the overexpression of FOXM1, which results in the activation of metastatic programs. Therefore, these patients might have survival benefits if they receive adjuvant therapies. As several adjuvant therapies might be beneficial for HCC patients after surgical resection,[37] integration of the EOB-AFP classification system into current staging practices may provide additional therapeutic options for early-stage HCC patients who will receive surgery.

A limitation of the present study is that we used three different cohorts to reveal the molecular portraits associated with clinical imaging and prognosis (i.e., the microarray cohort of 238 HCCs of various stages for the evaluation of molecular profiling; Cohort 1 for the validation of molecular profiling and EOB-MRI findings in various stages of HCC; and Cohort 2 for evaluating the utility of EOB-MRI and serum AFP in predicting the prognosis of early-stage HCCs), which made the molecular and prognostic analyses complex. Another limitation of this study was in the evaluation of prognostic utility because it uses small retrospective cohorts. Direct evaluation of the molecular profiles and prognostic values of hyperintense HCCs should be performed in a prospective study using a large-scale HCC cohort.

Taken together, the present study demonstrates for the first time that the combined approach of noninvasive Gd-EOB-DTPA-enhanced MRI and serum AFP levels can be used preoperatively to classify resectable HCCs into three subgroups with distinct prognoses. This classification is molecularly related to the stem/maturation status of HCCs regulated by HNF4α and FOXM1. The multicenter early-stage HCC cohort that received radical resection revealed that the EOB-AFP classification is clinically useful to determine the prognosis of early-stage HCC patients. On the basis of these observations, we propose that the EOB-AFP classification system be incorporated into current HCC staging practices, especially for the management of early-stage HCCs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

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ARTICLE

Selenoprotein P as a diabetes-associated hepatokine that impairs angiogenesis by inducing VEGF resistance in vascular endothelial cells

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Abstract

Aims/hypothesis Impaired angiogenesis induced by vascular endothelial growth factor (VEGF) resistance is a hallmark of vascular complications in type 2 diabetes; however, its molecular mechanism is not fully understood. We have previously identified selenoprotein P (SeP, encoded by the *SEPP1* gene in humans) as a liver-derived secretory protein that induces insulin resistance. Levels of serum SeP and hepatic expression of *SEPP1* are elevated in type 2 diabetes. Here, we investigated the effects of SeP on VEGF signalling and angiogenesis.

Methods We assessed the action of glucose on *Sepp1* expression in cultured hepatocytes. We examined the actions of SeP on VEGF signalling and VEGF-induced angiogenesis in HUVECs. We assessed wound healing in mice with hepatic SeP overexpression or SeP deletion. The blood flow recovery after ischaemia was also examined by using hindlimb ischaemia model with *Sepp1*-heterozygous-knockout mice.

Kazuhide Ishikura, Hirofumi Misu and Masafumi Kumazaki contributed equally to this work.

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Results Treatment with glucose increased gene expression and transcriptional activity for *Sepp1* in H4IIEC hepatocytes. Physiological concentrations of SeP inhibited VEGF-stimulated cell proliferation, tubule formation and migration in HUVECs. SeP suppressed VEGF-induced reactive oxygen species (ROS) generation and phosphorylation of VEGF receptor 2 (VEGFR2) and extracellular signal-regulated kinase 1/2 (ERK1/2) in HUVECs. Wound closure was impaired in the mice overexpressing *Sepp1*, whereas it was improved in $SeP^{-/-}$mice. $SeP^{+/-}$mice showed an increase in blood flow recovery and vascular endothelial cells after hindlimb ischaemia.

Conclusions/interpretation The hepatokine SeP may be a novel therapeutic target for impaired angiogenesis in type 2 diabetes.

Keywords Angiogenesis · Hepatokine · ROS · Selenoprotein P · VEGF

Abbreviations

BSO	Buthionine sulfoximine
DCF	2′,7′-Dichlorofluorescein diacetate
ERK1/2	Extracellular signal-regulated kinase 1/2
MAPK	Mitogen-activated protein kinase
ROS	Reactive oxygen species
SeP	Selenoprotein P
VEGF(R)	Vascular endothelial growth factor (receptor)

Introduction

Type 2 diabetes is a chronic hyperglycaemic condition that causes various vascular complications, including damage to: small blood vessels, resulting in retinopathy, nephropathy and neuropathy; and large blood vessels, resulting in cardiovascular diseases. Earlier improved glycaemic control is associated with reduced risk for cardiovascular disease in people with type 2 diabetes [1]. However, more recent clinical trials have indicated that strict glycaemic control does not necessarily prevent vascular complications [2]. Hence, beyond glycaemic control, novel therapies to directly treat vascular disease are needed to improve the prognosis of people with type 2 diabetes.

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vascular structures and the subsequent formation of a vascular network. A number of abnormalities associated with angiogenesis have been observed in people with type 2 diabetes [3], and impaired angiogenesis is linked to the development of various vascular complications in diabetes mellitus. Compared with control individuals without diabetes, people with type 2 diabetes show poor development of coronary collateral vessels on coronary angiography [4]. Moreover, a previous study using autopsied hearts reported that people with diabetes have significantly lower capillary densities in areas of myocardial infarction [5]. These reports suggest that the angiogenic response to infarction and/or ischaemia is inhibited at the levels of capillaries and small arterioles in type 2 diabetes. Inadequate vascular formation could attenuate perfusion recovery in response to ischaemia, thereby partially accounting for the poor clinical outcomes in type 2 diabetic patients with coronary heart disease or peripheral artery disease [6, 7]. In addition, insufficient angiogenesis is involved in abnormal wound healing and the development of diabetic skin ulcers [8].

Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis under physiological and pathophysiological conditions. VEGF binds and phosphorylates its receptors, leading to the activation of a variety of signalling cascades such as the mitogen-activated protein kinase (MAPK) and Akt cascades. Angiogenic gene therapy using plasmids encoding VEGF has been attempted in patients with coronary or peripheral artery diseases [9]. However, diabetes mellitus people often show a poor response to therapeutic angiogenesis [10]. Therefore, VEGF resistance, a defect of VEGF-related signal transduction, has been postulated as a molecular basis for the dysregulated angiogenesis in diabetes mellitus [3, 11]. The molecular mechanisms underlying VEGF resistance in diabetes mellitus are not fully understood.

Selenoprotein P (SeP, encoded by *SEPP1* in humans and *Sepp1* in mice) is a secretory protein produced primarily in the liver [12, 13]. It contains ten selenocysteine residues and functions as a selenium supply protein [14]. We have previously reported that levels of serum SeP and hepatic gene expression of *SEPP1* are elevated in type 2 diabetes [15]. More recently, Yang et al have reported that serum levels of SeP are increased in people with impaired glucose tolerance [16]. SeP impairs insulin signal transduction and induces dysregulation of glucose metabolism in skeletal muscle and liver, indicating that SeP functions as a type 2 diabetes-associated hepatokine that causes insulin resistance and hyperglycaemia [15]. SeP has heparin-binding properties [17] and is associated with endothelial cells in rat tissues [18], suggesting that SeP exerts some actions on vascular endothelial cells. A previous study using in vitro techniques reported that SeP has an antioxidative action in vascular endothelial cells [19]. Nevertheless, it is unknown whether SeP plays a role in the angiogenic response.

We speculated that the liver-derived secretory protein SeP contributes to angiogenesis-associated vascular complications in type 2 diabetes by acting directly on vascular endothelial cells. In the current study, we investigated the effects of SeP on angiogenesis in normal conditions, independently of diabetes, using purified SeP protein and *Sepp1*-deficient mice without the induction of diabetes.

Methods

Cell culture HUVECs were cultured in HuMedia EG2 (Kurabo, Osaka, Japan). H4-II-E-C3 cells were cultured in 10% (vol./vol.) fetal bovine serum (FBS)/DMEM (Gibco, Carlsbad, CA, USA) as previously described [20]. All cellular experiments were approved by the Committee for Cellular Study at our Institute.

Animals The *Sepp1*-deleted mice were produced by homologous recombination with genomic DNA cloned from a Sv-129 P1 library [21]. All animal studies were approved by the Committee for Animal Studies at our Institute. See the electronic supplementary material (ESM) for further details.

Measurement of selenium Total selenium concentrations were determined using a modification of Watkinson's method [22, 23]. See the ESM for further details.

SEPP1 promoter assay The human *SEPP1* promoter region was cloned to a luciferase reporter vector, and luciferase activities were measured using the dual luciferase assay system (Promega, Madison, WI, USA) [20]. See the ESM for further details.

Cell proliferation assay HUVECs were quantified using Cell Counting Kit-8 (Wako, Osaka, Japan). See the ESM for further details.

Migration assay HUVECs were seeded in the upper chamber of polycarbonate filters, and the number of cells migrating across the filter was counted. See the ESM for further details.

Cell tubule formation assay HUVECs were seeded on plates coated with ECMatrix gel. Endothelial tubule formation was photographed under a microscope. See the ESM for further details.

Matrigel plug implantation assay This assay was performed using a directed in vivo angiogenesis assay inhibition kit (Trevigen, Gaithersburg, MD, USA). See the ESM for further details.

Western blot analysis HUVECs were pretreated with SeP for 24 h. After 2 h of starvation, HUVECs were stimulated with VEGF for 15 min. See the ESM for further details.

RNA preparation and quantitative real-time Real-time PCR was performed on an ABI-Prism 7900HT (Applied Biosystems, Carlsbad, CA, USA). See the ESM for further details.

Reactive oxygen species generation Intracellular reactive oxygen species (ROS) levels were measured using 2′,7′-dichlorofluorescein diacetate (DCF) and quantified using a fluorescent plate reader (Fluoroskan Ascent FL, Yokohama, Japan). See the ESM for further details.

Purification of SeP SeP was purified from human plasma using conventional chromatographic methods [14, 24]. See the ESM for further details.

Preparation of human SEPP1 plasmids and overexpression of SeP in mice The human *SEPP1* expression plasmids were provided by Kaketsuken (The Chemo-Sero-Therapeutic Research Institute, Tokyo, Japan). Plasmid was injected into the tail vein of mice. See the ESM for further details.

Measurement of serum human SeP in mice injected with human SEPP1 plasmid Serum levels of human SeP were measured by enzyme-linked immunosorbent assays using two monoclonal antibodies [15, 25].

Mouse wound healing model Full-thickness wound was created, and the extent of wound closure was examined. See the ESM for further details.

Hindlimb ischaemia model Mice underwent ligation and segmental resection of the left femoral vessel [26]. See the ESM for further details.

Identification of $CD31^+$ vessels An antibody to CD31 was used for immunostaining. See the ESM for further details.

Calculations and statistical analysis All data were analysed using SPSS version 11.0 (Japanese Windows Edition; SPSS, www.ibm.com/software/analytics/spss/). See the ESM for further details.

Results

Glucose increases gene expression and transcriptional activity for SeP in cultured hepatocytes To confirm the elevation of SeP in the livers of people and animal models with type 2 diabetes [15], we examined the action of glucose on *Sepp1* expression in H4-II-EC hepatocytes (Fig. 1). *Sepp1* mRNA expression was significantly increased by 25 mmol/l glucose in a time-dependent manner (Fig. 1a). Additionally, *SEPP1* promoter activity as measured by luciferase activity was increased by 25 mmol/l glucose compared with mannitol (Fig. 1b). These results are consistent with our previous findings showing that treatment with high glucose increases protein levels of SeP in mouse primary hepatocytes [15]. These results indicate that high concentrations of glucose increase the transcriptional activity of SeP genes in the cultured hepatocytes.

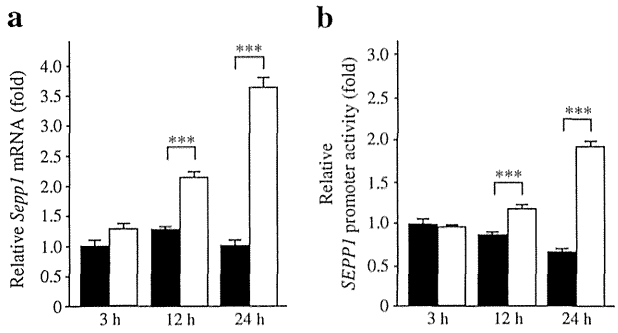

Fig. 1 Glucose increases gene expression and transcriptional activity for SeP in H4-II-EC3 hepatocytes. (**a**) Relative *Sepp1* mRNA expression normalised to β-actin. (**b**) Promoter activity for *SEPP1* in H4-II-EC3 hepatocytes treated with glucose and mannitol. Data are mean ± SD, $n=3$, ***$p<0.001$. White bars, glucose; black bars, mannitol

SeP impairs VEGF-induced angiogenesis in endothelial cells To assess the direct action of the liver-derived secretory protein SeP on vascular endothelial cells, we treated HUVECs with purified human SeP protein. HUVECs were treated with 5 or 10 μg/ml purified human SeP protein, corresponding to serum levels of SeP in healthy individuals or people with type 2 diabetes [15]. In addition, we confirmed that levels of selenium were undetectable (less than 2.5 ng/ml) in all the culture media used for HUVECs. VEGF-induced proliferation of HUVECs was significantly suppressed by treatment with 10 μg/ml SeP (Fig. 2a). Co-administration of buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, partly rescued the suppressive effect of SeP.

Next, we examined the effects of SeP on VEGF-induced migration in HUVECs. VEGF promoted the migration of HUVECs across polycarbonate filters. This migration was inhibited by the addition of SeP in a concentration-dependent manner (Fig. 2b, c). In the absence of VEGF, treatment with SeP did not affect the migration of HUVECs, suggesting that SeP modulates VEGF-dependent migration of endothelial cells. We further examined the effects of SeP on tubule formation in HUVECs. HUVECs cultured on Matrigel containing VEGF showed morphological tubule formation, with a lumen surrounded by endothelial cells adhering to one another (Fig. 2d). SeP inhibited tubule formation of HUVECs in a concentration-dependent manner (Fig. 2d–e). These in vitro results indicate that SeP at physiological concentrations impairs VEGF-dependent angiogenesis of vascular endothelial cells.

SeP reduces VEGF-stimulated formation of new vessels in Matrigel The role of SeP in angiogenesis in vivo was further determined by Matrigel plug implantation assay. Matrigel was mixed with VEGF in the presence or absence of SeP protein and the plugs were implanted into the dorsal subcutaneous tissue of mice. After 10 days, angiogenesis inside the Matrigel was quantified. SeP markedly inhibited VEGF-stimulated

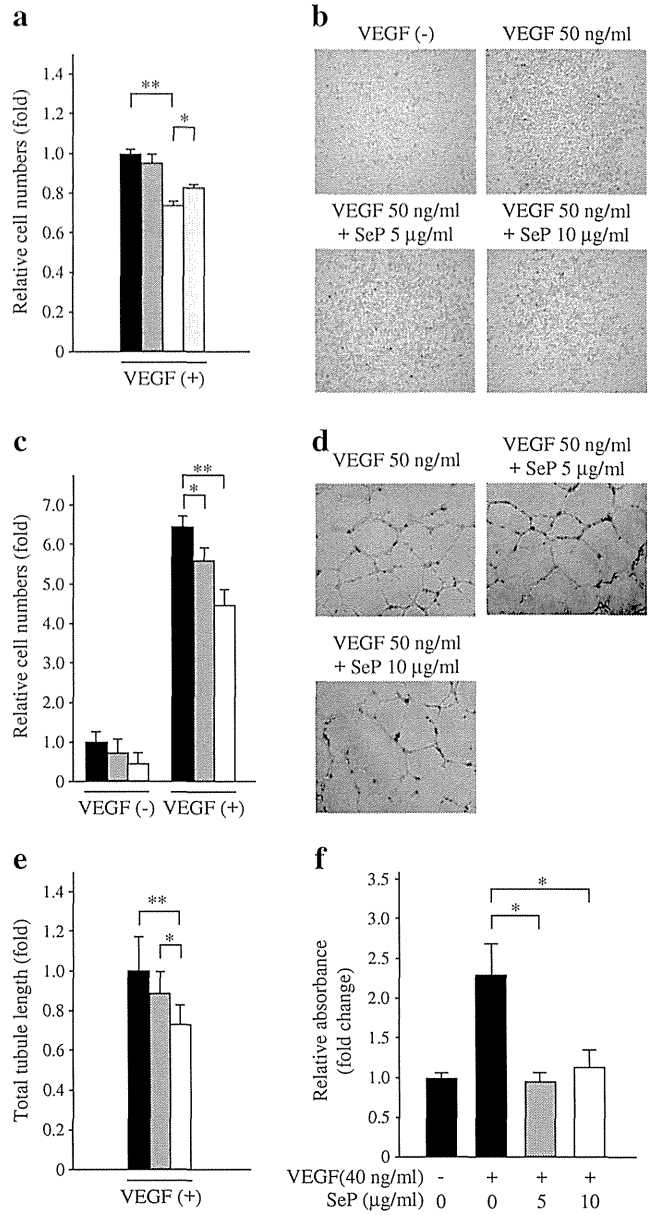

Fig. 2 SeP suppresses VEGF-stimulated angiogenesis in vascular endothelial cells. (**a**) Cell proliferation in HUVECs treated with VEGF for 48 h ($n=12$). (**b**) Representative images of HUVECs that migrated across the polycarbonate filters (magnification×200). (**c**) Quantification of HUVECs that migrated across the filters ($n=8$). (**d**) Representative images of HUVECs that were subjected to Matrigel tubule formation assay (magnification×400). (**e**) Quantification of total tubule length of HUVECs ($n=9$). (**f**) Matrigel implant assay in mice ($n=6$–8). Data are mean ± SEM, *$p<0.05$ and **$p<0.01$. 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

formation of new vessels in the Matrigel (Fig. 2f). These results further indicate that SeP impairs angiogenesis in vivo.

SeP impairs VEGF signal transduction in endothelial cells Next, we determined whether SeP affects VEGF signal transduction in endothelial cells. Pretreatment with SeP

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.

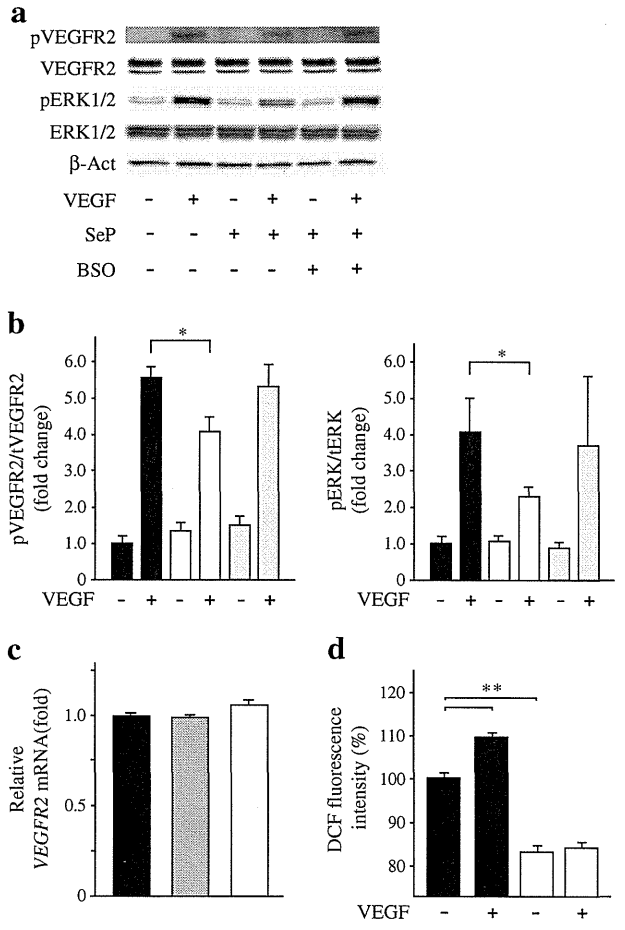

Fig. 3 SeP impairs VEGF signal transduction in endothelial cells. (**a**) VEGF signalling in HUVECs treated with SeP (10 μg/ml). (**b**) Quantification of phosphorylated VEGFR2 and ERK normalised to total VEGFR2 and total ERK in HUVECs (n=6). (**c**) Gene expression levels for *VEGFR2* in HUVECs treated 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

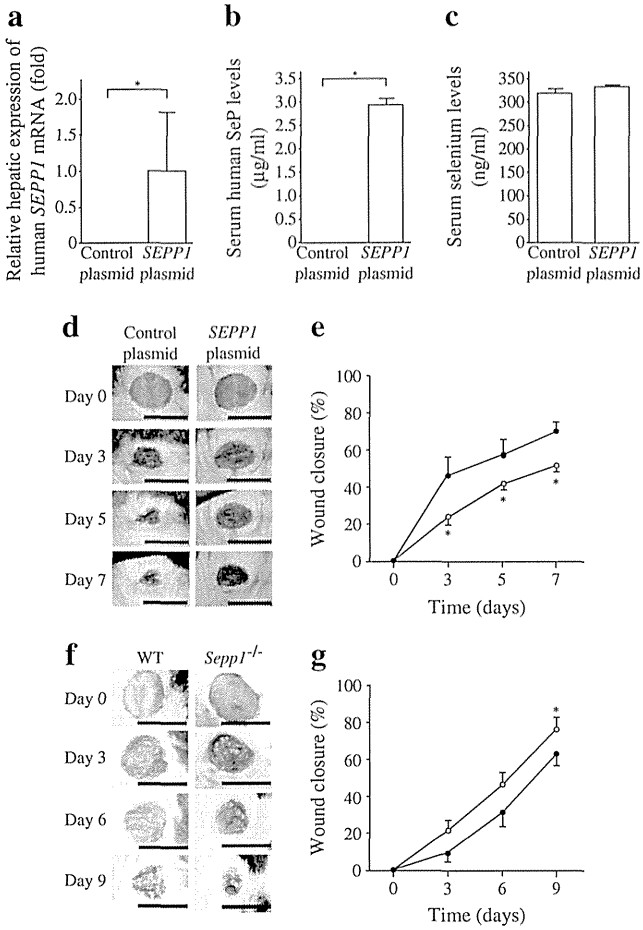

Fig. 4 Hepatic overexpression of SeP impairs wound healing in mice. (**a**) Levels of human *SEPP1* mRNA normalised to 18S rRNA in the livers of mice injected with plasmid DNA via the tail vein (n=9). (**b**) Serum human SeP levels in mice injected with a plasmid encoding *SEPP1* (n=9). (**c**) Serum 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

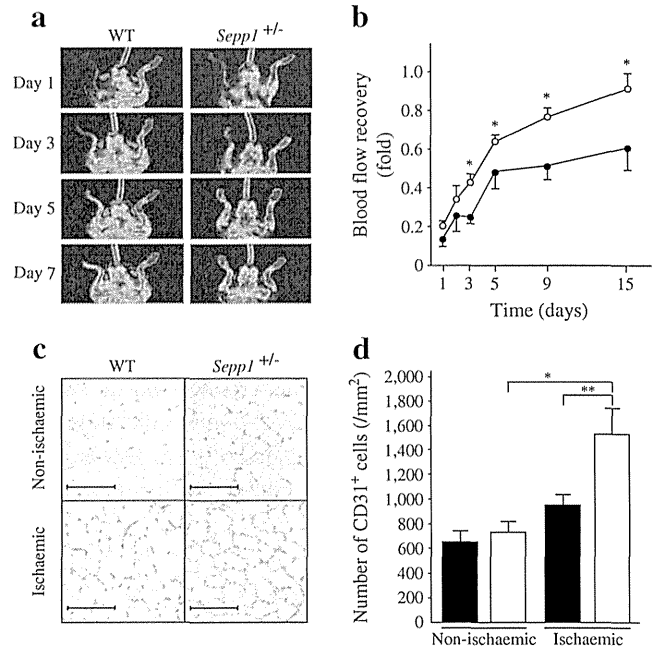

Fig. 5 *Sepp1*$^{+/-}$mice show enhanced angiogenesis during hindlimb ischaemia. (**a**) Representative images of perfusion recovery following hindlimb ischaemia in *Sepp1*$^{+/-}$mice. (**b**) Quantification of blood flow recovery in *Sepp1*$^{+/-}$mice (white circles) and control (black circles) (n=5). 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

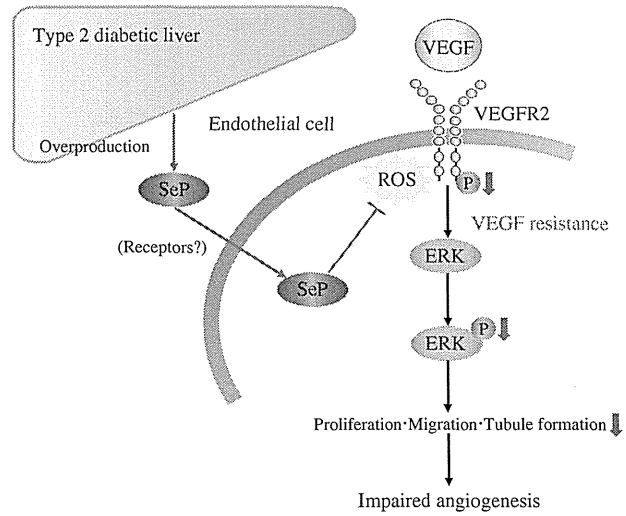

Fig. 6 Overproduction of SeP in type 2 diabetic liver induces VEGF resistance in vascular endothelial cells. SeP inhibits VEGF signal transduction by suppressing acute generation of ROS, resulting in the onset of impaired angiogenesis

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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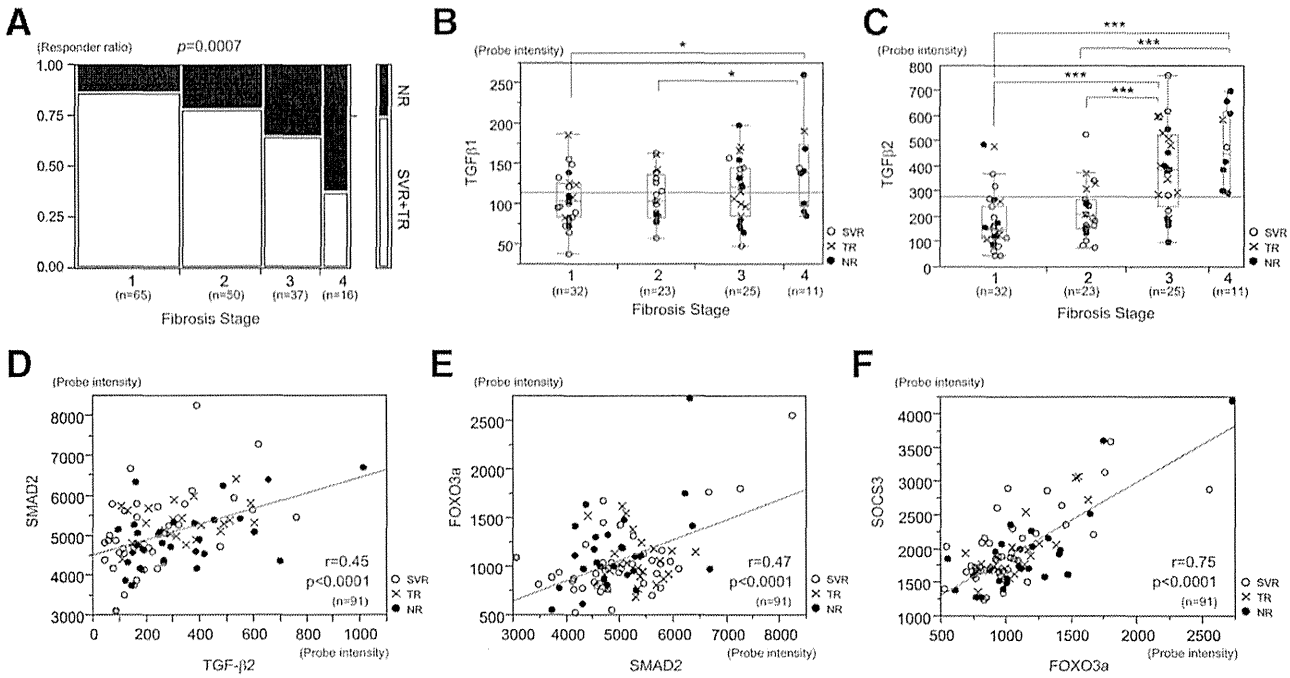

Fig. 1. Activation of TGF-β signaling in the liver of patients at the advanced fibrosis stage of CH-C. A: Significant increase in the NR ratio with the progression of fibrosis stage. B,C: Expression of TGF-β1 (B) and TGF-β2 (C) with the progression of fibrosis stage. D-F: Significant correlations of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

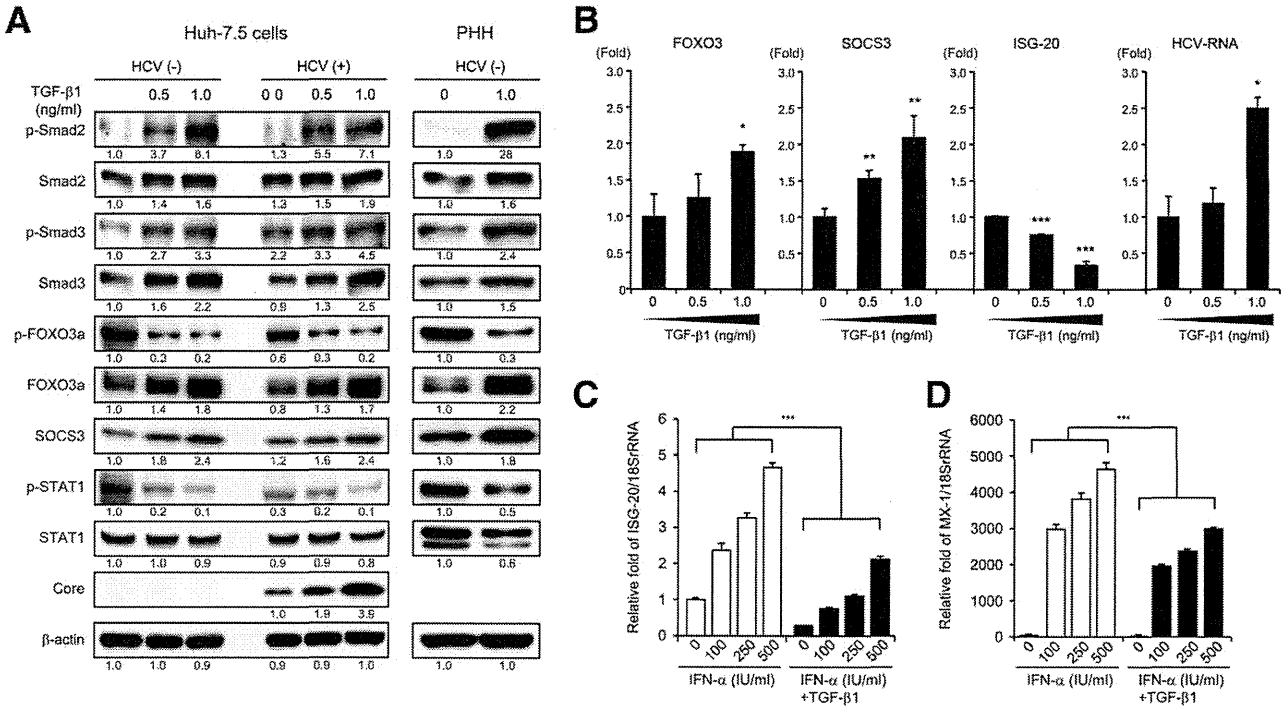

Fig. 2. Effect of TGF-β1 on IFN signaling in Huh-7.5 cells and PHH. A: Western blotting of TGF-β, Foxo3a-Socs3, and IFN signaling in Huh-7.5 cells and PHH treated with TGF-β1. Huh-7.5 cells were transfected with infectious HCV RNA, H77Sv3 GLuc2A prior to TGF-β1 treatment (Huh-7.5 HCV (+)). The experiments 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

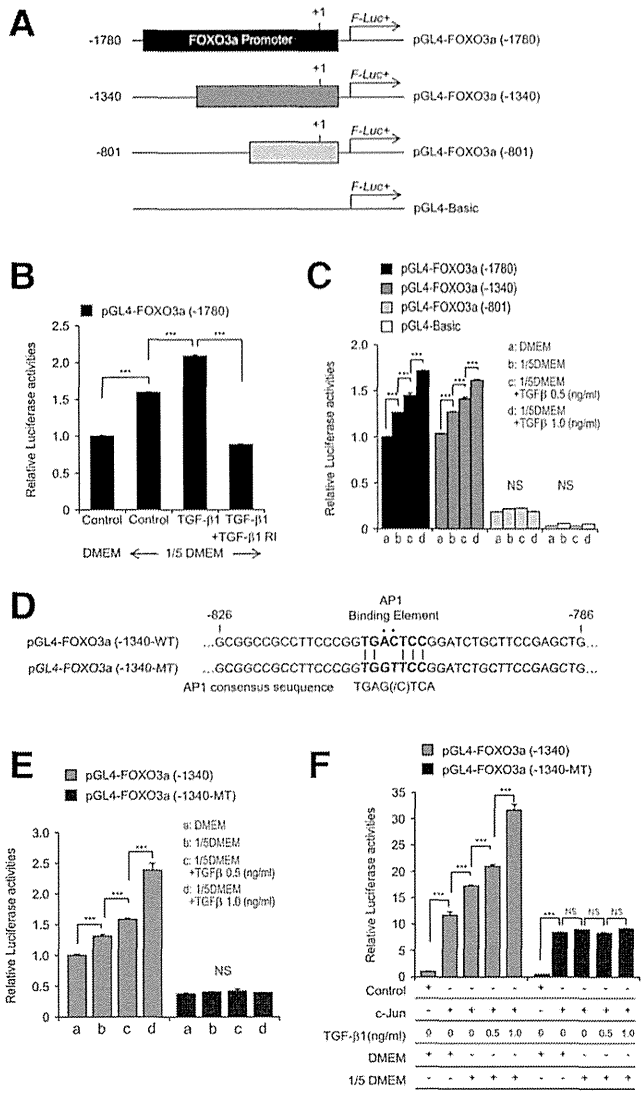

Fig. 3. Foxo3a promoter analysis. A: Foxo3a promoter-luciferase reporter constructs. B: Promoter activity of pGL4-FOXO3a (−1780) following amino acid depletion (1/5 DMEM), TGF-β1 treatment, and TGF-β1 RI treatment. C: Abolished regulation of the promoter activity of pGL4-FOXO3a (−801) by amino acid depletion (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

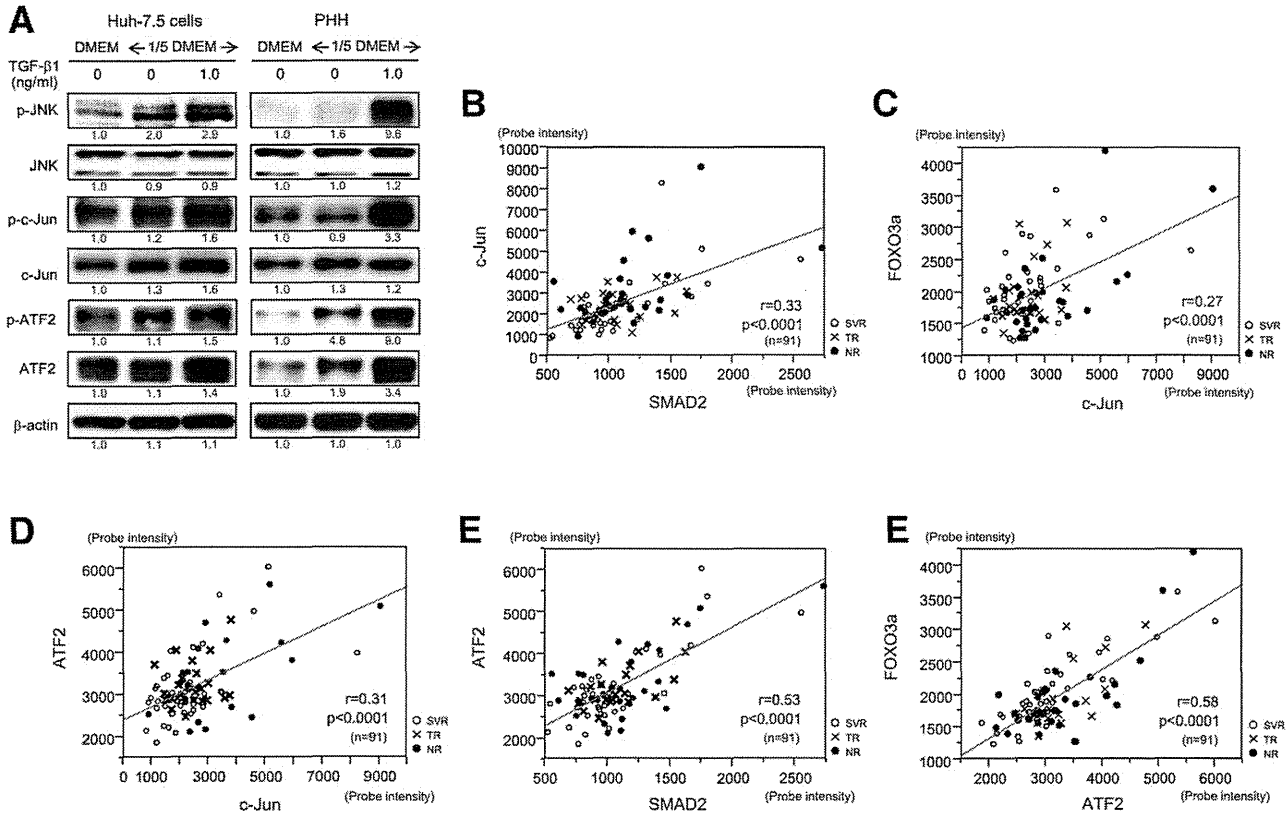

Fig. 4. TGF-β signaling up-regulates the expression of the transcription factors c-Jun and ATF2 in Huh-7.5 cells, PHH, and the liver of CH-C patients. A: Western blotting of JNK, c-Jun, and ATF2 in Huh-7.5 cells and PHH treated with amino acid depletion (1/5 DMEM) and TGF-β1. The experiments were repeated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

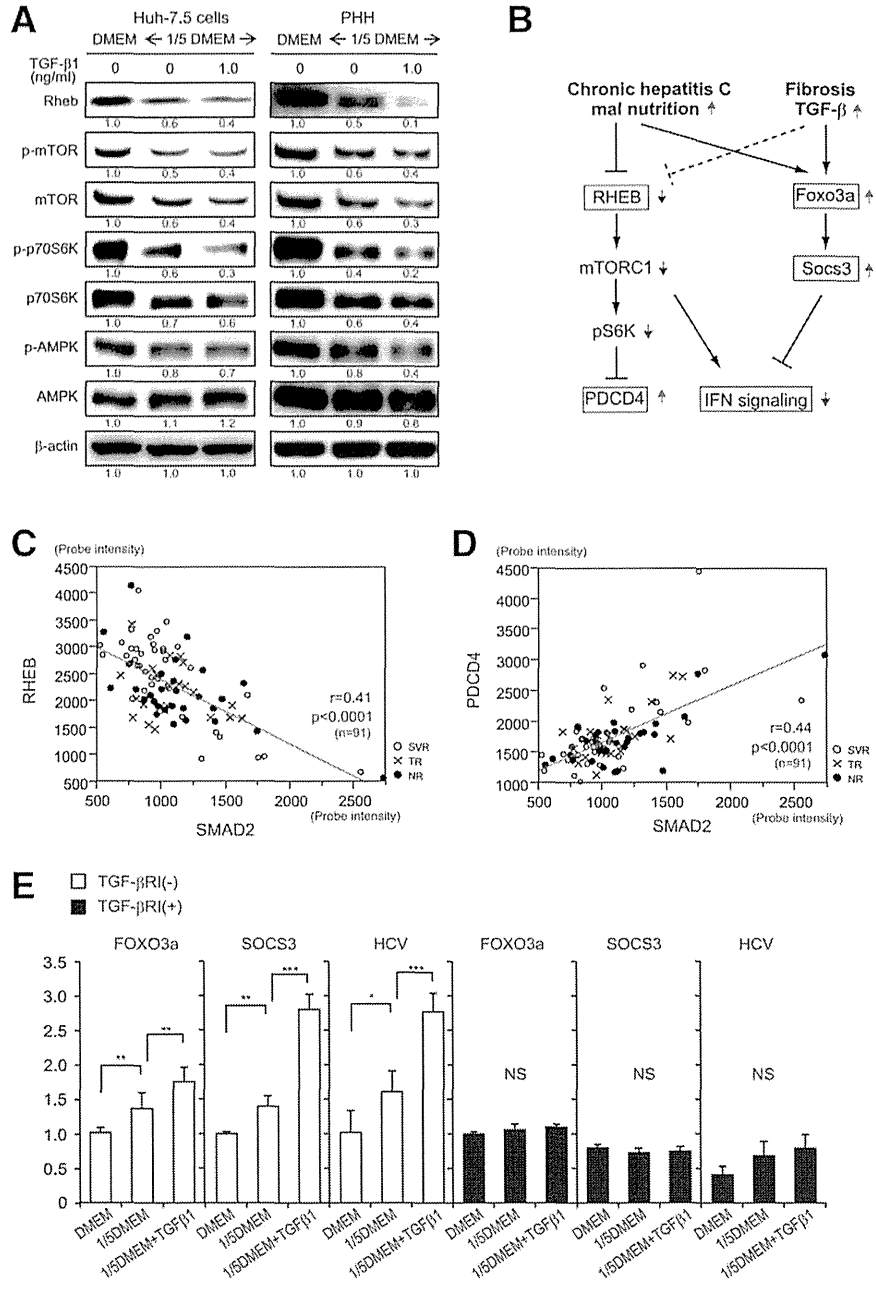

Fig. 5. TGF-β signaling represses mTORC1 signaling in Huh-7.5 cells, PHH, and the liver of CH-C patients. A: Western blotting of RHEB, mTOR, p70S8K, and AMPK in Huh-7.5 cells and PHH treated with amino acid depletion (1/5 DMEM) and TGF-β1. The experiments were repeated 3 times. B: Schematic representation 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

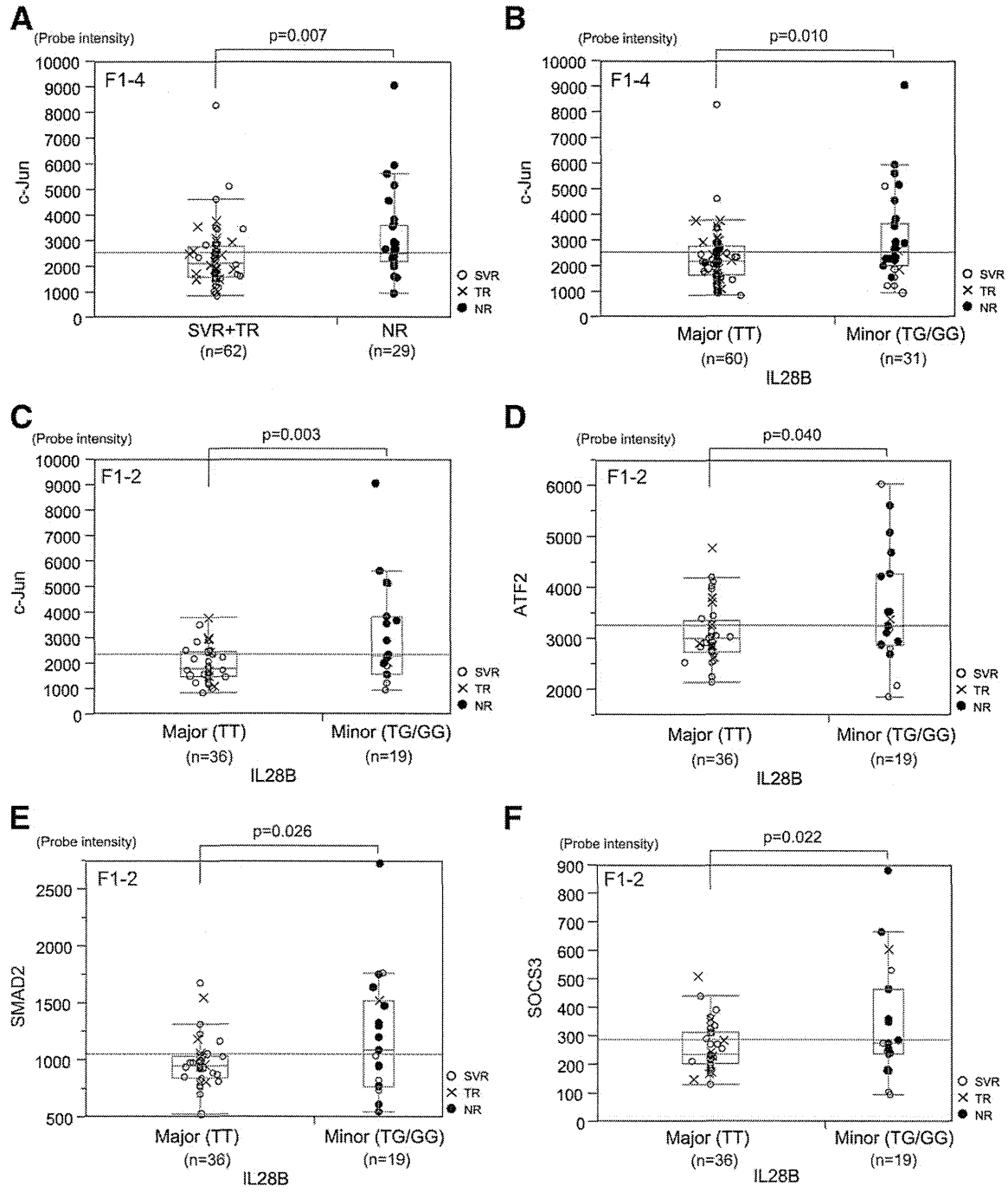

Fig. 6. Relationship between TGF-β signaling and treatment response and the IL28B genotype. The expression of c-Jun was up-regulated in NR (A) and IL28B minor genotype (TG/GG at rs8099917) (B) patients in all fibrosis stages (F1-4). The expression of ATF2 (D), Smad2 (E), and Socs3 (F) was up-regulated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

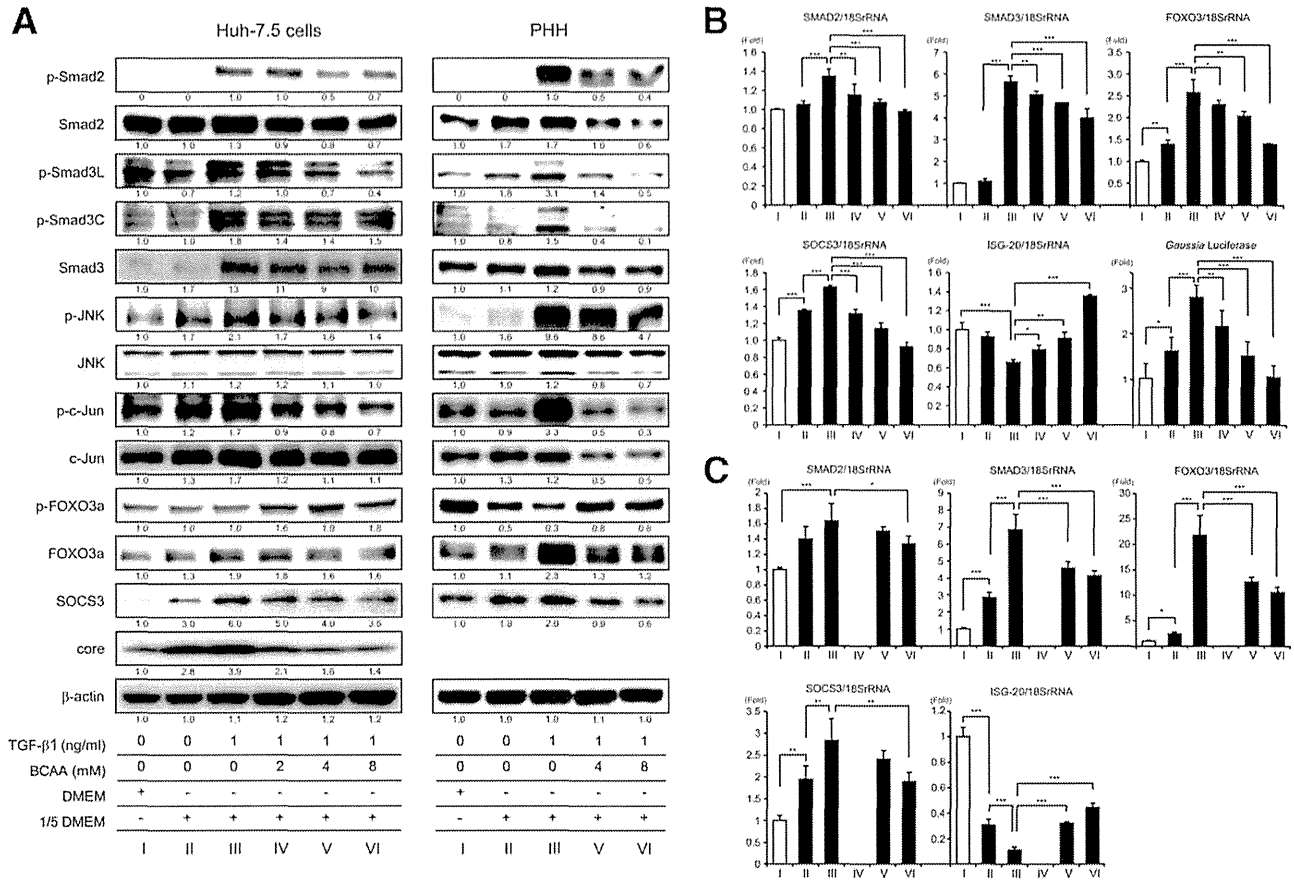

Fig. 7. BCAAs inhibit the effect of malnutrition and TGF-β signaling in Huh-7.5 cells and PHH. A: Western blotting of TGF-β and Foxo3a-Socs3 signaling in Huh-7.5 HCV (+) and PHH treated with amino acid depletion (1/5 DMEM), TGF-β1, and BCAAs. B,C: mRNA expression of TGF-β, Foxo3a-Socs3, and IFN signaling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