

Table 3 Performance of  $\alpha$ -fetoprotein (AFP) and des- $\gamma$ -carboxy prothrombin (DCP)

	Number	Sensitivity	Number	Specificity
<b>All patients</b>				
AFP $\geq$ 9 ng/mL	68/106	64.1%	163/201	81.0%
AFP $\geq$ 20 ng/mL	55/106	51.8%	181/201	90.0%
DCP $\geq$ 40 mAU/mL	38/106	35.8%	115/194	58.3%
DCP $\geq$ 100 mAU/mL	20/106	18.8%	140/194	71.6%
<b>Excluding PSC and PBC patients</b>				
AFP $\geq$ 9 ng/mL	66/104	63.4%	92/127	72.4%
AFP $\geq$ 20 ng/mL	55/104	52.8%	107/127	84.2%
DCP $\geq$ 40 mAU/mL	38/104	36.5%	88/122	72.1%
DCP $\geq$ 100 mAU/mL	20/104	19.2%	98/122	80.3%

PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

adopted the cut-off values of AFP  $\geq$  20 ng/mL according to the ROC curve. For DCP, we tentatively set the cut-off level at 100 mAU/mL, since the majority of non-HCC patients showed DCP levels  $\geq$  40 mAU/mL. Performance of DCP was inferior to that of AFP. When patients with HCC were sub-stratified according to previous history of HCC treatment, the sensitivities for AFP and DCP with each cut-off value did not differ.

After removing patients with primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) from the analysis, AUROC for AFP and DCP were 0.77 (95% CI: 0.71–0.83) and 0.56 (95% CI: 0.49–0.64), respectively. Although sensitivity and specificity of DCP was improved (Table 3), the performance of DCP was still inferior to that of AFP.

#### Factors associated with elevated tumor marker level in non-HCC group

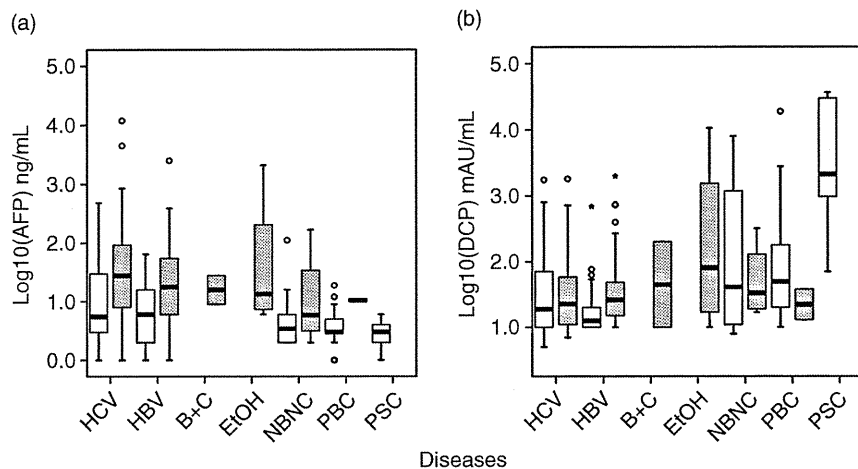
Associations between elevated serum AFP ( $\geq$ 20 ng/mL), and other factors were analyzed. By univariate analysis, hepatitis C virus (HCV;  $P < 0.001$ ) and male gender ( $P = 0.002$ ) were significantly associated with AFP  $\geq$  20 ng/mL among non-HCC patients. Multivariate analysis revealed both HCV (odds ratio [OR] 8.39,  $P < 0.001$ ) and male gender (OR 3.32,  $P = 0.035$ ) remained significant.

Associations between elevated serum DCP ( $\geq$ 100 mAU/mL), and other factors were then analyzed. Primary sclerosing cholangitis (PSC,  $P = 0.003$ ), hepatitis B disease ( $P = 0.031$ ), total bilirubin level ( $P = 0.015$ ), and albumin level ( $P = 0.012$ ) were significant factors. By multivariate analysis, PSC (OR 22.9,  $P = 0.004$ ) and lower albumin level (OR: 0.44 per 1.0 g/dL,  $P = 0.02$ ) remained significant factors associated with DCP levels  $\geq$  100 mAU/mL among non-HCC patients.

Since the association between DCP false positivity and the etiology of liver diseases was suggested, the differences in AFP and DCP levels between HCC and non-HCC groups were compared graphically as a box plot according to the etiology of liver disease (Fig. 2). AFP levels, shown as the log<sub>10</sub> transformation, were higher in patients with HCC than in those without in each etiology group (Fig. 2a). On the other hand, DCP levels, also shown as the log<sub>10</sub> transformation, were rather similar regardless of HCC status; instead, DCP levels were higher in patients with PSC, alcoholic liver disease, and cryptogenic cirrhosis (Fig. 2b) even without HCC.

#### Factors associated with elevated tumor marker level in HCC group

Among HCC group, the association between pathologic features and the level of tumor markers were analyzed. There was a positive association between AFP  $\geq$  20 ng/mL and the number of tumors ( $P = 0.021$ ), and moderately to poorly differentiated HCC ( $P = 0.036$ ) by univariate analysis, but the number of tumor (OR 1.22,  $P = 0.021$ ) remained significant by multivariate analysis. DCP  $\geq$  100 mAU/mL was associated with vascular invasion ( $P = 0.003$ ), size of HCC ( $P = 0.008$ ), number of tumors ( $P = 0.029$ ) and moderately to poorly differentiated HCC ( $P = 0.019$ ) by univariate analysis. Multivariate analysis showed a positive association between DCP  $\geq$  100 mAU/mL and vascular invasion (OR 4.95,  $P = 0.008$ ), size of HCC (OR 1.53,  $P = 0.022$ ), and moderately to poorly differentiated HCC (OR 5.76,  $P = 0.031$ ). These associations remained the same after removing 10 cases with necrotic HCC tissue (data not shown). None of these factors were associated with the level of CEA.



**Figure 2**  $\alpha$ -fetoprotein (AFP) (a) and des- $\gamma$ -carboxy prothrombin (DCP) (b) levels according to etiology of liver diseases is shown as box-plot. White bar indicates non-hepatocellular carcinoma (HCC) group, and grey bar indicates HCC group. Serum level of AFP was higher in HCC group regardless of etiology. In contrast, DCP level in HCC group was similar to that in non-HCC group for each etiology groups. Longitudinal axis represents tumor marker levels after  $\log_{10}$  transformation; upper horizontal line of box, 75th percentile; lower horizontal line, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, outliers are indicated by the circle (o), and extreme values are indicated by the asterisk. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non B non C cirrhosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

### Sequential changes in DCP levels by vitamin K administration

There were 74 cases whose DCP level was  $\geq 100$  mAU/mL, and 39 of these cases had more than two DCP measurements within one month before the transplantation. In 20 of these cases, either oral or parenteral vitamin K was administered after the first measurement of DCP until the last measurement (Table 4); DCP decreased in 19, and was unchanged in one. In the 19 patients without vitamin K administration, DCP levels increased in eight (two in the HCC group) and decreased in 11 (seven in the HCC group). Among those 19 cases, we could not find any specific markers that changed in parallel with or in inverse to the level of DCP. In cases 38 and 39, decrease of DCP levels might be related to transcatheter arterial chemoembolization (TACE), which was performed between the first and the last measurements of DCP.

### DISCUSSION

**I**N THE PRESENT study, we evaluated pretransplant serum AFP and DCP levels as indicators of HCC presence in liver transplant candidates using histopathologic examination of explanted liver as the gold standard. Because the study subjects consisted entirely of liver

transplantation candidates and none had advanced HCC, we had not expected high sensitivity for those tumor markers. On the other hand, specificity of DCP was revealed to be unexpectedly low, resulting in very low AUROC of 0.47, which indicates no diagnostic relevancy.

Des- $\gamma$ -carboxy prothrombin positivity in HCC patients is reportedly associated with advanced features of HCC, such as vascular invasion and poorer differentiation, as shown indeed also in the present study.<sup>26–28</sup> In contrast, the tumor marker is reported to be fairly specific, at least in surveillance for HCC among chronic viral hepatitis patients, except in the cases of warfarinization or antibiotics-induced vitamin K deficiency.<sup>19</sup> The subjects of the present study, especially those without HCC, had poorer liver function than average chronic hepatitis patients under HCC surveillance. The difference in liver function may explain the very low specificity of DCP in the present study, and, indeed, lower albumin concentration was associated with false positivity in patients without HCC.

In advanced stages of cholestatic liver diseases, such as PBC and PSC, many patients show deficiency of fat-soluble vitamins including vitamin K.<sup>29,30</sup> In the present study, 22 of 67 (32.8%) patients with PBC and 8 of 10 (80%) patients with PSC, who had no HCC in liver

**Table 4** Change of des- $\gamma$ -carboxy prothrombin (DCP) level in 39 patients with pre-transplant DCP  $\geq$  100 mAU/mL

Case	Age/Sex	Etiology	DCP level (mAU/mL)		Interval (days)	Vitamin K administration	HCC	TACE for HCC†
			First DCP	Last DCP				
1	25/M	BA	118	35	10	K2, parenteral	No	
2	59/F	NBNC	709	17	12	K2, parenteral	No	
3	40/F	NBNC	8 041	15	28	K2, parenteral	No	
4	48/F	NBNC	1 198	47	13	K2, parenteral	No	
5	48/M	PBC	18 890	4 604	6	K2, parenteral	No	
6	50/F	PBC	1 257	387	8	K2, parenteral	No	
7	65/F	PBC	1 144	27	13	K2, parenteral	No	
8	56/F	PBC	524	59	24	K2, parenteral	No	
9	44/F	PSC	36 613	13 645	5	K2, parenteral	No	
10	57/M	PSC	2 853	1 571	3	K2, parenteral	No	
11	53/F	HCV	135	65	6	K2, parenteral	Yes	Yes
12	22/F	BA	7 490	26	27	K2, oral	No	
13	28/M	HCV	805	109	14	K2, oral	No	
14	55/F	PBC	397	389	14	K2, oral	No	
15	51/F	PBC	2 159	23	30	K2, oral	No	
16	38/F	PBC	603	21	26	K2, oral	No	
17	24/M	PSC	2 958	10	29	K1, oral	No	
18	33/F	PSC	972	699	14	K1, oral	No	
19	53/F	PSC	1 088	65	28	K1, oral	No	
20	42/M	HCV	1 089	105	19	K1, oral	Yes	No
21	24/M	BA	365	21	18	–	No	
22	48/M	HBV	692	369	30	–	No	
23	46/M	HCV	104	106	28	–	No	
24	59/F	HCV	33	287	21	–	No	
25	47/M	HCV	146	12	7	–	No	
26	54/F	HCV	340	378	26	–	No	
27	40/F	NBNC	128	310	30	–	No	
28	47/F	PBC	355	1 915	8	–	No	
29	29/F	PSC	593	919	30	–	No	
30	19/M	PSC	2 031	1 006	7	–	No	
31	57/M	Alcoholic	13 248	577	29	–	Yes	No
32	55/M	HBV	250	269	30	–	Yes	No
33	60/M	HBV	399	210	6	–	Yes	No
34	56/M	HBV	1 111	726	14	–	Yes	No
35	66/M	HCV	227	219	7	–	Yes	No
36	54/M	HCV	42	109	28	–	Yes	No
37	55/F	NBNC	323	168	14	–	Yes	No
38	54/M	HBV	1 994	682	6	–	Yes	Yes
39	44/M	HBV	302	40	20	–	Yes	Yes

†TACE performed only between the 1<sup>st</sup> and the last measurement of DCP was included.

BA, biliary atresia; DCP, des- $\gamma$  carboxy prothrombin; F, female; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; K1, vitamin K1 (phyloquinone 15 mg q day); K2, vitamin K2 (menatetrenone 20 mg q day); M, male; NBNC, non B non C cirrhosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; TACE, transcatheter arterial chemoembolization.

explant, showed elevated levels of DCP (>100 mAU/mL). Thus, DCP is practically useless in the pre-transplant screening of HCC in patients with advanced cholestatic liver diseases. Furthermore, specificity of DCP was still low, 98/122 (80.3%), among pre-

transplant patients after excluding those with PBC or PSC. Although elevated DCP levels were statistically associated with tumor factors suggesting poorer prognosis in patients with HCC, we cannot deny the possibility that elevated DCP levels were not related to HCC but

spurious and related to poor liver function. Although Liebman *et al.*<sup>12</sup> reported that vitamin K administration did not alter serum DCP levels in HCC patients, two patients with HCC in the present study showed decrease in DCP levels after vitamin K administration. The utility of DCP in pre-transplant screening seems to be very limited.

Vitamin K was administered to 20 patients with elevated DCP and the DCP levels decreased in 19 of them, suggesting that the increase in DCP levels was due to vitamin K deficiency. Except for patients with cholestatic liver diseases, who are likely to have vitamin K malabsorption, the mechanism of vitamin K deficiency is not clear. Possibilities include altered vitamin K metabolism, malnutrition, and prophylactic antibiotic use.<sup>19</sup> In addition, increase in serum DCP levels with normal serum concentration of vitamin has been reported in patients with alcoholic liver diseases,<sup>31</sup> although all patients with alcoholic cirrhosis in the present study had HCC and we do not have data concerning this issue. Several patients with HCC showed a decrease in DCP levels after vitamin K administration. Previous reports *in vitro* and *in vivo* have indicated suppression of HCC by the vitamin.<sup>32–34</sup> However, it is not clear whether tumor suppression by vitamin K contributed to the decrease in DCP levels in these patients.

Diagnosis of HCC should be based primarily on imaging studies. However, some HCC nodules fail to show typical enhancement patterns, requiring tumor biopsy. The guideline of American Association for the Study of Liver Diseases and that of European Association of the Study of Liver Diseases both state that biopsy of liver nodule is not required if serum AFP is greater than 200 ng/mL.<sup>2,3</sup> For DCP, however, data are insufficient regarding whether elevation of DCP could complement inconclusive imaging findings. Previous reports on the use of DCP as a prognostic factor showed that DCP levels over 300 mAU/mL predict histologic vascular invasion and tumor recurrence after transplantation.<sup>26,27,35</sup> However, among 23 patients in the present study with serum DCP levels over 1000 mAU/mL, only three (13%) had HCC in their explanted livers (data not shown).

In conclusion, DCP levels are associated with vascular invasion, poorer histologic grade, and a larger size of hepatocellular carcinoma among liver transplant recipients with HCC. Elevated DCP in pre-transplant patients with severe hepatic impairment levels, however, did not correlate with the presence of HCC in the explant. To screen for HCC in patients awaiting liver transplanta-

tion, repeated imaging studies would be desirable. Elevated DCP levels may lead to further imaging studies to detect HCC, but the cost-effectiveness of using DCP as a screening mode requires further investigation.

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# Hepatocarcinogenesis in Hepatitis C: HCV Shrewdly Exacerbates Oxidative Stress by Modulating both Production and Scavenging of Reactive Oxygen Species

Hidetake Fujinaga Takeya Tsutsumi Hiroshi Yotsuyanagi Kyoji Moriya  
Kazuhiko Koike

Department of Internal Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

## Key Words

Hepatitis C · Hepatocellular carcinoma · Oxidative stress · Transgenic mouse · Core protein

## Abstract

Persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). One of the characteristics of HCV infection is the unusual augmentation of oxidative stress, which is exacerbated by iron accumulation in the liver, as observed frequently in hepatitis C patients. Using a transgenic mouse model, in which HCC develops late in life after the preneoplastic steatosis stage, the core protein of HCV was shown to induce the overproduction of reactive oxygen species (ROS) in the liver. In excessive generation of ROS, HCV affects the steady-state levels of a mitochondrial protein chaperone, i.e. prohibitin, leading to an impaired function of the mitochondrial respiratory chain with the overproduction of ROS. Insulin resistance and hepatic steatosis, which frequently accompany HCV infection, exacerbate ROS production. On the other hand, HCV compromises some of the antioxidant systems, including heme oxygenase-1 and NADH dehydrogenase quinone 1, resulting in the provocation of oxidative stress, together with ROS overproduction, in the liver with HCV infection. Thus,

HCV infection not only induces ROS but also hampers the antioxidant system in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis. Combination with the other activated pathway, including an alteration in the intracellular signaling cascade of MAP kinase, along with HCV-associated disturbances in lipid and glucose metabolism would lead to the unusual mode of hepatocarcinogenesis, i.e. very frequent and multicentric development of HCC, in persistent HCV infection.

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## Introduction

Approximately 200 million people are infected with hepatitis C virus (HCV) worldwide. More than two thirds of those with acute HCV infection suffer from persistent infection causing active or inactive chronic hepatitis, and approximately 30% of patients with chronic hepatitis are assumed to develop cirrhosis within their lifetime. Once HCV infection develops into cirrhosis, hepatocellular carcinoma (HCC) develops at an annual rate of 7% [1]. The strong association of oxidative stress with HCV infection has been demonstrated and can explain at least part of the clinical progression of the disease. The patho-

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Kazuhiko Koike, MD, PhD  
Department of Gastroenterology, Graduate School of Medicine  
The University of Tokyo  
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655 (Japan)  
Tel. +81 3 5800 8800, E-Mail [kkoike-tky@umin.ac.jp](mailto:kkoike-tky@umin.ac.jp)

genesis of chronic hepatitis C is not merely ascribed to inflammation caused by viral infection; the role of viral proteins in the pathogenesis has also been reported [2]. Of the proteins constituting HCV, the core protein in particular has various functions with respect to host cells and is closely related to oxidative stress. In this article, the relationship between HCV infection and oxidative stress is analyzed focusing on the pathological effect of the core protein of HCV, and the significance of oxidative stress in the pathogenesis of liver disease is discussed.

### **HCV Infection and Hepatocarcinogenesis**

The mechanism underlying hepatocarcinogenesis in HCV infection is not fully understood yet. Inflammation induced by an immune response to HCV should be considered, of course, in a study on hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in HCV infection with such a high incidence (90% in 15 years) or in a multicentric fashion? The other role of HCV would have to be weighed against a rare occurrence of HCC, even after the development of cirrhosis, in patients with autoimmune hepatitis in which severe inflammation in the liver persists. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C *in vivo* using transgenic mouse technology.

### **Transgenic Mouse Model for HCV-Related HCC**

One of the major issues regarding the pathogenesis of HCV-associated liver lesions is whether the HCV proteins have direct effects on pathological phenotypes. For this purpose, several lines of mice have been established

which are transgenic for the HCV cDNA. We have engineered transgenic mouse lines carrying the HCV genome by introducing the genes from the cDNA of the HCV genome of genotype 1b [3, 4]. Four different kinds of transgenic mouse lines are established, and they carry the core gene, envelope genes, the entire nonstructural (NS) genes, or the NS5A gene, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages [4]. The envelope gene transgenic mice did not develop HCC despite high expression levels of both E1 and E2 proteins [5], and the transgenic mice carrying the entire NS or NS5A gene developed no HCC.

Early in life, core gene transgenic mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damages [6]. Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. It is important to note that no significant inflammation is observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the structural genes including the core gene [4, 7, 8]. These outcomes indicate that the core protein *per se* of HCV has an oncogenic potential when expressed *in vivo*.

### **Augmentation of Oxidative Stress in Hepatitis C**

There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei [4]. On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in the core gene transgenic mouse model in the absence of inflammation in the liver [4]. The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage [2].

Augmentation of oxidative stress is implicated in the pathogenesis of liver disease in HCV infection as shown by a number of clinical and basic studies [2, 9]. Reactive

oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [10, 11]. Recent studies have shown that oxidative stress is more augmented in hepatitis C than in other types of hepatitis such as hepatitis B [9].

Thus, a major role in the pathogenesis of HCV-associated liver disease has been attributed to oxidative stress augmentation, but little is known regarding the mechanism of increased oxidative stress in HCV infection. Hence, it is important to understand the mechanism of oxidative stress augmentation, in terms of both generation and scavenging of ROS, which may allow us to develop new tools of therapies for chronic hepatitis C.

## Oxidative Stress and the Liver

### *Oxidative Stress and Reactive Oxygen*

The main source of ROS in hepatocytes is the mitochondria. Outside of hepatocytes, ROS also originate from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase in Kupffer cells and inflammatory cells. A large percentage of consumed oxygen is constantly converted into ROS in the mitochondria accompanied by oxygen consumption in the electron transport system (ETS). Hepatocytes contain many mitochondria and therefore have a high ROS production. Generated ROS are very unstable and highly reactive and attack biomolecules such as DNA, lipids, and proteins. The liver not only produces much ROS but is also the center of the antioxidative effect in the form of protein synthesis. Oxidative stress refers to the oxidation-reaction-dominant state of the living body induced by an imbalance between the oxidation reaction caused by ROS and the antioxidation reaction. Main ROS include superoxide ( $\cdot\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and the hydroxyl radical ( $\text{HO}\cdot$ ). ROS are mainly produced from  $\cdot\text{O}_2^-$  and converted into stable  $\text{H}_2\text{O}_2$  through a dismutation reaction.  $\text{H}_2\text{O}_2$  is converted into highly reactive  $\text{HO}\cdot$  in the presence of a transition metal.

### *The Antioxidant System and Oxidative Stress Markers*

Antioxidants include glutathione (GSH), thioredoxin (TRX), vitamin E, vitamin C, and  $\beta$ -carotene. Reactive oxygen elimination enzymes include superoxide dismutase (SOD), GSH peroxidase, heme oxygenase (HO)-1, and catalase. SOD is induced by oxidative stress and dis-

mutates  $\cdot\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  and oxygen. Catalase in peroxisomes also decomposes  $\text{H}_2\text{O}_2$  to water and oxygen. TRX is also a protein induced by oxidative stress and is reduced via S-S binding of the substrate protein by two SH groups in TRX and acts on the  $\text{H}_2\text{O}_2$  elimination system via peroxiredoxins. HO-1 is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin with the release of carbon monoxide. Biliverdin is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities.

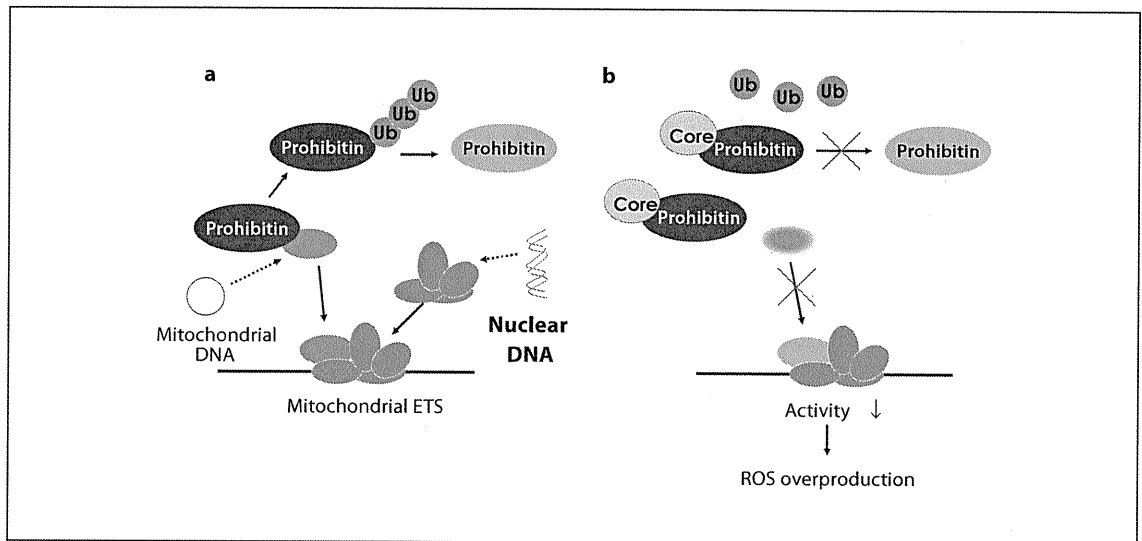
ROS cause various forms of cellular damage. 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are the peroxidation reaction products of lipids, and 8-hydroxydeoxyguanosine (8-OHdG) is the product of DNA base modification. These products serve as oxidative stress markers.

## The Origin of ROS Production in HCV Infection

Then, where is the place for oxidative stress overproduction in the liver of hepatitis C patients? The core protein is mostly localized to the endoplasmic reticulum, but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice [12]. In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core gene transgenic mice. Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid  $\beta$ -oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of ROS. In livers of transgenic mice harboring the core gene, increased ROS production has been observed [2]. A recent study found, via proteomic profiling of biopsy specimens, that impairment of key mitochondrial processes including fatty acid oxidation and oxidative phosphorylation and of the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis [13]. Therefore, it is probable that the HCV core protein affects mitochondrial functions since such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. We performed a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and





**Fig. 1.** The HCV core protein binds prohibitin and impairs its chaperone function leading to ROS overproduction. **a** Mitochondrial proteins consist of nuclear DNA-encoded proteins as well as mitochondrial DNA-encoded ones. Prohibitin acts as a protein chaperone for the mitochondrial proteins that are encoded by mitochondrial DNA by stabilizing newly synthesized mitochondrial translation products through direct interaction. **b** The HCV core

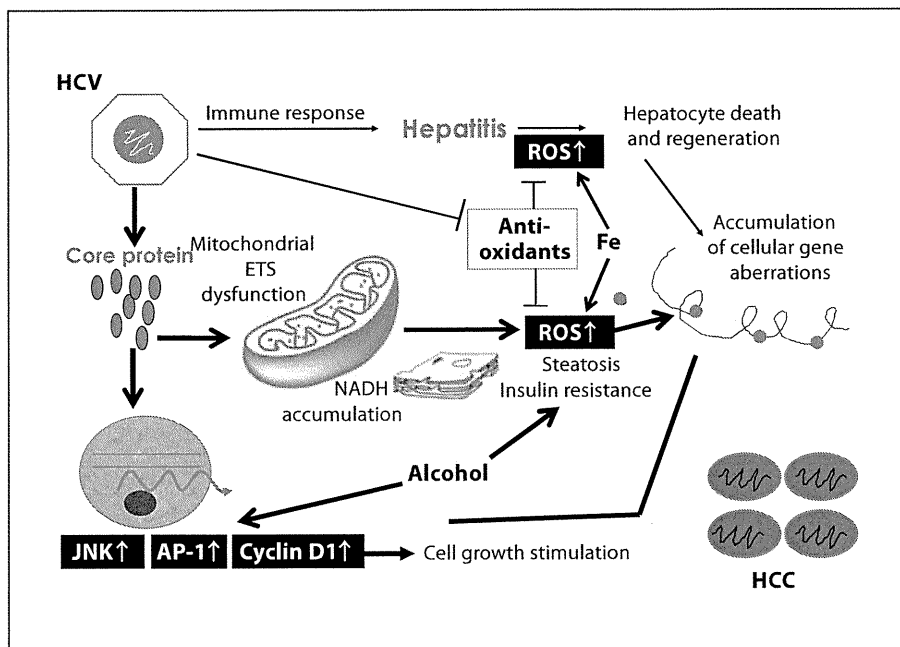
interacts with prohibitin, disturbing its molecular chaperone function, and leads to a decrease in the activity of ETS complex IV, COX. Subunit II of COX is encoded by the mitochondrial DNA, while other subunits are encoded by the nuclear DNA. This is a new mechanism for oxidative stress overproduction in viral infection in that HCV induces mitochondrial ETS dysfunction by inhibiting chaperone function. Ub = Ubiquitin.

identified several proteins of different expressions when compared with control HepG2 cells. Among upregulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperone, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity.

Prohibitin, a mitochondrial protein chaperone, was identified as an upregulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell cycle progression and cellular proliferation by attenuating DNA synthesis [14]. It is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells. Mitochondrial prohibitin acts as a protein chaperone by stabilizing newly synthesized mitochondrial translation products through direct interaction [15]. We examined the interaction between prohibitin and the

mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX) [15] (fig. 1). Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. In respect to the complex I function, we previously found a decreased complex I activity in core-expressing cells. Other groups have also shown that complex I activity is decreased in cultured cells [16]. Based on these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of prohibitin function has been shown to result in an increased production of ROS [17], a phenomenon observed in the core-expressing cells used in this study as well as in the liver of core-gene transgenic mice [2]. Interestingly, Shelly Lu et al. [18] recently reported that the liver-specific deletion of prohibitin resulted in morphological abnormality and HCC.

**Fig. 2.** Molecular pathogenesis of HCC development in HCV infection. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus itself contributes to hepatocarcinogenesis via two pathways. In one pathway, the core protein acts on the function of the mitochondrial ETS, leading to the overproduction of oxidative stress. The core protein also compromises some antioxidants and exacerbates ROS generation. Fe accumulation is an aggravating factor. The presence of steatosis and insulin resistance augments oxidative stress production. The other pathway is the modulation of cellular gene expression including the JNK pathway, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of HCC in HCV infection.



This is a new mechanism for ROS overproduction in viral infection in that HCV induces mitochondrial dysfunction through the inhibition of chaperone function in the mitochondria [19].

### HCV Compromises the Antioxidant System

As discussed above, chronic hepatitis C is characterized by its prominent augmentation of oxidative stress. Related to this, iron accumulation in the liver has been shown to aggravate the oxidative stress as shown by the increase in the amount of DNA adducts in the liver [2, 9]. Iron is accumulated in the liver of HCV core gene transgenic mice [20]. The accumulation of iron observed in the liver of the core gene transgenic mice fed with normal chow corroborates the observation in chronic hepatitis C patients [9, 10]. Then, the impact of iron overloading on the oxidant/antioxidant system was examined using this mouse model and cultured cells. Iron overloading caused the induction of ROS as well as antioxidants. However, some of the key antioxidant enzymes, including HO-1 and NADH dehydrogenase quinone 1 (NDQ-1), were not augmented sufficiently by iron overloading, while other antioxidant enzymes such as catalase and GST were augmented more strongly in the iron-overloaded core gene transgenic mice than in the iron-overloaded control or non-iron-overloaded core gene transgenic mice. The at-

tenuation of iron-induced augmentation of HO-1 was also confirmed in HepG2 cells expressing the core protein. HO-1 catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin, which is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities [21]. In addition, HO-1 has been also suggested to be a central antioxidant in conditions of GSH depletion [22]. Thus, HO-1 is an essential protective endogenous mechanism against oxidative stress, particularly in the case of iron overload. Therefore, it is probable that the attenuation of HO-1 and NQO-1 would hamper the antioxidant system and lead to a robust production of oxidative stress in HCV infection.

Thus, HCV infection not only induces ROS but also hampers antioxidant activation in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis.

### Conclusion

Pathways other than oxidative stress provocation in HCV-related hepatocarcinogenesis are alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)- $\alpha$  and interleukin-1 $\beta$  have been found transcriptionally activated [23]. The mitogen-activated pro-

tein kinase (MAPK) cascade, which is involved in numerous cellular events including cell proliferation, is also activated in the liver of the core gene transgenic mouse model. In the liver prior to HCC development, only the c-Jun N-terminal kinase (JNK) route is activated. Downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced [23, 24]. Far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes. The combination of these pathways that are activated in HCV infection, i.e. ROS overproduction, attenuation of antioxidants, cell growth stimulation via MAPK activation, metabolic disturbances such as hepatic steatosis, and insulin resistance [25], which are all induced by HCV itself, would contribute to hepatocarcinogenesis, together with moderate but long-lasting inflammation in chronic hepatitis C (fig. 2).

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations [26]. Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis. On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcino-

genesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute. The overall effect achieved by expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events which occur in HCV carriers. It no longer seem so difficult to determine why HCC develops in persistent HCV infection with an outstandingly high incidence. Our theory may also give an account of the multicentric *de novo* occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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### Disclosure Statement

The authors have nothing to disclose.

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# Receptor for Activated Protein Kinase C: Requirement for Efficient MicroRNA Function and Reduced Expression in Hepatocellular Carcinoma

Motoyuki Otsuka<sup>1\*</sup>, Akemi Takata<sup>1</sup>, Takeshi Yoshikawa<sup>1</sup>, Kentaro Kojima<sup>1</sup>, Takahiro Kishikawa<sup>1</sup>, Chikako Shibata<sup>1</sup>, Mutsuhiro Takekawa<sup>2</sup>, Haruhiko Yoshida<sup>1</sup>, Masao Omata<sup>1‡</sup>, Kazuhiko Koike<sup>1</sup>

<sup>1</sup> Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, <sup>2</sup> Department of Cell Signaling and Molecular Medicine, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan

## Abstract

MicroRNAs (miRNAs) are important regulators of gene expression that control physiological and pathological processes. A global reduction in miRNA abundance and function is a general trait of human cancers, playing a causal role in the transformed phenotype. Here, we sought to newly identify genes involved in the regulation of miRNA function by performing a genetic screen using reporter constructs that measure miRNA function and retrovirus-based random gene disruption. Of the six genes identified, RACK1, which encodes “receptor for activated protein kinase C” (RACK1), was confirmed to be necessary for full miRNA function. RACK1 binds to KH-type splicing regulatory protein (KSRP), a member of the Dicer complex, and is required for the recruitment of mature miRNAs to the RNA-induced silencing complex (RISC). In addition, RACK1 expression was frequently found to be reduced in hepatocellular carcinoma. These findings suggest the involvement of RACK1 in miRNA function and indicate that reduced miRNA function, due to decreased expression of RACK1, may have pathologically relevant roles in liver cancers.

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\* E-mail: [otsukamo-ky@umin.ac.jp](mailto:otsukamo-ky@umin.ac.jp)

‡ Current address: Yamanashi Prefectural Hospital Organization, Yamanashi, Japan

## Introduction

MicroRNAs (miRNAs) are short (20–23-nt), endogenous, single-stranded RNA molecules, that regulate gene expression and control physiological and pathological processes, such as development and cancer. Mature miRNAs and Argonaute (Ago) proteins form the RNA-induced silencing complex (RISC), a ribonucleoprotein complex that mediates post-transcriptional gene silencing [1] and then, complementary base-pairing of miRNAs guides the RISC to target messenger mRNAs, which are subsequently destabilized and sequestered from the translational machinery by Ago proteins [2–5]. Although insights into the regulatory function of miRNAs are beginning to emerge, their mechanisms of action and the genes involved in miRNA pathway have not yet been fully determined [6,7].

A large body of evidences suggests that the multigene regulatory capacity of miRNAs is dysregulated and exploited in cancer. Although several miRNAs are upregulated in specific tumors [8], a global reduction of miRNA abundance appears a general trait of human cancers, playing a causal role in the transformed phenotype [9,10]. In fact, the enzymes and cofactors involved in miRNA processing pathways may be targets of genetic disruption,

further enhancing cellular transformation [9]. Moreover, the disruption of Dicer in mice promotes hepatocarcinogenesis [11] and the truncating mutations in TARBP2, which causes a defect in the processing of miRNAs, were identified in sporadic and hereditary colon carcinomas [12].

Retroviral insertion-mediated random gene disruption can be used to generate null alleles, resulting in diminished endogenous gene expression [13]. The use of such retroviral integration methods, combined with appropriate reporter constructs, has provided an efficient, comprehensive gene screening method [14,15]. In this study, we sought to identify new genes involved in miRNA function and determine its role in live cancers. To this end, we established reporter cell lines in which cellular miRNA function could be assessed by expression of a drug resistance gene. Using these cell lines and a random gene disruption method, we identified genes that have not previously been implicated in the regulation of miRNA function. We subsequently determined the role of one of these genes, RACK1, which encodes “receptor for activated protein kinase C” (RACK1), in miRNA function, as well as its expression in liver cancers. Collectively, our data suggest the potential involvement of RACK1 in pathological processes.

## Results

### Identification of the genes required in the miRNA pathway by random gene disruption

To identify genes involved in miRNA pathways especially in liver cells, we constructed a reporter carrying a hygromycin resistance gene with two miR122-responsive elements in its 3'-UTR (Fig. 1A). We chose miR122 because it is the most abundant and tissue-specific miRNA in the liver [16]. Binding of miR122 reduces the expression of this hygromycin resistance gene in this construct. However, if miR122 function is impaired by the disruption of genes that are important for miRNA signaling, hygromycin resistance gene expression increases. Cells carrying such disrupted genes will therefore survive hygromycin treatment. Additionally, to enhance the effects of miR122, we co-transfected a miR122 precursor-expressing plasmid (Fig. 1A) with the reporter construct and selected monoclonal cells containing both constructs to minimize the effects of their random integration. After infection with retroviruses carrying a blasticidin resistance gene to produce random gene disruption in the selected reporter cells, cells surviving hygromycin treatment were harvested. The disrupted genes in the surviving cells were identified by 3' RACE. We infected  $\sim 10^6$  Huh7-pBS-Hygro-miR122 cells established from Huh7 cells and obtained  $\sim 10^4$  clones with random gene disruptions (confirmed by resistance to blasticidin) (Fig. 1B). After hygromycin selection, ten clones in which miRNA function was apparently impaired were obtained. In these ten clones, six disrupted genes were successfully identified (Table 1). One of them, RACK1 (also known as GNB2L1), appeared in duplicate and was found to be disrupted in two clones.

### Requirement of RACK1 for microRNA function

Of the genes identified, RACK1 was found to be disrupted in two independent clones (Table 1). RACK1 was previously identified as the binding partner of eIF6, a ribosome inhibitory protein known to prevent proper assembly of the 80S ribosome, and contributes to miRNA silencing by associating with RISC [17–19]. However, the requirement for eIF6 in miRNA function is controversial because other models, in which miRNA silencing is mediated by Ago2 (eIF2C2) and an interaction with eIF4E [20], or by GW182 [21], have more recently been described.

To confirm the requirement for RACK1 in miRNA function, we measured miRNA activity using a reporter assay involving transient expression of an siRACK1 construct and an miRNA precursor-overexpression plasmid. Transient knockdown of RACK1 reduced the function of three miRNAs: miR122, miR140, and miR185 (Fig. 2A). To examine these effects using a natural 3'-UTR containing miR122 binding sites, we used a *CatA*-Luc reporter that carried the 3'-UTR of the *CAT1* (cationic amino acid transporter 1) gene and a luciferase gene [22]. The *CAT1* 3'-UTR contains three predicted miR122 binding sites [22]. The requirement for RACK1 in miRNA function was confirmed using this reporter construct (Fig. 2B).

Next, we established stable RACK1-knockdown Huh7 cells (Fig. 2C) and compared the effects of miRNAs in control and RACK1-knockdown cells. Consistent with the results of the transient transfection assays, RACK1-knockdown cells showed weaker miRNA mediated-inhibition of target gene expression in a reporter system involving miR185 precursor-expressing plasmids and its reporter constructs (Fig. 2D). To measure changes in endogenous miRNA function in RACK1-knockdown cells, control and RACK1-knockdown cells were transfected with reporter constructs specific for several miRNAs and luciferase activity was then measured. We confirmed the inhibition of endogenous miRNA function in RACK1-knockdown cells (Fig. 2E). These

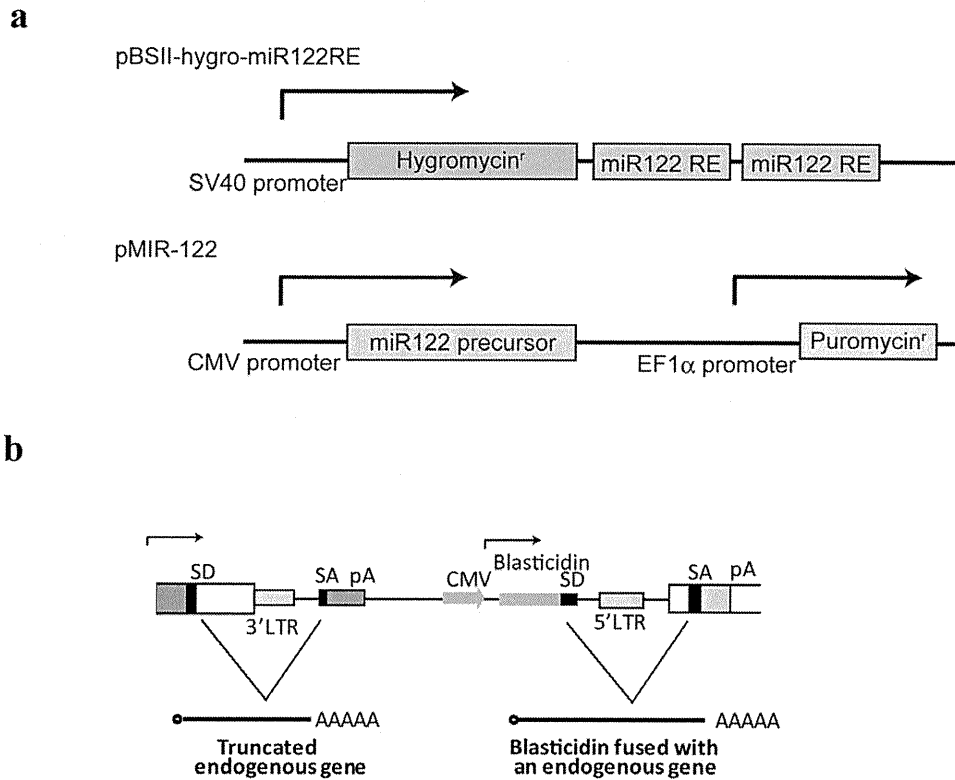
results suggest that RACK1 is indeed required for miRNA function.

### RACK1 may function after miRNA maturation but before the expression-inhibitory machinery

To identify the point at which RACK1 enhances miRNA function, mature miRNA levels were first measured in stable RACK1-knockdown cells. Levels of endogenous mature miR122, miR22, miR140-5p, -3p, and miR185, which are expressed at relatively high levels in liver cells [16], were comparable in control and RACK1-knockdown cells (Fig. 3A and Figure S1). This suggests that RACK1 may not be involved in miRNA maturation. We next showed that overexpression of artificial synthetic miRNA oligonucleotides replicated normal miRNA-mediated inhibition of gene expression in RACK1-knockdown cells (Fig. 3B). These results suggest that, *in vivo*, RACK1 may function after miRNA maturation, but before the expression-inhibiting machinery in the natural miRNA pathway, although the finding that the synthetic mature miRNAs were functional even in Ago2-knockdown cells (Figure S2A, B) indicates that their function might be Ago2-independent. Additionally, the expression of Droscha, DGCR8, Dicer, TRBP, Ago1, Ago2, Ago3, Ago4, and eIF4E, all of which are known to be involved in the miRNA pathway, were almost similar in control and RACK1-knockdown cells (Fig. 3C). Furthermore, the localization and number of the p-bodies, which may be involved in miRNA-mediated silencing [23,24], were not markedly affected by RACK1-knockdown (as determined by staining for the intracellular marker GW182) [23] (Fig. 3D). These results suggest that RACK1 functions after miRNA maturation, but before mature miRNAs exert their expression-inhibitory effects.

### RACK1 interacts with KSRP and is required for the full recruitment of mature miRNAs to the RISC

To determine whether RACK1 interacts with miRNA pathway related-molecules, transiently-transfected myc-tagged RACK1 was first immunoprecipitated. While myc-tagged RACK1 was efficiently precipitated (Fig. 4A), RISC constituent proteins such as Dicer, DDX20 (Gemin3) and Gemin4 [25] were found not to interact with RACK1 (Fig. 4A). GW182 did not interact with RACK1 (Fig. 4A). eIF6, which was previously reported to interact with RACK1 [18] and which may be involved in miRNA silencing [19], did not show an interaction with RACK1 in the present study (Fig. 4A). However, Ago2 weakly interacted with RACK1 (Fig. 4A), as reported recently [26]. Moreover, KH-type splicing regulatory protein (KSRP; also known as KHSRP), a key mediator of mRNA degradation [27,28] and a component of the Dicer complex that promotes the maturation of a subset of miRNAs [29], interacted with RACK1, especially when Dicer was overexpressed (Fig. 4A and Figure S3). This interaction was also confirmed by immunoprecipitation of endogenous RACK1 (Fig. 4B). The interactions of RACK1 with Ago2 and KSRP were insensitive to RNase A treatment (Figure S4), suggesting that they were not mediated by RNAs. Next, because it seemed that RACK1 functioned after miRNA maturation and before mature miRNA recruitment into the RISC, we measured levels of miRNAs in complexes immunoprecipitated using an anti-Ago2 antibody. Levels of mature miRNAs examined in the Ago2-containing complexes were lower in RACK1-knockdown cells than in control cells (Fig. 4C and Figure S5). To determine the possible causes of impaired mature miRNA loading into Ago2-related complexes in RACK1-knockdown cells, we determined the intracellular localization of KSRP and Ago2 in RACK1 knockdown cells (Fig. 4D). While KSRP is distributed both in the nucleus and cytoplasm and Ago2 localizes mainly in



**Figure 1. Constructs used in the study.** **A**, Reporter and miR122 precursor-expressing constructs. A construct containing an SV40 promoter-driven hygromycin resistance gene with two tandem miR122-responsive elements (miR122 RE) in its 3'-UTR was used to assess miRNA function. To express miR122, a construct carrying a CMV promoter-driven miR122 precursor was used in conjunction with puromycin selection. **B**, pDisrupt vector structure and gene products resulting from viral integration. Splicing occurs between the SD (splicing donor) and SA (splicing acceptor) sites (i.e., between the 3'-end of the endogenous gene exon and the retroviral SA site, as well as between the retroviral SD at the 3'-end of the blastidicin gene and the endogenous SA site at the 5'-end of the downstream gene exon). pA, polyadenylation signal.  
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cytoplasm in control cells, KSRP localizes more in the nucleus in RACK1-knockdown cells (Fig. 4D). The changes in the subcellular localization of KSRP in RACK1-knockdown cells were also confirmed by Western blotting using cell lysates prepared after subcellular fractionation (Fig. 4E). The function of synthetic mature miRNAs was not affected by KSRP knockdown (Figure S6A, B), suggesting that KSRP itself is not required for RISC activity. Thus, these localization changes with RACK-1 may be related to the impaired mature miRNA loading into Ago2 complexes from the KSRP-associated complexes, although, because the binding of KSRP

to Ago2 could not be detected in our coimmunoprecipitation study (Figure S7), the precise mechanisms remain to be elucidated. Nonetheless, these results suggest that RACK1 interacts with KSRP and that the recruitment of mature miRNAs into the RISC is impaired in RACK1-knockdown cells.

**RACK1 expression is frequently decreased in liver cancers**

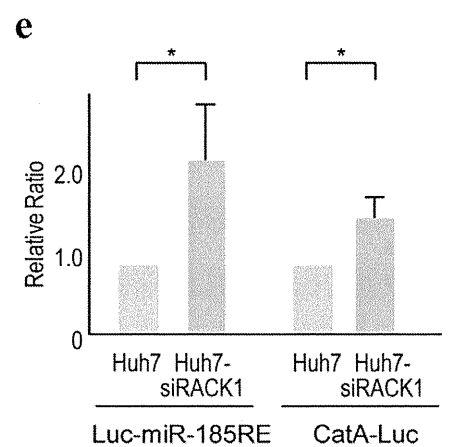
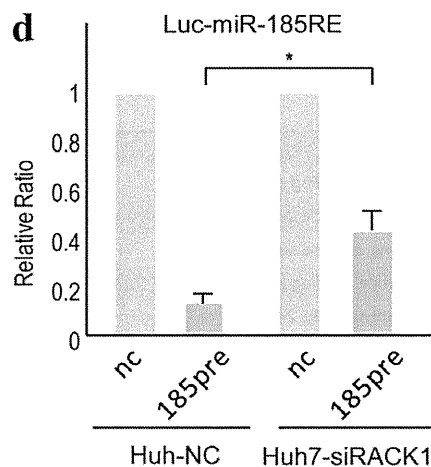
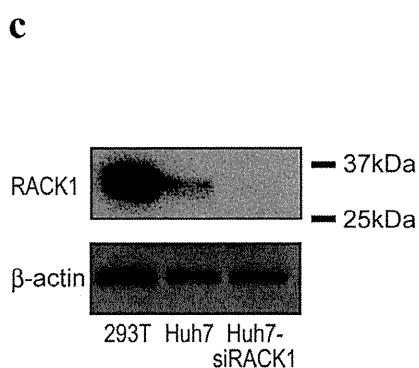
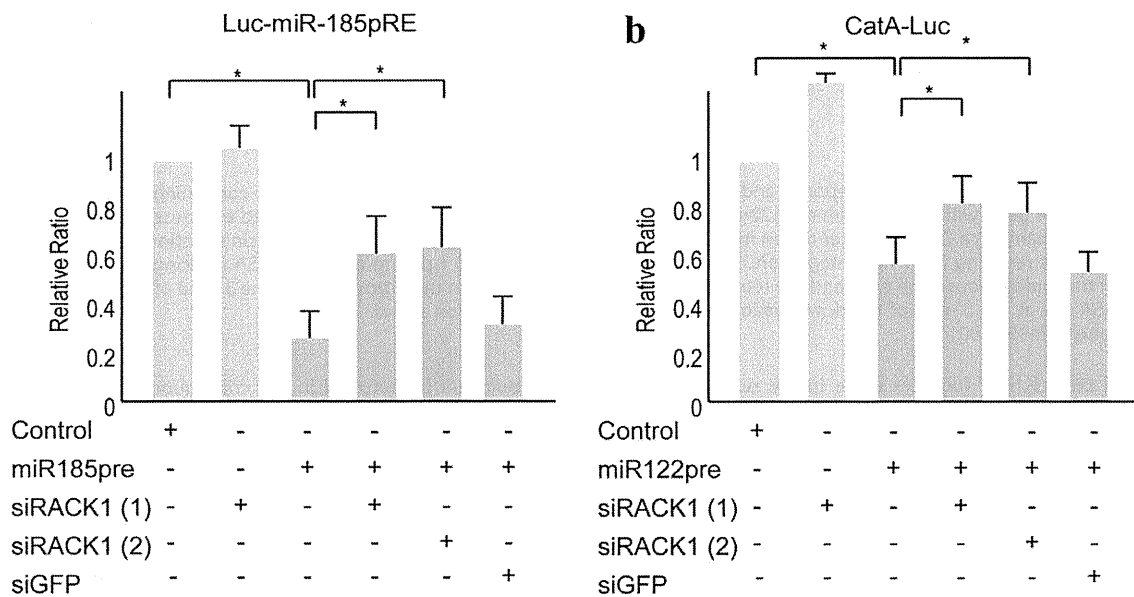
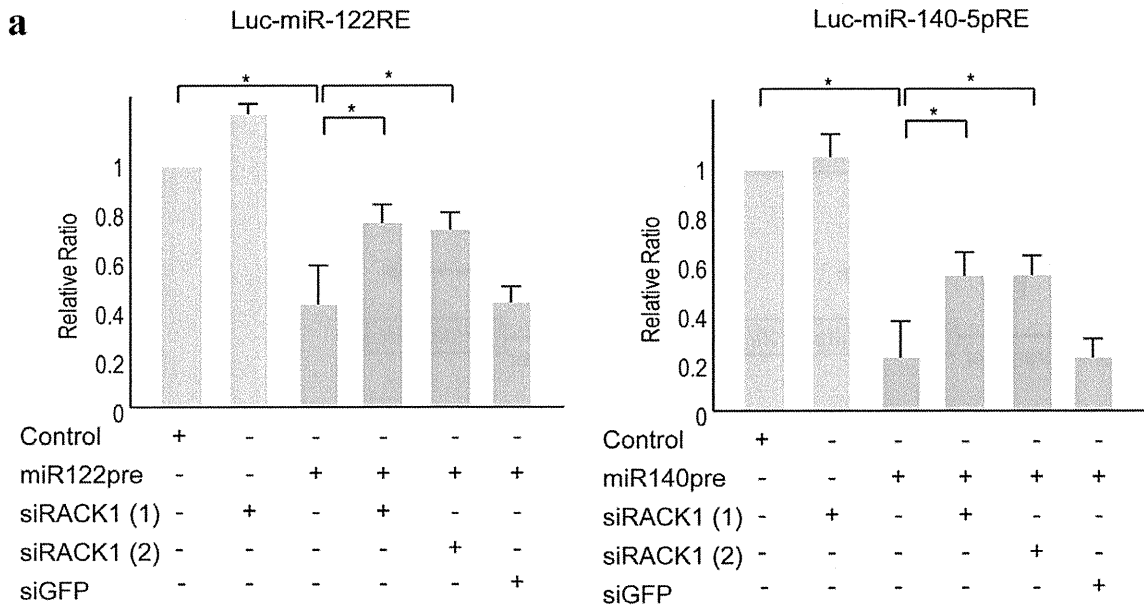
Recent results linking reduced global expression of miRNAs and reduced miRNA function with tumorigenesis [12,30] encouraged us to examine RACK1 expression in cancers. To this end, we

**Table 1.** List of genes identified in this screening.

Gene ID	Gene symbol	Gene title	Known function	Also known as
100288263	LOC100288263	hypothetical protein	Unknown	
10399	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	Translation	RACK1, PIG21
10055	SAE1	SUMO1 activating enzyme subunit 1	Sumoylation	AOS1, SUA1, UBLE1A
10399	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	Translation	RACK1, PIG21
6228	RPS23	ribosomal protein S23	Ribosome 40S	FLJ35016
6135	RPL11	ribosomal protein L11	Ribosome 60S	DBA7, GIG34
5358	PLS3	plastin 3	Actin binding	T-plastin

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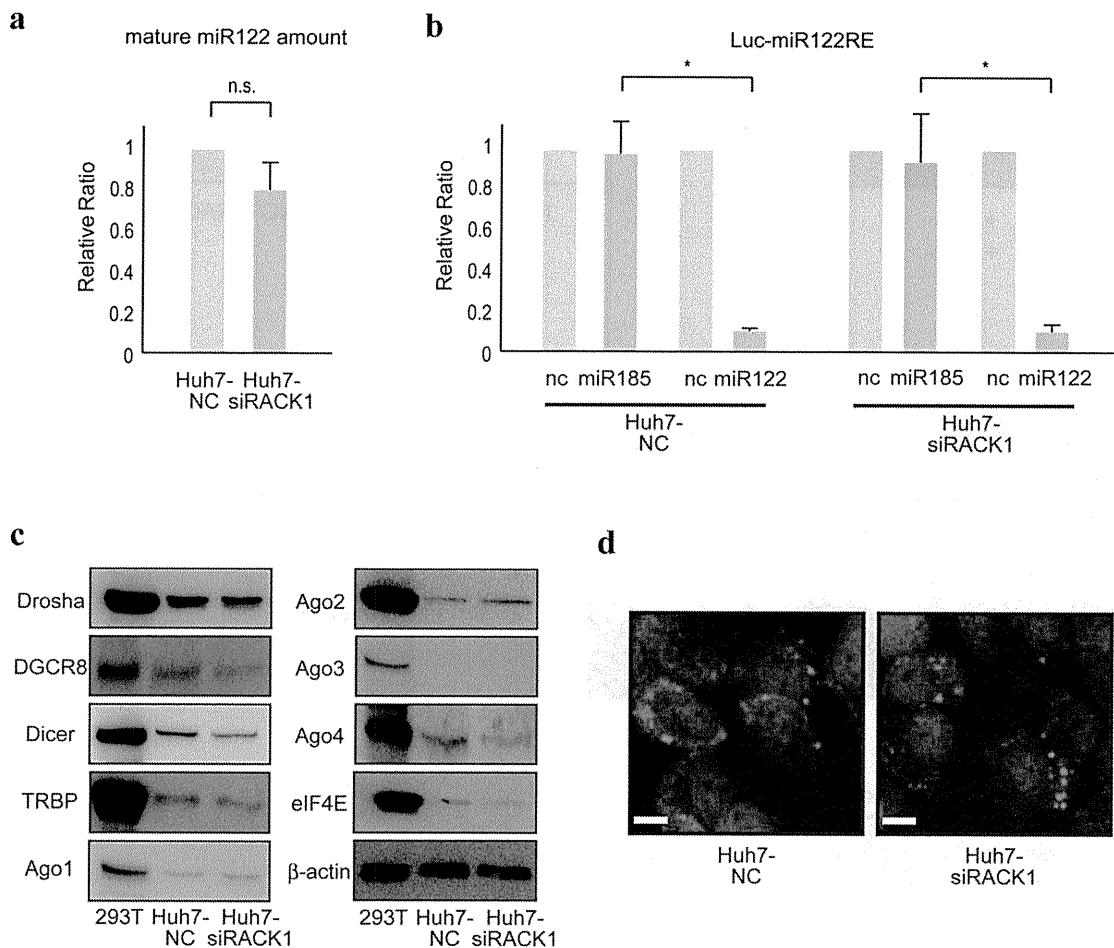




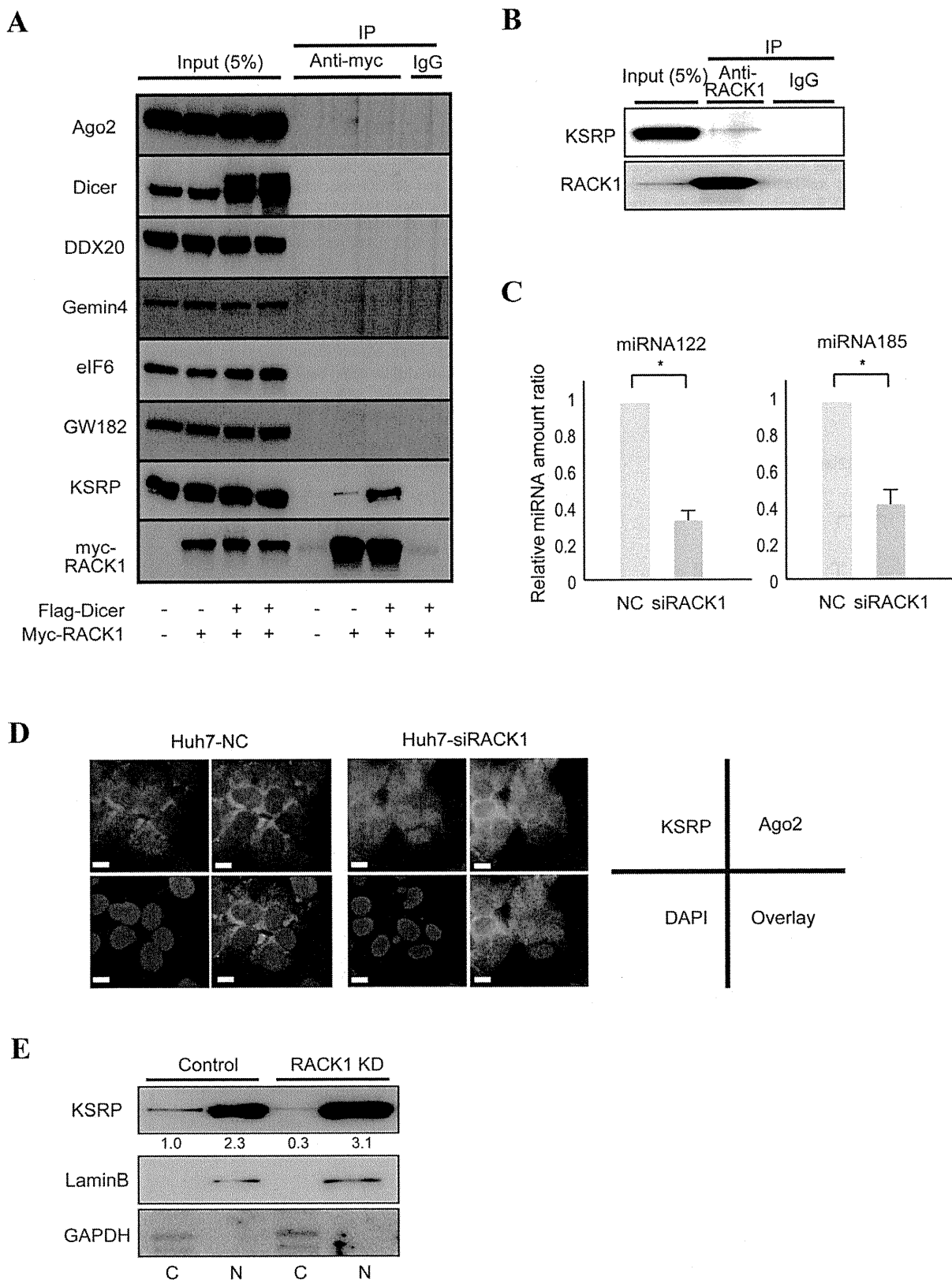
**Figure 2. RACK1 is required for miRNA function.** **A**, Overexpression of miR122, miR140, and miR185 precursors suppressed activity of the corresponding reporters. RACK1 knockdown partially blocked these effects. miRNA reporter plasmids were transfected, with or without the corresponding miRNA precursor- and two types of siRACK1-expressing plasmids (siRACK1 (1) and (2)), into Huh7 cells. Values were normalized to those obtained from cells transfected with a miRNA precursor-non-expressing control vector, which were set to 1. Data represent the mean  $\pm$  SD of three independent experiments. siGFP was used as a control, and it had no effect. Similar results were obtained using PLC/PRF/5 cells. **B**, CatA-Luc plasmids, which contain endogenous miR122 target sites derived from the CAT1 gene in its 3'-UTR, were transfected, with or without miR122 precursor- and siRACK1-expressing plasmids, into Huh7 cells. Data were generated and are presented as described in (A). Similar results were obtained using PLC/PRF/5 cells. **C**, Confirmation of the efficient knockdown of RACK1 expression in stable RACK1-knockdown Huh7 cells. 293T cell lysates were used as a positive control. **D**, miRNA function is impaired in stable RACK1-knockdown cells. miRNA185 reporter plasmids were transfected, with or without miR185 precursor-expressing plasmids, into control and RACK1-knockdown cells. Data were generated and are presented as described in (A). **E**, Endogenous miRNA function is impaired in RACK1-knockdown cells. miRNA185 reporter plasmids and CatA-Luc plasmids were transfected without miRNA precursor-expressing plasmids into control and RACK1-knockdown cells to assess endogenous miRNA function. Values were normalized to those obtained from control cells, which were set to 1. Data represent the mean  $\pm$  SD of three independent experiments. \*,  $p < 0.05$ .  
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determined RACK1 expression in various cancers and in healthy tissues by immunohistochemistry. While RACK1 expression was essentially normal in most cancers, it was frequently reduced in hepatocellular carcinoma (HCC) (Fig. 5A, B, C), consistent with a

previous report [31]. KSRP expression levels were relatively higher in liver tissues than in other organs, but no remarkable expression differences between HCC and adjacent tissues were observed (Figure S8A, B). These results suggest that decreased



**Figure 3. RACK1 functions after miRNA maturation and before the silencing machinery.** **A**, miRNA maturation was not impaired in RACK1-knockdown cells. Total RNA was isolated from control and RACK1-knockdown cells. Levels of mature miR122 were measured and normalized to the level of U6 snRNA. Relative ratios were calculated by adjusting the value for each miRNA in control cells to 1. Data represent the mean  $\pm$  SD of six independent experiments. **B**, Artificial synthetic miRNA oligonucleotides function appropriately in RACK1-knockdown cells. Control Huh7 cells and RACK1-knockdown cells were transfected with miR122 reporter plasmids with or without synthetic corresponding miR122 oligonucleotides and non-corresponding miR185 oligonucleotides (to verify specificity). Values were normalized to those obtained from the cells transfected with control synthetic oligonucleotides, which were set to 1. Data represent the mean  $\pm$  SD of three independent experiments. \*,  $p < 0.05$ . Similar results were obtained using PLC/PRF/5 cells. **C**, Expression of proteins involved in the miRNA pathway was normal in RACK1-knockdown cells. 293T cell lysates were used as a positive control. **D**, Localization and expression of P-bodies were normal in RACK1-knockdown cells. Control and RACK1-knockdown cells were immunostained for the p-body marker GW182. Scale bar, 50  $\mu$ m.  
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**Figure 4. RACK1 binds to KSRP and may be involved in the recruitment of mature miRNAs to the RISC.** **A**, Huh7 cells were transiently transfected with a control vector or a myc-tagged RACK1-expressing plasmid with or without a flag-tagged Dicer-expressing plasmid. Myc-tagged RACK1 was immunoprecipitated using anti-myc agarose. Normal mouse IgG was used as a control for immunoprecipitation. Co-precipitated proteins were blotted using antibodies against the indicated proteins. Five percent of the total cell lysates was loaded as "input." Representative results from two independent experiments are shown. Similar results were obtained using 293T cells. **B**, Endogenous RACK1 in Huh7 cells was immunoprecipitated using anti-RACK1 antibody and Protein A/G Sepharose. Normal mouse IgG was used as a control for immunoprecipitation.

Coprecipitated proteins were blotted using antibodies against the indicated proteins. Five percent of the total cell lysate was loaded as “input.” Representative results from two independent experiments are shown. **C**, Levels of mature miR122 and miR185 in Ago2-containing complexes were reduced in RACK1-knockdown cells. Mature miRNA levels were measured in RNA samples isolated from Ago2-containing complexes from control Huh7 (NC) cells and RACK1-knockdown (siRACK1) cells. miRNA levels were calculated as relative ratios. Data represent the mean  $\pm$  SD of six independent experiments. \*,  $p < 0.05$ . **D**, More KSRP localizes in the nucleus in RACK1-knockdown cells. Intracellular localization of KSRP (red) and Ago2 (green) were examined in control and RACK1-knockdown cells. KSRP was distributed both in the nucleus and cytoplasm, but more KSRP localized in RACK1-knockdown cells. Scale bar, 50  $\mu$ m. **E**, Greater localization of KSRP to the nucleus in RACK1-knockdown (RACK1 KD) cells was confirmed by Western blotting. Cytoplasmic (C) and nuclear (N) fractions were blotted with anti-KSRP antibody. The numbers below the panel indicate relative KSRP protein levels. GAPDH (a cytoplasm marker) and Lamin B (a nucleus marker) were blotted to confirm the appropriate fractionation.

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RACK1 expression and, consequently, decreased miRNA function may play an important role in HCC.

## Discussion

Here, we have shown that RACK1 is required for miRNA function through comprehensive gene screening using a random gene disruption method. Our results show that RACK1 interacts with KSRP and plays an important role in recruiting mature miRNAs to the RISC. Additionally, the expression of RACK1 is frequently decreased in HCC and may play a role in its pathogenesis.

RACK1 was initially identified as a major component of active ribosomes [18]. It binds directly to eIF6, which keeps the 40S and 60S ribosomal subunits apart, preventing the formation of a translationally competent complex. RACK1 bridges PKC and eIF6. Subsequent phosphorylation of eIF6 by PKC causes eIF6 to be released, an event that triggers 60S subunit activation. More recently, eIF6 was reported to be a component of a large TRBP-containing complex involved in miRNA-mediated post-transcriptional silencing [19]. However, the importance of eIF6 in miRNA-mediated silencing remains controversial [21]. In this study, we report that, although RACK1 is involved in miRNA-mediated silencing, it did not influence of translational machinery complexes containing eIF6, but instead appears to recruit mature miRNAs to the RISC.

The importance of RACK1 in regulating miRNA function was independently reported during the preparation of this manuscript [26]. Similar to our cases, they reported that RACK1 is required for miRNA function; however, the mechanism they reported differed from ours. They found that RACK1 contributes to the recruitment of RISC to the site of translation through binding with Ago2. While we could not detect the effects of RACK1 on miRNA-mediated translational repression as described above, our results were based on the use of synthesized mature miRNAs, which appear functionally Ago2-independent. Due to the fact that we also detected binding between RACK1 and Ago2, their proposed mechanism remains a possibility [26]. In addition, the importance of these diverse mechanisms might be dependent on different types of miRNAs. For example, the maturation of miRNAs used in our study, i.e., miR122, miR140, and miR185, is KSRP-independent [29]. Taking these points into consideration, in the future it may be necessary to determine if RACK1 is involved in different types of miRNA function under the same mechanisms, using various kinds of miRNAs.

We found that RACK1 interacts with KSRP particularly when Dicer is overexpressed. KSRP is a key mediator of AU-rich element-containing mRNA degradation [27,28] that has also been reported to bind to and promote the maturation of a subset of miRNA precursors [29]. In our study, however, maturation of miRNAs examined was preserved and the transfected synthetic miRNAs functioned appropriately in RACK1-knockdown cells. Moreover, levels of miRNAs in Ago2-containing complexes were

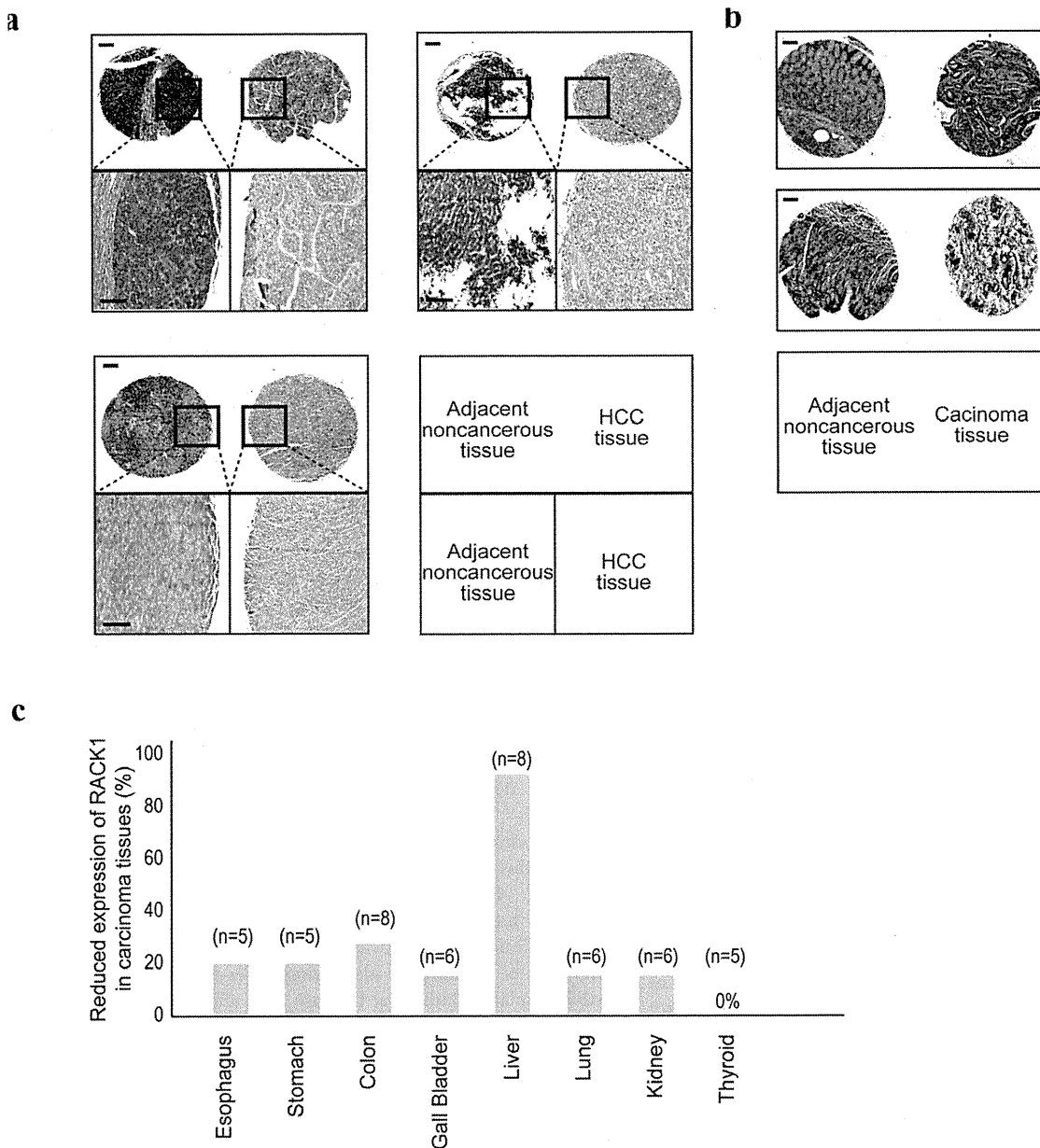
decreased in RACK1-knockdown cells. Based on these observations, we speculate that RACK1 is only involved in the recruitment of mature miRNAs to RISCs from miRNA maturation to the point at which they exert their effects in vivo. Although the precise mechanisms by which RACK1 contributes to the recruitment of miRNAs to RISCs and the biological significance of its binding to KSRP remains to be determined, these results suggest that RACK1 may regulate miRNA function as a component of a KSRP complex and during the maturation of miRNAs and their recruitment to RISCs.

Translationally repressed mRNA accumulates in discrete cytoplasmic foci known as p-bodies [24] or in another class of cytoplasmic aggregates, stress granules [32]. Although RACK1 has been reported to mediate stress granule formation [33], no change in the localization of p-bodies or expression of the p-body marker GW182 was detected in RACK1-knockdown cells in the present study. Additionally, no interaction between p-bodies and RACK1 was observed. We thus conclude that RACK1 does not produce p-body-related effects in the miRNA pathway.

Retroviral insertion-mediated random gene disruption screening in this study identified several genes involved in miRNA function. Functional genomics approaches involving RNAi libraries have recently been used to identify genes involved in certain functional pathways [34–37]. However, RNAi libraries may not be suitable for identifying genes involved in miRNA- or siRNA-related pathways, because they affect the pathways being examined. Thus, random gene disruption, as used here, may be a useful alternative option for the functional screening of such genes.

While identified genes here did not include well-known components of the miRNA pathway such as Dicer and Ago2, these genes may be critical for cell survival, which would make their selection by survival screening impossible. Alternatively, it may be that the screening required a greater number of cells. Nonetheless, we newly identified six genes that may potentially be involved in miRNA function. Of the genes identified, two, RPS23 and RPL11, are ribosome-related molecules, encoding 40S and 60S subunit proteins, respectively. In view of the fact that one of RACK1's roles is as a mediator of the synthesis of 80S ribosomes (composed of 40S and 60S subunits) [18], these results may suggest the importance of the ribosomal machinery in miRNA function.

Consistent with recent evidence linking global microRNA depletion with oncogenesis [12,30], RACK1 expression was found to be reduced in HCC. Because RACK1 staining in non-cancerous liver tissues was stronger than that in other tissues, the involvement of RACK1 in physiological functions may be more relevant in the liver. These results suggest that the miRNA functional impairment plays an important role in oncogenesis in the liver and/or the maintenance of oncogenicity in HCC, similar to the deficiency of miRNA expression [11].



**Figure 5. RACK1 expression is reduced in HCC.** **A**, Immunohistochemical analysis of RACK1 protein expression in HCC and non-cancerous surrounding tissues. While strong staining was observed in the cytoplasm of hepatocytes in non-cancerous liver tissues (upper left (one panel)), HCC cells were stained more weakly (upper right). Lower panels: magnified images of the highlighted regions in the corresponding upper panels. Three representative cases are shown. Scale bar, 500  $\mu$ m. **B**, Comparable expression of RACK1 in colon carcinoma tissues (right images) and non-cancerous surrounding tissues (left). Two representative cases are shown. Scale bar, 500  $\mu$ m. **C**, Comparison of RACK1 staining in cancers and healthy surrounding tissues. Eight types of cancers were examined. Percentages of cases in which RACK1 expression was lower in cancerous tissues than in healthy tissues were calculated. The number of cases of each type of cancer studied is indicated. doi:10.1371/journal.pone.0024359.g005

**Materials and Methods**

**Cell culture**

Huh7 and PLC/PRF/5 cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). 293T cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Stable cell lines derived from Huh7 and PLC/PRF/5 cells were established by retroviral or lentiviral infection. Clones were selected through the addition of 6  $\mu$ g/mL

puromycin, 400  $\mu$ g/mL hygromycin, or 5  $\mu$ g/mL blasticidin to the culture medium, unless otherwise specified.

**Random gene disruption**

The use of a pDisrupt retroviral vector carrying a blasticidin resistance gene for random gene disruption has been described previously [13]. Retroviruses were produced by transfection of the retroviral vector into virus-packaging Platinum A cells (Orbiden, San Diego, CA). Then, 48 h after transfection, the supernatants were harvested and viruses collected. To prepare reporter cell