In this study, we observed augmented expression of RIG-I, a cytoplasmic double-stranded RNA sensor molecule, and its adaptor molecule IPS-1 in CHC B cells (fig. 2). To our knowledge, this is the first study describing the enhanced expression of a cytoplasmic HCV sensor in CHC B cells. Miyazaki et al. [39] recently reported similar findings on examining myeloid dendritic cells in CHC patients, thus suggesting that HCV infection augments the expression of a cytoplasmic double-stranded RNA sensor regardless of the cell type. The same may be true for expression of the RIG-I adaptor molecule IPS-1, because Miyazaki et al. [39] also reported enhanced IPS-1 expression in myeloid dendritic cells in CHC patients. These results support the notion that HCV triggers innate immunity by immune cells, including peripheral B cells.

The primary cause for the failure to induce innate immunity in CHC B cells appears to be a defect in promoting the IRF-3 activation cascade (fig. 3). HCV is thought to evade an innate antiviral response by the actions of the viral NS3/4A protease-helicase complex, which inhibits RIG-I signaling via proteolytic cleavage and IPS-1 inactivation [28], thereby preventing its downstream activation (i.e. IRF-3 activation). This hypothesis was essentially derived from in vitro experiments using human hepatoma cell lines such as HuH-7. In the present study, IPS-1 cleavage was analyzed for the first time in peripheral B cells of CHC patients. The results shown in figure 4 indicate only marginal cleavage of IPS-1 in CHC B cells, such that this could not be the main cause of the defect in downstream IRF-3 activation. We therefore sought additional explanations for the IRF-3 activation defect in CHC B cells.

The results shown in figure 5 indicate markedly enhanced expression of TBK1 and IKKε in CHC B cells compared with normal B cells, thus suggesting that IPS-1 signaling is mainly intact and not completely abolished by HCV infection as expected. In the present study, it was not possible to identify the mechanism underlying the expression of TBK1 and IKKε induced by HCV infection. However, as shown in figure 3, IRF-3 activation was markedly suppressed in CHC B cells. The defect in IRF-3 activation in CHC B cells may be linked to the enhanced expression of SIKE and the reduced expression of Hsp90 (fig. 6a), which together may affect the kinase activities of both TBK1 and IKKε. Consequently, IRF-3 phosphorylation (i.e. the first step of the IRF-3 activation cascade) would not be successfully executed. However, the possibilities that IPS-1 signaling is disrupted along the cascade despite the existence of the signal molecule and/or that IRF-3 activation is directly blocked by HCV proteins should also be taken into consideration.

It was recently demonstrated that the expression of DDX3X, a DEAD box RNA helicase [32], enhanced IFNβ promoter induction by TBK1/IKKε, whereas its knockdown inhibited IRF3 activation [33]. The results shown in figure 6a confirm that DDX3X expression levels were significantly downregulated in CHC B cells, which is in agreement with previous studies. However, Ariumi et al. [40] reported that DDX3X was required for HCV RNA replication, which seems contradictory to our results. We currently do not know the reasons for this discrepancy; however, the fact that in their study they used the HuH-7 hepatoma cell line, while we examined naturally occurring HCV-infected B cells, may account for these conflicting results.

The role of VAP proteins in HCV replication is another interesting aspect. HCV NS5A is associated with a range of cellular proteins, including VAP-A [41], which are involved in cell signaling pathways. VAP-B has been identified as another NS5A-binding protein by screening human libraries using the yeast two-hybrid system with NS5A as bait [35]. Both VAP-A and VAP-B are involved in HCV replication via interactions with NS5A and NS5B [35], while VAP-C inhibits the association between VAP-A/B and NS5B, which results in reduced HCV replication efficiency [38]. Therefore, the absence of VAP-C expression in B cells, similar to hepatocytes, may be favorable for HCV replication. This could be another strategy for maintaining persistent HCV infection in B cells. In fact, our recent study demonstrated that a high copy number of HCV RNA was detected in CHC B cells, but not in CHC non-B cells [15], suggesting that the absence of VAP-C expression in B cells is, at least in part, responsible for the HCV replication. Further analyses are required to examine if the forced expression of VAP-C could inhibit HCV replication in B cells.

Several lines of evidence indicate that chronic infection with HCV can induce molecular alterations in lymphocytes that may subsequently play a role in the multistep process of malignant lymphocyte transformation with the induction of clonal B cell expansion. Lymphoid cells from patients with chronic HCV overexpress the antiapoptotic protein Bcl-2 with a high incidence of *t* (14;18) translocations involving the *bcl-2* gene [42, 43]. Our recent study revealed that the expression level of activation-induced cytidine deaminase, which promotes B cell lymphomagenesis by its overexpession [44, 45], in CHC B cells was significantly increased [15]. Furthermore, enhanced expression of putative lymphomagenesis-related

genes such as cyclin D1, cyclin D2, B aggressive lymphoma gene, serine/threonine kinase 15 and galectin-3 was observed in CHC B cells [15]. In addition, expression of HCV core protein and NS3 was detected in CD19+ B cells of CHC patients [15]. HCV core protein has been demonstrated to promote immortalization in different cell lines as well as being capable of blocking c-myc-induced apoptosis [46]. NS3 has also been shown to promote oncogenic transformation and to interact with p53 and interfere with apoptosis [47]. Thus, persistent infection with HCV via the suppression of innate immunity responses in CHC B cells would cause functional disorders and lead to B cell lymphoma.

Interestingly, it has been shown that interaction between HCV E2 and CD81 on B cells triggers enhanced expression of activation-induced cytidine deaminase, which induces double-strand DNA breaks and hypermutation, specifically in the VH gene of B cells [48]. Stamataki et al. [49] demonstrated that peripheral blood B cells could bind infectious HCV in the cell strain JFH-1. Accordingly, it seems likely that mere interaction between envelope proteins of HCV and signaling receptors on the surface of B cells could generate lymphoproliferative disorders.

The difficulty in collecting an adequate number of purified B cells from CHC patients prevented us from analyzing a large sample size in each experiment, which may weaken the impact of these results. However, in the light of the fact that potentially heterogeneous patient samples were examined in this study, we believe that our considerably homogeneous results do have a certain biological impact. In addition, it may be worth noting that most CHC patients enrolled in this study (21 of 24) were infected with HCV of genotype 1b, which is prevalent in Asia. It would have been ideal to perform these experiments with different genotypes to observe if this phenomenon is noted across all genotypes or if it is specific only to genotype 1b. A study enrolling CHC patients infected with other HCV genotypes will be required in order to draw more robust conclusions, although it is very difficult to enroll such CHC subjects in Japan.

Taken together, the present results strongly suggest that HCV utilizes B cells as an extrahepatic reservoir for persistent infection. Whether the apparent suppression of innate immune responses in B cells is restricted to HCV infection or if this is a phenomenon seen in other B cell tropic viruses such as Epstein-Barr virus is currently unknown. This intriguing question could be answered by further elucidating the suppression mechanisms in CHC B cells as well as by investigating innate immune responses in Epstein-Barr virus-infected B cells in future studies.

We assume that memory B cells are the main reservoir of HCV infection because of their long life span. In support of this proposal, one of our current studies indicated that CD19+CD27+ cells (i.e. memory B cells) are recruited to the livers of CHC patients via interactions between CXCR3 expressed on CD19+CD27+ cells and IP-10 produced in the liver [Mizuochi et al., in press]. This would be a robust strategy for HCV in order to secure sites for long-lasting infection. Interestingly, a recent study by Stamataki et al. [49] indicated that HCV associated with B cells had the potential to transfect HuH-7.5 in vitro. Our results strongly suggest that such HCV transfection may occur in vivo under physiological conditions. This would offer new therapeutic insights for HCV clearance by eliminating peripheral B cells with anti-B cell antibodies and drugs such as rituximab in conjunction with a combination therapy using peginterferon and ribavirin.

In conclusion, we propose that peripheral B cells serve as a reservoir for persistent HCV infection. Based on this proposal and from a therapeutic perspective, it may be beneficial to eliminate peripheral B cells in CHC patients. Together with antiviral treatment to eliminate circulating HCV in the blood, this could lead to a synergistic effect for HCV clearance in CHC patients.

Acknowledgments

We would like to thank Drs. Miho Suzuki and Kenji Ikebuchi for providing the CHC blood samples used in this study. We would also like to thank Drs. Koji Onomoto, Mitsutoshi Yoneyama and Takashi Fujita for their valuable suggestions during the course of this study.

This study was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan.

References

1 Suzuki T, Ishii K, Aizaki H, Wakita T: Hepatitis C viral life cycle. Adv Drug Deliv Rev 2007;59:1200–1212.

2 Lauer GM, Walker BD: Hepatitis C virus infection. N Engl J Med 2001;345:41–52.

3 Agnello V, Mecucci C, Casato M: Regression of splenic lymphoma after treatment of hepatitis C virus infection. N Engl J Med 2002; 347:2168–2170, author reply 2168–2170.

4 Zuckerman E, Zuckerman T, Levine AM, Douer D, Gutekunst K, Mizokami M, Qian DG, Velankar M, Nathwani BN, Fong TL: Hepatitis C virus infection in patients with B-cell non-Hodgkin lymphoma. Ann Intern Med 1997;127:423–428.

5 de Sanjose S, Benavente Y, Vajdic CM, Engels EA, Morton LM, Bracci PM, Spinelli JJ, Zheng T, Zhang Y, Franceschi S, Talamini R, Holly EA, Grulich AE, Cerhan JR, Hartge P, Cozen W, Boffetta P, Brennan P, Maynadie M, Cocco P, Bosch R, Foretova L, Staines A, Becker N, Nieters A: Hepatitis C and non-Hodgkin lymphoma among 4784 cases and 6269 controls from the international lymphoma epidemiology consortium. Clin Gastroenterol Hepatol 2008;6:451–458.
6 Turner NC, Dusheiko G, Jones A: Hepatitis C and B-cell lymphoma. Ann Oncol 2003;14:1341–1345.
7 Mazzaro C, Franzin F, Tulissi P, Pussini E, Crovatto M, Carniello GS, Efremov DG, Burrone O, Santini G, Pozzato G: Regression of monoclonal B-cell expansion in patients affected by mixed cryoglobulinemia responsive to alpha-interferon therapy. Cancer 1996;77:2604–2613.
8 Caussin-Schwemling C, Schmitt C, Stoll-Keller F: Study of the infection of human blood derived monocyte/macrophages with hepatitis C virus in vitro. J Med Virol 2001;65:14–22.
9 Goutagny N, Fatmi A, De Ledinghen V, Penin F, Couzigou P, Inchauspe G, Bain C: Evidence of viral replication in circulating dendritic cells during hepatitis C virus infection. J Infect Dis 2003;187:1951–1958.
10 Navas MC, Fuchs A, Schvoerer E, Bohbot A, Aubertin AM, Stoll-Keller F: Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. J Med Virol 2002;67:152–161.
11 Blackard JT, Kemmer N, Sherman KE: Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. Hepatology 2006;44:15–22.
12 Ducoulombier D, Roque-Afonso AM, Di Liberto G, Penin F, Kara R, Richard Y, Dussaix E, Feray C: Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. Hepatology 2004;39:817–825.
13 Morsica G, Tambussi G, Sitia G, Novati R, Lazzarin A, Lopalco L, Mukenge S: Replication of hepatitis C virus in B lymphocytes (CD19+). Blood 1999;94:1138–1139.
14 Muller HM, Kallinowski B, Solbach C, Theilmann L, Goeser T, Pfaff E: B-lymphocytes are predominantly involved in viral propagation of hepatitis C virus (HCV). Arch Virol Suppl 1994;9:307–316.
15 Ito M, Murakami K, Suzuki T, Mochida K, Suzuki M, Ikebuchi K, Yamaguchi K, Mizuochi T: Enhanced expression of lymphomagenesis-related genes in peripheral blood B cells of chronic hepatitis C patients. Clin Immunol 2010;135:459–465.
16 Foy E, Li K, Wang C, Sumpter R Jr, Ikeda M, Lemon SM, Gale M Jr: Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 2003;300:1145–1148.
17 Foy E, Li K, Sumpter R Jr, Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM, Gale M Jr: Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc Natl Acad Sci USA 2005;102:2986–2991.
18 Dansako H, Ikeda M, Kato N: Limited suppression of the interferon-beta production by hepatitis C virus serine protease in cultured human hepatocytes. FEBS J 2007;274:4161–4176.
19 Dansako H, Naka K, Ikeda M, Kato N: Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells. Biochem Biophys Res Commun 2005;336:458–468.
20 Ikeda M, Sugiyama K, Mizutani T, Tanaka T, Tanaka K, Sekihara H, Shimotohno K, Kato N: Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. Virus Res 1998;56:157–167.
21 Li K, Chen Z, Kato N, Gale M Jr, Lemon SM: Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J Biol Chem 2005;280:16739–16747.
22 Pattyn F, Robbrecht P, De Paepe A, Speleman F, Vandesompele J: RTPrimerDB: the real-time PCR primer and probe database, major update 2006. Nucleic Acids Res 2006;34(Database issue):D684–D688.
23 Imaizumi T, Yagihashi N, Kubota K, Yoshida H, Sakaki H, Yagihashi S, Kimura H, Satoh K: Expression of retinoic acid-inducible gene-I (RIG-I) in macrophages: possible involvement of RIG-I in atherosclerosis. J Atheroscler Thromb 2007;14:51–55.
24 Xu J, Yang Y, Sun J, Ding Y, Su L, Shao C, Jiang B: Expression of Toll-like receptors and their association with cytokine responses in peripheral blood mononuclear cells of children with acute rotavirus diarrhoea. Clin Exp Immunol 2006;144:376–381.
25 Seth RB, Sun L, Chen ZJ: Antiviral innate immunity pathways. Cell Res 2006;16:141–147.
26 Hiscott J, Pitha P, Genin P, Nguyen H, Heylbroeck C, Mamane Y, Algarte M, Lin R: Triggering the interferon response: the role of IRF-3 transcription factor. J Interferon Cytokine Res 1999;19:1–13.
27 Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S: IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 2005;6:981–988.
28 Li XD, Sun L, Seth RB, Pineda G, Chen ZJ: Hepatitis C virus protease NS3/4a cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci USA 2005;102:17717–17722.
29 Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T: IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491–496.
30 Huang J, Liu T, Xu LG, Chen D, Zhai Z, Shu HB: SIKE is an IKK epsilon/TBK1-associated suppressor of TLR3- and virus-triggered IRF-3 activation pathways. EMBO J 2005;24:4018–4028.
31 Yang K, Shi H, Qi R, Sun S, Tang Y, Zhang B, Wang C: Hsp90 regulates activation of interferon regulatory factor 3 and TBK-1 stabilization in Sendai virus-infected cells. Mol Biol Cell 2006;17:1461–1471.
32 Cordin O, Banroques J, Tanner NK, Linder P: The DEAD-box protein family of RNA helicases. Gene 2006;367:17–37.
33 Schroder M, Baran M, Bowie AG: Viral targeting of dead box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. EMBO J 2008;27:2147–2157.
34 Soulat D, Burckstummer T, Westermayer S, Goncalves A, Bauch A, Stefanovic A, Hantschel O, Bennett KL, Decker T, Superti-Furga G: The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response. EMBO J 2008;27:2135–2146.
35 Hamamoto I, Nishimura Y, Okamoto T, Aizaki H, Liu M, Mori Y, Abe T, Suzuki T, Lai MM, Miyamura T, Moriishi K, Matsuura Y: Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J Virol 2005;79:13473–13482.
36 Loewen CJ, Levine TP: A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins. J Biol Chem 2005;280:14097–14104.
37 Nishimura Y, Hayashi M, Inada H, Tanaka T: Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. Biochem Biophys Res Commun 1999;254:21–26.
38 Kukihara H, Moriishi K, Taguwa S, Tani H, Abe T, Mori Y, Suzuki T, Fukuhara T, Taketomi A, Maehara Y, Matsuura Y: Human VAP-C negatively regulates hepatitis C virus propagation. J Virol 2009;83:7959–7969.
39 Miyazaki M, Kanto T, Inoue M, Itose I, Miyatake H, Sakakibara M, Yakushijin T, Kakita N, Hiramatsu N, Takehara T, Kasahara A, Hayashi N: Impaired cytokine response in myeloid dendritic cells in chronic hepatitis C virus infection regardless of enhanced expression of Toll-like receptors and retinoic acid inducible gene-I. J Med Virol 2008;80:980–988.
40 Ariumi Y, Kuroki M, Abe K, Dansako H, Ikeda M, Wakita T, Kato N: DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J Virol 2007;81:13922–13926.

41 Tu H, Gao L, Shi ST, Taylor DR, Yang T, Mircheff AK, Wen Y, Gorbalenya AE, Hwang SB, Lai MM: Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology 1999;263:30–41.
42 Zuckerman E, Zuckerman T, Sahar D, Streichman S, Attias D, Sabo E, Yeshurun D, Rowe J: bcl-2 and immunoglobulin gene rearrangement in patients with hepatitis C virus infection. Br J Haematol 2001;112:364–369.
43 Zignego AL, Giannelli F, Marrocchi ME, Mazzocca A, Ferri C, Giannini C, Monti M, Caini P, Villa GL, Laffi G, Gentilini P: t(14;18) translocation in chronic hepatitis C virus infection. Hepatology 2000;31:474–479.
44 Komeno Y, Kitaura J, Watanabe-Okochi N, Kato N, Oki T, Nakahara F, Harada Y, Harada H, Shinkura R, Nagaoka H, Hayashi Y, Honjo T, Kitamura T: AID-induced T-lymphoma or B-leukemia/lymphoma in a mouse BMT model. Leukemia 2010;24:1018–1024.
45 Robbiani DF, Bunting S, Feldhahn N, Bothmer A, Camps J, Deroubaix S, McBride KM, Klein IA, Stone G, Eisenreich TR, Ried T, Nussenzweig A, Nussenzweig MC: AID produces DNA double-strand breaks in non-Ig genes and mature B cell lymphomas with reciprocal chromosome translocations. Mol Cell 2009;36:631–641.
46 Ray RB, Meyer K, Ray R: Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 1996;226:176–182.
47 Sakamuro D, Furukawa T, Takegami T: Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J Virol 1995;69:3893–3896.
48 Machida K, Cheng KT, Pavio N, Sung VM, Lai MM: Hepatitis C virus E2-CD81 interaction induces hypermutation of the immunoglobulin gene in B-cells. J Virol 2005;79:8079–8089.
49 Stamataki Z, Shannon-Lowe C, Shaw J, Mutimer D, Rickinson AB, Gordon J, Adams DH, Balfe P, McKeating JA: Hepatitis C virus association with peripheral blood B lymphocytes potentiates viral infection of liver-derived hepatoma cells. Blood 2009;113:585–593.

View Online

Network based analysis of hepatitis C virus Core and NS4B protein interactions†

Lokesh P. Tripathi,[a] Chikako Kataoka,[b] Shuhei Taguwa,[b] Kohji Moriishi,[b] Yoshio Mori,[b] Yoshiharu Matsuura[b] and Kenji Mizuguchi*[a]

Received 15th July 2010, Accepted 21st September 2010
DOI: 10.1039/c0mb00103a

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Here we attempt to further our understanding of the biological context of protein interactions in HCV pathogenesis, by investigating interactions between HCV proteins Core and NS4B and human host proteins. Using the yeast two-hybrid (Y2H) membrane protein system, eleven human host proteins interacting with Core and 45 interacting with NS4B were identified, most of which are novel. These interactions were used to infer overall protein interaction maps linking the viral proteins with components of the host cellular networks. Core and NS4B proteins contribute to highly compact interaction networks that may enable the virus to respond rapidly to host physiological responses to HCV infection. Analysis of the interaction networks highlighted enriched biological pathways likely influenced in HCV infection. Inspection of individual interactions offered further insights into the possible mechanisms that permit HCV to evade the host immune response and appropriate host metabolic machinery. Follow-up cellular assays with cell lines infected with HCV genotype 1b and 2a strains validated Core interacting proteins ENO1 and SLC25A5 and host protein PXN as novel regulators of HCV replication and viral production. ENO1 siRNA knockdown was found to inhibit HCV replication in both the HCV genotypes and viral RNA release in genotype 2a. PXN siRNA inhibition was observed to inhibit replication specifically in genotype 1b but not in genotype 2a, while SLC25A5 siRNA facilitated a minor increase in the viral RNA release in genotype 2a. Thus, our analysis can provide potential targets for more effective anti-HCV therapeutic intervention.

1. Introduction

Hepatitis C virus (HCV) is the causative agent of chronic liver disease including liver steatosis, cirrhosis and hepatocellular carcinoma (HCC) and infects nearly 3% of the population worldwide. HCV is a positive single-stranded RNA virus with a single 9600 nucleotide ORF flanked by 5′ and 3′-UTRs. The HCV ORF encodes a 3000 amino acid polyprotein, which undergoes proteolytic processing by host and viral proteases to yield four structural (Core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins.[1–3] HCV variants span six genotypes that display phylogenetic heterogeneity, differences in infectivity and interferon sensitivity.[4] However, despite a wealth of concerted research, a precise understanding of the molecular mechanisms underlying HCV pathology remains elusive.

Most genes and proteins function in a complex web of interactions. Thus, the study of protein–protein interactions (PPIs) is critical to understanding the cellular networks that regulate the physiology of a living organism. The increasing availability of PPI data for human and host–pathogen interactions has led to increasing efforts in understanding the network basis of human diseases and pathogenesis.[5,6] In particular, the increasing availability of large scale interaction data between viral and human host proteins is likely to lead to a better understanding of viral pathogenesis and help identify novel targets for experimental and therapeutic intervention.[7–9] Comprehensive analyses of yeast two-hybrid (Y2H) screens have been employed to investigate the interactions of HCV,[7] Epstein–Barr virus[10] herpesviral[11] proteins with host factors. Analysis of such interactions suggests that viral (and bacterial) pathogens preferably interact with host proteins either engaged in a large number of interactions or critical to the integrity of the host cellular networks.[7,10]

Here, we report the host biological processes likely to be perturbed by HCV Core and NS4B proteins by virtue of inferred PPI networks. Core, also known as capsid protein, is spliced from the polyprotein by signal peptidase and further processed into a highly conserved 21 kDa mature form by the signal peptide peptidase; this processing facilitates its transfer to the detergent-resistant membrane fraction where virus replication and assembly take place.[12] Core also localises to the nucleus, which is essential for efficient viral propagation and development of HCV pathogenicity.[3,13] Core is a multifunctional protein implicated in RNA binding and as a pathogenic factor, which induces steatosis and HCC and thus, a promising target for anti-HCV therapy.[14,15] NS4B, the least

[a] *National Institute of Biomedical Innovation, 7-6-8 Asagi-Saito, Ibaraki-City, Osaka, 567-0085, Japan. E-mail: kenji@nibio.go.jp*
[b] *Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, 565-0871, Japan*

† Electronic supplementary information (ESI) available: PPI networks analysed in the study and their functional associations. See DOI: 10.1039/c0mb00103a

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

characterised HCV protein, is a 27 kDa non-structural integral membrane protein located in the ER membrane, which induces membrane changes and facilitates HCV replication in the host cells, though recent reports suggest that it may function in HCV pathogenesis and viral assembly.[14,16] Since Core and NS4B proteins are primarily associated with the ER membrane, they were employed as baits to screen against a library of human cDNAs using the Y2H membrane protein approach, which identifies PPIs involving integral membrane proteins and membrane-associated proteins in an *in vivo* setting.[17] We identified 11 interactions for Core and 45 interactions for NS4B, nearly all of which are previously uncharacterised. By extending these interactions to include human protein interaction data, our analysis provided insights into the functional pathways likely to be associated with HCV–host interactions in HCV pathogenesis, a better understanding of which may help identify new targets for anti-HCV therapeutic intervention.

2. Results and discussion

2.1 Identifying host proteins that interact with HCV Core and NS4B proteins

Since Core and NS4B are primarily localised to the ER membrane, to investigate their biological associations, we performed a series of Y2H screens customised for characterising the PPIs involving integral membrane and membrane-associated proteins (see Materials and Methods). Analysis of positive colonies revealed 11 interactors for Core protein and 45 interactors for NS4B protein (Table 1).

Nine of the 11 host proteins interacting with Core (Table 1) are novel findings but the other two interactions are known; signal peptide peptidase (HM13), an ER localised protein, is crucial for the intramembrane processing of the Core protein, facilitating its localisation and viral propagation;[12] proteasome subunit alpha type 7 (PSMA7) is involved in regulating HCV internal ribosome entry site (IRES), which is essential for HCV replication.[18] These results suggest that the PPIs detected by our screening approach may closely reflect Core interactions *in vivo*. Among the other interacting proteins, four localise to mitochondria and are likely involved in oxidative electron transfer (ETFB; NDUFS2) and solute transport (SLC25A5; TOMM20), which may be a consequence of known Core localisation to the mitochondrial outer membrane.[19] Additionally, Core interacting proteins Alpha Enolase (ENO1), Ferritin light chain (FTL) and SLC25A5 are perturbed in cancerous tissues from HCC patients with HCV infection.[20–22] These observations suggest potential roles for the above-identified Core protein interactions in HCV infection.

NS4B protein was found to interact with 45 host proteins (Table 1), nearly all of which are novel interactions. A significant proportion of these mapped to either the membrane component (GO:0016020; 17 of 45, 38%; $p = 0.04243$), or the extracellular region (GO:0005576; 12 of 45, 27%; $p = 3.64 \times 10^{-4}$), while five (APOA1, APOB, F2, FGG, LRG1) localise to both compartments. The NS4B interactions with a large number of host proteins (especially membrane proteins) may be crucial to its ability to induce membrane alterations termed membranous webs (MW), which host the HCV replication complex. It appears consistent with the suggested role of NS4B protein as an important hub in the virus–host interaction network.[16,23]

The absence of overlap between the PPIs identified in our approach and a previous large scale study[7] may be attributed to differences in screening approaches and experimental settings. Since our approach seeks to investigate the interactions associated with Core and NS4B in their membrane setting, it is more likely to fish out associations that may not be easily detected using the standard Y2H screening and co-immunoprecipitation assays such as those employed by de Chassey *et al.*[7] This situation would be especially true for NS4B, which unlike Core is not detected outside the membrane fraction, thus explaining the 45 interactions for NS4B reported by our approach compared to the one reported previously.[7] Our observations also highlight the significance of employing specific approaches to investigating different aspects of host–pathogen interactions in general.

2.2 Topological analysis of Core and NS4B protein interaction networks

To further understand the biological processes likely targeted by HCV, we expanded the Y2H-derived interactions by incorporating the secondary interactors of the human proteins that interact with the Core and NS4B proteins to derive extended PPI networks (Fig. 1a and b). The Core extended PPI network was made up of 208 entities (genes) with 1063 interactions between them (Table S1–S3, ESI†). For comparison, we also derived an extended PPI network for Core interactions reported by de Chassey *et al.*[7] (Table S4, ESI†). The NS4B extended PPI network was made up of 253 entities (genes) with 481 interactions between them (Table S1–S3, ESI†). First, we computed *node degree distribution* and *characteristic/average path length* measures to capture the topology of the Core and NS4B extended PPI networks (Fig. 3A and B). The degree of a protein, which corresponds to the number of its interacting partners, provides some insights into its biological relevance, since a higher degree may likely correspond to a higher ability to influence biological networks. It is also a useful measure to distinguish real world and random networks. In most interactome networks, a few nodes called "hubs" have a high degree and most nodes have a low degree, while in random networks the degree is uniformly distributed. Average path lengths provide an approximate measure of the relative ease and speed of dissemination of signalling information among network components.

Our analysis revealed that the average degree of the Core membrane protein yeast two-hybrid (MY2H) network (9.75) is on par with the human interactome (9.3), though shorter than the average degree estimated for the Core de Chassey extended network (14.7) (Table S4, ESI†). The Core MY2H network has a shorter characteristic path length vis-à-vis the human interactome (2.9 *versus* 4.04) and on par with that of Core de Chassey network (2.97), which is consistent with previous observations on the HCV protein infection network.[7] While the average degree of the NS4B MY2H network (3.4) is substantially lower than that of the

This journal is © The Royal Society of Chemistry 2010

Table 1 List of host proteins interacting with HCV Core and NS4B proteins, identified by Y2H screens

List of host proteins interacting with the Core protein		
Gene ID	Official symbol	Description
1937	EEF1G	Eukaryotic translation elongation factor 1 gamma
1964	EIF1AX	Eukaryotic translation initiation factor 1A, X-linked
2023	ENO1	Enolase 1 (alpha)
2109	EFTB	Electron-transfer-flavoprotein, beta polypeptide
2512	FTL	Ferritin, light polypeptide
292	SLC25A5	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5
4720	NDUFS2	NADH dehydrogenase (ubiquinone) Fe–S protein 2, 49 kDa (NADH-coenzyme Q reductase)
5265	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
5688	PSMA7	Proteasome (prosome, macropain) subunit, alpha type, 7
81502	HM13	Histocompatibility (minor) 13
9804	TOMM20	Translocase of outer mitochondrial membrane 20 homolog (yeast)
List of host proteins interacting with the HCV NS4B protein		
Gene ID	Official symbol	Description
10130	PDIA6	Protein disulfide isomerase family A, member 6
10682	EBP	Emopamil binding protein (sterol isomerase)
116844	LRG1	Leucine-rich alpha-2-glycoprotein 1
1209	CLPTM1	Cleft lip and palate associated transmembrane protein 1
132299	OCIAD2	OCIA domain containing 2
1528	CYB5A	Cytochrome b5 type A (microsomal)
154467	C6orf129	Chromosome 6 open reading frame 129
1571	CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1
196410	METTL7B	Methyltransferase like 7B
200185	KRTCAP2	Keratinocyte associated protein 2
2013	EMP2	Epithelial membrane protein 2
2147	F2	Coagulation factor II (thrombin)
2220	FCN2	Ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin)
2266	FGG	Fibrinogen gamma chain
2267	FGL1	Fibrinogen-like 1
27173	SLC39A1	Solute carrier family 39 (zinc transporter), member 1
2731	GLDC	Glycine dehydrogenase (decarboxylating)
286451	YIPF6	Yip1 domain family, member 6
334	APLP2	Amyloid beta (A4) precursor-like protein 2
335	APOA1	Apolipoprotein A-I
338	APOB	Apolipoprotein B (including Ag(x) antigen)
3732	CD82	CD82 molecule
4267	CD99	CD99 molecule
4513	COX2	Cytochrome c oxidase subunit II
4538	ND4	NADH dehydrogenase, subunit 4 (complex I)
4924	NUCB1	Nucleobindin 1
51075	TMX2	Thioredoxin-related transmembrane protein 2
51643	TMBIM4	Transmembrane BAX inhibitor motif containing 4
517	ATP5G2	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)
5265	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
5355	PLP2	Proteolipid protein 2 (colonic epithelium-enriched)
5446	PON3	Paraoxonase 3
54657	UGT1A4	UDP glucuronosyltransferase 1 family, polypeptide A4
54658	UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1
5479	PPIB	Peptidylprolyl isomerase B (cyclophilin B)
563	AZGP1	Alpha-2-glycoprotein 1, zinc-binding
56851	C15orf24	Chromosome 15 open reading frame 24
57817	HAMP	Hepcidin antimicrobial peptide
5950	RBP4	Retinol binding protein 4, plasma
6048	RNF5	Ring finger protein 5
6522	SLC4A2	Solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1)
7905	REEP5	Receptor accessory protein 5
84975	MFSD5	Major facilitator superfamily domain containing 5
9204	ZMYM6	Zinc finger, MYM-type 6
967	CD63	CD63 molecule

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

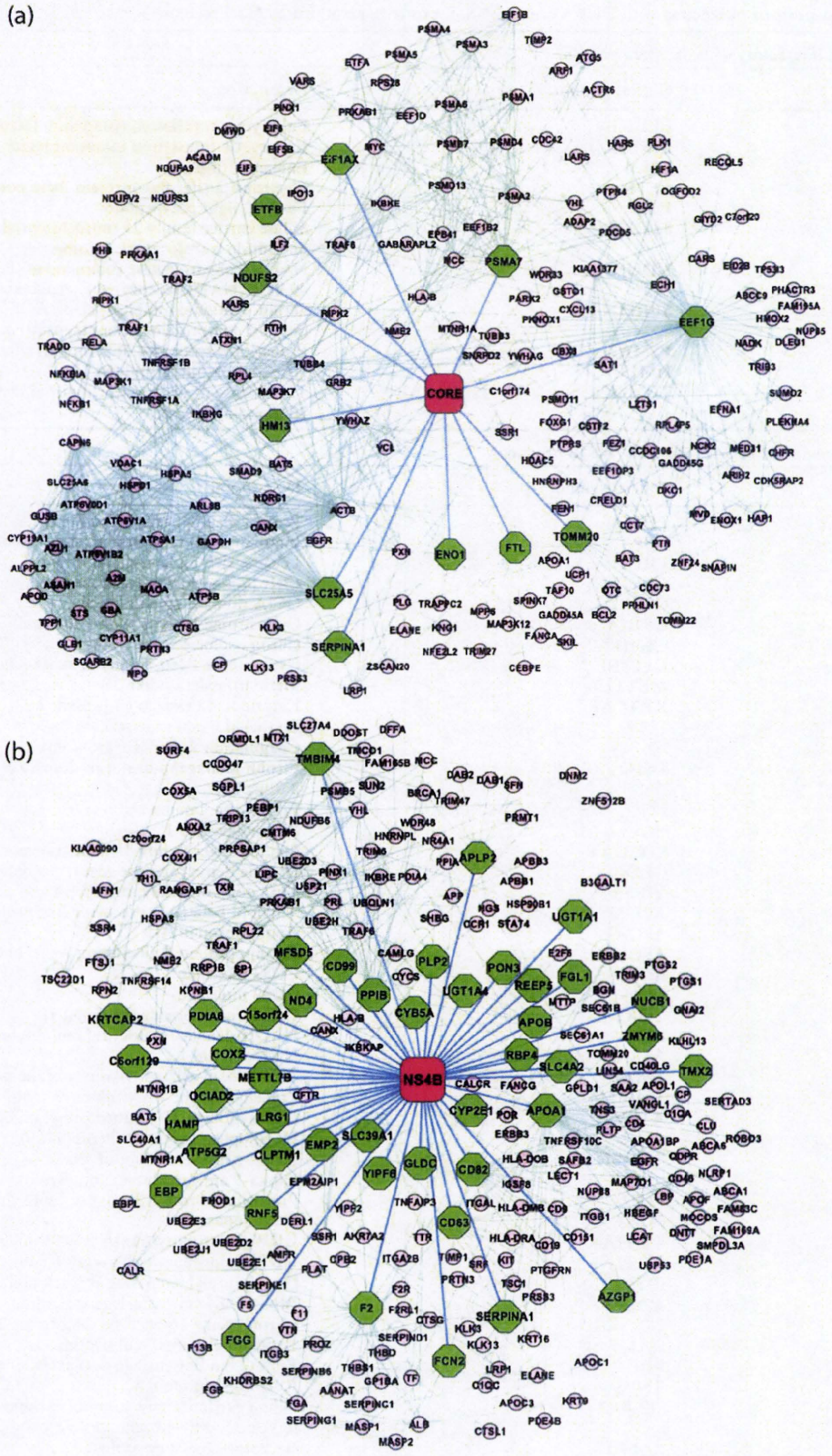

Fig. 1 Graphical representation of HCV (a) Core protein and (b) NS4B protein extended protein–protein interaction (PPI) networks. Red node: Core and NS4B protein; blue edge: Core and NS4B yeast two-hybrid (Y2H) interactions; green node: host proteins identified as interacting partners of Core and NS4B by Y2H membrane protein system; pink node: secondary interactors of the host proteins interacting with Core and NS4B; grey edge: interactions between human proteins. The node sizes differ for better clarity and do not reflect any topological attributes.

This journal is © The Royal Society of Chemistry 2010

View Online

Downloaded by Osaka University on 26 October 2010
Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A

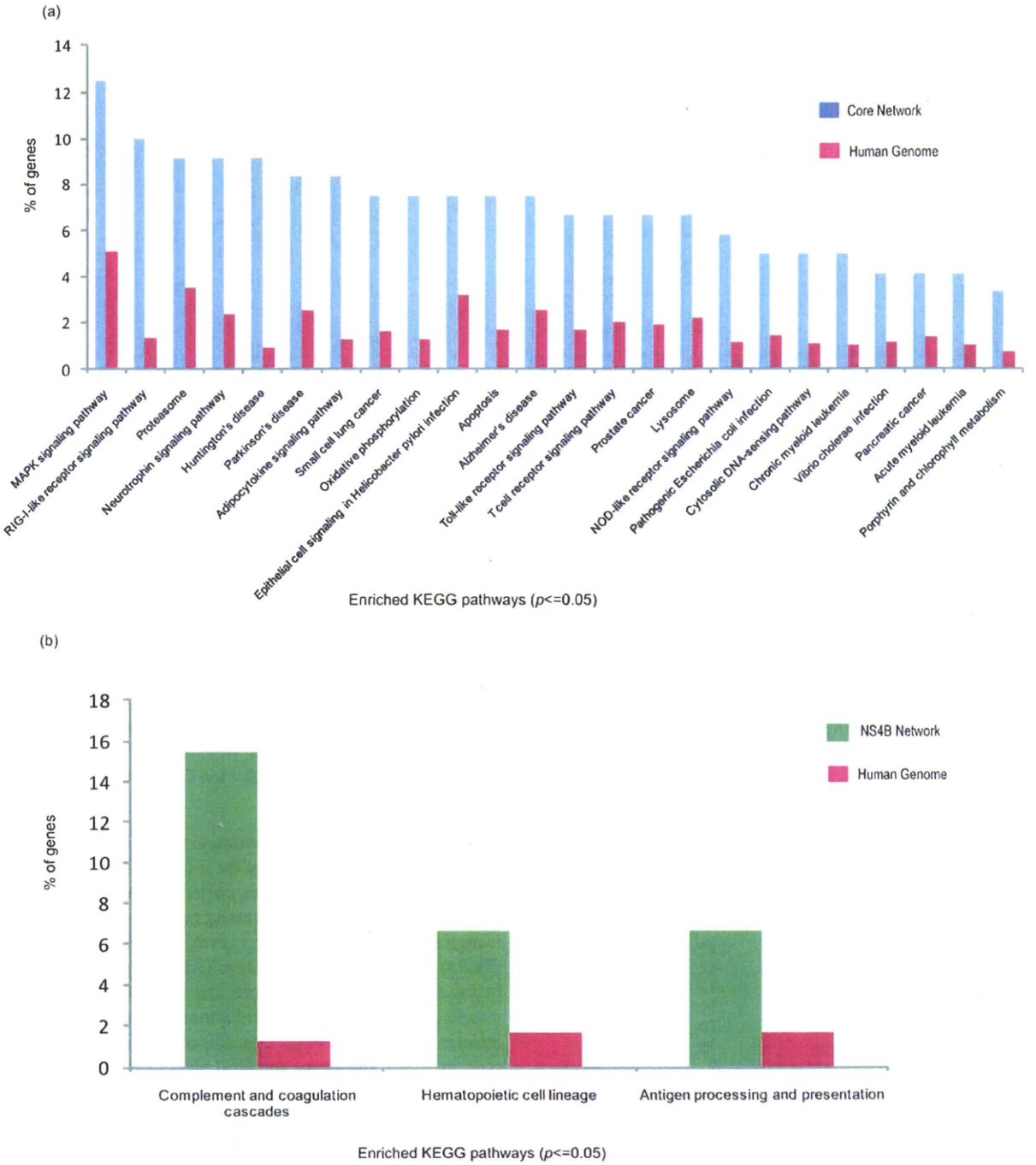

Fig. 2 KEGG pathway associations enriched in (a) Core and (b) NS4B extended PPI networks. The pathway labels are mapped to *x*-axis while the *y*-axis represents the % of genes mapped to a given KEGG pathway within the network and in the human genome.

Core MY2H network (9.75) and the human interactome (9.3), the characteristic path length is on par with the former (3.3 *versus* 2.9) and shorter than the human interactome (3.3 *versus* 4.04). Our observations suggest that the compactness of Core and NS4B interaction networks may facilitate rapid propagation of signaling information and allow the virus to respond rapidly to host mobilisation against HCV infection.

2.3 Functional analysis of Core and NS4B interactions with host proteins

Next, we investigated the extended networks for enrichment of specific biological associations (KEGG pathways,[24] OMIM phenotypes[25] and Gene Ontology [GO][26] terms). The analysis revealed that Core and NS4B protein networks were enriched in largely non-overlapping functional associations, indicating

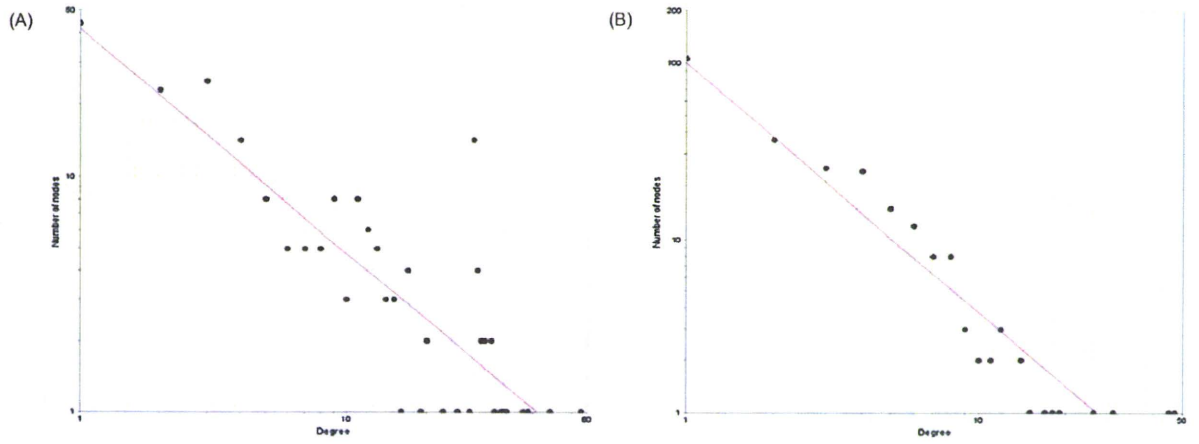

Fig. 3 Graphical representation of node degree distribution for Core and NS4B extended PPI networks. The node degree k is represented on the x-axis while the number of nodes mapped to a specific degree is represented on y-axis. The inverse trend between degree distribution and the number of proteins indicates a non-random network. The graphs correspond to (A) Core extended PPI network (R-squared value 0.727) and (B) NS4B extended network (R-squared value 0.895).

that they occupy different niches in HCV infection (Table 2; Fig. 2). Below, we first describe our observations on the Core network followed by the NS4B network.

2.3.1 Functional analysis of Core interaction network. The analysis of the extended Core interaction network revealed an enrichment of 24 KEGG pathways ($p \leq 0.05$) (Table 2), encompassing host immune response and oxidative and non-oxidative metabolism, which are likely to be significantly affected by HCV infection. Seventeen of the 24 enriched

Table 2 The number of proteins mapped to KEGG pathways significantly enriched ($p \leq 0.05$) in Core and NS4B extended PPI networks

KEGG pathway	Core network	NS4B network
Complement and coagulation cascades	—	21
MAPK signaling pathway	15	—
RIG-I-like receptor signaling pathway	12	—
Huntington's disease	11	—
Neurotrophin signaling pathway	11	—
Proteasome	10	—
Adipocytokine signaling pathway	10	—
Parkinson's disease	9	—
Alzheimer's disease	9	—
Antigen processing and presentation	—	9
Apoptosis	9	—
Epithelial cell signaling in *Helicobacter pylori* infection	9	—
Hematopoietic cell lineage	—	9
Oxidative phosphorylation	9	—
Small cell lung cancer	9	—
Lysosome	8	—
Prostate cancer	8	—
T cell receptor signaling pathway	8	—
Toll-like receptor signaling pathway	8	—
NOD-like receptor signaling pathway	7	—
Chronic myeloid leukemia	6	—
Cytosolic DNA-sensing pathway	6	—
Pathogenic *Escherichia coli* infection	6	—
Acute myeloid leukemia	5	—
Pancreatic cancer	5	—
Vibrio cholerae infection	5	—
Porphyrin and chlorophyll metabolism	4	—

associations overlapped with the enriched KEGG pathway associations inferred for the Core de Chassey network (Table S4, ESI†). Furthermore, to assess the robustness of enriched KEGG pathway associations, we explored the overlap of PPIs in the Core network with those documented in the I2D database.[27] We observed that 559 of the 1052 secondary interactions (see Material and Methods) in the Core interaction network were represented in I2D; the genes associated with these interactions were mapped to 22 enriched KEGG pathways, of which 20 were shared with the enriched KEGG pathway associations for the Core interaction network (data not shown).

2.3.1.1 Immune response. Immune response to HCV infection includes the recognition of the HCV RNA and proteins as pathogen associated molecular patterns (PAMPs) by macrophages and dendritic cells expressing germline-encoded pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), a family of RNA helicases. These events induce the production of Type I interferons (IFN-α/β) and inflammatory cytokines in the infected hepatocytes, which then activate downstream processes such as T-cell signalling for viral clearance.[28–32] The persistence of HCV in the host is attributed to its ability to hinder and evade the host immune response, which is regulated by the interplay between the HCV proteins and the components of the host immune system.[31,33,34]

Analysis of the Core interaction network revealed that six of the 11 Core interacting proteins interact with proteins mapped to one or more of the following KEGG pathways: "RIG-I-like receptor signaling" (12 of 208, $p = 4.0117 \times 10^{-7}$), "T cell receptor signaling" (8 of 208, $p = 0.0072$), "NOD-like receptor signaling" (7 of 208, $p = 0.00267$), "Toll-like receptor signaling" (8 of 208, $p = 0.0054$) and "Adipocytokine signaling" (10 of 208, $p = 1.637 \times 10^{-5}$). All of these pathways are related to innate and adaptive immunity (Fig. 1a and 4a, Table 2, Table S3, ESI†). Thus, Core protein may partly cause perturbations in the host immune response by virtue of these interactions. Several genes involved in these processes are implicated in

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

This journal is © The Royal Society of Chemistry 2010

Downloaded by Osaka University on 26 October 2010
Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A

HCV infection. For instance, hepatic RELA mRNA levels are suppressed in chronic hepatitis C patients and associated with increased liver fibrosis,[35] while RELA and TRAF2 knockdowns with siRNA resulted in a substantial reduction in HCV replication.[36] Additionally, the interaction of HCV protein NS5A with TRAF2 inhibits NFKB activation and thereby disrupts the host immune response,[37] which also suggests probable links between HCV replication and NFKB activation.[36] The expression of NFKBIA, a critical regulator of NFKB activation, is known to be increased by HCV Core protein, resulting in suppression of pro-inflammatory genes downstream of NFKB.[38] IKBKG (IKK Gamma), an anti-apoptotic protein, is essential for NFKB activation and modulates tumour necrosis factor (TNF)-mediated apoptosis.[39] IKBKG mutations are associated with immune deficiency phenotype (Table S5, ESI†) and disruption of IKBKG activity may contribute to impaired immune response in HCV infection.

Some genes (PRKAA1 and PRKAB1) associated with the pro-inflammatory "Adipocytokine signaling pathway" also function in "Insulin signaling pathway", the disruption of which may contribute to insulin resistance (IR). IR is commonly observed in HCV infection and is associated with steatosis and fibrosis progression and impaired response to interferon-α anti-HCV therapy[40,41] and overexpression of HCV Core protein can induce IR in transgenic mice.[42] PRKAA1 expression is implicated in HCV infection,[43] suggesting that it may function in Core-induced IR.

The bulk of these genes (RELA, NFKB1, IKBKG, NFKBIA, TRAF2, PRKAA1) interact with SLC25A5 (Fig. 4a), suggesting that SLC25A5 may play an important role in Core perturbation of host innate immune response and liver fibrosis and possibly HCV replication.

2.3.1.2 Oxidative stress. HCV and other pathogens have evolved mechanisms to modulate host metabolism to facilitate their survival and propagation. ER stress, oxidative stress and mitochondrial dysfunction are some characteristic features associated with chronic hepatitis C infection.[44–46] Overexpression of HCV Core, NS3 and NS5A proteins is associated with increased production of reactive oxygen species (ROS), disrupted mitochondrial electron transport and altered Ca^{2+} homeostasis leading to perturbed Cytochrome c release from the mitochondria. This reaction together with induced insulin resistance eventually leads to accelerated fibrosis, HCC and DNA damage, while ensuring cell survival.[1,46–48]

Our Y2H screening identified NDUFS2, a mitochondrial protein essential for NADH to ubiquinone electron transfer, and ETFB, a mitochondrial electron-transfer flavoprotein as interacting partners of Core protein (Table 1). These interactions may permit Core protein to perturb oxidative electron transfer and consequently induce mitochondrial aberrations, which would be consistent with oxidative modification of mitochondrial respiratory complexes in pathogen infection.[49] Mutations in NDUFS2 (KEGG pathway "Oxidative phosphorylation" (10 of 208, $p = 0.0072$)) are associated with the OMIM phenotype "Mitochondrial complex I deficiency" (Table S5, ESI†), which causes several clinical disorders including liver disease. ETFB and its interacting partner ETFA are involved in beta-oxidation of fatty acids; ETFA displays a decreased activity during HCV replication, possibly contributing to steatosis,[50] suggesting that its interaction with ETFB may permit Core protein to interfere with mitochondrial fatty acid metabolism. This interference may contribute to lipid accumulation in hepatocytes, which facilitates viral entry, replication and assembly, insulin resistance and steatosis.[40,51–53]

In addition, Core may also induce mitochondrial perturbations *via* SLC25A5 protein which interacts with the subunits of ATP synthase (ATP5A1, ATP5B) and ATPase (ATP6V1A, ATP6V1B2, ATP6V0D1) enzymes. Interestingly, SLC25A5 (ANT2) in association with ATP synthase is involved in glycolytic ATP import into mitochondria, thus facilitating a shift to almost exclusively glycolytic metabolism in cancer cells.[54] Thus, Core interactions with SLC25A5 may provide an important link between oxidative stress and a shift in energy metabolism in HCV infection (see below).

2.3.1.3 Host energy metabolism and cell adhesion. HCV induction of oxidative stress is accompanied by a shift towards non-oxidative glucose metabolism to facilitate viral growth and is often characterised by elevated levels of glycolytic enzymes in the infected cells.[22,55] Our Y2H screening identified a novel interaction between Core protein and Alpha Enolase (ENO1), a key enzyme in the glycolytic pathway implicated in several disorders including metastatic cancer.[56] ENO1 was upregulated in response to HCV infection[22] and may be a key regulator in the shift towards glycolytic metabolism and viral replication. However, there is little understanding of the role of ENO1 in HCV infection and to the best of our knowledge no physical interaction between HCV proteins and ENO1 had been reported earlier.

Our network analysis revealed that some proteins interacting with ENO1 (ACTB, PXN) mapped to KEGG "Focal adhesion" pathway (Fig. 4b; Table 1, Table S3, ESI†). Focal adhesion regulates cell migration and its deregulation is linked to tumour progression and probably HCV propagation in the host.[7] Paxillin (PXN) is involved in cytoskeleton remodelling and a disruption in its activity by human papilloma virus (HPV) E6 protein is an important aspect of HPV pathogenesis.[57] Thus, the Core interaction with ENO1 may be important in HCV mediated cytoskeleton remodelling to facilitate viral propagation. SLC25A5 (ANT2), also implicated in cancer cell glycolysis,[54] interacts with proteins (ACTB, EGFR, PXN, VCL) involved in "Focal adhesion" (Fig. 4b; Table 1, Table S3, ESI†). Most of these interactions are associated with reasonable confidence levels.[58,59] Enhancement of EGFR signalling by HCV NS3/4A and NS5A proteins is important for viral replication and persistence.[60,61] Interestingly, EGFR is upregulated in lymph node metastasis in HCC, while SLC25A5 levels are downregulated,[20] suggesting that the Core interaction with SLC25A5 may be involved in regulating EGFR activity and host energy metabolism and consequently HCV replication and propagation.

HCV infection is also characterised by elevated hepatic iron levels (induced by ROC), which contributes to abnormalities in glucose metabolism and induction of insulin resistance, eventually leading to fibrosis.[62,63] Our Y2H screening identified interaction between Core and FTL, a subunit of the

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

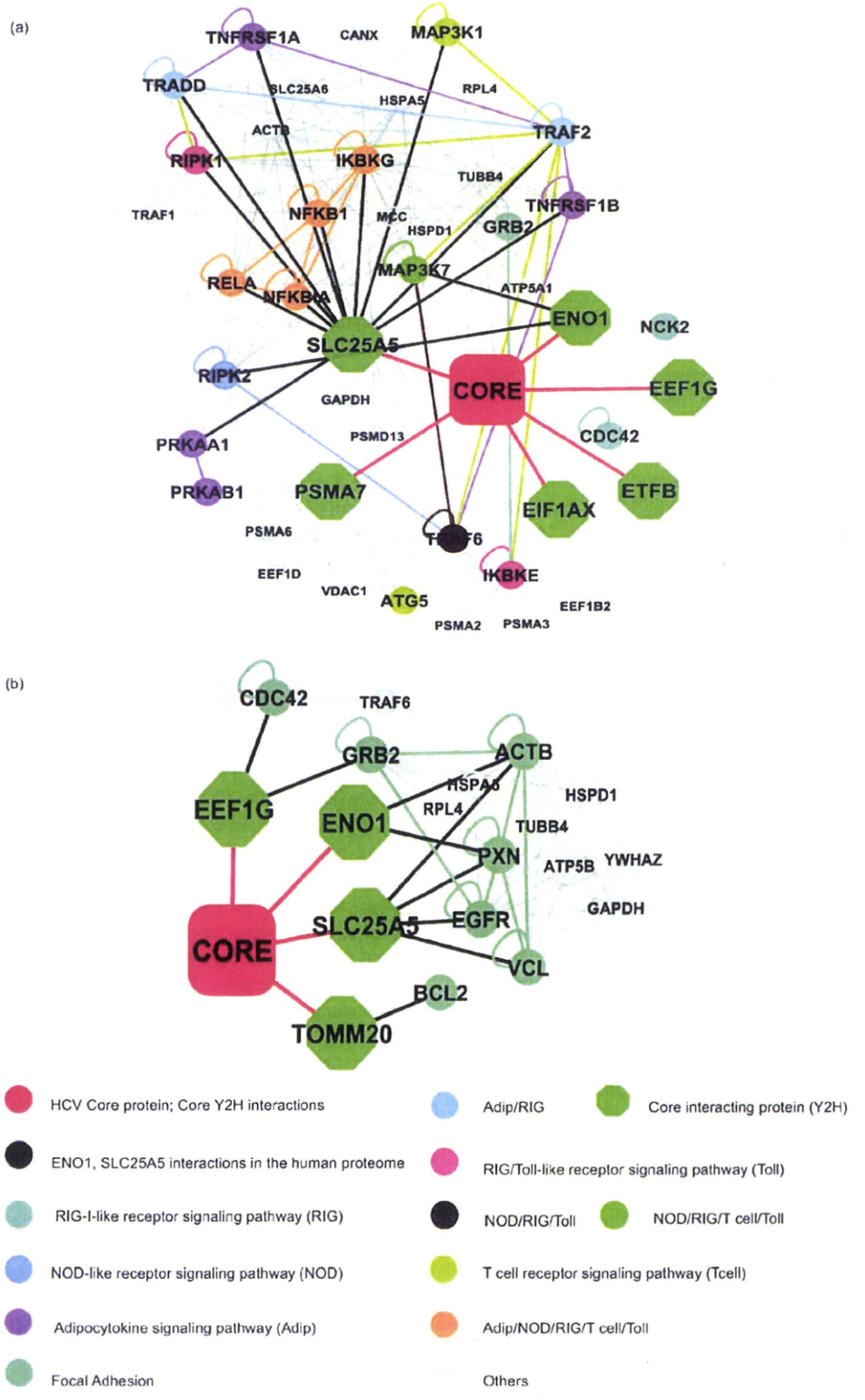

Fig. 4 Functional associations of the Core network. (a) Network illustration of interactions between HCV Core interacting proteins and host proteins mapped to five KEGG pathways— "RIG-I-like receptor signaling", "T cell receptor signaling", "NOD-like receptor signaling", "Toll-like receptor signaling" and "Adipocytokine signaling". SLC25A5 and ENO1 interactions are highlighted in black. (b) Network illustration of interactions between HCV Core interacting proteins and host proteins mapped to KEGG "Focal adhesion" pathway. The node sizes differ for better clarity and do not reflect any topological attributes.

intracellular iron storage protein Ferritin, which may facilitate Core induced disruptions in host iron metabolism.

2.3.2 Functional analysis of NS4B interaction network. To further understand the biological context of NS4B interactions, we examined the overrepresentation of specific biological associations within the NS4B PPI network. The analysis revealed an enrichment of three KEGG pathways ($p \leq 0.05$) "Complement and coagulation cascades", "Hematopoietic cell lineage" and "Oxidative phosphorylation" likely to be significantly affected by NS4B interactions in HCV infection (Table 2). To assess the robustness of enriched KEGG pathway associations, we explored the overlap of PPIs in the NS4B network with those documented in the I2D database.[27] We observed that 396 of the 436 secondary interactions (see Materials and Methods) in the NS4B interaction network were represented in I2D;[27] the genes associated with these interactions were mapped to two enriched KEGG pathways ("Complement and coagulation cascades" and "Hematopoietic cell lineage"; data not shown), both of which were among the enriched KEGG pathway associations for the NS4B interaction network.

2.3.2.1 Complement and coagulation. A significant number of proteins in the NS4B network mapped to KEGG pathway "Complement and coagulation cascades" (21 of 254, 5%; $p = 2.49 \times 10^{-14}$; Fig. 5, Table 2, Table S3, ESI†), which functions in the host innate immune response against pathogen invasion and clearance of viral antigens from the blood of the infected hosts. Overexpression of HCV Core protein activates the coagulation pathway *via* hepatic inflammation, which contributes to fibrosis, disruption of host T-cell mediated

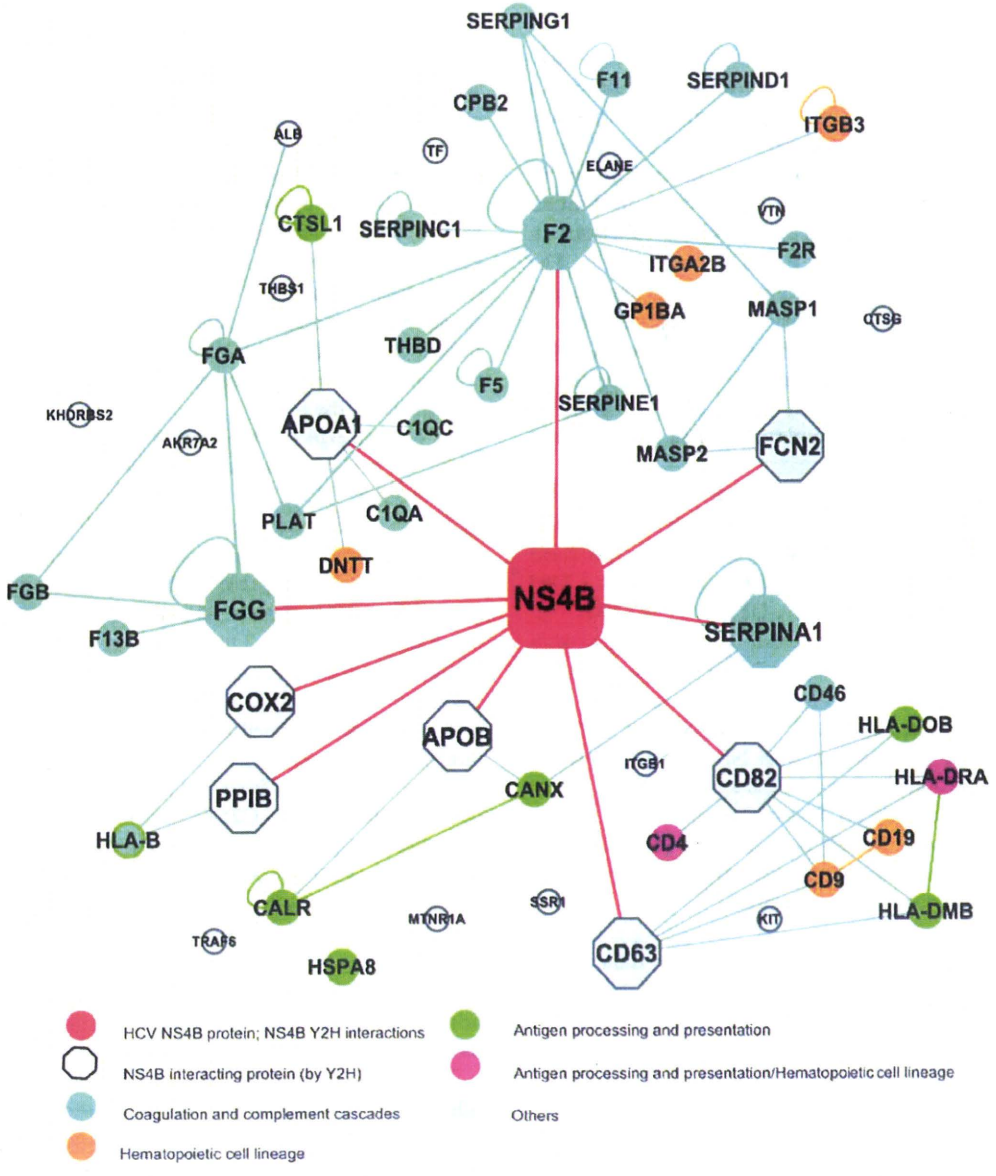

Fig. 5 Network illustration of HCV NS4B interactions associated with KEGG pathways Complement and coagulation cascades, Hematopoietic cell lineage and Antigen processing and presentation. The node sizes differ for better clarity and do not reflect any topological attributes.

Downloaded by Osaka University on 26 October 2010
Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

immune response and viral persistence. Blocking complement activation can ameliorate the effects of HCV induced hepatic inflammation, suggesting the complement pathway as an attractive target for anti-HCV therapy.[64–67]

Our Y2H screening identified thrombin (F2), serine protease inhibitor SERPINA1 and fibrinogen gamma chain (FGG) as primary interacting partners of NS4B (Table 1, Fig. 2 and 5). Thrombin is implicated in liver cell fibrosis by inducing hepatic stellate cell proliferation,[68] while SERPINA1 defects are implicated in chronic liver disease, hepatitis C and HCC[69–71] and elevated FGG expression and plasma fibrinogen levels are associated with HCC progression.[72] To the best of our knowledge, however, no interactions between these proteins and NS4B had been reported earlier. Interactions with thrombin and SERPINA1 may allow NS4B to directly perturb the host immune response *via* complement activation and thus contribute to HCV pathogenesis and HCC.

2.3.2.2 Hematopoietic development and antigen presentation. Chronic HCV infection induces a reduced natural killer (NK) cell frequency and activity, resulting in impaired cytokine secretion, a stunted immune response and viral persistence.[73] Analysis of the NS4B network revealed an enrichment of proteins (9 of 254, 4%; $p = 0.0213$) mapped to KEGG pathway "Hematopoietic cell lineage", which functions in the generation of NK cells and T and B lymphocytes. The T and B lymphocytes play an important role in innate and adaptive immune response.[74] Our analysis suggested that interactions with host proteins CD63, CD82 and APOA1 (Apolipoprotein A-I) that physically interact with the components of "Hematopoietic cell lineage" may permit NS4B to influence and possibly impair NK cell development and host immune response (Fig. 5).

We also observed an enrichment of proteins (9 of 254, 4%; $p = 0.0155$) mapped to the KEGG pathway "Antigen processing and presentation". Our network analysis showed that these proteins interact with four NS4B interacting proteins: CD63 and CD82 (interacting with HLA-DMB; HLA-DOB and HLA-DRB); APOB (Apolipoprotein B; interacting with CALR, CANX and HSPA8) and APOA1 (interacting with CTSL1 and DNTT) (Fig. 5). The dysfunction of CD63 and CD82 is implicated in cell-to-cell transmission of HIV-I,[75] suggesting that their interaction with NS4B may be crucial to the spread of HCV in the host. APOB associated cholesterol is positively associated with HCV assembly and entry[76,77] thus, NS4B interactions with APOB (and possibly APOA1) may modulate host lipid metabolism and immune response to facilitate HCV pathogenesis and steatosis.[16]

2.3.2.3 Oxidative stress. NS4B overexpression induces ER stress, unfolded protein response (UPR) and production of ROS, which eventually triggers oxidative stress.[78] However, whether NS4B may induce mitochondrial dysfunction by direct associations remains unclear.

Our Y2H screening identified three mitochondrial proteins COX2 (MT-CO2; Cytochrome c oxidase II), ND4 (mitochondrially encoded NADH dehydrogenase 4) and ATP5G2 (ATP synthase subunit C2) to interact with NS4B (Table 1). These proteins map to the KEGG pathway "Oxidative phosphorylation" and are components of the mitochondrial oxidative machinery. Disruptions in ND4 activity[79] and Cytochrome c oxidase deficiency[80] are associated with oxidative stress. Thus, NS4B interactions with the mitochondrial oxidative machinery may allow the virus to influence host oxidative metabolism and potentially induce insulin resistance, steatosis and fibrosis.

The analysis of NS4B interactions may help unravel further associations in HCV infection. Our Y2H screening identified RBP4 (retinol binding protein 4; Table 1) to interact with NS4B, which is suggested to be inversely correlated with chronic HCV infection.[81] However, a precise understanding of the significance of the interactions identified here would be apparent only with further experimental investigations.

2.4 Validation of novel interactions for their role in HCV replication and release

Traditionally, viral and host proteins associated with various steps in HCV lifecycle (internalisation, replication, assembly and release) have been the primary targets in studies focused on anti-HCV strategies. Due to the lack of a suitable model system for HCV infection, cell culture-based systems for HCV RNA replication and infectious viral particle production have been extensively exploited to understand HCV–host interactions and identify potential anti-HCV drug targets.[4,82–84] Our observations by virtue of extended PPI networks suggested novel and potentially crucial roles of host proteins ENO1, PXN, SLC25A5 and VCL (vinculin), an important component of cell–cell junctions,[85] in HCV replication and persistence in the host.

To further explore the roles of these proteins in HCV life cycle, we performed cellular assays to assess the impact of ENO1, PXN, SLC25A5 and VCL siRNA knockdowns on HCV replication and release. Since HCV-production systems established with HCV JFH1 infectious strain (genotype 2a) isolates alone are capable of both efficient replication and production of infectious viral particles,[86,87] JFH1 was used to infect the Huh7OK1 cell line 24 h after transfection with each siRNA (see Materials and Methods). The infected cells were harvested after 72 h post-infection and the expression of each host protein was assessed by qRT-PCR (Fig. 6A). Supernatant viral RNA was significantly decreased by the knockdown of ENO1, but was not affected by the knockdown of PXN in the infected cells, while SLC25A5 knockdown resulted in a slight but statistically significant increase in the amount of the supernatant viral RNA (Fig. 6B). Intracellular viral RNA was significantly reduced by the knockdown of ENO1, but was unaffected by the knockdown of PXN and SLC25A5 (Fig. 6C), suggesting that ENO1 and SLC25A5 regulate HCV replication and assembly/secretion, respectively. VCL knockdown had no significant effect on the intracellular and supernatant virus RNA in the infected cells (data not shown).

To assess the impact of the knockdown of these genes on other HCV genotypes, we repeated the HCV replication assays using Huh-7 cells including HCV replicons derived from JFH1 and Con1 (genotype 1b) infectious strains. Unlike Huh7OK1 cells infected with JFH1, these replicon systems facilitate studies on HCV replication but not infectious virus

This journal is © The Royal Society of Chemistry 2010

View Online

Downloaded by Osaka University on 26 October 2010
Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A

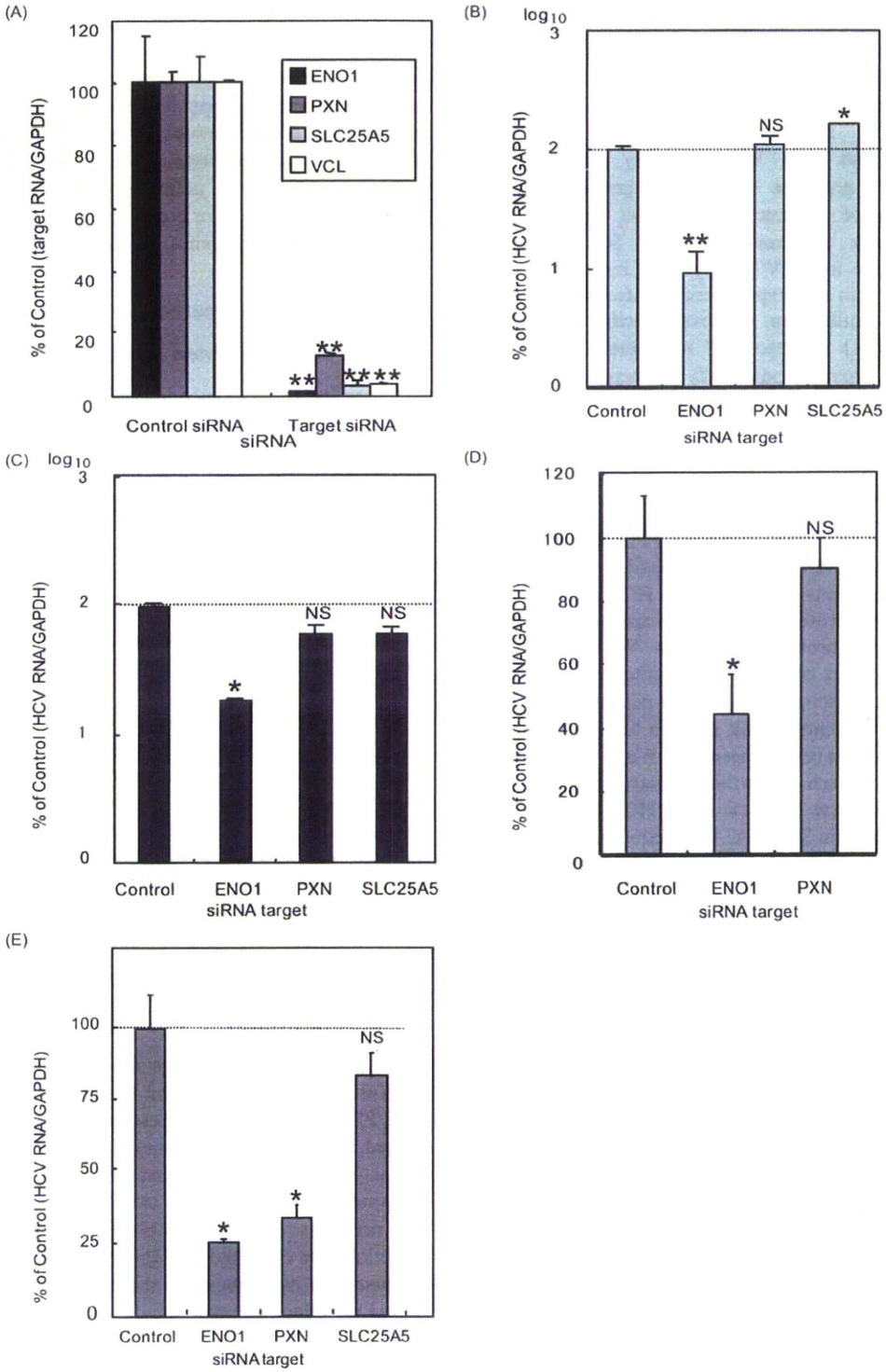

Fig. 6 Effects of knockdown of host protein candidates on HCV propagation and replication. Host proteins ENO1, PXN, SLC25A5 and VCL were suppressed by RNAi in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a; A, B and C) and in Huh-7 cells including JFH1 replicon RNA (D) or Con1 replicon (genotype 1b; E). Amounts of mRNA of the intracellular host proteins (A), the supernatant viral RNA (B) and the intracellular viral RNA (C, D and E) were estimated. Each value was represented as the percentage of the corresponding quantity measured for the cells transfected with the control siRNA. $*P < 0.05$; $**P < 0.01$; NS: not statistically significant.

secretion.[88] ENO1 knockdown suppressed HCV replication in Huh-7 cells, including either subgenomic replicon RNA of JFH1, which does not encode structural proteins (Fig. 6D), or of Con1, which encodes all viral proteins (Fig. 6E). On the other hand, PXN knockdown in Huh-7 cells including Con1 impaired HCV replication significantly (Fig. 6E), in contrast

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

to no effect in HuhOK1 cells including JFH1, as described above. This result was not due to the differences in cell lines and the expression of Core protein, because PXN knockdown in Huh-7 cells including JFH1 had no significant effect on intracellular HCV RNA production (Fig. 6D). This observation suggests that PXN possibly regulates the replication of HCV genotype 1b but not of genotype 2a. The standard therapy of pegylated interferon-α plus rebavirin treatment typically achieves less than 50% sustained virological response in HCV genotype 1b infected patients compared to 80% in genotype 2 and 3 infected patients.[89] Therefore, the identification of novel specific (PXN) and non-specific (ENO1) regulators of replication in different HCV genotypes provides potentially attractive targets for developing more effective combinatorial therapies with interferon/RBV treatment.

3. Conclusions

We describe here our observations of PPIs between HCV encoded and host proteins. We first derived a set of experimentally determined interactions between HCV proteins Core and NS4B and host proteins *via* Y2H screens customised for detecting membrane protein interactions. We proceeded to map these interactions onto an overall interaction network that comprised a repertoire of connections potentially required for the two viral proteins to link up with and modulate the components of the host cellular networks. We then employed a network-based approach to further understanding the biological context of these connections in HCV pathogenesis. Core interacting protein SLC25A5 manifested as a potentially important link between the Core protein and the host immune machinery. ENO1, by virtue of gathered interactions, may also function in regulating HCV replication and propagation. We identified 45 previously uncharacterised interactions for NS4B protein that may be crucial for NS4B to function as an important hub in HCV–host interactions. Further investigation of these interactions may help expand substantially our understanding of NS4B function in viral pathogenesis and its potential as an anti-HCV target.

Our observations were then used to prioritise four of the 459 potential candidates in the two extended PPI networks for follow-up experimental investigations through cellular assays based on siRNA knockdowns for HCV genotypes 1b and 2a. These assays validated Core interacting protein ENO1 as a novel regulator of HCV replication and a potentially minor role of SLC25A5 in HCV secretion. In addition, our assays also suggested a genotype 1b specific role of the host protein PXN (which interacts with ENO1 and SLC25A5) in HCV replication. These observations highlighted the attractiveness of the selected host proteins as suitable targets in potentially more effective targeted anti-HCV strategies. The genetic variability of HCV has facilitated the emergence of drug resistance against antiviral drugs that target HCV components. Therefore, antivirals that target less mutable host proteins critical to viral pathogenesis, preferably with minimal adverse side effects, may provide attractive alternatives to existing therapies. That we were able to experimentally validate three of the four genes selected for experimental characterisation reinforces the strengths of elaborate network-based approaches employing diverse functional associations and knowledge-based inputs for identification and prioritisation of suitable targets for experimental and therapeutic investigation. Our study also provides a generic framework for investigating host–pathogen interactions. Such investigation may help identify common themes associated with pathogen (especially viral) infection and help develop effective broad spectrum strategies aimed at ameliorating pathogen (viral) infections.

4. Materials and methods

4.1 Y2H membrane protein assay

Screening for the genes encoding the host proteins that interact with HCV Core and NS4B proteins derived from genotype 1b Con1 strain was performed with a Y2H membrane protein kit system (MoBiTec, Göttingen, Germany) as per the manufacturers specifications. Human adult liver libraries that were constructed based on pPR3 were purchased from MoBiTec and expressed as a fusion protein fused to the N-terminal or the C-terminal end of Nub-G. The cDNA of the Core (or NS4B) encoding region of the HCV polyprotein from the Con1 strain (genotype 1b) was amplified by polymerase chain reaction (PCR) and cloned into the pBT3-N vector (MoBiTec). The screening process was repeated three times to maximise the confidence in the interactions. The total number of screened transformants was 4×10^6, which is about twice the amount of independent clones in the libraries employed for the screening. The clones including genes encoding the Core (or NS4B) interacting proteins were grown on the histidine- and adenosine-deficient culture plate containing a high concentration (10 mM) of 3-amino-1,2,4-triazole (3AT), to remove weak interactions and minimise false positive data. The positive colonies were identified from the blue colour by beta-galactosidase assay (data not shown).

4.2 PPI resources

Secondary interactors of the Core and NS4B interacting proteins were retrieved from BioGRID[90] (version 2.0.63) and PPIView[91] databases. These secondary interactions were merged, filtered for redundancy and appended to the Y2H interactions to infer an extended PPI network. To estimate the robustness of the interactions employed to construct extended PPI networks and infer enriched functional associations (see below), we examined the Human PPI dataset from the I2D database[27] for its overlap with the BioGRID[90]- and PPIView[91]-derived secondary interactions.

4.3 Network topology analysis

Network components were visualised using Cytoscape 2.6,[92] while the network properties such as *node degree distribution* and *average shortest path* measures were computed using Cytoscape NetworkAnalyzer plugin.[93] The degree of node v is defined as the number of nodes directly connected to it, *i.e.*, its first neighbours. Node degree distribution $P(k)$ is the number of nodes with a degree k for $k = 0,1,2\ldots$. By fitting a line on datasets, such as node degree distribution data, the

pattern of their dependencies can be visualised. NetworkAnalyzer considers only data points with positive coordinate values for fitting the line where the power law curve of the form $y = \beta x^{a}$ is transformed into a linear model $\ln y = \ln \beta + a \ln x$ and the *R*-squared value (coefficient of determination) is computed on logarithmised data, which provides a measure of how well the data points fit to the curve. The *average shortest path length*, also known as the *characteristic path length*, gives the expected distance between two connected nodes.

4.4 Functional analysis by characterisation of enriched biological associations

GO associations retrieved from the GO consortium,[26] biological pathway data from KEGG[24] and disease phenotype associations from OMIM[25] were used to assign functional annotations to the genes in the extended networks. The enrichment of specific biological associations within each network was estimated by Fisher's exact test ($p \leq 0.05$) using the module fisher.test from the R statistical package (http://www.R-project.org). The inferred *p*-values were further adjusted for multiple test correction to control the false discovery rate using the Benjamini and Hochberg procedure.[94,95]

4.5 RNAi and transfection

The siRNA pair targets to ENO1, PXN and SLC25A5 and VCL were purchased from Ambion (Ambion, Austin, TX). Stealth™ RNAi Negative Control Low GC Duplex (Invitrogen) was used as a control siRNA. Each siRNA duplex was introduced into the cell lines using lipofectamine RNAiMax (Invitrogen, Carlsbad, CA). Ambion ID numbers of siRNA duplex of ENO1, PXN, SLC25A5 and VCL were S4682, S44629, S1375 and S14764, respectively. The replicon cell line, described below, was transfected with each siRNA at a final concentration of 20 nM as per the manufacturer's protocol and then seeded at 2.5×10^4 cells per well of a 24-well plate. The transfected cells were harvested at 72 h posttransfection. The Huh7OK1 cell line, described below, was transfected with each siRNA at a final concentration of 20 nM as per the manufacturer's protocol and then seeded at 2.5×10^4 cells per well of a 24-well plate. The transfected cells were infected with HCV JFH1 infectious strain (genotype 2a) at a MOI of 0.05 at 24 h posttransfection. The resulting cells were harvested at the indicated times.

4.6 Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was prepared from cell and culture supernatant using the RNeasy mini kit (QIAGEN) and QIAamp Viral RNA Mini Kit (QIAGEN), respectively. First-strand cDNA was synthesised using High capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) with random primers. Each cDNA was estimated by Platinum SYBR Green qPCR Super Mix UDG (Invitrogen) as per the manufacturer's protocol. Fluorescent signals of SYBR Green were analysed with ABI PRISM 7000 (Applied Biosystems). The HCV internal ribosomal entry site (IRES) region and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were amplified with the primer pairs 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCACCCTATCA-3′, and 5′-GAAGGTCGGAGTCAACGGATT-3′ and 5′-TGATGACAAGCTTCCCGTTCTC-3′, respectively.[96] The quantities of the HCV genome and other host mRNAs were normalised with that of GAPDH mRNA. ENO1, PXN, and SLC25A5 genes were amplified using the primer pairs 5′-TGCCTCCTGCTCAAAGTCAACCAGA-3′ and 5′-GGTTTCTGAAGTTCCTGCCGGCAAA-3′; 5′-TACTGTCGCAAGGACTACTTCGAC-3′ and 5′-AAGAAGCTGCCGTTCACGAATG-3′; 5′-AGTCTGCCTCCTCTTTCAACATGAC-3′ and 5′-GGACCACGCAGTCTATAATGCCTTT-3′, respectively. VCL gene was amplified using the primer pair 5′-GGTATTTGATGAGAGGGCAGCTAAC-3′ and 5′-GGCTGAATGTTGGCCATAGCTAC-3′.

4.7 Cell lines and virus infection

Cells from the Huh7OK1 cell line are highly permissive to HCV JFH1 strain (genotype 2a) infection compared to Huh 7.5.1 and exhibit highest propagation efficiency for JFH1.[96] These cells were maintained at 37 °C in humidified atmosphere and 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with nonessential amino acids (NEAA), sodium pyruvate, and 10% fetal calf serum (FCS). The human hepatoma cell line Huh-7, harbouring the full genome of HCV Con1 strain (genotype 1b), was prepared as described by Pietschmann *et al.*[88] We also established Huh-7 cell line harbouring the subgenome of the JFH1 strain by the transfection of the plasmid pSGR-JFH1.[97] The Huh7-derived cell lines harbouring a full length HCV replicon were maintained in DMEM containing 10% FCS, nonessential amino acids, sodium pyruvate and 1 mg ml^{-1} G418 (Nakarai Tesque, Tokyo, Japan). The viral RNA of JFH1 was introduced into Huh7OK1 as described by Wakita *et al.*[86] The viral RNA of JFH1 derived from the plasmid pJFH1 was prepared as described by Wakita *et al.*[86]

Acknowledgements

This study was supported by the Industrial Technology Research Grant Program in 2007 from New Energy and Industrial Technology Development Organization (NEDO) of Japan and also by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Osaka University Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation. We gratefully acknowledge Dr Tadashi Imanishi of Biomedicinal Information Research Centre (AIST) for providing us with the PPIview interactions and permission for publishing the data and Dr T. Wakita for providing us with cell lines and plasmids. We also thank Yi-An Chen for technical assistance.

References

1 H. Tang and H. Grise, *Clin. Sci.*, 2009, **117**, 49–65.
2 H. Myrmel, E. Ulvestad and B. Asjo, *APMIS*, 2009, **117**, 427–439.
3 K. Moriishi, R. Mochizuki, K. Moriya, H. Miyamoto, Y. Mori, T. Abe, S. Murata, K. Tanaka, T. Miyamura, T. Suzuki, K. Koike

Downloaded by Osaka University on 26 October 2010
Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A

View Online

and Y. Matsuura, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 1661–1666.
4 D. Moradpour, F. Penin and C. M. Rice, *Nat. Rev. Microbiol.*, 2007, **5**, 453–463.
5 M. D. Dyer, T. M. Murali and B. W. Sobral, *PLoS Pathog.*, 2008, **4**, e32.
6 T. Ideker and R. Sharan, *Genome Res.*, 2008, **18**, 644–652.
7 B. de Chassey, V. Navratil, L. Tafforeau, M. S. Hiet, A. Aublin-Gex, S. Agaugue, G. Meiffren, F. Pradezynski, B. F. Faria, T. Chantier, M. Le Breton, J. Pellet, N. Davoust, P. E. Mangeot, A. Chaboud, F. Penin, Y. Jacob, P. O. Vidalain, M. Vidal, P. Andre, C. Rabourdin-Combe and V. Lotteau, *Mol. Syst. Biol.*, 2008, **4**, 230.
8 S. L. Tan, G. Ganji, B. Paeper, S. Proll and M. G. Katze, *Nat. Biotechnol.*, 2007, **25**, 1383–1389.
9 P. Georgel, C. Schuster, M. B. Zeisel, F. Stoll-Keller, T. Berg, S. Bahram and T. F. Baumert, *Trends Mol. Med.*, 2010, **16**, 277–286.
10 M. A. Calderwood, K. Venkatesan, L. Xing, M. R. Chase, A. Vazquez, A. M. Holthaus, A. E. Ewence, N. Li, T. Hirozane-Kishikawa, D. E. Hill, M. Vidal, E. Kieff and E. Johannsen, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 7606–7611.
11 P. Uetz, Y. A. Dong, C. Zeretzke, C. Atzler, A. Baiker, B. Berger, S. V. Rajagopala, M. Roupelieva, D. Rose, E. Fossum and J. Haas, *Science*, 2006, **311**, 239–242.
12 K. Okamoto, Y. Mori, Y. Komoda, T. Okamoto, M. Okochi, M. Takeda, T. Suzuki, K. Moriishi and Y. Matsuura, *J. Virol.*, 2008, **82**, 8349–8361.
13 K. Moriishi, I. Shoji, Y. Mori, R. Suzuki, C. Kataoka and Y. Matsuura, *Hepatology*, 2010, **52**(2), 411–420.
14 J. Dubuisson, *World J. Gastroenterol.*, 2007, **13**, 2406–2415.
15 Y. Mori, K. Moriishi and Y. Matsuura, *Int. J. Biochem. Cell Biol.*, 2008, **40**, 1437–1442.
16 J. Gouttenoire, F. Penin and D. Moradpour, *Rev. Med. Virol.*, 2010, **20**(2), 117–129.
17 J. Snider, S. Kittanakom, D. Damjanovic, J. Curak, V. Wong and I. Stagljar, *Nat. Protoc.*, 2010, **5**, 1281–1293.
18 M. Kruger, C. Beger, P. J. Welch, J. R. Barber, M. P. Manns and F. Wong-Staal, *Mol. Cell. Biol.*, 2001, **21**, 8357–8364.
19 B. Schwer, S. Ren, T. Pietschmann, J. Kartenbeck, K. Kaehlcke, R. Bartenschlager, T. S. Yen and M. Ott, *J. Virol.*, 2004, **78**, 7958–7968.
20 C. F. Lee, Z. Q. Ling, T. Zhao, S. H. Fang, W. C. Chang, S. C. Lee and K. R. Lee, *World J. Gastroenterol.*, 2009, **15**, 356–365.
21 Y. Kuramitsu and K. Nakamura, *Expert Rev. Proteomics*, 2005, **2**, 589–601.
22 D. L. Diamond, A. J. Syder, J. M. Jacobs, C. M. Sorensen, K. A. Walters, S. C. Proll, J. E. McDermott, M. A. Gritsenko, Q. Zhang, R. Zhao, T. O. Metz, D. G. Camp, 2nd, K. M. Waters, R. D. Smith, C. M. Rice and M. G. Katze, *PLoS Pathog.*, 2010, **6**, e1000719.
23 C. Welsch, M. Albrecht, J. Maydt, E. Herrmann, M. W. Welker, C. Sarrazin, A. Scheidig, T. Lengauer and S. Zeuzem, *J. Mol. Graphics Modell.*, 2007, **26**, 546–557.
24 K. F. Aoki-Kinoshita and M. Kanehisa, *Methods Mol. Biol.*, 2007, **396**, 71–91.
25 J. H. U. B. McKusick-Nathans Institute of Genetic Medicine, MD and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 2010.
26 M. Ashburner, C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, J. T. Eppig, M. A. Harris, D. P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J. C. Matese, J. E. Richardson, M. Ringwald, G. M. Rubin and G. Sherlock, *Nat. Genet.*, 2000, **25**, 25–29.
27 K. R. Brown and I. Jurisica, *GenomeBiology*, 2007, **8**, R95.
28 K. Hiroishi, T. Ito and M. Imawari, *J. Gastroenterol. Hepatol.*, 2008, **23**, 1473–1482.
29 T. Kawai and S. Akira, *Ann. N. Y. Acad. Sci.*, 2008, **1143**, 1–20.
30 T. Saito and M. Gale, Jr., *Hepatol. Res.*, 2008, **38**, 115–122.
31 G. Szabo and A. Dolganiuc, *Clin. Liver Dis.*, 2008, **12**, 675–692.
32 X. Zhang, J. Dou and M. W. Germann, *Med. Res. Rev.*, 2009, **29**, 843–866.
33 E. H. Sklan, P. Charuworn, P. S. Pang and J. S. Glenn, *Nat. Rev. Gastroenterol. Hepatol.*, 2009, **6**, 217–227.
34 D. R. Taylor and E. Silberstein, *Front. Biosci.*, 2009, **14**, 4950–4961.
35 P. Boya, E. Larrea, I. Sola, P. L. Majano, C. Jimenez, M. P. Civeira and J. Prieto, *Hepatology*, 2001, **34**, 1041–1048.
36 T. I. Ng, H. Mo, T. Pilot-Matias, Y. He, G. Koev, P. Krishnan, R. Mondal, R. Pithawalla, W. He, T. Dekhtyar, J. Packer, M. Schurdak and A. Molla, *Hepatology*, 2007, **45**, 1413–1421.
37 K. J. Park, S. H. Choi, S. Y. Lee, S. B. Hwang and M. M. Lai, *J. Biol. Chem.*, 2002, **277**, 13122–13128.
38 H. Nguyen, S. Sankaran and S. Dandekar, *Virology*, 2006, **354**, 58–68.
39 D. Legarda-Addison, H. Hase, M. A. O'Donnell and A. T. Ting, *Cell Death Differ.*, 2009, **16**, 1279–1288.
40 J. A. Del Campo and M. Romero-Gomez, *World J. Gastroenterol.*, 2009, **15**, 5014–5019.
41 M. W. Douglas and J. George, *World J. Gastroenterol.*, 2009, **15**, 4356–4364.
42 H. Miyamoto, K. Moriishi, K. Moriya, S. Murata, K. Tanaka, T. Suzuki, T. Miyamura, K. Koike and Y. Matsuura, *J. Virol.*, 2007, **81**, 1727–1735.
43 M. C. Ryan, P. V. Desmond, J. L. Slavin and M. Congiu, *Journal of Viral Hepatitis*, 2010, DOI: 10.1111/j.1365-2893.2010.01283.x.
44 C. Fierbinteanu-Braticevici, M. Mohora, D. Cretoiu, S. Cretoiu, A. Petrisor, R. Usvat and D. A. Ion, *Rom. J. Morphol. Embryol.*, 2009, **50**, 407–412.
45 S. Pal, S. J. Polyak, N. Bano, W. C. Qiu, R. L. Carithers, M. Shuhart, D. R. Gretch and A. Das, *J. Gastroenterol. Hepatol.*, 2010, **25**(3), 627–634.
46 C. Piccoli, G. Quarato, M. Ripoli, A. D'Aprile, R. Scrima, O. Cela, D. Boffoli, D. Moradpour and N. Capitanio, *Biochim. Biophys. Acta, Bioenerg.*, 2009, **1787**, 539–546.
47 T. Wang, R. V. Campbell, M. K. Yi, S. M. Lemon and S. A. Weinman, *Journal of Viral Hepatitis*, 2009, DOI: 10.1111/j.1365-2893.2009.01238.x.
48 M. Korenaga, T. Wang, Y. Li, L. A. Showalter, T. Chan, J. Sun and S. A. Weinman, *J. Biol. Chem.*, 2005, **280**, 37481–37488.
49 J. J. Wen and N. Garg, *Free Radical Biol. Med.*, 2004, **37**, 2072–2081.
50 R. Singaravelu, D. R. Blais, C. S. McKay and J. P. Pezacki, *Proteome Sci.*, 2010, **8**, 5.
51 M. Alaei and F. Negro, *Diabetes Metab.*, 2008, **34**, 692–700.
52 B. Bartosch, *J. Hepatol.*, 2009, **50**, 845–847.
53 G. H. Syed, Y. Amako and A. Siddiqui, *Trends Endocrinol. Metab.*, 2010, **21**, 33–40.
54 A. Chevrollier, D. Loiseau, B. Chabi, G. Renier, O. Douay, Y. Malthiery and G. Stepien, *J. Bioenerg. Biomembr.*, 2005, **37**, 307–316.
55 M. Ripoli, A. D'Aprile, G. Quarato, M. Sarasin-Filipowicz, J. Gouttenoire, R. Scrima, O. Cela, D. Boffoli, M. H. Heim, D. Moradpour, N. Capitanio and C. Piccoli, *J. Virol.*, 2010, **84**, 647–660.
56 H. J. Kang, S. K. Jung, S. J. Kim and S. J. Chung, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2008, **64**, 651–657.
57 J. W. Fei and E. M. de Villiers, *Int. J. Cancer*, 2008, **123**, 108–116.
58 O. K. Bernhard, A. L. Cunningham and M. M. Sheil, *J. Am. Soc. Mass Spectrom.*, 2004, **15**, 558–567.
59 C. L. de Hoog, L. J. Foster and M. Mann, *Cell (Cambridge, Mass.)*, 2004, **117**, 649–662.
60 E. D. Brenndorfer, J. Karthe, L. Frelin, P. Cebula, A. Erhardt, J. Schulte am Esch, H. Hengel, R. Bartenschlager, M. Sallberg, D. Haussinger and J. G. Bode, *Hepatology*, 2009, **49**, 1810–1820.
61 J. Mankouri, S. Griffin and M. Harris, *Traffic*, 2008, **9**, 1497–1509.
62 H. C. Isom, E. I. McDevitt and M. S. Moon, *Biochim. Biophys. Acta, Gen. Subj.*, 2009, **1790**, 650–662.
63 A. Lecube, C. Hernandez and R. Simo, *Diabetes/Metab. Res. Rev.*, 2009, **25**, 403–410.
64 C. L. Basiglio, S. M. Arriaga, F. Pelusa, A. M. Almara, J. Kapitulnik and A. D. Mottino, *Clin. Sci.*, 2009, **118**, 99–113.
65 V. Calvaruso, S. Maimone, A. Gatt, E. Tuddenham, M. Thursz, M. Pinzani and A. K. Burroughs, *Gut*, 2008, **57**, 1722–1727.
66 M. L. Chang, C. T. Yeh, D. Y. Lin, Y. P. Ho, C. M. Hsu and D. M. Bissell, *BMC Med. Genomics*, 2009, **2**, 51.
67 Z. Q. Yao, D. T. Nguyen, A. I. Hiotellis and Y. S. Hahn, *J. Immunol.*, 2001, **167**, 5264–5272.
68 M. D. Gaca, X. Zhou and R. C. Benyon, *J. Hepatol.*, 2002, **36**, 362–369.

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

This journal is © The Royal Society of Chemistry 2010

View Online

69 K. F. Kok, P. J. Wahab, R. H. Houwen, J. P. Drenth, R. A. de Man, B. van Hoek, J. W. Meijer, F. L. Willekens and R. A. de Vries, *Neth. J. Med.*, 2007, **65**, 160–166.
70 A. Topic, T. Alempijevic, A. S. Milutinovic and N. Kovacevic, *Upsala Journal of Medical Sciences*, 2009, **114**, 228–234.
71 M. Wang, R. E. Long, M. A. Comunale, O. Junaidi, J. Marrero, A. M. Di Bisceglie, T. M. Block and A. S. Mehta, *Cancer Epidemiol., Biomarkers Prev.*, 2009, **18**, 1914–1921.
72 W. L. Zhu, B. L. Fan, D. L. Liu and W. X. Zhu, *Anticancer Res.*, 2009, **29**, 2531–2534.
73 O. Dessouki, Y. Kamiya, H. Nagahama, M. Tanaka, S. Suzu, Y. Sasaki and S. Okada, *Biochem. Biophys. Res. Commun.*, 2010, **393**, 331–337.
74 M. D. Boos, K. Ramirez and B. L. Kee, *Immunol. Res.*, 2008, **40**, 193–207.
75 D. N. Krementsov, J. Weng, M. Lambele, N. H. Roy and M. Thali, *Retrovirology*, 2009, **6**, 64.
76 C. I. Popescu and J. Dubuisson, *Biol. Cell*, 2009, **102**, 63–74.
77 D. A. Sheridan, D. A. Price, M. L. Schmid, G. L. Toms, P. Donaldson, D. Neely and M. F. Bassendine, *Aliment. Pharmacol. Ther.*, 2009, **29**, 1282–1290.
78 S. Li, L. Ye, X. Yu, B. Xu, K. Li, X. Zhu, H. Liu, X. Wu and L. Kong, *Virology*, 2009, **391**, 257–264.
79 A. Dlaskova, L. Hlavata and P. Jezek, *Int. J. Biochem. Cell Biol.*, 2008, **40**, 1792–1805.
80 A. M. Pickrell, H. Fukui and C. T. Moraes, *J. Bioenerg. Biomembr.*, 2009, **41**, 453–456.
81 J. F. Huang, C. Y. Dai, M. L. Yu, S. J. Shin, M. Y. Hsieh, C. F. Huang, L. P. Lee, K. D. Lin, Z. Y. Lin, S. C. Chen, L. Y. Wang, W. Y. Chang and W. L. Chuang, *J. Hepatol.*, 2009, **50**, 471–478.
82 R. De Francesco and G. Migliaccio, *Nature*, 2005, **436**, 953–960.
83 C. L. Murray and C. M. Rice, *Nature*, 2010, **465**, 42–44.
84 N. Kato, K. Mori, K. Abe, H. Dansako, M. Kuroki, Y. Ariumi, T. Wakita and M. Ikeda, *Virus Res.*, 2009, **146**, 41–50.
85 X. Peng, L. E. Cuff, C. D. Lawton and K. A. DeMali, *J. Cell Sci.*, 2010, **123**, 567–577.
86 T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager and T. J. Liang, *Nat. Med. (N. Y.)*, 2005, **11**, 791–796.
87 Y. Bungyoku, I. Shoji, T. Makine, T. Adachi, K. Hayashida, M. Nagano-Fujii, Y. H. Ide, L. Deng and H. Hotta, *J. Gen. Virol.*, 2009, **90**, 1681–1691.
88 T. Pietschmann, V. Lohmann, A. Kaul, N. Krieger, G. Rinck, G. Rutter, D. Strand and R. Bartenschlager, *J. Virol.*, 2002, **76**, 4008–4021.
89 S. Zeuzem, *Nat. Clin. Pract. Gastroenterol. Hepatol.*, 2008, **5**, 610–622.
90 C. Stark, B. J. Breitkreutz, T. Reguly, L. Boucher, A. Breitkreutz and M. Tyers, *Nucleic Acids Res.*, 2006, **34**, D535–539.
91 C. Yamasaki, K. Murakami, Y. Fujii, Y. Sato, E. Harada, J. Takeda, T. Taniya, R. Sakate, S. Kikugawa, M. Shimada, M. Tanino, K. O. Koyanagi, R. A. Barrero, C. Gough, H. W. Chun, T. Habara, H. Hanaoka, Y. Hayakawa, P. B. Hilton, Y. Kaneko, M. Kanno, Y. Kawahara, T. Kawamura, A. Matsuya, N. Nagata, K. Nishikata, A. O. Noda, S. Nurimoto, N. Saichi, H. Sakai, R. Sanbonmatsu, R. Shiba, M. Suzuki, K. Takabayashi, A. Takahashi, T. Tamura, M. Tanaka, S. Tanaka, F. Todokoro, K. Yamaguchi, N. Yamamoto, T. Okido, J. Mashima, A. Hashizume, L. Jin, K. B. Lee, Y. C. Lin, A. Nozaki, K. Sakai, M. Tada, S. Miyazaki, T. Makino, H. Ohyanagi, N. Osato, N. Tanaka, Y. Suzuki, K. Ikeo, N. Saitou, H. Sugawara, C. O'Donovan, T. Kulikova, E. Whitfield, B. Halligan, M. Shimoyama, S. Twigger, K. Yura, K. Kimura, T. Yasuda, T. Nishikawa, Y. Akiyama, C. Motono, Y. Mukai, H. Nagasaki, M. Suwa, P. Horton, R. Kikuno, O. Ohara, D. Lancet, E. Eveno, E. Graudens, S. Imbeaud, M. A. Debily, Y. Hayashizaki, C. Amid, M. Han, A. Osanger, T. Endo, M. A. Thomas, M. Hirakawa, W. Makalowski, M. Nakao, N. S. Kim, H. S. Yoo, S. J. De Souza, F. Bonaldo Mde, Y. Niimura, V. Kuryshev, I. Schupp, S. Wiemann, M. Bellgard, M. Shionyu, L. Jia, D. Thierry-Mieg, J. Thierry-Mieg, L. Wagner, Q. Zhang, M. Go, S. Minoshima, M. Ohtsubo, K. Hanada, P. Tonellato, T. Isogai, J. Zhang, B. Lenhard, S. Kim, Z. Chen, U. Hinz, A. Estreicher, K. Nakai, I. Makalowska, W. Hide, N. Tiffin, L. Wilming, R. Chakraborty, M. B. Soares, M. L. Chiusano, C. Auffray, Y. Yamaguchi-Kabata, T. Itoh, T. Hishiki, S. Fukuchi, K. Nishikawa, S. Sugano, N. Nomura, Y. Tateno, T. Imanishi and T. Gojobori, *Nucleic Acids Res.*, 2008, **36**, D793–799.
92 M. S. Cline, M. Smoot, E. Cerami, A. Kuchinsky, N. Landys, C. Workman, R. Christmas, I. Avila-Campilo, M. Creech, B. Gross, K. Hanspers, R. Isserlin, R. Kelley, S. Killcoyne, S. Lotia, S. Maere, J. Morris, K. Ono, V. Pavlovic, A. R. Pico, A. Vailaya, P. L. Wang, A. Adler, B. R. Conklin, L. Hood, M. Kuiper, C. Sander, I. Schmulevich, B. Schwikowski, G. J. Warner, T. Ideker and G. D. Bader, *Nat. Protoc.*, 2007, **2**, 2366–2382.
93 Y. Assenov, F. Ramirez, S. E. Schelhorn, T. Lengauer and M. Albrecht, *Bioinformatics*, 2008, **24**, 282–284.
94 Y. Benjamini and Y. Hochberg, *J. R. Stat. Soc. Ser. B*, 1995, **57**, 289–300.
95 W. S. Noble, *Nat. Biotechnol.*, 2009, **27**, 1135–1137.
96 T. Okamoto, H. Omori, Y. Kaname, T. Abe, Y. Nishimura, T. Suzuki, T. Miyamura, T. Yoshimori, K. Moriishi and Y. Matsuura, *J. Virol.*, 2008, **82**, 3480–3489.
97 T. Kato, T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami and T. Wakita, *Gastroenterology*, 2003, **125**, 1808–1817.

Published on 18 October 2010 on http://pubs.rsc.org | doi:10.1039/C0MB00103A
Downloaded by Osaka University on 26 October 2010

Scandinavian Journal of Gastroenterology, 2010; 45: 1488–1496

ORIGINAL ARTICLE

Prolongation of interferon therapy for recurrent hepatitis C after living donor liver transplantation: Analysis of predictive factors of sustained virological response, including amino acid sequence of the core and NS5A regions of hepatitis C virus

TOMOKAZU KAWAOKA[1], NOBUHIKO HIRAGA[1], SHOICHI TAKAHASHI[1], SHINTARO TAKAKI[1], FUKIKO MITSUI[1], MASATAKA TSUGE[1], YUKO NAGAOKI[1], YUKI KIMURA[1], YOSHIMASA HASHIMOTO[1], YOSHIO KATAMURA[1], AKIRA HIRAMATSU[1], KOJI WAKI[1], MICHIO IMAMURA[1], YOSHIIKU KAWAKAMI[1], HIROSHI AIKATA[1], HIROTAKA TASHIRO[2], HIDEKI OHDAN[2] & KAZUAKI CHAYAMA[1]

[1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan, and* [2]*Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan*

Abstract

Objective. The aim of the present retrospective study was to evaluate the therapeutic efficacy and predictive factors of prolongation of treatment with peginterferon (PEGIFN) combined with ribavirin (RBV) for recurrent hepatitis C after living donor liver transplantation (LDLT). ***Methods.*** Fifty-three patients underwent LDLT due to HCV-related end-stage liver disease. Sixteen patients were removed from the study as a result of early death ($n = 14$), no recurrence of HCV ($n = 1$) and refusal of antiviral therapy ($n = 1$). Therapy is ongoing in another 10 patients. The remaining 27 patients were available to establish the efficacy of IFN therapy. HCV genotype was 1b in 24 patients. All patients with genotype 1b were treated with IFN therapy for at least 48 weeks after HCV RNA levels had become undetectable. Amino acid substitutions in the HCV core region and NS5A region were analyzed by direct sequencing before LDLT. ***Results.*** The rate of sustained virological response (SVR) was 37.0% (10/27). SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in patients with genotype 2. Most patients with genotype 1b whose HCV RNA reached undetectable levels achieved SVR (87.5%; 7/8). However, mutation of the HCV core region and number of ISDR mutations were not associated with SVR rate in LDLT in our study. ***Conclusions.*** Prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels might prevent virological relapse of HCV.

Key Words: *Core and NS5A regions, HCV, IFN, LDLT*

Introduction

Hepatitis C virus (HCV)-related end-stage liver disease is currently the leading indication for liver transplantation (LT). Unfortunately, prevention of HCV infection after transplantation is difficult and, unlike the situation with the prevention of hepatitis B virus after transplantation [1], HCV re-infection after LT is almost universal, with histological evidence of chronic hepatitis in approximately 50% of patients within 1 year and cirrhosis in about 30% after 5 years. This in turn yields an excess risk of death or

Correspondence: Shoichi Takahashi, MD, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima, 734-8551 Japan. Tel: +81 82 257 5192. Fax: +81 82 257 5194. E-mail: shoichit@hiroshima-u.ac.jp

(Received 26 April 2010; accepted 26 June 2010)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2010 Informa Healthcare
DOI: 10.3109/00365521.2010.505657

RIGHTS