fig. 6).

The attenuated VEGF signal transduction, VEGF resistance, has been postulated as the molecular mechanism underlying the dysregulation of angiogenesis in people with type 2 diabetes [3, 11]. Waltenberger et al reported that circulating monocytes show attenuation of VEGF-induced chemotaxis in people with diabetes mellitus [28] and that VEGF-stimulated phosphorylation of downstream molecules is reduced in monocytes from patients with type 2 diabetes [29]. In addition, Sasso et al found impaired VEGF signalling in the myocardium of patients with type 2 diabetes and coronary

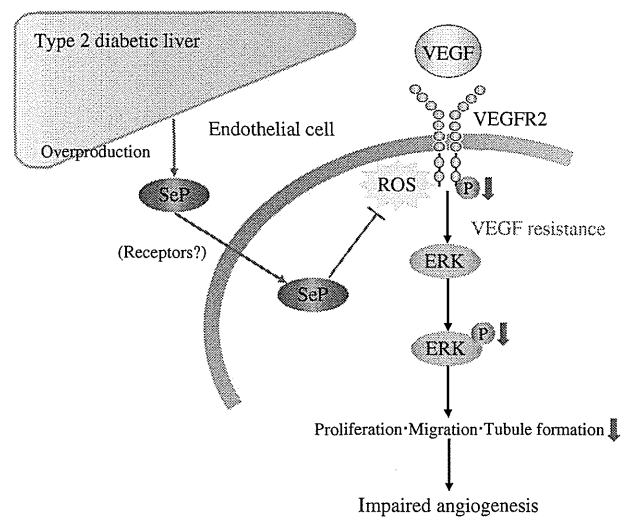

Fig. 6 Overproduction of SeP in type 2 diabetic liver induces VEGF resistance in vascular endothelial cells. SeP inhibits VEGF signal transduction by suppressing acute generation of ROS, resulting in the onset of impaired angiogenesis

heart disease [30], suggesting that diabetes induces VEGF resistance in not only monocytes but also other types of cells such as cardiomyocytes and endothelial cells. However, the molecular mechanisms by which VEGF resistance arises in diabetes mellitus have not been elucidated. The results of the present study suggest a novel molecular pathology of type 2 diabetes; elevation of circulating SeP induces VEGF resistance in vascular endothelial cells.

The liver is the production site of various secretory proteins. Recent work in our laboratory has indicated that genes encoding secretory proteins are abundantly expressed in the liver in type 2 diabetes [31]. Moreover, genes encoding angiogenic factors, fibrogenic factors and redox-associated factors are differentially expressed in the liver in type 2 diabetes, possibly contributing to the pathophysiology and clinical manifestations of this disease [32, 33]. The present study sheds light on a previously under-explored function of the liver; the liver may participate in the regulation of systemic angiogenesis by altering the production of angiogenesis-associated hepatokines such as SeP.

Our observation that SeP impairs angiogenic processes is noteworthy in the context of experimental data suggesting that SeP plays a role in the antioxidative defence system [13]. In fact, we have shown previously that SeP increases the activity of glutathione peroxidase 1 (GPX1), a representative antioxidative enzyme that requires selenium for its enzymatic action, in Jurkat E6-1 cells, a human T cell leukaemia cell line [14]. SeP-induced activation of GPX1 was also demonstrated in endothelial cells [19]. Accumulating evidence indicates that ROS stimulate the angiogenic response in order to initiate the tissue repair process in ischaemia–reperfusion lesions [34]. Among the growth factors involved in angiogenesis, VEGF plays a role in a ROS-dependent signal transduction system [27]. VEGF binding to VEGFR2 stimulates NADPH oxidase in endothelial cells, resulting in the acute generation of ROS such as hydrogen peroxide. This ROS burst oxidises and inactivates protein tyrosine phosphatases, which negatively regulate VEGF signalling and thereby promote VEGFR2 phosphorylation and the subsequent signalling cascade [27].

In combination with these previous reports, the present data suggest that SeP induces VEGF resistance in endothelial cells by increasing GPX1 and subsequently suppressing the VEGF-induced ROS generation that is required for VEGF signal transduction. This speculation was supported by our findings that the co-administration of BSO, an inhibitor of glutathione synthesis, rescued the inhibitory effects of SeP on VEGF signalling and the subsequent VEGF responsiveness. The identification of SeP receptor(s) in endothelial cells would provide further insight into the molecular mechanism by which SeP impairs VEGF signal transduction.

VEGF signalling is known to play paradoxical roles in the pathogenesis of diabetic complications. Both enhancement and suppression of angiogenesis are observed in different tissues in diabetic conditions [35]. In contrast to hindlimb ischaemia or wound healing, advanced diabetic retinopathy is characterised by VEGF-induced abnormal neovascularisation in the retina. Current management for diabetic retinopathy includes anti-VEGF therapy along with blood glucose control [36]. In addition to retinopathy, growing evidence indicates that VEGF-related abnormal angiogenesis plays a major role in diabetic nephropathy [37]. Moreover, a recent report showed that pharmacological inhibition of VEGF-B improves glucose tolerance and insulin resistance in rodent models with type 2 diabetes [38]. Additional studies are needed to determine the actions of SeP on the enhanced angiogenesis in diabetic retinopathy or nephropathy.

Unlike phosphorylation of VEGFR2 and ERK1/2, VEGF-induced phosphorylation of Akt, p38 MAPK and protein kinase, AMP-activated, α1 catalytic subunit (AMPK) was unchanged by SeP in HUVECs (data not shown). Although the detailed molecular mechanism by which SeP selectively impairs VEGFR2/ERK pathway in HUVECs is still unknown, SeP might act on ERK-selective MAPK phosphatases [39]. In fact, some MAPK phosphatases are inactivated by intracellular oxidative stress [39]. However, SeP-induced selective impairment of VEGFR2/ERK pathway should be confirmed in other vascular endothelial cells.

All the culture media we used for HUVECs in this study contained 5.5 mmol/l glucose, which corresponds to fasting plasma glucose levels in people with normal glucose tolerance. However, we confirmed that SeP also attenuated VEGF signalling of HUVECs in the presence of 25 mmol/l glucose (data not shown). These results suggest that SeP induces VEGF resistance in HUVECs in both normoglycaemic and hyperglycaemic conditions. However, additional experiments are clearly needed to determine whether SeP sufficiently removes hyperglycaemia-induced chronic oxidative stress in vascular endothelial cells.

We have shown that serum levels of total selenium were unchanged in the mice injected with *SEPP1* plasmid compared with the control animals (Fig. 4c), in spite of the significant elevation of serum SeP (Fig. 4b). Selenium content in forms other than SeP might decrease in the serum of the SeP-transgenic mice compensatively [14, 40]. Because a recent report showed that SeP exerts antioxidative actions independently of selenium supply [41], we speculate that the phenotype of the SeP-transgenic mice reflects the action of SeP itself, not the abnormal selenium distribution in mice.

Sepp1-heterozygous-knockout mice exhibited an increase in angiogenesis during hindlimb ischaemia without the induction of diabetes (Fig. 5), suggesting that the hepatokine SeP plays a role in the regulation of systemic angiogenesis, irrespective of diabetes status. For example, lipopolysaccharide-induced acute inflammation was reported to downregulate the production of SeP in mice [42]. Angiogenesis promoted by suppressed production of SeP might be beneficial in

inflammatory conditions. Further characterisation of *Sepp1*-deficient mice will provide insights into the involvement of SeP in the regulation of angiogenesis in normoglycaemic conditions.

Serum levels of human SeP in the mice injected with human *SEPP1* plasmid reached approximately 2.0 μg/ml (Fig. 4b). This corresponds with the incremental change in serum level of SeP from that of people with normal glucose tolerance to that of people with type 2 diabetes in the Japanese population [15, 25]. This strongly suggests that the phenotype observed in the SeP-transgenic mice reflects the physiological actions of SeP.

One limitation of the present study is that we examined the action of SeP on endothelial cells only. Various types of cell participate in the angiogenic processes. Further studies are necessary to determine whether SeP exerts effects on other cell types such as the monocytes or endothelial progenitor cells.

Another limitation of the present study is that we carried out all the experiments of *Sepp1*-deficient mice without the induction of diabetes with a high-fat diet or streptozotocin. Hence, we did not investigate the contribution of SeP in the development of the dysregulated angiogenesis seen in diabetes in vivo. However, our data indicate that treatment with purified SeP directly inhibits angiogenesis in both vascular endothelial cells and mice under euglycaemic conditions. Combined with the previous reports showing the elevated production of SeP in type 2 diabetes [15, 16], the current data suggest that overproduction of SeP contributes to the onset of impaired angiogenesis in type 2 diabetes. However, further studies in animals with diabetes are necessary to determine the degree of the contribution of SeP on the impaired angiogenesis observed in diabetes.

In summary, the present study indicates that the diabetes-associated hepatokine SeP impairs angiogenesis by reducing VEGF signal transduction in endothelial cells, and suggests that SeP may be a novel therapeutic target for treatment of VEGF resistance in people with type 2 diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement KI researched the data and wrote the manuscript. HM conceived and designed the experiments, researched the data, contributed to the discussion, wrote the manuscript and reviewed and edited the manuscript. MK researched the data, contributed to the discussion and reviewed and edited the manuscript. HT, NM-N, NTaj, KC, FL, HA, TO, MS, YT, KK, AF and KM designed the experiments, contributed to the discussion and reviewed the manuscript. YS, YO, YT, KT, HK, SKam and NTak conceived and designed the experiments, researched the data, contributed to the discussion and revised the manuscript critically for important intellectual content. SKan and TT conceived and designed the experiments, contributed to the discussion, wrote the manuscript and reviewed and edited manuscript. TT is the guarantor of this work, has full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis. All the authors have approved the final version of the manuscript.

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Impaired Interferon Signaling in Chronic Hepatitis C Patients With Advanced Fibrosis via the Transforming Growth Factor Beta Signaling Pathway

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Malnutrition in the advanced fibrosis stage of chronic hepatitis C (CH-C) impairs interferon (IFN) signaling by inhibiting mammalian target of rapamycin complex 1 (mTORC1) signaling. However, the effect of profibrotic signaling on IFN signaling is not known. Here, the effect of transforming growth factor (TGF)-β signaling on IFN signaling and hepatitis C virus (HCV) replication was examined in Huh-7.5 cells by evaluating the expression of forkhead box O3A (Foxo3a), suppressor of cytokine signaling 3 (Socs3), c-Jun, activating transcription factor 2, ras homolog enriched in brain, and mTORC1. The findings were confirmed in liver tissue samples obtained from 91 patients who received pegylated-IFN and ribavirin combination therapy. TGF-β signaling was significantly up-regulated in the advanced fibrosis stage of CH-C. A significant positive correlation was observed between the expression of TGF-β2 and mothers against decapentaplegic homolog 2 (Smad2), Smad2 and Foxo3a, and Foxo3a and Socs3 in the liver of CH-C patients. In Huh-7.5 cells, TGF-β1 activated the Foxo3a promoter through an AP1 binding site; the transcription factor c-Jun was involved in this activation. Foxo3a activated the Socs3 promoter and increased HCV replication. TGF-β1 also inhibited mTORC1 and IFN signaling. Interestingly, c-Jun and TGF-β signaling was up-regulated in treatment-resistant IL28B minor genotype patients (TG/GG at rs8099917), especially in the early fibrosis stage. Branched chain amino acids or a TGF-β receptor inhibitor canceled these effects and showed an additive effect on the anti-HCV activity of direct-acting antiviral drugs (DAAs). *Conclusion:* Blocking TGF-β signaling could potentiate the antiviral efficacy of IFN- and/ or DAA-based treatment regimens and would be useful for the treatment of difficult-to-cure CH-C patients. (HEPATOLOGY 2014;60:1519-1530)

A human liver infected with hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and in some instances, hepatocellular carcinoma (HCC). HCC develops frequently in the advanced fibrosis stage, and the annual incidence of HCC in patients with HCV-related liver cirrhosis is ~6-8%.[1] The eradication of HCV infection has been a promising prophylactic therapy for preventing the occurrence of HCC.

Interferon (IFN) and ribavirin (RBV) combination therapy has been a popular modality for eliminating HCV; however, its efficacy is limited in patients with advanced liver fibrosis.[2] The use of the recently developed direct-acting antiviral drugs (DAAs) telaprevir or

Abbreviations: AMPK, protein kinase, AMP-activated, alpha 1 catalytic subunit; CH-C, chronic hepatitis C; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IL28B, interleukin 28B; ISG-20, interferon-stimulated exonuclease gene 20; MX1, myxovirus resistance 1; NR, no response; RBV, ribavirin; RHEB, ras homolog enriched in brain; RIG-I, retinoic acid inducible gene I; SMAD, mothers against decapentaplegic homolog; TGF, transforming growth factor; TGF-RI, transforming growth factor-receptor inhibitor.

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boceprevir, combined with pegylated (PEG)-IFN plus RBV, significantly improved the sustained virologic response (SVR) rates; however, the SVR rate is reduced in patients with advanced liver fibrosis and the treatment-resistant interleukin 28B (IL28B) genotype,[3-5] in whom HCC can develop at a high frequency. Moreover, extended therapy should be avoided in these patients in terms of the high frequency of adverse effects.

The mechanism of treatment resistance in patients with advanced liver fibrosis has not yet been clarified completely. Previously, we reported that the malnutrition status of patients with advanced chronic hepatitis C (CH-C) is associated with IFN resistance, and Fischer's ratio (branched chain amino acids [BCAAs] / aromatic amino acids) is an independent predictor of treatment outcome of PEG-IFN plus RBV combination therapy. Furthermore, we showed that malnutrition impaired IFN signaling by inhibiting mammalian target of rapamycin complex 1 (mTORC1) and activating suppressor of cytokine signaling 3 (Socs3)-mediated IFN inhibitory signaling through the nutrition-sensing transcriptional factor forkhead box protein O3a (Foxo3a).[6] This report represented the first clue to disentangling the molecular links between advanced CH-C and poor treatment response; however, the association of profibrotic signaling and IFN signaling was not evaluated in detail.

In the present study, we investigated the interaction between the signaling of the profibrotic gene transforming growth factor (TGF)-β and IFN signaling in the liver of CH-C patients. We showed that blocking TGF-β signaling as well as improving the nutritional status of patients by using BCAAs restored IFN signaling and increased the treatment efficacy of anti-HCV therapy.

Materials and Methods

Cell Lines. A reversibly immortalized human hepatocyte cell line (TTNT) was established by transduction with a retroviral vector containing cDNA expressing hTERT for immortalization.[7] TTNT, 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's medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Primary human hepatocytes (PHH) were isolated from chimeric mice with a humanized liver (PXB-mice; PhoenixBio, Hiroshima, Japan).

Amino Acid-Free Medium and BCAAs. Amino acid-free medium and BCAAs were prepared as described previously. Details are given in the Supporting Materials and Methods.

TGF-β and IFN Treatment. Huh-7.5 cells or HCV-RNA-transfected Huh-7.5 cells were seeded at 1.0×10^5 cells/well in 12-well plates. After 24 hours, the cells were treated with TGF-β (Millipore, Billerica, MA). At 24 hours later, the cells were treated with the indicated international units of IFN-α for 24 hours (Schering-Plough, Tokyo, Japan).

BCAA Treatment. HCV-RNA-transfected Huh-7.5 cells were seeded at 1.0×10^5 cells/well in 12-well plates. After 24 hours, the cells were treated with TGF-β in low-amino-acid medium and the indicated concentration of BCAAs. At 48 hours after treatment, real-time detection, polymerase chain reaction (RTD-PCR), western blotting, and *Gaussia* luciferase assays were carried out as described previously.

TGF-β Receptor Inhibitor Treatment. HCV-RNA-transfected Huh-7.5 cells were seeded at 1.0×10^5 cells/well in 12-well plates. After 24 hours, the cells were treated with TGF-β in low-amino-acid medium and TGF-β Receptor Inhibitor (TGF-β RI; Millipore). At 24 hours after treatment, RTD-PCR, western blotting, and *Gaussia* luciferase assays were carried out as described previously.

DAA Treatment. DAAs (boceprevir and BMS-790052) were purchased from AdooQ Bioscience (Irvine, CA). HCV-RNA-transfected Huh-7.5 cells were seeded at 1.0×10^5 cells/well in 12-well plates. After 24 hours, the cells were treated with TGF-β in low-amino-acid medium and BCAAs and DAAs. At 24 hours after treatment, the *Gaussia* luciferase assay was carried out as described previously.

Patients' characteristics, HCV replication analysis, western blotting, quantitative RTD-PCR, and promoter analysis are described in the Supporting Materials and Methods.

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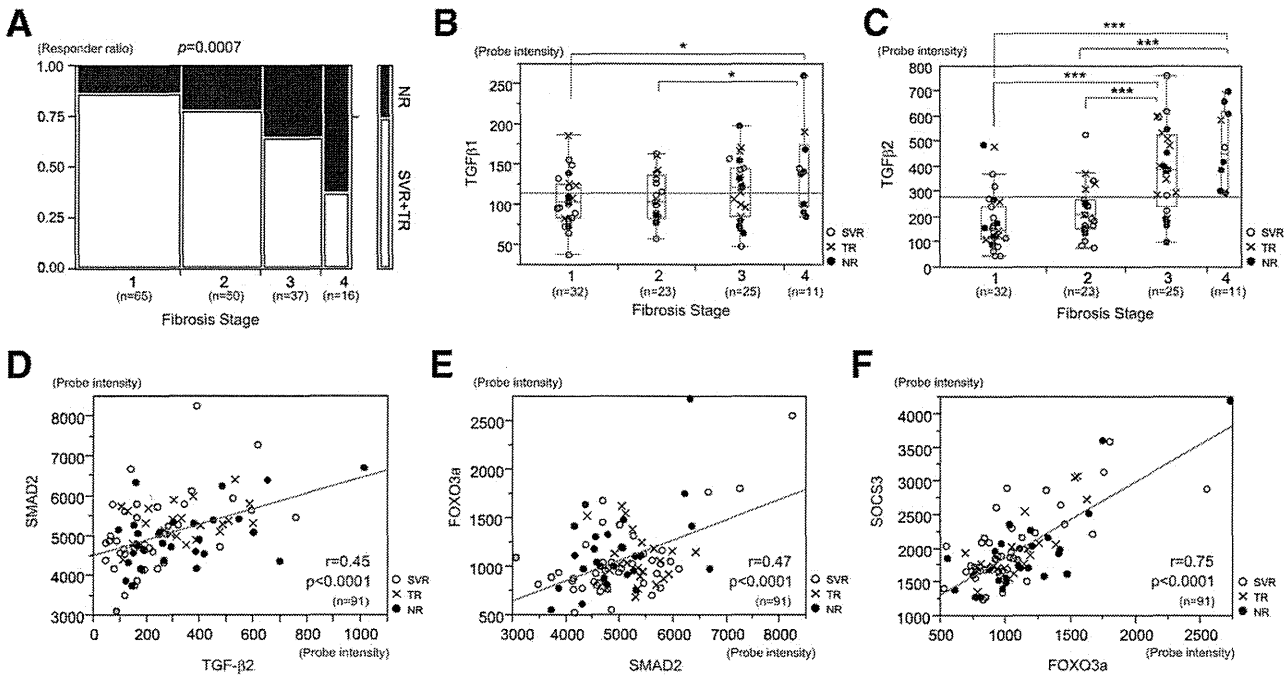

Fig. 1. Activation of TGF-β signaling in the liver of patients at the advanced fibrosis stage of CH-C. A: Significant increase in the NR ratio with the progression of fibrosis stage. B,C: Expression of TGF-β1 (B) and TGF-β2 (C) with the progression of fibrosis stage. D-F: Significant correlations of TGF-β2 and Smad2 (D), Smad2 and Foxo3a (E), and Foxo3a and Socs3 (F) expression in the liver of CH-C patients.

Statistical Analysis. The results are expressed as the mean value ± standard deviation. At least three samples were tested in each assay. Significance was tested by one-way analysis of variance with Bonferroni methods, and differences were considered statistically significant at $P < 0.05$.

Results

Up-Regulated TGF-β Signaling and Low Treatment Response in CH-C Patients With Advanced Liver Fibrosis Who Received PEG-IFN Plus RBV Combination Therapy. Previously, using a cohort of 168 CH-C patients who received PEG-IFN plus RBV combination therapy, we demonstrated that liver fibrosis stage and Fischer's ratio as well as IL28B genotype were independent significant factors associated with no response (NR) to treatment (Supporting Table 1).[6] The NR rate was significantly increased according to the increase in fibrosis stage ($P = 0.007$) (Fig. 1A). To reveal the molecular mechanism between profibrotic signaling and treatment resistance, we focused on TGF-β signaling in the liver of CH-C patients. The expression of TGF-β1 and TGF-β2, deduced from 91 CH-C patients whose liver tissues were analyzed previously using an Affymetrix GeneChip (Supporting Table 2),[6,8] was significantly up-regulated in the advanced fibrosis stage (Fig. 1B,C). In particular, the up-regulation of TGF-β2 in patients with stage 3 and 4 fibrotic livers was more prominent (Fig. 1C). There was a significant correlation between the expression of TGF-β2 and mothers against decapentaplegic homolog 2 (Smad2), a downstream signaling molecule of the TGF-β receptor, showing the activation of TGF-β signaling in the liver of CH-C patients. Interestingly, Smad2 expression was significantly correlated with Foxo3a expression, a nutrition-sensing transcription factor. Previously, we reported that Foxo3a increases the transcription of Socs3, an inhibitor of IFN signaling, through binding to the Socs3 promoter (Foxo3a-Socs3 signaling).[6] Foxo3a expression was significantly correlated with Socs3 expression in the CH-C patients (Fig. 1F).

TGF-β Signaling Activates Foxo3a-Socs3 Signaling in the Huh-7.5 Human Hepatoma Cell Line and PHH. The relationship between TGF-β and Foxo3a-Socs3 signaling was evaluated in PHH and the Huh-7.5 human hepatoma cell line without HCV replication (Huh-7.5 HCV (–)). This signaling was also evaluated in Huh-7.5 cells in which the infectious HCV clone H77Sv3 GLuc2A[6] was replicating (Huh-7.5 HCV (+)) (Fig. 2A). Treatment of these cells with TGF-β1 substantially increased the levels of phosphorylated (p)-Smad2 and p-Smad3. In this condition, the levels of p-Foxo3a, which is degraded through the proteasomal pathway,

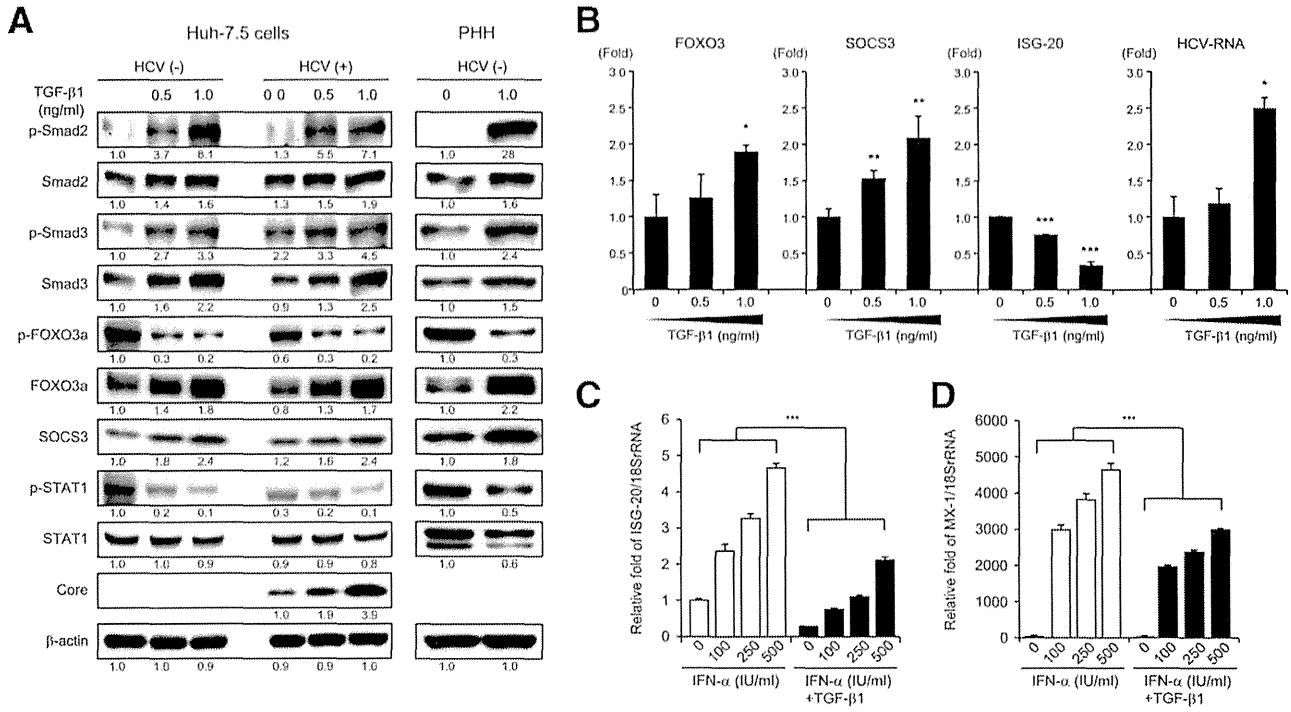

Fig. 2. Effect of TGF-β1 on IFN signaling in Huh-7.5 cells and PHH. A: Western blotting of TGF-β, Foxo3a-Socs3, and IFN signaling in Huh-7.5 cells and PHH treated with TGF-β1. Huh-7.5 cells were transfected with infectious HCV RNA, H77Sv3 GLuc2A prior to TGF-β1 treatment (Huh-7.5 HCV (+)). The experiments were repeated 3 times. B: RTD-PCR results for Foxo3a, Socs3, ISG-20, and HCV-RNA expression in Huh-7.5 HCV (+) treated with TGF-β1. C,D: Inhibition of IFN-α-induced ISG induction (ISG-20 [C] and MX1 [D]) by TGF-β1 in Huh-7.5 HCV (+). B-D: The experiments were performed in triplicate and repeated 3 times ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

decreased and total Foxo3a expression increased, and then Socs3 expression increased. Subsequently, the levels of phosphorylated signal transducer and activator of transcription 1 (p-STAT1) were decreased and the amount of HCV core protein increased in Huh-7.5 HCV (+). Thus, TGF-β signaling activated Foxo3a-Socs3 signaling and inhibited IFN signaling in hepatocytes, regardless of HCV replication and a loss-of-function mutation in retinoic acid inducible gene I (RIG-I).

These findings were also confirmed at the mRNA level in Huh-7.5 HCV (+). RTD-PCR showed that TGF-β1 treatment significantly increased Foxo3a and Socs3 expression, and decreased the expression of interferon-stimulated exonuclease gene 20 (ISG-20) in a dose-dependent manner. HCV-RNA was significantly increased in this condition (Fig. 2B). Moreover, the induction of interferon-stimulated genes (ISG-20 and myxovirus-resistance 1 [MX1]) by IFN-α treatment was significantly reduced in the presence of TGF-β1 (Fig. 2C,D).

When endogenous TGF-β signaling was compared between Huh-7.5 HCV (–) and Huh-7.5 HCV (+), TGF-β signaling was preactivated in Huh-7.5 HCV (+) before TGF-β1 treatment (Fig. 2A). To examine the role of endogenous TGF-β1 signaling on Foxo3a-Socs3 signaling and HCV replication, a small interfering (si) RNA specific to TGF-β1 was introduced to Huh-7 cells in which cell culture-derived infectious HCV HJ3-5 (HCVcc HJ3-5)[9] (Supporting Materials and Methods) was replicating. With the repression of TGF-β1, the levels of p-Smad2, p-Smad3, Foxo3a, and Socs3a decreased, while the levels of p-STAT1 increased. As a result, HCV replication deceased in both the amino acid-depleted (1/5 DMEM) and non-depleted (DMEM) conditions (Supporting Fig. 1).

AP1 Binding Site in the Foxo3a Promoter Is Responsible for the Induction of Foxo3a by TGF-β Signaling. To identify which transcription factors were involved in the induction of Foxo3a by TGF-β1, we cloned the upstream promoter region of Foxo3a and generated Foxo3a promoter-luciferase reporter constructs with various lengths of 5′-end deletions (−1780, −1340, and −801 nucleotides [nt]) (Fig. 3A). Luciferase activity deduced from pGL4-FOXO3a (−1780) increased by ~1.5-fold in the amino acid-depleted condition (1/5 DMEM) compared with the nondepleted condition (DMEM). TGF-β1 further stimulated the promoter activity of pGL4-FOXO3a (−1780) (Fig. 3B). A TGF-β1 RI canceled this stimulation (Fig. 3B). pGL4-FOXO3a (−1340) retained the regulation of promoter activity by amino acid depletion (1/5 DMEM) and

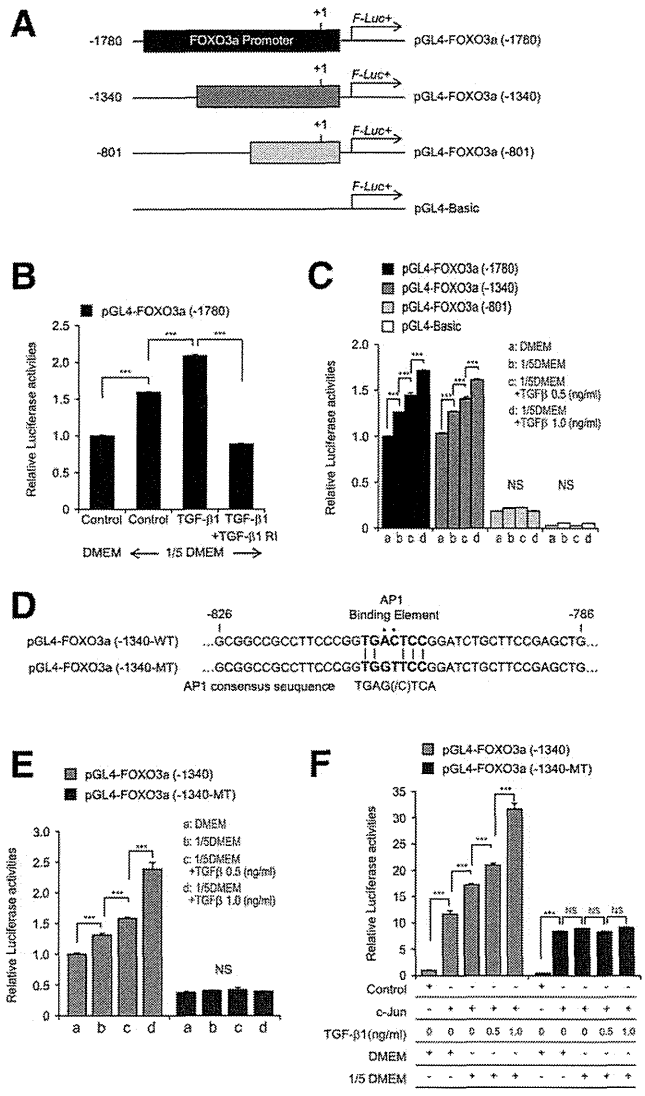

Fig. 3. Foxo3a promoter analysis. A: Foxo3a promoter-luciferase reporter constructs. B: Promoter activity of pGL4-FOXO3a (−1780) following amino acid depletion (1/5 DMEM), TGF-β1 treatment, and TGF-β1 RI treatment. C: Abolished regulation of the promoter activity of pGL4-FOXO3a (−801) by amino acid depletion (1/5 DMEM) and TGF-β1 treatment. D: Alignment of the AP1 binding element of pGL4-FOXO3a (−1340) and pGL4-FOXO3a (−1340-MT), in which the AP1 site was mutated. E: Abolished regulation of the promoter activity of pGL4-FOXO3a (−1340-MT) by amino acid depletion (1/5 DMEM) and TGF-β1 treatment. F: Overexpression of c-Jun, amino acid depletion (1/5 DMEM), and TGF-β1 treatment increased the promoter activity of pGL4-FOXO3a (−1340) by up to 32-fold, while these had less of an effect on the promoter activity of pGL4-FOXO3a (−1340-MT). The experiments were performed in triplicate and repeated 3 times (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

TGF-β1 treatment; however, pGL4-FOXO3a (−801) lost this regulation (Fig. 3C), suggesting the presence of a response element between −1340 and −801 nt. We identified an activator protein (AP) 1 transcription factor binding site at −810 to −804 nt. (Fig. 3D). We introduced two nucleotide mutations (AC to GT) in the AP1 consensus binding sequence, and the mutant construct, pGL4-FOXO3a (−1340-MT), lost the response to amino acid depletion (1/5 DMEM) and TGF-β1 treatment (Fig. 3E). These results were confirmed by using three different hepatocyte-derived cell lines (TTNT, Huh-7, and Huh-7.5 cells; Supporting Fig. 2A-C). Although RIG-I-dependent IFN signaling was active in TTNT cells (Supporting Fig. 2D), Foxo3a promoter activity in response to amino acid depletion (1/5 DMEM) and TGF-β1 treatment was not significantly different between these cell lines.

To confirm these findings further, we overexpressed c-Jun, a component of AP1, and evaluated Foxo3a promoter activity. The overexpression of c-Jun increased the promoter activity of pGL4-FOXO3a (−1340) to 12-fold, and amino acid depletion (1/5 DMEM) and TGF-β1 treatment further increased promoter activity up to 32-fold (Fig. 3F). Conversely, pGL4-FOXO3a (−1340-MT) lost the response to amino acid depletion (1/5 DMEM) and TGF-β1 treatment (Fig. 3F). These results confirmed that AP1 plays an important role in the induction of Foxo3a by these stimulatory factors.

Transcription Factor c-Jun Is Involved in the Induction of Foxo3a in the Liver of CH-C Patients. The AP1 transcription factor is mainly composed of Jun, Fos, and activating transcription factor (ATF) protein dimers.[10] Therefore, we evaluated the expression of c-Jun, ATF2, and c-Fos in Huh-7.5 cells and PHH under amino acid depletion (1/5 DMEM) and TGF-β1 treatment. Western blotting analysis showed that the levels of p-c-Jun and p-ATF2 were increased under these conditions, although the induction of p-c-Jun by amino acid depletion (1/5 DMEM) was not obvious in PHH (Fig. 4A). These findings were also confirmed by RTD-PCR. The mRNA expression of c-Jun and ATF2 increased significantly, while the expression of c-Fos decreased (Supporting Fig. 3A-C). The overexpression of c-Jun in Huh-7.5 cells induced Foxo3a and Socs3 expression at the protein and mRNA levels (Supporting Fig. 3D,E). In the liver of CH-C patients, there were significant correlations between the expression of Smad2 and c-Jun, and c-Jun and Foxo3a (Fig. 4B,C). ATF2 expression was significantly correlated with c-Jun expression (Fig. 4D). Similarly, there were significant correlations between the expression of Smad2 and ATF2, and ATF2 and Foxo3a (Fig. 4E,F). These results suggested that c-Jun and possibly ATF2, but not c-Fos, might be involved in TGF-β-Foxo3a signaling.

TGF-β Signaling Induces Socs3 Through the Induction of Foxo3a. Previously, we reported that Foxo3a increases the transcription of Socs3 through its binding to the Socs3 promoter region.[6] We confirmed

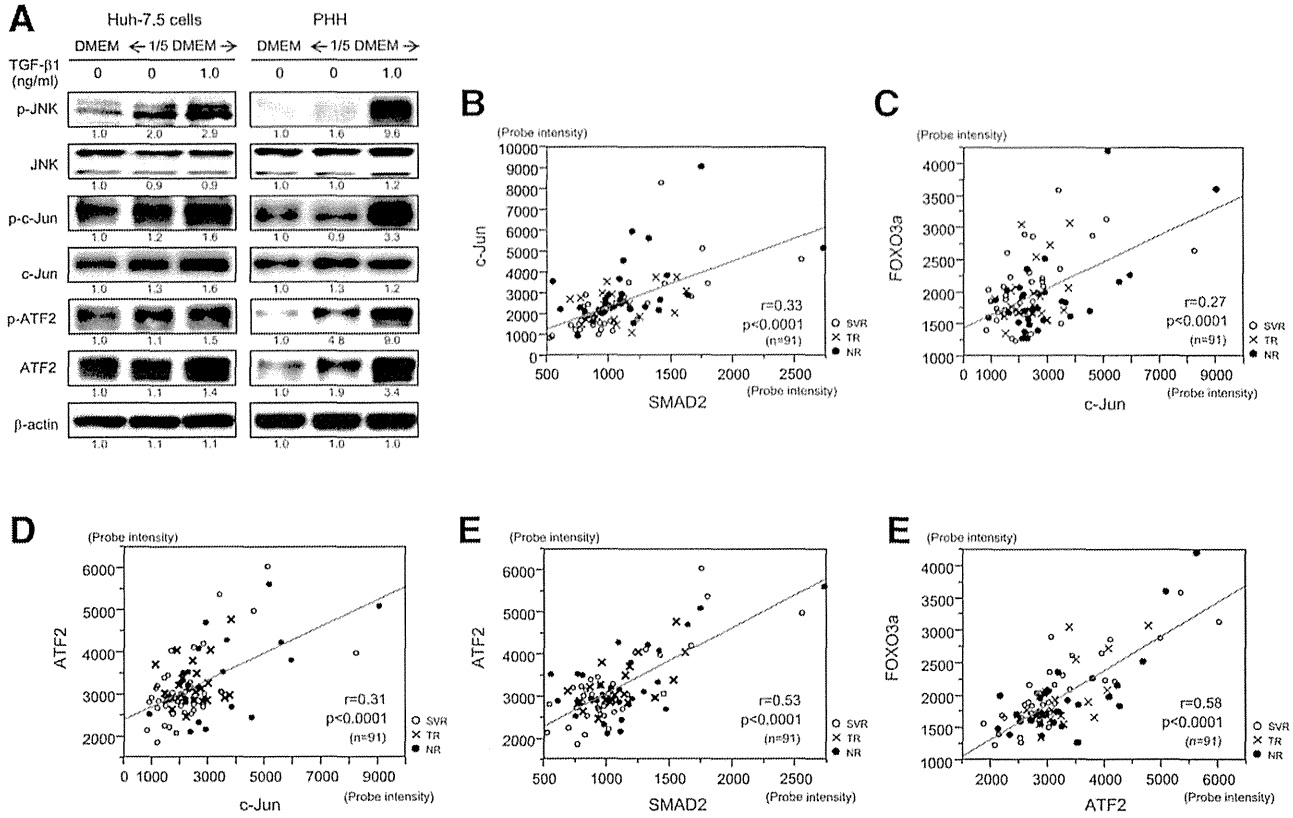

Fig. 4. TGF-β signaling up-regulates the expression of the transcription factors c-Jun and ATF2 in Huh-7.5 cells, PHH, and the liver of CH-C patients. A: Western blotting of JNK, c-Jun, and ATF2 in Huh-7.5 cells and PHH treated with amino acid depletion (1/5 DMEM) and TGF-β1. The experiments were repeated 3 times. B-F: Significant correlations of Smad2 and c-Jun (B), Foxo3a and c-Jun (C), c-Jun and ATF2 (D), Smad2 and ATF2 (E), and ATF2 and Foxo3a (F) expression in the liver of CH-C patients.

these findings in more detail in conjunction with TGF-β signaling. The overexpression of Foxo3a increased Socs3 expression in the nonamino acid-depleted condition (DMEM), and Socs3 was further induced in the amino acid-depleted condition (1/5 DMEM) and by TGF-β1 treatment (Supporting Fig. 4A). HCV-RNA was similarly increased in these conditions (Supporting Fig. 4B). Foxo3a mRNA expression, as deduced from RTD-PCR, was increased up to 7-fold in the combination of amino acid depletion (1/5 DMEM), c-Jun overexpression, and TGF-β1 treatment (Supporting Fig. 4C). Socs3 mRNA expression was up-regulated by 8-fold in the same conditions (Supporting Fig. 4D). The promoter activity of Socs3 was significantly increased by amino acid depletion (1/5 DMEM) and TGF-β1 treatment (pGL4-SOCS3-WT, Supporting Fig. 4E), while mutation of the Foxo3a binding site in the Socs3 promoter (pGL4-SOCS3-MT) abrogated this regulation. These results confirmed that TGF-β signaling up-regulated the expression of Socs3 through the induction of Foxo3a.

TGF-β Signaling Suppresses mTORC1 Signaling. Previously, we demonstrated that malnutrition decreased mTORC1 and IFN signaling using Huh-7 cells and clinical samples.[6] In the present study, we examined the effect of TGF-β signaling on mTORC1 signaling. In Huh-7.5 cells and PHH, amino acid depletion (1/5 DMEM) repressed mTORC1 signaling, as demonstrated by the decreased expression of ras homolog enriched in brain (RHEB),[11] a stimulator of mTORC1 signaling, p-mTOR, and p-p70S6K (Fig. 5A). Interestingly, TGF-β1 further decreased this expression. The decreased mTORC1 signaling was independent of AMP-activated, alpha 1 catalytic subunit (AMPK), a suppressor of mTORC1 signaling, as the levels of p-AMPK were rather decreased by amino acid depletion (1/5 DMEM) and TGF-β1 treatment in Huh-7.5 cells and PHH (Fig. 5A). It could be speculated that TGF-β signaling, combined with malnutrition, repressed the expression of RHEB and induced the expression of Foxo3a, which leads to the impaired IFN signaling observed in the advanced fibrosis stage of CH-C (Fig. 5B). In the liver of CH-C

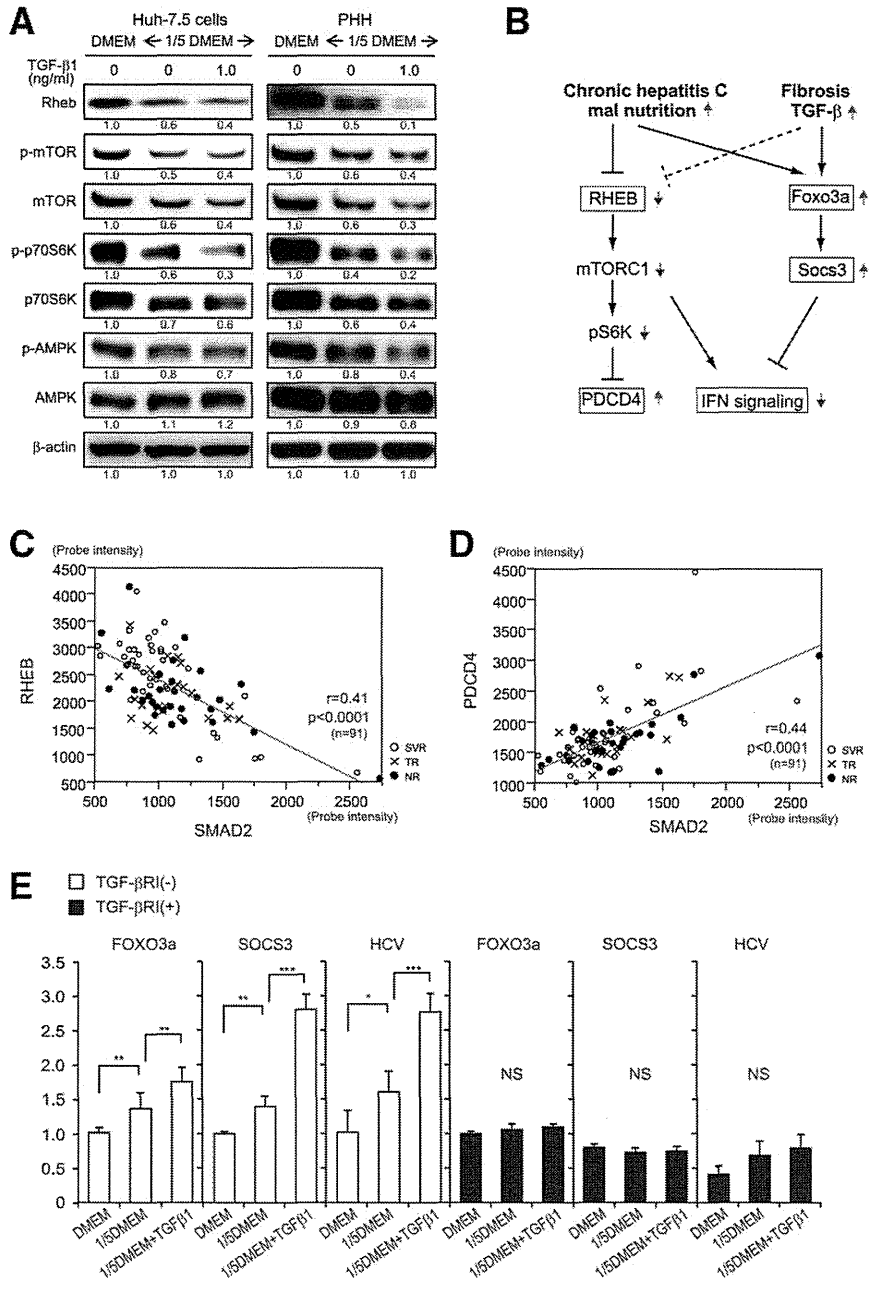

Fig. 5. TGF-β signaling represses mTORC1 signaling in Huh-7.5 cells, PHH, and the liver of CH-C patients. A: Western blotting of RHEB, mTOR, p70S8K, and AMPK in Huh-7.5 cells and PHH treated with amino acid depletion (1/5 DMEM) and TGF-β1. The experiments were repeated 3 times. B: Schematic representation of the effects of malnutrition and TGF-β signaling on IFN signaling. C,D: Significant correlations of Smad2 and RHEB (C), and Smad2 and PDCD4 (D) expression in the liver of CH-C patients. E: Blocking TGF-β signaling by TGF-β1 RI treatment abolishes the increase in Foxo3a, Socs3, and HCV replication by amino acid depletion (1/5 DMEM) and TGF-β1 treatment. The experiments were performed in triplicate and repeated 3 times ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

patients, Smad2 expression was significantly negatively correlated with RHEB expression. The expression of programmed cell death 4 (PDCD4), which is negatively regulated by mTORC1 signaling at the transcriptional level (Fig. 5C),[12] was significantly positively correlated with Smad2 expression (Fig. 5D).

We further examined the effect of TGF-β1 on IFN signaling by using TGF-β RI. TGF-β RI substantially repressed the levels of p-Smad2 and p-Smad3 (Supporting Fig. 5). TGF-β RI abolished the induction of Foxo3a expression and the subsequent induction of Socs3 by amino acid depletion (1/5 DMEM) and TGF-β1 treatment (Fig. 5E). HCV replication in normal medium (DMEM), as deduced from *Gaussia* luciferase activity, was repressed by TGF-β RI, and the increase in HCV replication by amino acid depletion (1/5 DMEM) and TGF-β1 treatment was abrogated (Fig. 5E).

c-Jun Is Up-Regulated in the Liver of NR and Treatment-Resistant IL28B Minor Genotype Patients. We evaluated the clinical significance of c-Jun for treatment response. The expression of c-Jun was significantly higher in NR patients than in responder patients (SVR+TR) (Fig. 6A). Furthermore, c-Jun expression was significantly higher in patients with the treatment-resistant IL28B minor genotype

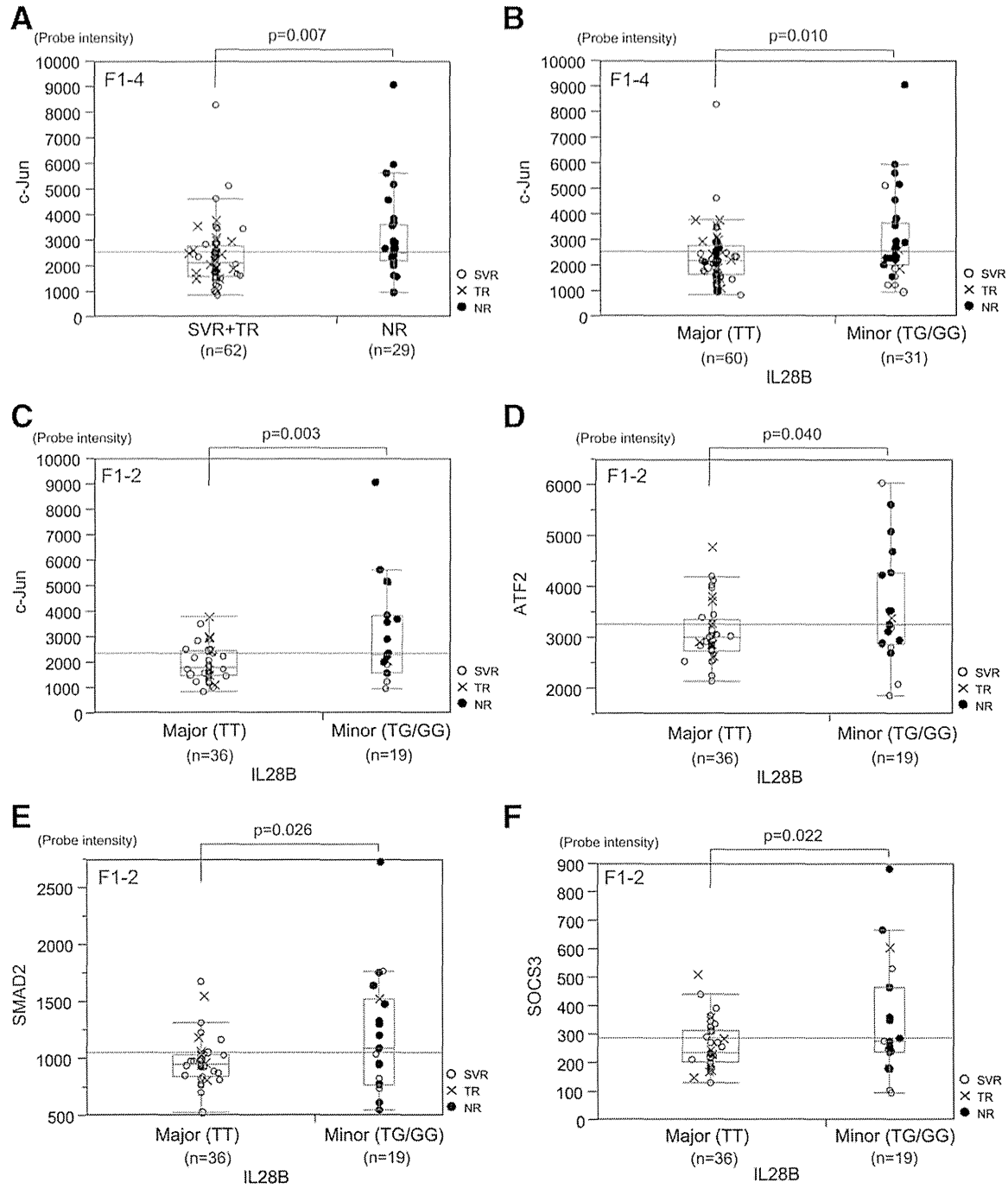

Fig. 6. Relationship between TGF-β signaling and treatment response and the IL28B genotype. The expression of c-Jun was up-regulated in NR (A) and IL28B minor genotype (TG/GG at rs8099917) (B) patients in all fibrosis stages (F1-4). The expression of ATF2 (D), Smad2 (E), and Socs3 (F) was up-regulated in IL28B minor genotype (TG/GG at rs8099917) patients at early fibrosis stages (F1-2).

(TG/GG at rs8099917) than in those with the treatment-sensitive IL28B major genotype (TT) (Fig. 6B).[5] Interestingly, TGF-β signaling was more activated in patients with the treatment-resistant IL28B minor genotype at an early stage of liver fibrosis (F1 and F2). The expression of c-Jun, ATF2, Smad2, and Socs3 was significantly higher in IL28B minor genotype patients (Fig. 6C-F).

BCAAs Inhibit TGF-β Signaling and Restore IFN Signaling. Previously, we reported that BCAAs restored IFN signaling in the amino acid-depleted condition (1/5 DMEM) by activating mTORC1 signaling and suppressing Foxo3a-Socs3 signaling.[6] In the present study, we examined whether BCAAs could inhibit TGF-β signaling and restore IFN signaling. Western

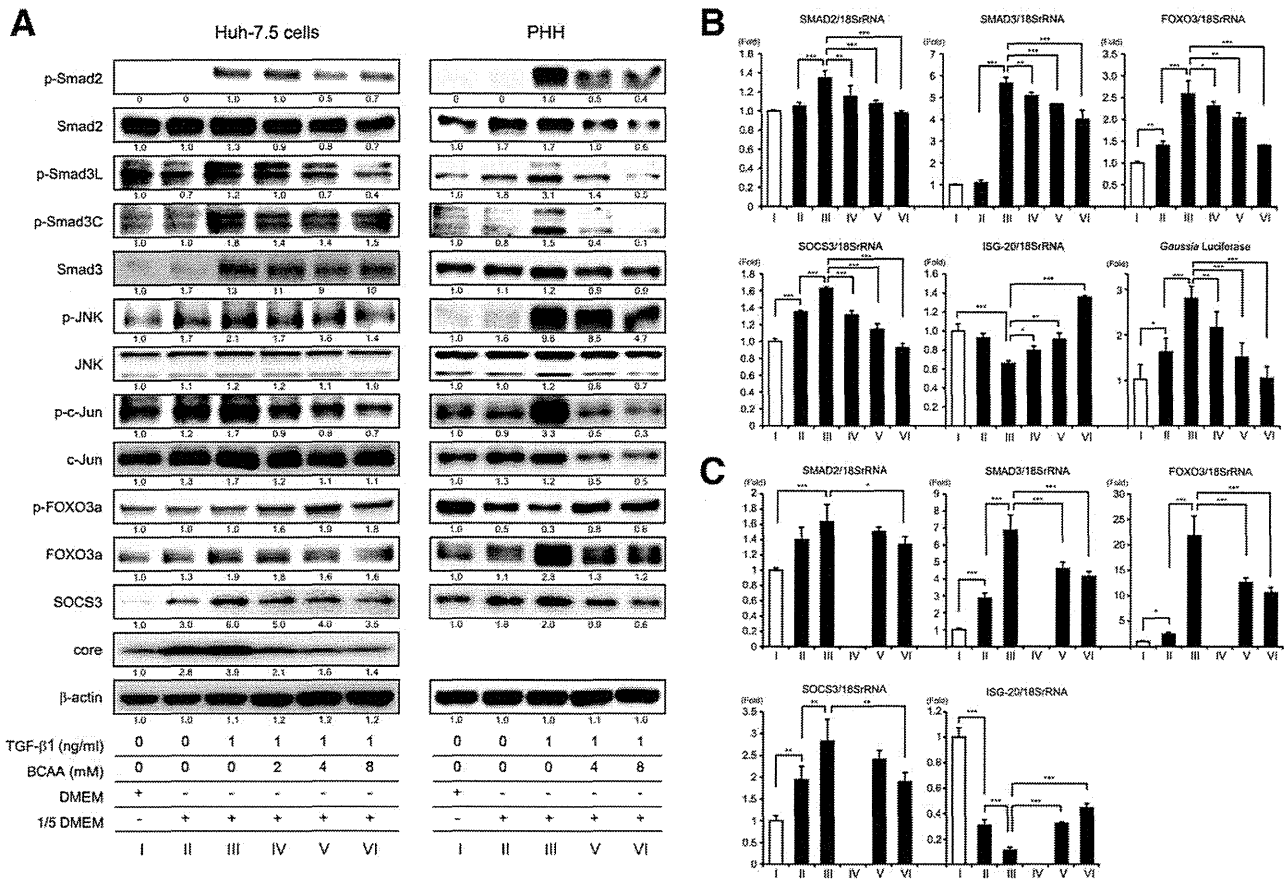

Fig. 7. BCAAs inhibit the effect of malnutrition and TGF-β signaling in Huh-7.5 cells and PHH. A: Western blotting of TGF-β and Foxo3a-Socs3 signaling in Huh-7.5 HCV (+) and PHH treated with amino acid depletion (1/5 DMEM), TGF-β1, and BCAAs. B,C: mRNA expression of TGF-β, Foxo3a-Socs3, and IFN signaling in Huh-7.5 HCV (+) (B) and PHH (C) treated with amino acid depletion (1/5 DMEM), TGF-β1, and BCAA.

blotting analysis showed that BCAAs dose-dependently repressed the expression of p-Smad3L, p-Smad3C, p-JNK, p-c-Jun, Foxo3a, Socs3 (in Huh-7.5 cells and PHH), and HCV core protein (in Huh-7.5 cells), which was induced by amino acid depletion (1/5 DMEM) and TGF-β1 treatment (Fig. 7A). RTD-PCR demonstrated similar mRNA expression patterns (Smad2, Smad3, Foxo3a, and Socs3a) to those obtained by western blotting (Fig. 7B,C), and BCAAs induced the expression of ISG-20 (in Huh-7.5 cells and PHH) and decreased HCV replication in a dose-dependent manner (in Huh-7.5 cells) (Fig. 7B). These results were also confirmed in HCVcc HJ3-5-infected Huh-7 cells (Supporting Fig. 6).

BCAAs and TGF-β RI Potentiate the Anti-HCV Activity of DAAs. Finally, we examined whether BCAAs or TGF-β RI potentiate the anti-HCV activity of DAAs. Amino acid depletion (1/5 DMEM) and TGF-β1 treatment significantly increased HCV replication (deduced from *Gaussia* luciferase activity), and BCAAs (8 mM) and boceprevir (250 nM; NS3 protease inhibitor) inhibited HCV replication to 64% and 50%, respectively (Fig. 8A, black bars). The combination of BCAAs (8 mM) and boceprevir (250 nM) further inhibited HCV replication to 10% and canceled the effect of amino acid depletion (1/5 DMEM) and TGF-β1 treatment, which supported HCV replication (Fig. 8A, compare white and black bars). Similarly, TGF-β RI (10 μM) repressed HCV replication to 60%, and its combination with boceprevir (250 nM) decreased HCV replication to 16% (Fig. 8B, black bars) and canceled the effect of amino acid depletion (1/5 DMEM) and TGF-β1 treatment (Fig. 8A, compare white and black bars). Thus, BCAAs and TGF-β RI had an additive effect on the anti-HCV activity of boceprevir and would be useful for CH-C patients with advanced fibrosis and the IL28B treatment-resistant genotype. A similar effect was obtained by using the NS5A inhibitor BMS-790052; however, its effect was less than that of boceprevir (Supporting Fig. 7).

Discussion

The recently developed DAAs have significantly improved the efficacy of anti-HCV therapy. Triple

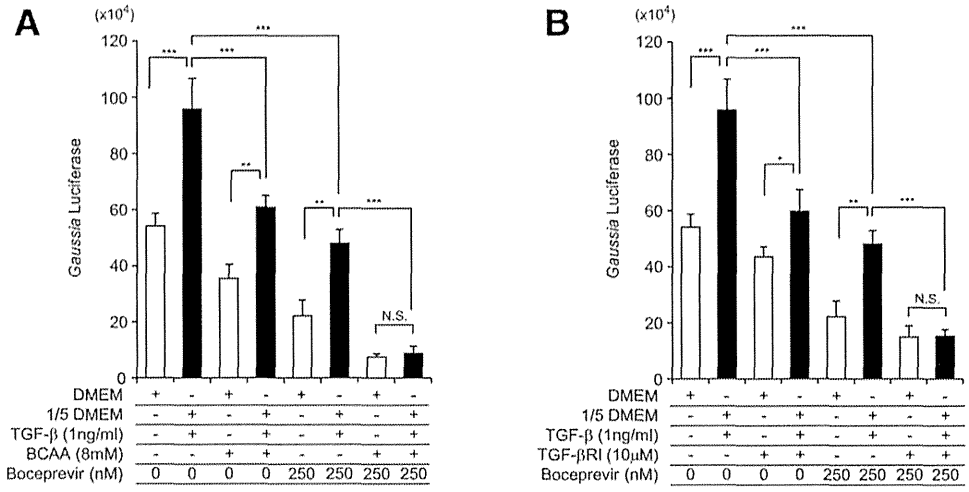

Fig. 8. Anti-HCV activity of boceprevir in combination with BCAAs (A) and TGF-β1 RI (B). HCV replication in Huh-7.5 cells was deduced by *Gaussia* luciferase activity. Boceprevir in combination with BCAAs (A) and TGF-β1 RI (B) efficiently repressed HCV replication in Huh-7.5 cells treated with amino acid depletion (1/5 DMEM) and TGF-β1. The experiments were performed in triplicate and repeated 3 times ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

therapy comprising PEG-IFN, RBV, and DAA (e.g., telaprevir or boceprevir) has significantly increased SVR rates; however, its efficacy is poor in difficult-to-cure patients such as those with cirrhosis and the IL28B treatment-resistant genotype.[2,4] An IFN-free regimen using a combination of DAAs would be effective to treat these difficult-to-cure patients; however, the emergence of multiple drug resistant viruses and the high cost of these therapies should be considered carefully in the future. Therefore, standard PEG-IFN plus RBV combination therapy is still useful as an alternative therapy for CH-C.

Previously, we reported that malnutrition in patients with the advanced fibrosis stage of CH-C is associated with IFN resistance and impaired IFN signaling by inhibiting mTORC1 and activating Socs3-mediated IFN inhibitory signaling through the nutrition-sensing transcriptional factor Foxo3a.[6] However, the effect of profibrosis signaling on IFN signaling was not addressed in our previous study. In the present study, using clinical samples and cell lines, we clearly showed that TGF-β signaling inhibits IFN signaling by activating Foxo3a-Socs3-mediated IFN inhibitory signaling (Figs. (1 and 4)) and inhibiting mTORC1 signaling (Fig. 5).

Using Foxo3a promoter-luciferase reporter constructs, we showed that TGF-β1 activated Foxo3a promoter activity through an AP1 transcription factor binding site. Among the components of AP1, c-Jun and probably ATF2, but not c-Fos, were involved in this induction. Previous reports showed that c-Jun and ATF2 were induced by amino acid depletion[13,14] and TGF-β1 treatment,[15,16] although the induction of c-Jun by amino acid depletion was not obvious in PHH in this study. It could be considered that malnutrition and profibrotic signaling cooperatively activated the Foxo3a promoter through the AP1 site and that c-Jun induction was more specifically regulated by TGF-β1 in normal hepatocytes. Mutation of the AP1 binding site (pGL4-FOXO3a [-1340-MT]) abolished the response to amino acid depletion (1/5 DMEM) and TGF-β1 treatment (Fig. 3E; Supporting Fig. 2). Conversely, c-Jun overexpression combined with amino acid depletion (1/5 DMEM) and TGF-β1 treatment activated the Foxo3a promoter by 32-fold (Fig. 3F). In addition, we showed that TGF-β1 inhibited mTORC1 signaling, as demonstrated by the decreased expression of RHEB, p-mTOR, and p-p70S6K (Fig. 5A).

These results were in concordance with gene expression in the liver of CH-C patients. The expression of c-Jun and ATF2 was significantly correlated with Smad2 and Foxo3a expression, respectively (Fig. 4), while the expression of RHEB was significantly negatively correlated with Smad2 expression in the liver of CH-C patients (Fig. 5C). In this study, TGF-β1 and TGF-β2 expression was up-regulated in advanced liver fibrosis, and the expression of TGF-β2 was well correlated with the downstream signaling molecule Smad2 (Fig. 1B-D). Although we could not address the biological differences in TGF-β isoforms in this study, TGF-β1 and TGF-β2 reportedly mediate a similar signaling pathway to induce profibrotic responses.[17] Collectively, TGF-β signaling inhibited IFN signaling by activating Foxo3a-Socs3 IFN inhibitory signaling and

inhibiting mTORC1-IFN stimulating signaling *in vitro* and *in vivo*. Recently, Lee et al. showed that Foxo3a regulates the TGF-β1 promoter directly.[18] Combining their data and ours, there must be positive feedback regulation between TGF-β1 and Foxo3a. Moreover, they identified a polymorphism in Foxo3a (rs12212067: T > G) in which the minor (G) allele was involved in the increased production of TGF-β1 and associated with the inflammatory response.[18] We genotyped the Foxo3a rs12212067 polymorphism in three cell lines and observed TT in Huh-7 and Huh-7.5 and GG in TTNT (Supporting Table 3). Although we could not find a significant difference in Foxo3a promoter activity in response to TGF-β1 among these cell lines (Supporting Fig. 2), further studies should be performed to compare Foxo3a-Socs3 IFN inhibitory signaling among them. Furthermore, it is worthwhile to examine the relationship between the genotype at rs12212067 and treatment response and severity of liver disease in CH-C patients in the future.

Another interesting finding in this study was that TGF-β signaling was related to the IL28B genotype (Fig. 6). The expression of c-Jun was significantly higher in IL28B treatment-resistant minor genotype (TG/GG at rs8099917) patients than in IL28B treatment-sensitive major genotype (TT) patients. Moreover, the expression of c-Jun, Smad2, ATF2, and Socs3 was up-regulated more in IL28B minor genotype patients than in IL28B major genotype patients, especially in those with early stage liver fibrosis (F1-2). The underlying mechanisms of these findings are not known so far; however, we recently reported that the noncanonical WNT signaling ligand WNT5A is up-regulated in the liver of IL28B minor genotype patients and plays a role in treatment resistance.[19] WNT5A reportedly mediates downstream signaling through c-Jun and ATF2 in *Xenopus* cells and human osteosarcoma cells.[20,21] It could be speculated that WNT5A potentiates TGF-β signaling through these transcription factors, although this hypothesis should be tested in the future.

We examined whether BCAAs and TGF-β RI improve the IFN inhibitory signaling induced by malnutrition and TGF-β signaling (Fig. 7). Previously, we demonstrated that BCAAs improved the IFN signaling that was inhibited by malnutrition.[6] In the present study, we found that BCAAs blocked TGF-β signaling by decreasing the levels of p-Smad3L, p-JNK, and c-Jun (Fig. 7A). Consequently, BCAAs decreased the expression of Foxo3a, Socs3, and HCV core protein (Fig. 7). In addition, we found that the combination of BCAAs or TGF-β RI and the NS3 protease inhibitor boceprevir efficiently inhibited HCV replication and canceled the positive effects of malnutrition and TGF-β1 on HCV replication (Fig. 8). A recent report showed that the NS3 protease of HCV mimics TGF-β2 and activates the TGF-β type I receptor.[22] Therefore, the anti-HCV effect of boceprevir could be potentiated in combination with BCAAs or TGF-β RI, which blocked TGF-β signaling and increased IFN signaling. Therefore, the combination of BCAAs or TGF-β RI with DAAs could be useful for the treatment of difficult-to-cure CH-C patients with advanced liver fibrosis and the IL28B treatment-resistant genotype.

In conclusion, we clarified that TGF-β signaling inhibits IFN signaling and is related to the treatment-resistant phenotype of CH-C patients with advanced liver fibrosis and the IL28B treatment-resistant genotype. Furthermore, blocking TGF-β signaling by BCAAs or TGF-β RI could potentiate the anti-HCV effect of DAAs. An oral TGF-β RI small compound, LY2157299, is now being assessed in a phase II trial for the treatment of advanced-stage HCC. Further studies should be performed to address the significance of these compounds for the eradication of HCV in patients with advanced liver fibrosis for preventing HCC.

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Supporting Information

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REVIEW

Orchestration of hepatocellular carcinoma development by diverse liver cancer stem cells

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Abstract Hepatocellular carcinoma (HCC) is one of the world's most aggressive diseases and carries a poor prognosis for patients. Recent evidence suggests that HCC is organized by cancer stem cells (CSCs), which are a subset of cells with stem cell-like features. CSCs are considered a pivotal target for the eradication of cancer, and liver CSCs have been investigated using various stem cell markers. Several hepatic stem/progenitor markers have been shown to be useful for isolating putative CSCs from HCC, although the expression patterns and phenotypic diversity of CSCs purified by these markers remain obscure. Recently, we found that liver CSCs defined by different markers show unique features of tumorigenicity and metastasis, with phenotypes closely associated with committed liver lineages. Furthermore, our data suggest that these distinct CSCs collaborate to orchestrate the tumorigenicity and metastasis of HCC. In this review article, we summarize the recent advances in understanding the pathogenesis and heterogeneity of liver CSCs.

Keywords Hepatocellular carcinoma · Cancer stem cell · Tumorigenicity · Metastasis

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide [1]. Its prevalence is mostly attributed to hepatitis B virus or hepatitis C virus infection, and high incidence is observed in Asia and Africa [2]. Increasing occurrences and mortality from HCC have also been observed in most industrialized countries [3]. Therefore, there is an urgent need to develop effective diagnostic and treatment strategies against this disease.

HCC is a heterogeneous disease in terms of morphology, biological behavior, response to treatment, and molecular profile [4]. This heterogeneity has traditionally been explained by the clonal evolution of tumor cells resulting from the progressive accumulation of multiple genetic and epigenetic changes [5, 6]. However, recent studies suggest that its heterogeneity may result from the hierarchical organization of tumor cells by a subset of cells with stem and progenitor cell features known as cancer stem cells (CSCs) [7]. CSCs are highly tumorigenic, metastatic, chemo- and radiotherapy resistant, responsible for tumor relapse after therapy, and able to divide symmetrically or asymmetrically to orchestrate the tumor mass [8]. Therefore, they are considered to be a pivotal target for eradicating HCC [9]. In this review, we summarize recent findings on liver CSCs in terms of heterogeneity and discuss an HCC treatment strategy that targets them.

CSC hypothesis

Cancer cells and stem cells have similar capabilities with respect to self-renewal, limitless division, and the generation of heterogeneous cell populations. The observation of these similarities many years ago led to the proposal that

cancer might be a type of abnormal stem cell disease [10], a concept which has recently been revisited [11]. The generally acknowledged definition of a CSC is a cell within a tumor that possesses the ability to self-renew and to give rise to heterogeneous lineages of cancer cells that comprise tumors in immunodeficient mice [11]. Experimentally, putative CSCs have been isolated using cell surface markers specific for normal stem cells. Stem cell-like features of CSCs have been confirmed by functional in vitro clonogenicity and in vivo tumorigenicity assays. Moreover, accumulating evidence suggests that CSCs play a role in perpetuating various cancers including leukemia and solid tumors [12–18].

In HCC, several markers are reported to enrich the CSC population, including the epithelial cell adhesion molecule (EpCAM), CD133, CD90, CD44, CD24, CD13, and oval cell marker OV6, as well as Hoechst dye efflux or aldehyde dehydrogenase activities [19–25]. Most of these markers are expressed in normal hepatic progenitors known as oncofetal markers [20–22, 26–35]. These marker-positive cells were experimentally confirmed to be more tumorigenic than marker-negative cells in immunodeficient mice using cell lines [9]. Among them, calcium channel α2δ1 isoform5, EpCAM, CD90, and CD133 are the markers confirmed thus far to enrich CSCs from primary HCCs [36, 37]. Recent studies have shown that some of these liver CSC markers are also functionally involved in the maintenance of CSC features (Table 1). EpCAM enhances Wnt signaling in ES cells and cancer [38, 39], and CD133 expression may maintain $CD133^+$ liver CSCs through the activation of neurotensin/IL-8/CXCL1 signaling [40]. CD44 regulates the redox status [41], while CD13 decreases cell damage induced by oxidative stress after exposure to genotoxic reagents [19]. Furthermore, a recent study demonstrated that the calcium channel α2δ1 isoform5, recognized by a monoclonal antibody 1B50-1, is expressed in liver CSCs and regulates calcium influx and ERK signaling [37]. Thus, the functional involvement of most liver CSC markers potentially makes them a good target for the eradication of liver CSCs. In particular, cell surface markers detected in liver CSCs may be good targets for immunotherapy.

Table 1 Cell surface markers in liver CSCs

Cell surface markers	Function in CSCs
Calcium channel α2δ1 isoform5	Calcium influx and activation of ERK signaling
CD13	ROS-induced DNA damage reduction
CD133	Neurotensin-interleukin-8-CXCL1 signaling
CD24	STAT3 mediated NANOG regulation
CD44	Regulation of redox status through xCT
CD90	Unknown
DLK1	Unknown
EpCAM	Activation of Wnt signaling
OV6	Unknown

Heterogeneity of liver CSCs

As described above, various hepatic progenitor markers have been detected in the population of liver CSCs. Purified cell populations using certain stem cell markers show CSC features such as high tumorigenicity, an invasive nature, and chemo- and radiotherapy resistance. However, it is unclear how these markers are expressed in primary HCC tissues or HCC cell lines. It is also unclear whether the CSCs expressing these markers exist in all HCCs or are restricted to a certain subtype. This is an especially important issue when treating HCC patients using molecularly targeted therapy against certain marker-positive CSCs.

In normal fetal livers, hepatoblasts express the biliary markers CK19 and EpCAM, as well as the hepatocyte markers albumin and alpha fetoprotein (AFP) [26, 27, 42, 43]. In addition, numerous studies have demonstrated that hepatic progenitor cells express a variety of markers putatively detected in various ectodermal or mesodermal lineages, including nestin, NCAM, CD34 and c-Kit, CD133, CD90, E-cadherin, and Dlk1 [44]. Hepatoblasts are also considered a heterogeneous population potentially organized in a hierarchical manner with various degrees of differentiation that may be related to their expression of stem cell markers [45]. Indeed, recent studies demonstrated that the characteristics of hepatic progenitors expressing different markers show distinct natures [32, 46]. Normal $EpCAM^+$ and $CD90^+$ oval cells represent two distinct populations: the former expresses classical oval cell markers such as AFP, OV-1, and CK19, and the latter expresses desmin and a-SMA but not AFP, OV-1, or CK19, which indicates that $CD90^+$ populations are more likely to be mesenchymal cells.

We explored the expression patterns of the representative liver CSC markers CD133, CD90, and EpCAM in primary HCC, and found that $EpCAM^+$ and $CD90^+$ CSCs show different gene expression patterns and cell morphology [36]. We further explored the tumorigenic capacity of sorted cells isolated from 15 primary HCCs and 7 liver cancer cell lines [36]. Although the number of samples analyzed was small, tumorigenic $EpCAM^+$, $CD133^+$, or $CD90^+$ CSCs were obtained in 26.6 % ($n = 4$), 20 % ($n = 3$), and 13.3 % ($n = 2$) of 15 HCCs, respectively, when xenotransplanted into NOD/SCID mice.

Interestingly, no EpCAM/CD90 double positive cells were detected in primary HCC, and $EpCAM^+$ and $CD90^+$ cells were distinctive with different tumorigenic/metastatic

capacities; that is, $EpCAM^+$ cells were associated with a high tumorigenic capacity and hepatic epithelial stem cell features, while $CD90^+$ cells had a metastatic propensity with mesenchymal vascular endothelial cell features. Importantly, the existence of $EpCAM^+$ cells correlated with high serum AFP values with a tendency for portal vein invasion, whereas the existence of $CD90^+$ cells was associated with a high incidence of distant organ metastasis. Furthermore, $CD90^+$ CSCs abundantly expressed c-Kit and showed chemosensitivity against the c-Kit inhibitor imatinib mesylate, whereas $EpCAM^+$ CSCs showed no such chemosensitivity. These data demonstrate that liver CSCs are not a single entity but exist heterogeneously with distinct CSC marker expression, suggesting that no common liver CSCs expressing particular stem cell markers exist in all HCCs. Our data also indicate that the presence of distinct CSCs is a key determinant of cancer phenotypes in terms of tumorigenicity and metastatic propensity, which may influence the clinical outcome of HCC.

The distinct nature of $EpCAM^+$ and $CD90^+$ liver CSCs raises the question whether these different types of CSCs originate from the same or different type of cells. This question remains elusive, but a recent study investigating three independent cell clones established from the same HCC specimen revealed that these clones maintain common karyotype abnormality but express EpCAM, CD90, and CD133 distinctively with different chemosensitivities against sunitinib [47], suggesting that distinct liver CSCs expressing different markers may originate from the same type of cells. In terms of liver CSC origin, a recent study demonstrated that acquisition of liver CSC properties is independent of the cell of origin, and liver CSCs can originate from hepatic progenitor cells, hepatoblasts, or adult hepatocytes in mice by forced H-Ras/SV40LT induction and subsequent oncogenic reprogramming [48]. In addition, another study has demonstrated the unexpected plasticity of normal mature hepatocytes to dedifferentiate into progenitor cells in rats [49], and this type of plasticity has also been reported in breast non-CSCs [50, 51]. Given the cellular plasticity reported in normal and cancer cells described above, it is reasonable to speculate that a similar plasticity may exist in $EpCAM^+$ and $CD90^+$ CSCs that can convert their tumorigenic/metastatic phenotypes and marker expression status. Further studies are required to clarify the role of cell plasticity on heterogeneity of HCC [36].

Interaction of distinct cell lineages in liver organogenesis and hepatocarcinogenesis

Embryogenesis is characterized by the ordered emergence of an organism made up of a multitude of stem and differentiated cells. Various signaling pathways play crucial roles in the dynamic cell proliferation and motility of organogenesis [52]. For example, in liver organogenesis, liver specification signaling is activated at the ventral endoderm (hepatic endoderm) by the paracrine secretion of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) from the cardiac mesoderm and septum transversum, respectively [53–55]. Wnt/beta-catenin signaling may also induce hepatic specification [56]. Activation of these signaling pathways results in the formation of the liver bud from the hepatic endoderm. The liver bud is considered to be the earliest developmental stage of liver organogenesis, which coincides with the expression of albumin and AFP [57].

Once the hepatic endoderm is specified and the liver bud begins to grow, the cells become hepatoblasts and have the ability to differentiate into hepatic and biliary lineages as bipotent progenitors. Epithelial and mesenchymal cells located in the endoderm and/or mesoderm collaborate to orchestrate liver organogenesis [58] (Fig 1a). The importance of this was elegantly demonstrated in a recent in vitro study generating liver buds using induced pluripotent stem cells, human umbilical vascular endothelial cells, and mesenchymal stem cells [59].

Embryogenesis and tumorigenesis share similar features including autonomous cell proliferation, motility, homing, dynamic morphologic changes, cellular heterogeneity, and interactions with the microenvironment. Liver cancer development may partially recapitulate fetal liver development in terms of the emergence of cells expressing certain stem cell markers and the activation of signaling pathways during liver development (Fig 1b). Indeed, signaling pathways activated in normal liver development are known to be activated and may be involved in the development and maintenance of liver CSCs. FGF and Wnt signaling has also been implicated in the development of HCC [60–63], with the latter shown to regulate the self-renewal of hepatoblasts and liver CSCs [20, 31, 64–68].

Moreover, as observed in the process of normal liver development, the collaboration of CSCs with epithelial or mesenchymal cell features may play an important role in the tumorigenicity and metastasis of HCC (Fig 1b). Our data indicate that $EpCAM^+$ CSCs have no metastatic capacity for distant sites when subcutaneously injected into NOD/SCID mice. However, when $CD90^+$ CSCs were co-injected with $EpCAM^+$ CSCs, $EpCAM^+$ cells could metastasize to the lung, whereas subcutaneous primary tumors showed no difference in size [36]. Furthermore, although imatinib mesylate treatment had little effect on the size of primary subcutaneous tumors, it significantly suppressed lung metastasis potentially through the suppression of $CD90^+$ CSCs.

We found that the effect of $CD90^+$ CSCs on the enhanced cell motility of $EpCAM^+$ cells was mediated, at least in part,