

Fig. 6 Quantitation of the FGF2 mRNA level in BMCs cultured with HSLCs (+) in comparison with BMCs cultured alone (-). Real-time PCR analysis

not involved in the proliferation of HSLCs but might play a role in the differentiation of HSLCs into the hepatocyte lineage in vitro.

Discussion

The transplantation of BMCs has considerable potential as a future cell therapy for the regeneration of liver tissue. The degree of liver function and fibrosis and survival rate have been shown to improve significantly after BMC transplantation in animal models of severe liver injury (Sakaida et al. 2004; Terai et al. 2003). A recent human study has also shown that autologous BMCs transplanted intravenously

into patients with cirrhosis improve many parameters of liver function (Terai et al. 2006). However, with regard to the mechanisms of liver regeneration resulting from BMC transplantation, many physiological and regenerative roles of transplanted BMCs remain unclear.

In the present study, we have focused on the interaction of BMCs with hepatic stem cells, because so far data on this topic have been lacking. The co-culture system that we have established, in which neither cell migration nor cell contact can occur, has enabled us to investigate the changes in cell characteristics that occur during interaction between HSLCs and BMCs via the stimulating factors produced by each cell type.

Using this co-culture system, we have clearly demonstrated that both proliferation and differentiation of HSLCs into the hepatocyte lineage occur in the presence of BMCs. This suggests that BMCs interact with HSLCs to stimulate HSLC proliferation and their differentiation into the hepatocyte lineage in vitro. Although many factors are postulated to be involved in this phenomenon, we have been able to identify one of them produced by BMCs in the present study. DNA microarray analysis of BMCs co-cultured with HSLCs to clarify the upregulated genes that might influence the changes in the characteristics of HSLCs has revealed the expression of various genes, particularly of *FGF2* whose mRNA expression is upregulated 7.5-fold in comparison with that in BMCs cultured alone. HSLCs showing the expression of mRNA for *albumin* and *tryptophan-2,3-dioxygenase* proliferate on day 3 of co-culture with BMCs. The differentiation of HSLCs into the hepatocyte lineage is enhanced by FGF2, whose mRNA expression is upregulated in BMCs co-cultured with HSLCs, even though no direct contact occurs between the two cell types. These findings suggest that FGF2 produced by BMCs acts as a factor to stimulate the hepatocyte differentiation of HSLCs in vitro. The limitation of this in vitro study is that, although HSLCs cultured with FGF2 begin to express mRNA for liver-specific proteins, they are not mature hepatocytes. Additional cultures of hepatic stellate cells producing various growth factors and ECM (Nagai et al. 2002; Miura et al. 2003), other growth factors, and culture on ECM or the

Fig. 7 *Left* Evidence of FGF receptor 2 (*FGFR2*) mRNA expression in HSLCs and in mature hepatocytes; RT-PCR analysis. *Right* Immunofluorescence staining for FGFR2 protein in HSLCs revealed by confocal laser scanning microscopy. Magnification ×400

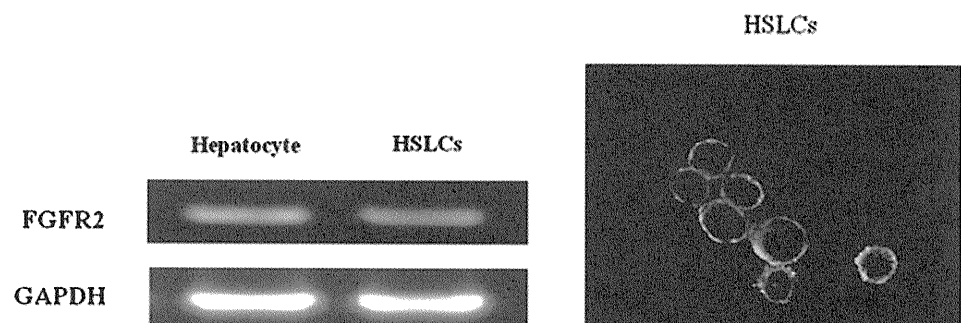
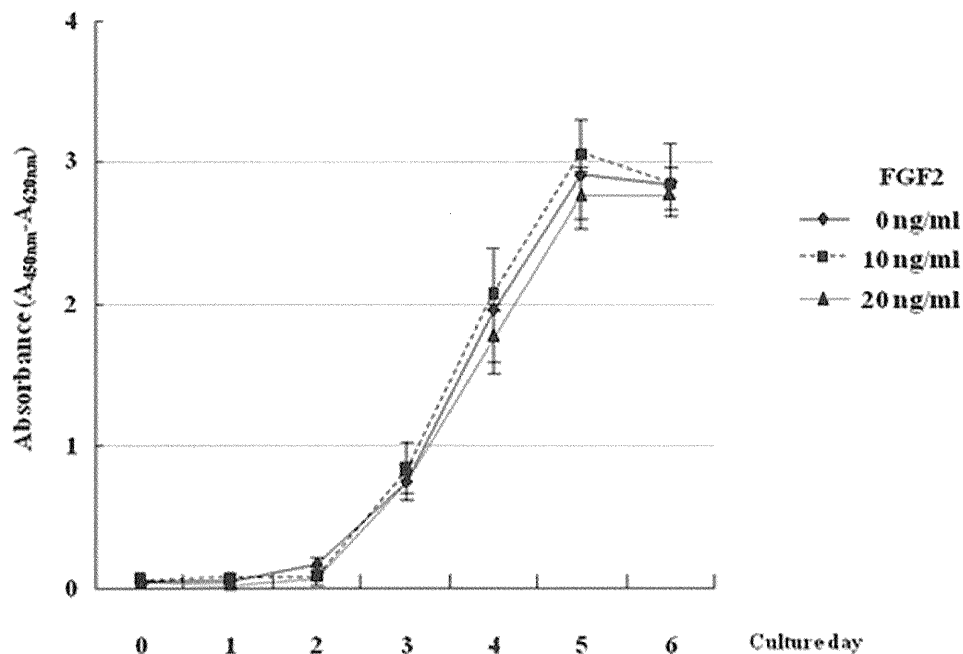


Fig. 8 Proliferation of HSLCs cultured with FGF2 at three different concentrations. The WST-1 assay was repeated eight times



injection of these cells in vivo would be necessary for the induction of mature hepatocytes from HSLCs.

FGF2 has an important role in organ regeneration; it stimulates angiogenesis and is expected to be a promising drug for the treatment of blood circulation disorders. Clinical trials of FGF2 in the treatment of intermittent claudication (Laham et al. 2005), ischemic heart disease, and chronic cutaneous wounds have previously been performed (Lederman et al. 2002; Fu et al. 2002; Goodman et al. 2003). As is well known, FGF2 plays an important role in development of the liver (Jung et al. 1999; Lemaigre and Zaret 2004; Zhao and Duncan 2005). FGF2 is produced primarily in liver sinusoidal cells, such as Ito cells, and promotes the production of ECM and vascularization, thus assisting in the repair of liver injury

(Hioki et al. 1996). It induces DNA synthesis in primary cultured fetal and adult rat hepatocytes (Hoffmann and Paul 1990) and has also been shown to promote the trans-differentiation of mouse BMCs into hepatic lineage cells, in association with the induction of transcription factors, including hepatocyte nuclear factors and GATA family proteins (Saji et al. 2004).

Administration of FGF2 in combination with BM transplantation synergistically improves liver fibrosis in models of liver injury induced by carbon tetrachloride (Ishikawa et al. 2007). However, the role of FGF2 in liver regeneration has not been clearly elucidated, and in particular, its effect on hepatic stem cell differentiation has not been studied previously. Interestingly, the present study has revealed that the expression of FGF2 mRNA in BMCs cultured with HSLCs is markedly upregulated in comparison with BMCs cultured alone, and that FGF2 enhances the differentiation of HSLCs into the hepatocyte lineage. Thus, FGF2 is induced in BMCs, as is the case in sinusoidal cells, and acts as a stimulating factor for the hepatocyte differentiation of HSLCs. However, the addition of FGF2 to the culture medium does not induce HSLC proliferation. Other stimulating factors that are released from BMCs and that might influence the proliferation of HSLCs are now being investigated in our laboratory.

In conclusion, our results suggest that BMCs stimulate both the proliferation and differentiation of HSLCs into the hepatocyte lineage, and that FGF2 is one of the factors that is produced by interacting BMCs and that stimulates the hepatocyte differentiation of HSLCs in vitro. Cross-talk between BM stem cells and hepatic stem cells may underlie

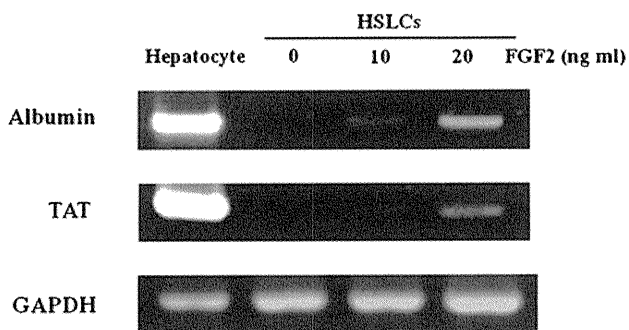


Fig. 9 RT-PCR analysis of mRNA expression for liver-specific proteins including albumin and tyrosine aminotransferase (TAT) in HSLCs cultured with FGF2 at three different concentrations (0, 10, 20 ng/ml) on culture day 3

the process of liver regeneration. This will be an area of interest for future investigations.

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Conflicts of interest We have no conflicts of interest to declare.

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Serum metabolomics reveals γ -glutamyl dipeptides as biomarkers for discrimination among different forms of liver disease

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Background & Aims: We applied a metabolome profiling approach to serum samples obtained from patients with different liver diseases, to discover noninvasive and reliable biomarkers for rapid-screening diagnosis of liver diseases.

Methods: Using capillary electrophoresis and liquid chromatography mass spectrometry, we analyzed low molecular weight metabolites in a total of 248 serum samples obtained from patients with nine types of liver disease and healthy controls.

Results: We found that γ -glutamyl dipeptides, which were biosynthesized through a reaction with γ -glutamylcysteine synthetase, were indicative of the production of reduced glutathione, and that measurement of their levels could distinguish among different liver diseases. Multiple logistic regression models facilitated the discrimination between specific and other liver diseases and yielded high areas under receiver-operating characteristic curves. The area under the curve values in training and independent validation data were 0.952 and 0.967 in healthy

controls, 0.817 and 0.849 in drug-induced liver injury, 0.754 and 0.763 in asymptomatic hepatitis B virus infection, 0.820 and 0.762 in chronic hepatitis B, 0.972 and 0.895 in hepatitis C with persistently normal alanine transaminase, 0.917 and 0.707 in chronic hepatitis C, 0.803 and 0.993 in cirrhosis type C, and 0.762 and 0.803 in hepatocellular carcinoma, respectively. Several γ -glutamyl dipeptides also manifested potential for differentiating between nonalcoholic steatohepatitis and simple steatosis.

Conclusions: γ -Glutamyl dipeptides are novel biomarkers for liver diseases, and varying levels of individual or groups of these peptides have the power to discriminate among different forms of hepatic disease.

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Keywords: γ -Glutamyl dipeptides; Metabolomics; Biomarker; Capillary electrophoresis mass spectrometry; Oxidative stress; Glutathione; Hepatocellular carcinoma; Nonalcoholic steatohepatitis; Hepatitis C virus.

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Abbreviations: HCC, hepatocellular carcinoma; AST, aspartate transaminase; ALT, alanine transaminase; γ -GTP, γ -glutamyl transpeptidase; CT, computed tomography; NAFLD, nonalcoholic fatty liver disease; SS, simple steatosis; NASH, non-alcoholic steatohepatitis; CE-TOFMS, capillary electrophoresis time-of-flight mass spectrometry; GSH, reduced glutathione; GC, gastric cancer; GCS, γ -glutamylcysteine synthetase; C, healthy control; DI, drug-induced liver injury; AHB, asymptomatic hepatitis B virus infection; CHB, chronic hepatitis B; CNALT, hepatitis C with persistently normal alanine transaminase; CHC, chronic hepatitis C; CIR, cirrhosis type C; HBs, hepatitis B surface; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α -fetoprotein; PIVKA, protein induced by vitamin K antagonist; LC-MS/MS, liquid chromatography-electrospray tandem mass spectrometry; MLR, multiple logistic regression; APAP, acetaminophen; GS, glutathione synthetase; BSO, buthionine sulfoximine; DEM, diethylmaleate; ROS, reactive oxygen species.

Introduction

Acute or chronic viral hepatitis affects populations around the world, and the disease often progresses from chronic hepatitis and cirrhosis to hepatocellular carcinoma (HCC) [1]. Accurate diagnosis at earlier stages is necessary for improved therapeutic outcome. However, the diagnostic procedures are laborious and not risk-free. Patients with suspected liver damage are initially subjected to liver function tests that include the assessment of aspartate transaminase (AST), alanine transaminase (ALT), and γ -glutamyl transpeptidase (γ -GTP) serum levels. If these levels are abnormal, patients are then subjected to diagnostic imaging, such as ultrasound and computed tomography (CT), and assays to determine the presence of antibodies against hepatitis virus. Finally, a liver biopsy may be recommended to evaluate the severity of inflammation or fibrosis and to confirm the indications for antiviral therapy.

Recently, nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in western countries. It

encompasses a wide spectrum of conditions associated with over-accumulation of fat in the liver, ranging from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH), and cirrhosis [2]. Although SS typically follows a benign non-progressive clinical course, NASH may eventually develop into cirrhosis and HCC. To date, a liver biopsy remains the gold standard for the diagnosis of NASH [3]. However, since the biopsy procedures carry the risk of mortality [4,5], the noninvasive identification of biomarkers, that can provide reliable differential diagnoses for the characterization of liver diseases, is desirable.

Metabolomics, which can be defined as measurement of the levels of all cellular metabolites, has emerged as a powerful new tool for discovering new low molecular weight biomarkers. Its utility has been demonstrated by the identification of new biomarkers for prostate cancer [6], Parkinson's disease [7], type 2 diabetes mellitus [8], acute myocardial ischemia [9], and pre-eclampsia [10].

Recently, we developed new metabolomic profiling approaches based on capillary electrophoresis mass spectrometry [11] and capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) [12–14]. The efficacy of CE-TOFMS was demonstrated by the discovery of ophthalmate (γ -glutamyl-2-aminobutyrylglycine) as a biomarker; in mice, reduced glutathione (GSH) depletion produced acetaminophen-induced hepatotoxicity [12,14]. In this study, to discover new noninvasive biomarkers for human liver diseases, we comprehensively analyzed the serum metabolites in a total of 248 samples from patients with nine types of liver disease or gastric cancer (GC) and from normal individuals using our metabolomic approaches, and found increased levels of γ -glutamyl dipeptides in the majority of the liver diseases. Moreover, we found that γ -glutamyl dipeptides were synthesized via the ligation of glutamate with various amino acids and amines by the γ -glutamylcysteine synthetase (GCS), an enzyme that is feedback-inhibited by GSH, and that the levels of γ -glutamyl dipeptides were indicative of the amount of GSH production. The concentrations of serum γ -glutamyl dipeptides varied with the stage and type of liver disease and can, therefore, act as new biomarkers for liver diseases. Here, we report that a highly specific set of γ -glutamyl dipeptides, alone or in combination with transaminases and methionine sulf-oxide, can effectively distinguish specific liver diseases from other hepatic injuries and healthy control samples.

Materials and methods

Serum samples

A total of 248 serum samples were obtained from three institutes, Yamagata University Hospital (YUH; Yamagata, Japan), University of Tokyo Hospital (UTH; Tokyo, Japan) and Shonai Hospital (SH; Tsuruoka, Japan). The 162 YUH cases comprised 53 healthy controls (C) and patients with drug-induced liver injury (DI; $n = 10$), asymptomatic hepatitis B virus infection (AHB; $n = 9$), chronic hepatitis B (CHB; $n = 7$), hepatitis C with persistently normal alanine transaminase (CNALT; $n = 10$), chronic hepatitis C (CHC; $n = 24$), cirrhosis type C (CIR; $n = 10$), HCC ($n = 19$), SS ($n = 9$) and NASH ($n = 11$). The 75 UTH cases comprised four controls and patients with DI ($n = 17$), AHB ($n = 7$), CHB ($n = 7$), CNALT ($n = 8$), CHC ($n = 11$), CIR ($n = 8$) and HCC ($n = 13$). The 11 SH cases were all GC patients. Written informed consent was obtained from all the participants and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review boards of YUH, UTH, and SH. The study subjects were patients with viral liver diseases, drug-induced hepatotoxicity or NAFLD who were referred to the Department of Gastroenterology and Hepatology at YUH, UTH, or SH.

All the healthy controls had normal liver function and no viral hepatitis infection, and none were alcoholics. The AHB and CNALT patients were confirmed to have normal liver function and to be positive for hepatitis B surface (HBs) antigen and hepatitis B virus (HBV) DNA, or for anti-hepatitis C virus (HCV) antibodies and HCV RNA, respectively. DI was diagnosed based on abnormal values on biochemical tests, absence of other hepatic diseases, and a history of treatment with drugs suspected of being probable causes of DI. The suspected medications were different, and the biochemical test results in each patient normalized after their withdrawal.

CHC and CIR were diagnosed on the basis of physical examination, biochemical tests, ultrasonography, and CT findings. Some patients with chronic hepatitis provided informed consent for a liver biopsy, and the procedure was performed to confirm the accuracy of the diagnosis. The diagnosis of CHB and CHC was based on increased ALT levels (above the upper limit of the normal range) in at least two blood samples assayed over a 6-month period, and the presence of detectable HBs antigen and HBV DNA or detectable anti-HCV antibodies and HCV RNA, respectively. HCV infection was causative in all cirrhosis patients, and they manifested symptoms of portal hypertension, such as splenomegaly, esophageal varices, encephalopathy, or ascites.

The diagnosis of HCC was based on ultrasonography, CT, and MRI findings that revealed features typical of HCC. HCV was causative in all cases, and the α -fetoprotein (AFP) and protein induced by vitamin K antagonist (PIVKA)-II levels were assayed in all HCC patients.

All of the SS and NASH patients underwent liver biopsy. The tissue samples were stained with hematoxylin-eosin, reticulin, and Masson trichrome; and examined by the same experienced pathologist who was blinded to the clinical data. The histological criterion for the diagnosis of NAFLD was the presence of fatty changes in hepatocytes. When hepatocytes exhibited macrovesicular steatosis, the differential diagnosis was SS or NASH. The criteria for a diagnosis of steatohepatitis were the presence of lobular inflammation and either ballooning cells or perisinusoidal/pericellular fibrosis, in addition to steatosis in the liver specimen. No patient with autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α 1-antitrypsin deficiency, Wilson's disease, or alcoholic liver injury was included. All patients with GC were diagnosed by pathologic studies of biopsy tissues.

Analytical and statistical technologies for biomarker discovery

Using a total of 237 samples from YUH (training cohort, $n = 162$) and UTH (validation cohort, $n = 75$) (Table 1), we performed CE-TOFMS for a comprehensive analysis of the metabolite changes to discover new biomarkers in the diagnosis of human liver diseases. To facilitate peak identification and quantification, we analyzed 162 metabolic standards listed in the KEGG LIGAND database [15] before analyzing the samples. Global mass scanning over a 50–1000 m/z range was applied in the CE-TOFMS mode [12]. To focus on γ -glutamyl peptides, we employed a highly sensitive method using liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS) with multiple reactions monitoring for analyses of the patient serum samples. The Kruskal-Wallis test and Dunn's post-test were used to assess the statistical significance of differences among C, DI, AHB, CHB, CNALT, CHC, CIR, and HCC. The Mann-Whitney test was used to evaluate the statistical significance of differences between SS and NASH. The algorithm of the feature selection for the multiple logistic regression (MLR) models is described in the Supplementary data.

Results

Discovery of γ -glutamyl dipeptides in serum by metabolomic profiling

The CE-TOFMS analysis quantified the levels of 49 metabolites in the serum samples (Supplementary Tables 1 and 2) and revealed increases in many compounds in most liver diseases. We identified these compounds as γ -glutamyl dipeptides (e.g., γ -Glu-Gly, γ -Glu-Ala, γ -Glu-Ser, γ -Glu-Val, γ -Glu-Thr, γ -Glu-Taurine, γ -Glu-Leu, γ -Glu-Gln, γ -Glu-Lys, γ -Glu-Glu, γ -Glu-Met, γ -Glu-His, γ -Glu-Phe, γ -Glu-Arg, γ -Glu-Citrulline, γ -Glu-Tyr, and γ -Glu-Trp) by comparing their migration times and exact molecular

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Table 1. Summary of patient information.

Clinical information		Training cohort (n = 162)	Defect no.	Validation cohort (n = 75)	Defect no.	p value
Age (years)						
	Median	61	0	66	0	0.47
	Interquartile range	51-73	0	55-70	0	
Sex (n)						0.0007*
	Male	73	0	52	0	
	Female	89	0	23	0	
AST (UL ⁻¹)						
	C	21.5 ± 5.40	0	25.3 ± 3.60	0	0.074
	DI	274 ± 567	0	81.2 ± 84.9	0	0.15
	AHB	25.0 ± 6.81	2	23.9 ± 6.90	0	0.71
	CHB	109 ± 164	0	150 ± 146	0	0.0059
	CNALT	24.1 ± 3.80	0	23.8 ± 6.00	0	0.72
	CHC	62.8 ± 65.3	0	110 ± 51.0	0	0.0010
	CIR	54.6 ± 27.1	0	58.0 ± 26.1	0	0.69
	HCC	71.3 ± 52.8	0	35.0 ± 24.5	0	0.0010
	SS	41.2 ± 11.5	0			
	NASH	78.6 ± 48.0	0			
ALT (UL ⁻¹)						
	C	17.7 ± 4.70	0	25.0 ± 8.30	0	0.062
	DI	253 ± 343	0	115 ± 132	0	0.15
	AHB	26.6 ± 18.6	2	23.1 ± 5.60	0	0.40
	CHB	117 ± 162	0	173 ± 131	0	0.0060
	CNALT	17.9 ± 4.10	0	21.5 ± 3.60	0	0.074
	CHC	79.4 ± 81.0	0	160 ± 116	0	0.0036
	CIR	40.7 ± 21.9	0	57.3 ± 42.4	0	0.69
	HCC	57.9 ± 58.8	0	25.0 ± 21.6	0	0.0026
	SS	72.2 ± 24.5	0			
	NASH	121 ± 140	0			
γ-GTP						
	C	20.7 ± 8.60	0	—	4	—
	DI	190 ± 236	0	46.2 ± 29.5	5	0.010
	AHB	31.1 ± 24.1	2	—	7	—
	CHB	52.8 ± 38.1	1	—	7	—
	CNALT	150 ± 5.70	0	—	8	—
	CHC	48.5 ± 36.4	0	—	11	—
	CIR	28.8 ± 17.9	0	49.6 ± 53.1	0	0.17
	HCC	51.2 ± 31.1	0	—	13	—
	SS	61.8 ± 43.7	0			
	NASH	98.7 ± 99.1	0			
AFP						
	CHC	6.40 ± 7.40	3	—	11	—
	CIR	35.1 ± 71.8	0	14 ± 15.6	0	0.63
	HCC	9.79 × 10 ² ± 1.73 × 10 ³	0	7.04 × 10 ³ ± 2.52 × 10 ⁴	0	0.024
PIVKA-II						
	HCC	1.57 × 10 ² ± 1.87 × 10 ²	0	7.78 × 10 ³ ± 2.77 × 10 ⁴	0	0.022

*Chi-square test. The others p values were obtained by the Mann-Whitney U-test.



Fig. 1. Heat map representing the hierarchical clustering of 67 compounds in serum samples from controls and patients with various types of liver disease in both cohorts. Each row shows data for a specific metabolite or transaminase, and each column shows data for the healthy controls and patients with liver diseases. The compound concentration in each individual was divided by the average concentration in the healthy controls and the obtained values were then averaged again for each disease. The metabolites highlighted in blue showed large fold changes (disease/control ratios of >2.5) in an average of seven liver diseases. **p* < 0.05, ***p* < 0.01, ****p* < 0.0001, significance difference by the Kruskal–Wallis test. The compounds were clustered based on elucidation distances. Red and green denote relatively high and low concentrations, respectively, compared with the average concentration.

weights with those of the standards. Significant differences were observed among controls and liver diseases (*p* < 0.0001; Kruskal–Wallis test) except for γ -Glu-Met in the validation data (Supplementary Tables 1 and 2). Correlational cluster analyses of 67 compounds showed that all the γ -glutamyl dipeptides except for γ -Glu-Tyr and γ -Glu-Trp were clustered with AST, ALT, and metabolites involved in oxidative stress responses, namely glucosamine [16] and methionine sulfoxide [17–19] (Fig. 1).

Statistical analysis and validation for biomarker discovery

From the serum samples obtained at YUH, we selected 89 liver disease patients including DI, AHB, CHB, CNALT, CHC, CIR, and HCC patients, and 53 healthy controls with no significant differences in the age distribution between the training and validation cohorts (Table 1). As shown in the whisker box plots for the training cohort (Fig. 2), the levels of γ -glutamyl dipeptides and of AST and ALT, as commonly used hepatocyte biomarkers, were increased in different patterns in comparison with C. For example, the AST and ALT levels were significantly increased in patients with DI, CHB, CHC, CIR, and HCC (*p* < 0.05; Dunn’s post-test), but not in those with AHB and CNALT (Fig. 2). On the other hand, significant increases were observed in the levels of γ -Glu-Ser, γ -Glu-Val, γ -Glu-Thr, γ -Glu-Leu, and γ -Glu-Phe (*p* < 0.05; Dunn’s post-test) in AHB and in the levels of all the γ -glutamyl derivatives of amino acids (*p* < 0.05; Dunn’s post-test) except for ophthalmate, γ -Glu-Thr, and γ -Glu-Trp in CNALT (Fig. 2 and Supplementary Table 1). Oxidative metabolites, methionine sulfoxide, and glucosamine were significantly increased in all diseases (*p* < 0.05; Dunn’s post-test) and in CHB, CNALT, and CHC (*p* < 0.0001; Dunn’s post-test), respectively (Fig. 2).

To assess their abilities to discriminate specific liver diseases from other liver diseases, we developed MLR models using combinations of several components of the γ -glutamyl dipeptides, transaminases, and oxidative metabolites using the training dataset. For example, an MLR model incorporating four selected biomarkers (γ -Glu-Ala, γ -Glu-Citrulline, γ -Glu-Thr, and γ -Glu-Phe) was able to differentiate HCC from the other groups (C, DI, AHB, CHB, CNALT, CHC, and CIR) with an area under the receiver-operating characteristic (ROC) curve (AUC) value of 0.762 (95% CI 0.647–0.877, *p* = 0.00025). The probability (*p*) of HCC is calculated by: $\log(p/(1 - p)) = -1.87 - 1.13 \times \gamma\text{-Glu-Ala} + 3.51 \times \gamma\text{-Glu-Citrulline} - 1.65 \times \gamma\text{-Glu-Thr} + 6.99 \times \gamma\text{-Glu-Phe}$ (Table 2). When the concentrations of γ -Glu-Ala, γ -Glu-Citrulline, γ -Glu-Thr, and γ -Glu-Phe are 1.7, 0.84, 0.54, and 0.34 μ M, respectively, the probability of HCC is 65.5%. All the MLR models achieved high AUC values at statistically significant levels (between 0.754 and 0.972, *p* < 0.011) (Fig. 3, Table 2 and Supplementary Table 3).

The developed MLR models were evaluated in a blinded manner using an independent cohort (YUH) consisting of 75 individuals who were not members of the training cohort (Supplementary Table 2). We found that all of the MLR models also produced high AUC values at statistically significant levels (between 0.707 and 0.993, *p* < 0.023) (Fig. 3, Table 2 and Supplementary Table 3). Although C, CHB, and CHC were each differentiated from the other groups by a single γ -glutamyl dipeptide (γ -Glu-Phe, γ -Glu-Thr, and γ -Glu-Lys, respectively), the MLR models for the other diseases required multiple biomarkers to achieve accurate discrimination (Table 2). The odds ratios of ALT, AST, and methionine sulfoxide were close to 1.0 compared with the odds ratios of the γ -glutamyl dipeptides, indicating their

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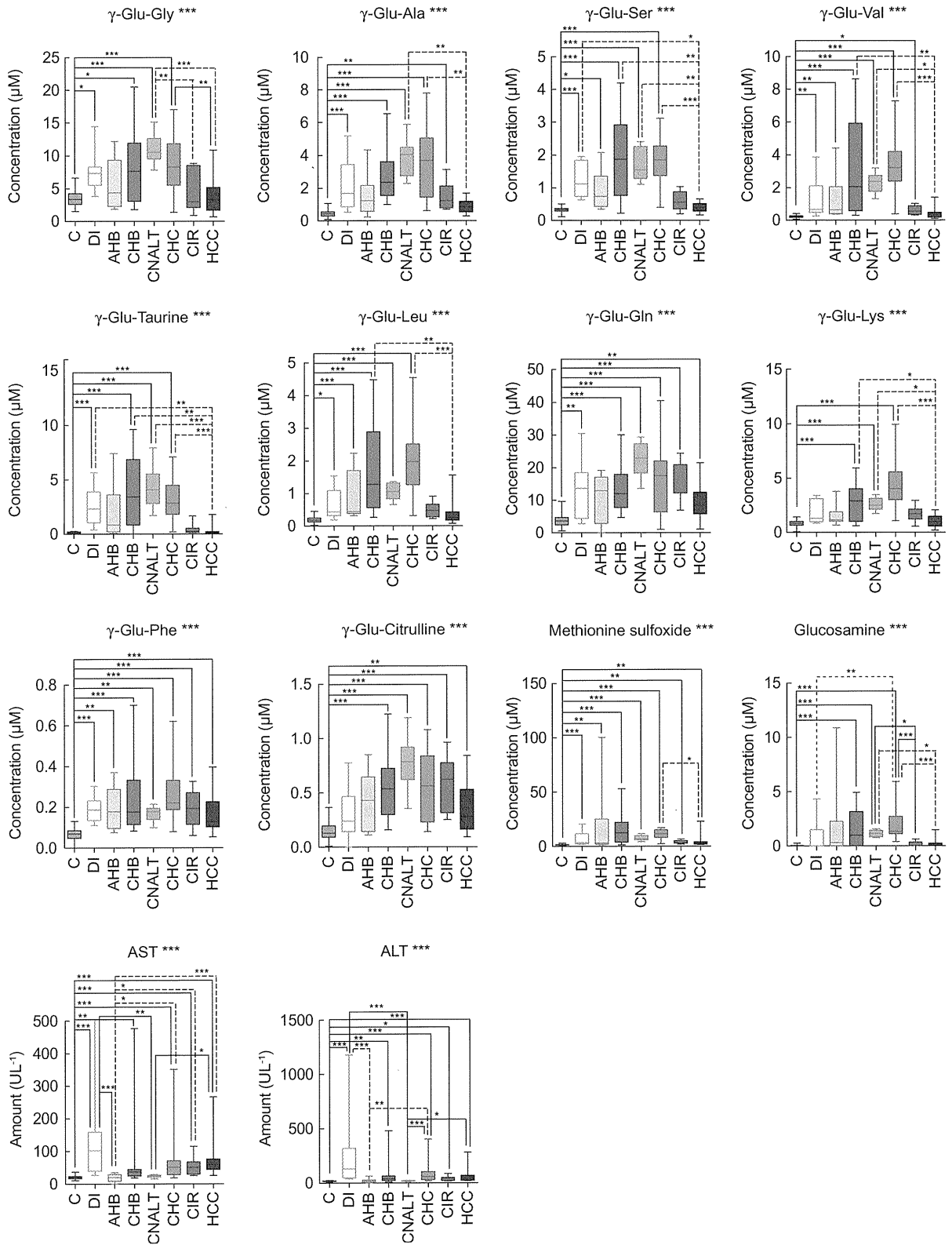


Table 2. Biomarkers for discriminating each liver disease selected by MLR models.

Group	Biomarker	Coefficient	95% CI		Odds ratio	95% CI		p value
C	(Intercept)	5.77	3.84	8.32	—	—	—	<0.0001
	γ-Glu-Phe	-58.2	-84.3	-39.0	5.16 × 10 ⁻²⁶	2.47 × 10 ⁻³⁷	1.15 × 10 ⁻¹⁷	<0.0001
DI	(Intercept)	-3.08	-4.49	-1.94	—	—	—	<0.0001
	ALT	0.020	7.89 × 10 ⁻³	0.034	1.02	1.01	1.03	2.00 × 10 ⁻³
	γ-Glu-Citrulline	-1.55	-5.01	1.13	0.21	6.68 × 10 ⁻³	3.11	0.31
AHB	(Intercept)	-1.52	-3.35	0.63	—	—	—	0.12
	AST	-0.057	-0.15	-4.96 × 10 ⁻³	0.94	0.86	1.00	0.12
	Methionine sulfoxide	0.072	0.018	0.15	1.08	1.02	1.17	0.047
	(Intercept)	-4.52	-6.33	-3.24	—	—	—	<0.0001
CHB	(Intercept)	-4.52	-6.33	-3.24	—	—	—	<0.0001
	γ-Glu-Thr	1.52	0.65	2.63	4.58	1.91	13.9	2.30 × 10 ⁻³
CNALT	(Intercept)	-0.76	-3.15	1.94	—	—	—	0.55
	ALT	-0.16	-0.34	-0.049	0.85	0.71	0.95	0.032
	γ-Glu-Taurine	0.80	0.43	1.31	2.23	1.54	3.72	3.00 × 10 ⁻⁴
CHC	(Intercept)	-4.73	-6.39	-3.47	—	—	—	<0.0001
	γ-Glu-Lys	1.27	0.85	1.82	3.57	2.34	6.14	<0.0001
CIR	(Intercept)	-2.79	-4.05	-1.55	—	—	—	<0.0001
	γ-Glu-Ala	1.80	0.42	3.52	6.05	1.52	33.7	0.020
	γ-Glu-Leu	-0.066	-3.06	2.24	0.94	0.047	9.42	0.96
	γ-Glu-Ser	-1.35	-5.35	1.86	0.26	4.77 × 10 ⁻³	6.44	0.41
	γ-Glu-Taurine	-2.28	-5.07	-0.33	0.10	6.27 × 10 ⁻³	0.72	0.064
HCC	(Intercept)	-1.87	-2.90	-0.90	—	—	—	2.00 × 10 ⁻⁴
	γ-Glu-Ala	-1.13	-2.44	-0.14	0.32	0.087	0.87	0.050
	γ-Glu-Citrulline	3.51	0.45	7.00	33.4	1.57	1.10 × 10 ³	0.033
	γ-Glu-Thr	-1.65	-5.12	0.49	0.19	5.95 × 10 ⁻³	1.63	0.27
	γ-Glu-Phe	6.99	-0.52	14.7	1.09 × 10 ³	5.92 × 10 ⁻¹	2.50 × 10 ⁶	0.063

Note: The en-dashes in the 95% CI columns indicate that these values could not be calculated. Biomarker and coefficients are used in MLR model to calculate the probability of each disease. Intercept indicates the constant term in MLR models.

relatively lower contributions to the separation ability of the MLR models (Table 2). Overall, for all types of liver diseases, the MLR models mostly based on γ-glutamyl dipeptides provided complementary results, even in the second (validation) cohort.

γ-Glutamyl dipeptides as biomarkers for HCC and NAFLD

To evaluate the diagnostic potential of γ-glutamyl dipeptides for HCC, we compared their diagnostic abilities with that of AFP, an established marker for HCC (Fig. 4). We found that the MLR models using four γ-glutamyl dipeptides (γ-Glu-Ala, γ-Glu-Citrulline, γ-Glu-Thr, γ-Glu-Phe) (Table 2) were better at distinguishing HCC from CHC and CIR (AUC = 0.881) than AFP (AUC = 0.760) (Fig. 4).

We further investigated the biomarker specificities by comparing the serum γ-glutamyl dipeptide levels in GC and HCC patients (Supplementary Fig. 2 and Table 4). The analyses

revealed significant differences, with the exception of γ-Glu-Phe, and the levels of γ-glutamyl dipeptides were notably low in GC.

Differences in the levels of γ-glutamyl dipeptides were also observed in NAFLD. The levels of six γ-glutamyl dipeptides (γ-Glu-Val, γ-Glu-Thr, γ-Glu-Leu, γ-Glu-His, γ-Glu-Phe, and γ-Glu-Arg) were significantly higher (p < 0.05; Mann-Whitney test) in SS than in NASH (Supplementary Fig. 3 and Table 5). Although further investigations are necessary, these dipeptides can be used as noninvasive biomarkers in rapid screening for SS and NASH.

Mechanism of γ-glutamyl dipeptide biosynthesis

To confirm the γ-glutamyl dipeptide biosynthesis pathway, the hepatic metabolism was investigated using a mouse model. In

Fig. 2. Representative whisker box plots of the serum levels of detected transaminases and metabolites in the training cohort. The horizontal lines indicate the upper median, median, and lower median, and the whiskers show the maximum and minimum levels. One plot for AST was outside the range (>500 U/L). *p < 0.05, **p < 0.01, ***p < 0.0001, significance difference by the Kruskal-Wallis test and Dunn's post-test for each marker and two groups in each marker, respectively.

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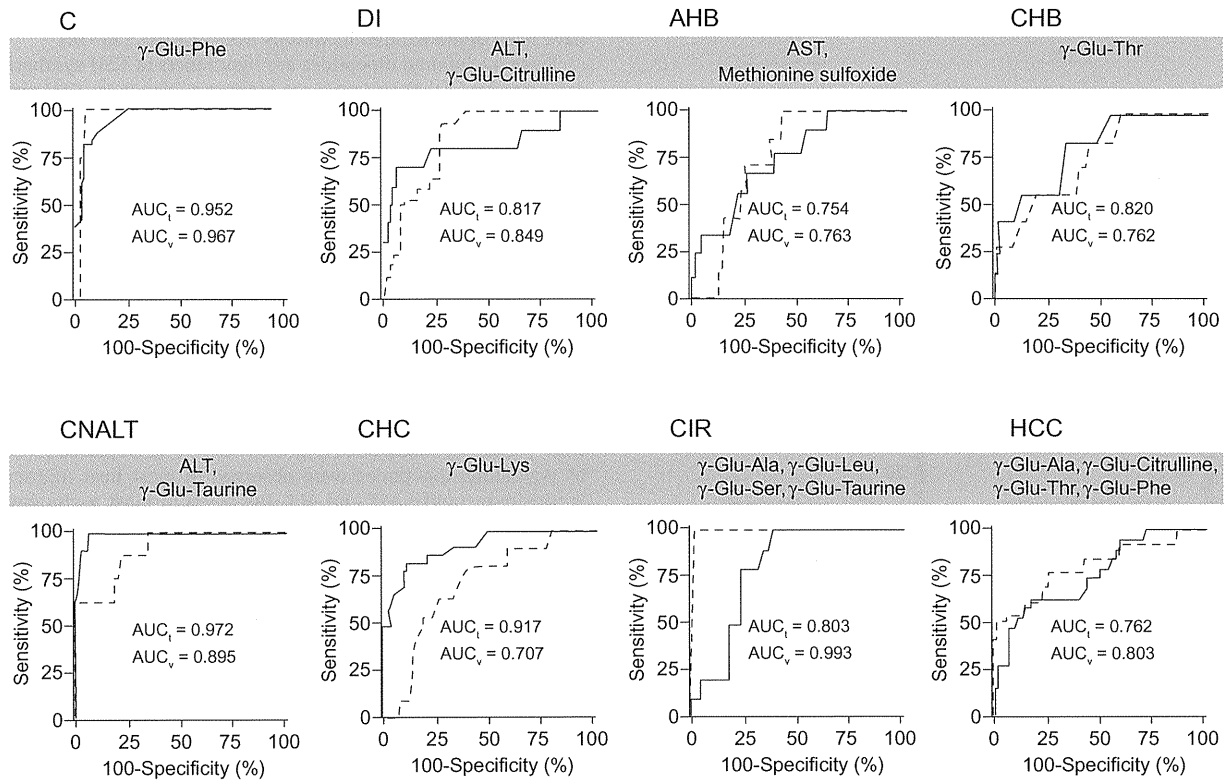


Fig. 3. ROC curve analyses of the ability of γ -glutamyl peptides alone or in combination with AST, ALT and methionine sulfoxide to discriminate each group from all other liver diseases and healthy controls. The solid and dashed curves represent the ROC curves for the training and validation cohorts, respectively. AUC_t and AUC_v in each panel indicate the AUC values in the training and validation cohorts, respectively. The group label indicates the discriminated group from all the other groups by an MLR model. The biomarkers in each panel were used in the MLR model for discriminating the group, e.g., ALT and γ -Glu-Taurine were the biomarkers for discriminating CNALT from the other groups. The coefficients and constant term of the MLR model of these biomarkers were summarized in Table 2.

acetaminophen (APAP)-treated mice [12], ophthalmate, a γ -glutamyl tripeptide, was synthesized through consecutive reactions with GCS and glutathione synthetase (GS), the same enzymes that play a role in GSH synthesis [12] (Fig. 5). Therefore, we investigated the alterations in the levels of hepatic amino acids, amines, γ -glutamyl dipeptides, and tripeptides after administration of buthionine sulfoximine (BSO), diethylmaleate (DEM) or APAP (Supplementary Fig. 4). BSO treatment resulted in GCS inhibition [20] and marked reductions in most of the hepatic γ -glutamyl dipeptide and tripeptide levels (Fig. 5 and Supplementary Fig. 4A). In contrast, DEM treatment led to GSH depletion by oxidation of the thiol group in GSH [21], resulting in GCS activation and considerable increases in the hepatic γ -glutamyl dipeptide and tripeptide levels compared with the controls (Fig. 5 and Supplementary Fig. 4A). The hepatic levels of several γ -glutamyl dipeptides and tripeptides were increased with concurrent GSH depletion in APAP-treated mice (Supplementary Fig. 4B and C). These results indicated that in mice, γ -glutamyl dipeptides and tripeptides were certainly synthesized via the ligation of glutamate by various amino acids through consecutive reactions with GCS and GS when GSH was depleted (Fig. 5). The identification details for the γ -glutamyl dipeptide biosynthetic pathway are described in the Supplementary data.

Discussion

Our analyses of 237 serum samples from patients with liver diseases and healthy controls revealed that γ -glutamyl dipeptides were increased in liver injuries and could provide specific information for different liver diseases. In APAP-induced liver injury in mice, ophthalmate, a γ -glutamyl tripeptide, was markedly increased as a byproduct of GSH synthesis [21] (Fig. 5 and Supplementary Fig. 4B). However, in liver diseases in humans, many γ -glutamyl dipeptides were primarily synthesized and secreted from hepatocytes into the blood (Figs. 1 and 5). Although the reason for the difference is unclear, it may be attributable to species differences in the levels and activities of enzymes and transporters [22,23].

In all types of liver disease, oxidative stress resulting from an imbalance between the production of reactive oxygen species (ROS) and the ability of a biological system to detoxify reactive intermediates plays a crucial role in the induction and progression of liver damage independently of its etiology [1]. In patients with hepatitis, oxidative stress is produced by inflammation induced by immunological mechanisms. Upon viral infection, NADPH oxidase produces ROS in neutrophils and macrophages, and ROS are also generated from free iron through the Fenton reaction [24–26]. ROS are further produced in hepatocytes upon

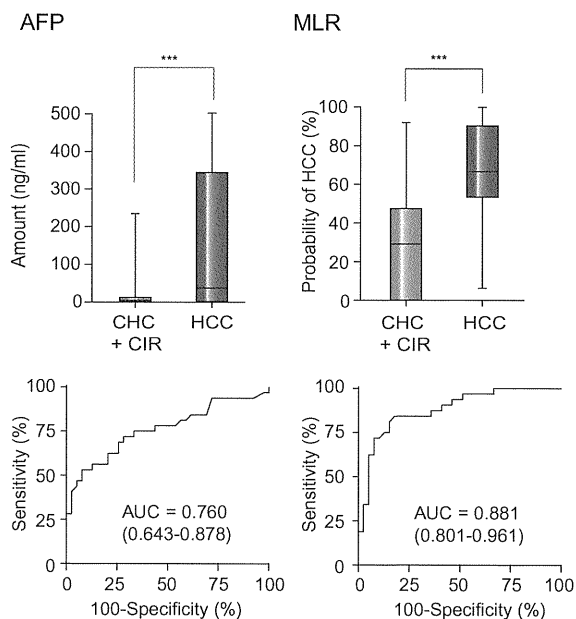


Fig. 4. Whisker box plots and ROC curves of AFP and MLR analyses based on γ -Glu-Ala, γ -Glu-Citrulline, γ -Glu-Thr and γ -Glu-Phe for discriminating patients with HCC ($n = 32$) from patients with CHC ($n = 35$) and CIR ($n = 18$).

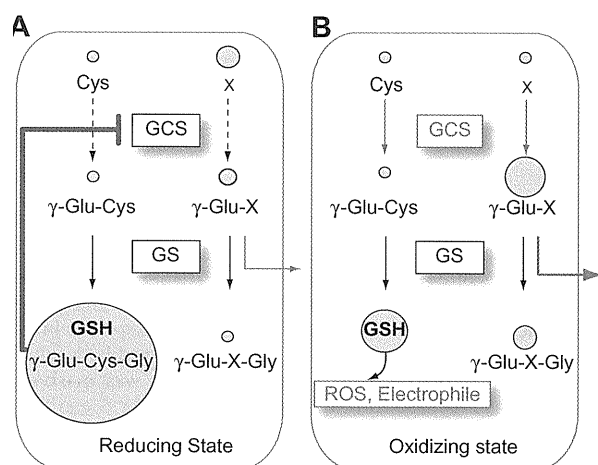


Fig. 5. Biosynthetic mechanism of γ -glutamyl peptides in hepatocytes under (A) reducing conditions and (B) oxidative stress. GCS is feedback-inhibited by GSH under reducing conditions and small amounts of γ -glutamyl dipeptides are synthesized. During oxidative stress, GSH is consumed, leading to GCS activation. This could result in biosynthesis of γ -glutamyl dipeptides, which are then effluxed across the hepatocellular membrane. γ -Glutamyl dipeptides and tripeptides are indicated by γ -Glu-X and Glu-X-Gly, respectively (X = amino acid or amine).

the release of inflammatory cytokines, such as tumor necrosis factor- α and interleukin-1 β from inflammatory cells [27]. GSH is the most abundant antioxidant in hepatocytes, and helps to protect cells against ROS. Upon depletion of GSH, ROS induce oxi-

dativ stress resulting in liver damage, and reduced GSH levels have been demonstrated in various liver diseases [28–30].

Since γ -glutamyl dipeptides are byproducts of GSH synthesis catalyzed by GCS, their levels are indirect evidence for GSH production (Fig. 5). Different levels of γ -glutamyl dipeptides were observed in different types of liver disease and each γ -glutamyl dipeptide showed a somewhat different variation pattern among liver diseases (Fig. 2). This might be attributed to differences in hepatic levels of amino acids (the substrate of GCS) among liver diseases, though further studies are necessary to understand the details of this observation.

In healthy controls, the γ -glutamyl dipeptide levels were low. This occurred because under reducing conditions, the level of hepatic GSH was high and a small amount of GSH was biosynthesized (Fig. 5A). However, in the patients with liver diseases, GSH was consumed to neutralize the generated ROS, which in turn led to GCS activation, resulting in the biosynthesis of GSH together with γ -glutamyl dipeptides (Fig. 5B). Therefore, increased levels of γ -glutamyl dipeptides were observed in most liver injuries. Surprisingly, unlike AST and ALT, the levels of most γ -glutamyl dipeptides were markedly increased in asymptomatic individuals with AHB and CNALT (Fig. 2 and Supplementary Fig. 1), possibly because viral infection induced ROS generation followed by GSH depletion, which led to the biosynthesis of GSH and γ -glutamyl dipeptides (Fig. 5). We hypothesize that sufficiently high levels of GSH production neutralized ROS, resulting in lower incidences of AHB and CNALT.

There are relationships between liver diseases attributable to HCV infection and oxidative stress parameters, such as ROS, antioxidants, and inflammation. Oxidative stress increased with hepatic disease progression in HCV-infected patients [31]. Consistent with that report, among all the patients with HCV-related liver diseases, the serum levels of γ -glutamyl dipeptides, as indicators of hepatic GSH production, were markedly increased in CNALT and tended to decrease with disease progression (CNALT \geq CHC > CIR > HCC) (Fig. 2). These observations led us to conclude that at the time of viral infection (CNALT), a sufficient amount of GSH production can neutralize ROS and thus weaken the pathogenesis of liver damage. However, when GSH production falls below ROS generation, oxidative stress followed by inflammation is induced, resulting in the development and progression of liver diseases. Similarly, the levels of several γ -glutamyl dipeptides were significantly lower in NASH patients than in SS patients (Supplementary Fig. 3), indicating low levels of GSH production in NASH patients. Based on the present observations, we suggest that NASH is susceptible to oxidative stress and progression to liver fibrosis and cirrhosis.

HCC is one of the most common cancers in humans, and primarily develops in patients with chronic liver disease. Its early detection is important because effective treatments are available for the management of non-advanced cancers [32]. Until now, the diagnosis of HCC has relied on combinations of imaging techniques and measurements of the serum levels of AFP [33] and PIVKA-II [34]. Although they are reliable tumor markers for the diagnosis and monitoring of primary HCC, high levels of serum AFP and plasma PIVKA-II have also been observed in some gastric carcinomas [34,35]. However, the serum γ -glutamyl dipeptide levels in GC and HCC patients revealed significant differences, and the levels of several γ -glutamyl dipeptides was notably low in GC (Supplementary Fig. 2). We suspect that this occurred through differences in the tissue activities of the glutathione

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system, since GSH is mainly synthesized *de novo* in the liver, and hypothesize that the γ -glutamyl dipeptide levels may reflect hepatic dysfunction.

Drug-induced hepatotoxicity is a frequent cause of liver injury, and the predominant clinical presentation is acute hepatitis and/or cholestasis. Overdoses of APAP, the most commonly used analgesic and antipyretic, can lead to possibly fatal hepatitis and several hundred deaths attributable to this drug occur annually in the United States. Our DI samples were from patients with so-called idiosyncratic hepatotoxicity, and the underlying mechanisms of this disease remain unclear. Interestingly, the changes in the serum levels of γ -glutamyl dipeptides were similar among the DI samples although the causative drugs differed widely and the mechanisms responsible for the development of hepatotoxicity may also be different. Our findings revealed that the amount of γ -glutamyl dipeptide production attributable to a reduction in the hepatocellular GSH concentration was a common feature in drug-induced idiosyncratic hepatotoxicity. With AUC values of 0.817 (training data) and 0.849 (validation data) (Supplementary Table 3), the serum levels of ALT and γ -Glu-Citrulline could be used to distinguish between DI patients on the one hand and patients with viral hepatitis infection and healthy controls on the other (Table 2). Therefore, we suggest that these compounds represent noninvasive biomarkers that facilitate rapid screening for DI.

In summary, our CE-TOFMS and LC-MS/MS metabolomics-based analyses of serum samples from patients with liver diseases showed quantitative differences in γ -glutamyl dipeptides in various liver diseases. Our highly specific set of γ -glutamyl dipeptides, transaminases, and methionine sulfoxide enabled us to discriminate among liver diseases including DI, AHB, CHB, CNALT, CHC, CIR, and HCC, indicating that they can be used as multiple biomarkers in rapid screening for different types and stages of liver disease. Furthermore, we have shown that γ -glutamyl dipeptide synthesis was catalyzed by GCS, the enzyme that is feedback-inhibited by GSH, and thus the levels of these biomarkers were indicative of hepatic GSH production. As observed in patients with HCV-related liver diseases and NAFLD, the serum γ -glutamyl dipeptide levels tended to decrease during the course of liver disease progression, indicating an increase in oxidative stress resulting from decreased GSH production during liver disease progression. Therefore, γ -glutamyl dipeptide measurement can potentially provide valuable information about the hepatic reduction-oxidation state to gain insights into the role of oxidative stress in the pathogenesis and progression of liver diseases.

Conflict of interest

The Authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.01.031.

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Short communication

Intracellular reactivation of transcription factors fused with protein transduction domain

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Oct3/4

Sox2

TEV protease

ABSTRACT

Induction of a desired cell type by defined transcription factors (TFs) using iPS technology can be used for cell replacement therapy. However, to overcome problems such as tumor formation, genomic insertional mutagenesis by viral transduction in the induction process needs to be avoided using alternative approaches. One approach could be the direct delivery of TF protein by a protein transduction system, whereby a protein transduction domain (PTD) is fused to facilitate the penetration of cell membrane. However, fusion proteins, including TFs, are reported to be biologically less active through the interference of PTD with proper protein folding. Here, we report a proof-of-concept study in which TF proteins fused with PTDs could be reactivated by removal of PTDs from cells. We demonstrated that Sox2 and Oct3/4 proteins fused with PTD were less active in mouse embryonic stem cells. Removal of PTD by a site-specific protease, derived from tobacco etch virus (TEV), substantially restored the functionality of these proteins, proved by enhanced rescue ability for differentiation induced by endogenous Sox2 and Oct3/4 repression. These results suggest that, by removing a PTD inside the cells, directly delivered TF proteins may exert substantially enhanced function than presently considered.

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1. Introduction

Transcription factors (TFs) regulate gene expression, and some play pivotal roles in the determination of a cellular differentiation status. In a pioneer study, Davis et al. (1987) showed that the basic helix–loop–helix (bHLH) transcription factor MyoD induced muscle-specific properties in fibroblasts. Recent examples include induction of pancreatic β -cells from adult pancreatic exocrine cells by Ngn3, Pdx1, and Mafa (Zhou et al., 2008), neural cells from fibroblasts by Ascl1, Brn2, and Myt11 (Vierbuchen et al., 2010), macrophages from fibroblasts by PU.1 and Cebpa (Feng et al., 2008), and myocardial cells from fibroblasts by Gata4, Mef2c, and Tbx5 (Ieda et al., 2010). In addition to lineage switching between differentiated cell types, introduction of TFs can induce dedifferentiation by four TFs including Oct3/4 and Sox2 (Takahashi and Yamanaka, 2006). Retrovirus or lentivirus is employed for introduction of these TFs; however integration into the host genome can lead to insertional mutagenesis and unpredictable gene activation (Okita et al., 2007). Thus, at present, these methods are unsuitable for clinical

trials or pathological analysis (Hanna et al., 2007; Wernig et al., 2008).

As an alternative to viral transduction, a protein transduction system for direct delivery of TFs into target cells has been developed. To facilitate the penetration of cell membrane, fusion with a high proportion of basic amino acids, termed a protein transduction domain (PTD), is required. PTDs, reported to date include TAT derived from HIV virus (Frankel and Pabo, 1988), Antennapedia transcription factor from *Drosophila* (Derossi et al., 1996), VP22 structural protein of Herpes-simplex virus 1 (Phelan et al., 1998), and poly-arginine (Matsushita et al., 2001; Wender et al., 2000). Although protein-fused PTD such as 11R (arginine)-P53 or VP22-P53 could penetrate cells (Phelan et al., 1998; Takenobu et al., 2002), it was also pointed out that these fusion proteins were biologically less active because of the improper folding of the protein caused by PTD (Ye et al., 2002). The efficiency of establishing iPS cells by Oct3/4, Sox2, Klf4, and c-Myc protein-fused 8R (arginine) was quite low (Kim et al., 2009). Therefore, we hypothesized that PTD should be removed from cells, especially PTD fused with TF.

In this study, we employed a site-specific protease, derived from tobacco etch virus (TEV), to dissociate PTD from fusion protein in the cell. Because of the stringent sequence specificity, TEV protease has been employed in cleaving genetically engineered fusion proteins in vitro (Dougherty et al., 1988). We demonstrated that TEV

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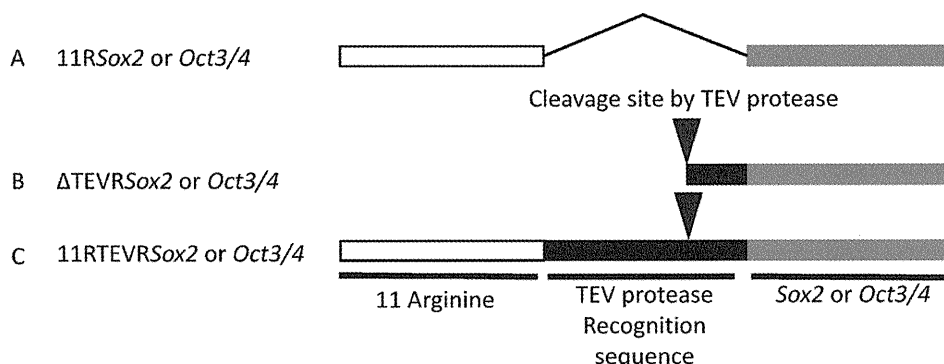


Fig. 1. Expression vectors used in the rescue experiments. (A) An eleven arginine (11R) sequence codon was ligated with *Sox2* or *Oct3/4* cDNA, (B) the residual sequence after cleavage of TEV protease was ligated with *Sox2* or *Oct3/4* cDNA, and (C) a TEV protease recognition sequence was inserted between the 11R sequence and *Sox2* or *Oct3/4* cDNA.

colonies increases only when exogenously introduced *Sox2* is functional (Masui et al., 2007). The normalized ratio of colony number, termed rescue index, reflects the relative activity of exogenous *Sox2*. When 11R or VP22 as a PTD was fused with *Sox2*, the rescue indices were greatly reduced compared with wild-type *Sox2*, suggesting that PTD interfered with the function of *Sox2* (Table 1). Conversely, *Sox2* fused with the residual sequence of the TEV protease recognition site showed an enhanced rescue index, suggesting that cleavage between PTD and *Sox2* by TEV protease will be able to restore the function.

Next, to examine whether PTD disturbs the function of another TF, we assayed *Oct3/4* fused with PTD in ZHBTc4, which represses endogenous *Oct3/4* expression in *Tc*⁺ and loses colony-forming capacity. As in the case of *Sox2*, *Oct3/4* fusion with 11R greatly reduced its function, whereas in the case of *Oct3/4* without 11R (with the residual sequence of the TEV protease recognition site), the function was restored substantially (Table 1). These results suggest that the addition of PTD to TFs compromises the function of TFs, whereas intracellular removal of PTD by TEV protease restores the function.

3.2. Expression of TEV protease in ES cells does not compromise pluripotent stem cell specific gene expression

Wild-type TEV protease is known to be unstable because of its self-cleaving activity. More stable and efficient TEV protease mutants have been developed (Kapust et al., 2001), which are reported to be active in a variety of species including *E. coli* (Kapust and Waugh, 2000), yeast (Rigaut et al., 1999), *Drosophila* (Pauli

et al., 2008), *Xenopus* (Wawersik et al., 2005) and rat (Wehr et al., 2006). To establish mouse ES cells expressing TEV protease, we infected mouse ES cells (2TS22C or ZHBTc4) with retroviruses carrying three types of TEV protease mutant (TEV-S219D, TEV-S219P or TEV-S219V). We confirmed that these ES cells expressed transgenes for TEV protease mutants (Fig. 2A). We then checked for the expression level of *Nanog*, marker gene for pluripotency, and found that they were maintained at almost the same level as the wild-type (Fig. 2B), suggesting that TEV protease did not cleave endogenous proteins necessary to maintain cellular identity.

3.3. TEV protease-expressing ES cells can restore the function of *Sox2* fused with PTD

To address whether TEV protease can function in ES cells and contribute to restoring the function of TF by removing PTD, we introduced 11R*Sox2*, ΔTEVR*Sox2*, or 11RTEVR*Sox2* (containing TEV protease recognition site between 11R and *Sox2*) into wild-type 2TS22C or 2TS22C expressing the TEV protease mutants (2TS22C-TEVS219D, 2TS22C-TEVS219P or 2TS22C-TEVS219V). As

Table 1
PTDs interfere with TF function.

Vectors	Tc ⁺ (<i>Sox2</i> null)	Tc ⁻	Rescue index
(A) 2TS22C			
pPyCAGIB	22	344	0
pPyCAGSox2IB	270	286	1.0
pPyCAGIB/VP22 <i>Sox2</i>	49	466	0.07
pPyCAGIB/11R <i>Sox2</i>	57	436	0.09
pPyCAGIB/Δ TEVR- <i>Sox2</i>	162	258	0.63
Vectors	Tc ⁺ (<i>Oct3/4</i> null)	Tc ⁻	Rescue index
(B) ZHBTc4			
pPyCAGIP	3	603	0
pPyCAGOct3/4IP	256	285	1.0
pPyCAGIP/11ROct3/4	8	216	0.03
pPyCAGIP/Δ TEVR-Oct3/4	248	298	0.93

The results of assay in 2TS22C (A) and ZHBTc4 (B). Colony numbers are shown in Tc⁺ and Tc⁻ column.

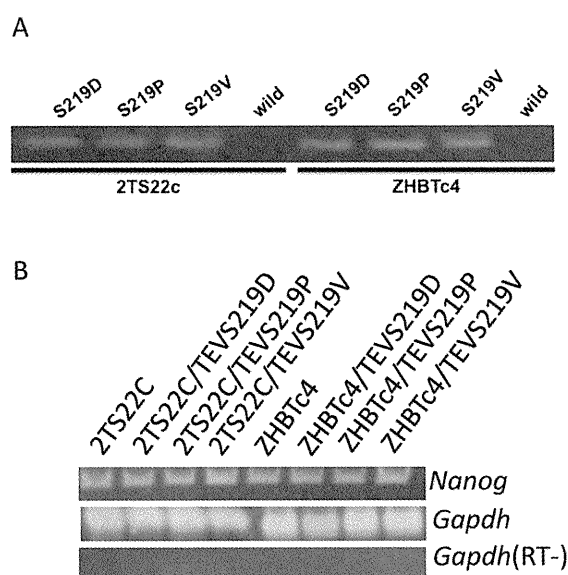


Fig. 2. Expression of TEV protease in ES cells does not compromise pluripotent stem cell specific gene expression. (A) RT-PCR analysis to confirm expression of transgenes for TEV protease mutants (S219D, S219P, S219V) and (B) RT-PCR analysis for *Nanog* in the ES cells.

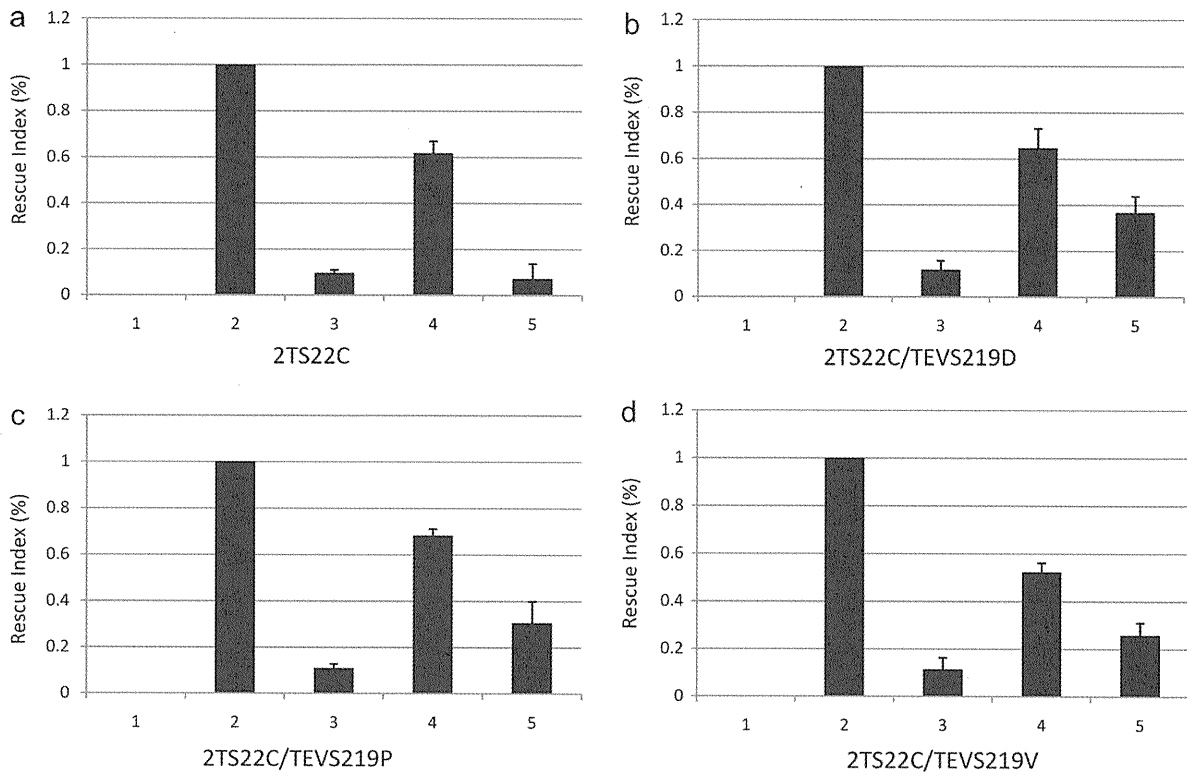


Fig. 3. TEV protease-expressing ES cells can restore the function of Sox2 fused with PTD. 1, pPyCAG-IB, empty vector; 2, pPyCAGSox2-IB; 3, pPyCAG-IB/11RSox2, 11R-containing Sox2; 4, pPyCAG-IB/ Δ TEVRSox2, truncated form of Sox2 with residual fusion protein; 5, pPyCAG-IB/11RTEVRSox2, 11R-TEV recognition sequence-Sox2.

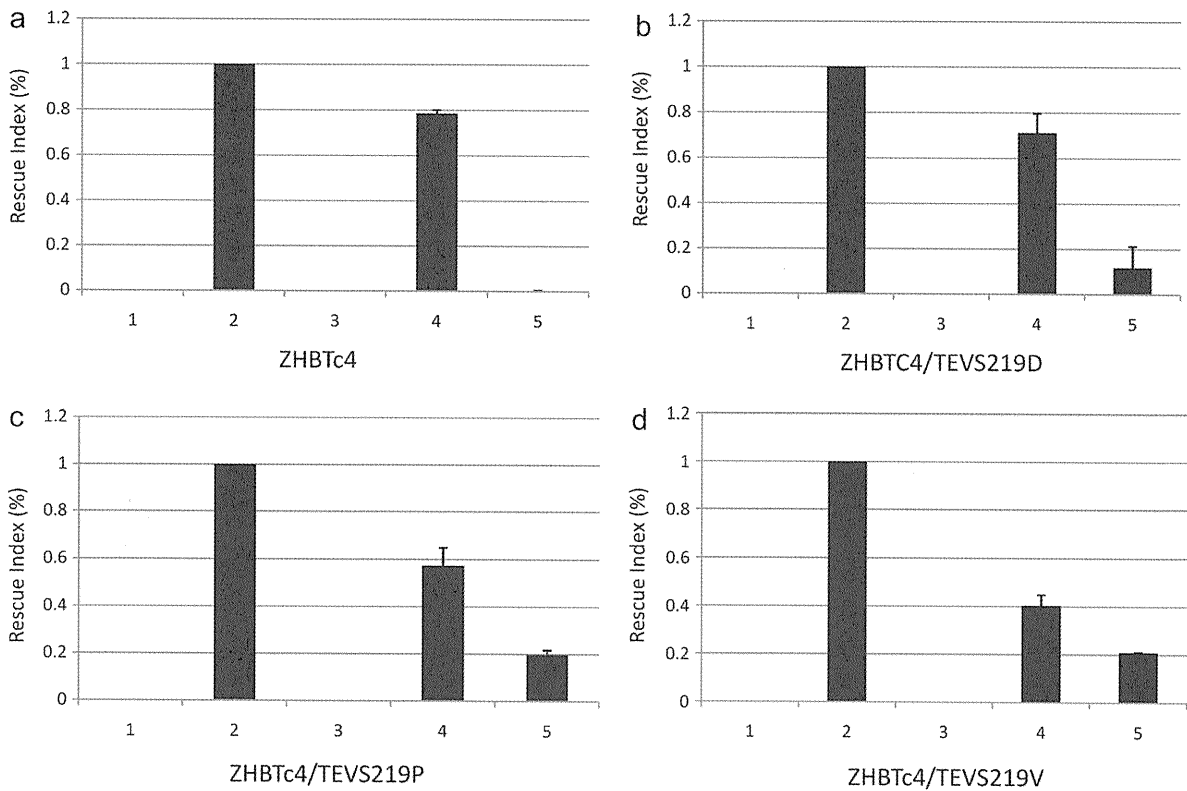


Fig. 4. TEV protease-expressing ES cells can restore the function of Oct3/4 fused with PTD. 1, pPyCAG-IP, empty vector; 2, pPyCAGOct3/4-IP; 3, pPyCAG-IP/11ROct3/4, 11R-containing Oct3/4; 4, pPyCAG-IP/ Δ TEVROct3/4, truncated form of Oct3/4 with residual fusion protein; 5, pPyCAG-IP/11RTEVROct3/4, 11R-TEV recognition sequence-Oct3/4.

expected, the rescue index of 11RSox2 was greatly reduced (Fig. 3a–d, lane 3). In contrast, 11RTEVRSox2 substantially enhanced rescue index to a level comparable to that of Δ TEVRSox2 (Fig. 3b–d, lanes 4 and 5). This restoration of function was due to the activity of TEV protease, since no enhancement was observed in 2TS22C without the TEV protease transgene (Fig. 3a, lanes 4 and 5). These results indicate that the TEV protease cleaved 11RTEVRSox2 protein to remove PTD, which restored Sox2 function. The efficacy of the three TEV protease mutants was comparable.

3.4. TEV protease-expressing ES cells can restore the function of Oct3/4 fused with PTD

To determine whether the function of another TF can be recovered by removing the PTD, we introduced 11ROct3/4, Δ TEVROct3/4 or 11RTEVROct3/4 into wild-type ZHBTc4 or ZHBTc4 expressing the TEV protease mutants (ZHBTc4-TEVS219D, ZHBTc4-TEVS219P and ZHBTc4-TEVS219V). As expected, the rescue index of 11ROct3/4 was greatly reduced (Fig. 4a–d, lane 3). In contrast, 11RTEVROct3/4 substantially enhanced the rescue index to a level comparable to that of Δ TEVROct3/4 (Fig. 4b–d, lanes 4 and 5). This functional restoration was due to the activity of TEV protease, as no enhancement was observed in ZHBTc4 without the TEV protease transgene (Fig. 4a, lanes 4 and 5). These results indicate that TEV protease cleaved 11RTEVROct3/4 protein to remove PTD, which restored Oct3/4 function. The efficacy of the three TEV protease mutants was comparable.

Collectively, these results suggest that a PTD interferes with the function of TFs, which can be restored by intracellular removal of PTD by TEV protease.

4. Discussion

To date, there are three approaches to circumvent insertional mutagenesis in cellular induction experiments. (i) In a non-integration type DNA-based approach such as an episomal vector, occasional integration into the genome has been observed (Okita et al., 2008). Thus it is desirable to avoid introducing exogenous DNA into the cell. (ii) In an RNA-based approach, such as mRNA transfection (Warren et al., 2010), expression level may not be strong and continuous enough to induce global change in transcriptional profile. This may limit usefulness to general applications. In fact, a number of mRNA transfections are required for induction of iPS cells (Warren et al., 2010). Conversely, in Sendai virus (SeV), the RNA virus that does not integrate with the genome, transgene expression is known to be strong and continuous because SeV replicates in the cell (Li et al., 2000). Although it is known that SeV in the cells can be eliminated by using temperature-sensitive replication mutants of SeV (Inoue et al., 2003), for clinical use it will be difficult to achieve complete elimination from all the cells. This may result in unexpected effects of transgene TFs a long time after cell replacement therapy. (iii) A protein-based approach such as protein transduction, does not produce residual exogenous TFs upon withdrawal from the medium. In addition, unlike mRNA transfection, protein transduction does not require a transfection reagent, which is generally toxic to cells, so that TFs can be continuously added to the medium at a high concentration, enabling strong and continuous expression. However, their use has been hampered by the interference of PTDs with the function of TFs (Ye et al., 2002). In this study, we demonstrated that intracellular removal of PTD indeed restored the function of TFs using TEV protease as an example of this strategy. The activity of TEV protease was probably sufficient for processing most TFs, since quite a high level of exogenous Sox2 or Oct3/4 is known to be required for rescuing the repression of endogenous counterparts in ES cells. In future, it is conceivable

that combination of PTD-TEVR-TFs introduced by protein transduction with TEV protease expressed by an RNA-based system (e.g., SeV) may achieve efficient cellular induction. In addition, this may reduce concern for any unexpected effect of expression of residual TFs (compared with all TFs being introduced by SeV), and may considerably reduce safety verification steps for clinical use.

In summary, we demonstrated that removal of PTD fused with Sox2 or Oct3/4 within ES cells restored protein function. This suggests that if PTD is removed from cells, directly delivered TF proteins, in general, may be able to exert substantially enhanced function than presently considered. This may enable induction of most cell types by protein transduction, and TEV protease may be a useful tool for this approach.

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