

**Figure 1.** Graphical representation of the shortest path length distribution for (A) CoreTGvsWT extended network, (B) PA28 $\gamma^{-/-}$ CoreTGvsWT extended network, (C) PA28 $\gamma^{-/-}$ CoreTGvsCoreTG extended network, and (D) human protein interactome. The path length is represented on the x-axis, while the y-axis describes the frequency, i.e., the percentage of node (protein) pairs within the PPI network with a given shortest path length. For simplicity, only the node frequencies for path lengths 1–5 are displayed.

summarized in Table 2. Specific functional associations for the CoreTGvsWT and PA28 $\gamma^{-/-}$ CoreTGvsWT networks, except when discussed below, are detailed in the Supporting Information. It will highlight the biological significance of the differentially expressed proteins, their interactions, and their probable roles in HCV infection and help identify potentially novel regulators of and biomarkers for HCV pathogenesis.

### Vesicular Transport

HCV infection involves the formation of the HCV replication complex in the detergent-resistant membrane (DRM) fraction or lipid rafts. These subcellular membrane fractions are utilized by some pathogens including viruses to facilitate viral entry and assembly.<sup>46–49</sup> HCV infection induces modifications in the host lipid raft proteome, which directly impacts HCV replication in the infected cells.<sup>50</sup> Core targeting to the early and late endosomes and the viral particle production requires the components of the endosome-based secretory pathways.<sup>51,52</sup>

**CoreTGvsWT Extended Network.** The PowerBlot analysis revealed the two endosomal proteins VTI1A and STX8 (KEGG Pathway “SNARE interactions in vesicular transport”;  $p = 0.023$ ; Table S5) that were upregulated 3.25- and 1.88-fold, respectively, in CoreTGvsWT (Table 1). SNAREs are membrane-anchored proteins involved in membrane trafficking.<sup>53</sup> Some SNAREs may function in HCV egress by possibly facilitating the fusion of the late endosomes that carry HCV particles with the plasma membrane resulting in their release into the extracellular environment.<sup>52</sup> VTI1A is a SNARE involved in the vesicular transport from the late endosomes to the trans-Golgi network and forms a SNARE complex with STX16 and VAMP4 (Table S4).<sup>54,55</sup> STX8 is involved in the protein trafficking from the early to the late endosomes and exocytosis and forms a SNARE complex with STX7, VAMP8,

and VTI1B.<sup>55,56</sup> A reduction in the expression of STX7, which interacts with both VTI1A and STX8 (Figure 2; Table S4), decreases HCV replication.<sup>50</sup> Taken together, the increased abundances of VTI1A and STX8 in CoreTGvsWT, but not PA28 $\gamma^{-/-}$ CoreTGvsWT, suggest potentially crucial roles of the two proteins in the HCV life cycle.

**PA28 $\gamma^{-/-}$ CoreTGvsCoreTG Extended Network.** Syntaxin 11 (STX11), a SNARE, was upregulated 3.52-fold in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG (Table 1) and was mapped to the enriched KEGG Pathway “SNARE interactions in vesicular transport” ( $p = 0.003$ ; Table S5). STX11 associates with the late endosomes and functions in the essential trafficking pathways (such as cytokine secretion) in the immune cells, with enhanced STX11 expression contributing to increased NK-cell mediated cytotoxicity.<sup>57–61</sup> STX11 binds with the SNARE VTI1B (Figure 3, Table S4) and regulates its participation in the Q-SNARE complexes and, thus, the endocytic and exocytic trafficking in the macrophages. Overexpression of STX11 alters the VTI1B binding to STX6 and STX8 and likely reduces the endosomal transport to the cell surface.<sup>57</sup>

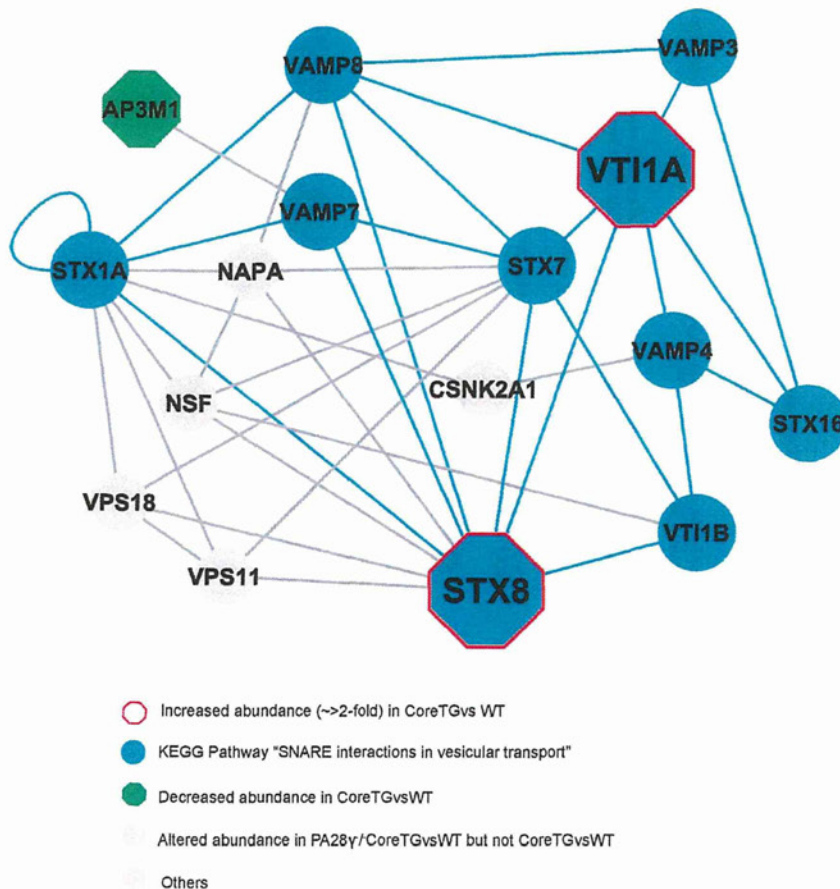
USO1, a Golgi-associated peripheral membrane protein, was decreased 2.05-fold in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG (Table 1) and was identified as a significant linking component of the PA28 $\gamma^{-/-}$ CoreTGvsCoreTG SNARE network (Figure 3). USO1 plays an important role in ER to Golgi trafficking and its knockdown leads to the disintegration of the Golgi complex.<sup>62,63</sup> Decreased USO1 levels in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG may, therefore, significantly impact the endosomal pathways associated with HCV release.

PA28 $\gamma$  knockdown impairs the production of the infectious HCV particles (but not replication) in the JFH1 (HCV genotype 2a) infected cells, largely due to the deregulation of the E6AP-dependent Core degradation, which contributes to an antiviral response.<sup>14</sup> Our analysis suggests a potentially novel

Table 2. Functional Analysis of the Extended Protein Interaction Networks<sup>a</sup>

proteins	data set <sup>b</sup>	KEGG pathways	prior involvement with HCV pathogenesis and HCC	probable associations with Core/PA28 $\gamma$ functions in HCV pathogenesis
AKT1	I (-)	Insulin signaling pathway ( $p = 5.72 \times 10^{-12}$ )	reduced levels associated with insulin resistance (IR) in rats; <sup>105</sup> Akt phosphorylation is suppressed in the CoreTG mice and contributes to IR. <sup>12</sup>	Core-induced suppression of AKT1 levels may contribute to IR in HCV pathogenesis.
BAX	I (+)	Apoptosis ( $p = 1.58 \times 10^{-18}$ )	interacts with NSSA; disrupts mitochondrial homeostasis leading to abnormal cytochrome <i>c</i> release and apoptosis in HCV infection. <sup>45,90,92,93</sup>	Core-mediated BAX upregulation may induce host cell apoptosis.
CASP8	I (+)	Apoptosis ( $p = 1.58 \times 10^{-18}$ )	activated in HCV infection. <sup>90</sup>	Core-mediated CASP8 upregulation may induce host cell apoptosis.
CDH1	I (+)	Adherens junction ( $p = 1.45 \times 10^{-20}$ )	downregulated in the rat liver during hepatocarcinogenesis <sup>106</sup>	Core-mediated decrease in CDH1 levels may contribute to HCC
COMT	I (+); II (+)	Steroid hormone biosynthesis ( $p = 0.002$ )	upregulated in the lipid rafts in HCV infection; <sup>50</sup> COMT siRNA knockdown decreases HCV replication <sup>103</sup>	Core-induced activation of COMT may play an important role in HCV entry and replication
CSNK2B	II (+); III (+)	Adherens junction ( $p = 1.4 \times 10^{-13}$ ); tight junction ( $p = 4.46 \times 10^{-7}$ )	interacts with the HCV NS3 protein; <sup>45</sup> regulates NSSA phosphorylation and hence infectious HCV particle production. <sup>87</sup>	loss of PA28 $\gamma$ activity may perturb CK2-mediated NSSA phosphorylation leading to decreased viral propagation.
EPS15	I (+); III (-)	Endocytosis ( $p = 2.08 \times 10^{-22}$ )	elevated in human and mouse HCC; <sup>107</sup> mediates human enterovirus 71 entry via clathrin-mediated endocytosis, <sup>108</sup> which also mediates HCV internalization. <sup>109</sup>	elevated EPS15 levels may facilitate HCC; decreased EPS15 levels may contribute to the lack of HCC in the PA28 $\gamma^{-/-}$ CoreTG. May facilitate HCV entry via clathrin-mediated endocytosis.
MCM2	I (+)	Cell cycle ( $p = 1.82 \times 10^{-25}$ )	increased hepatocyte MCM2 expression linked with fibrosis progression in HCV infection. <sup>110</sup>	Core-mediated enhanced MCM2 activity may contribute to fibrosis in HCV pathogenesis.
PTPN11	II (+)	Natural killer cell mediated cytotoxicity ( $p = 3.64 \times 10^{-10}$ ); Jak-STAT signaling pathway ( $p = 5.74 \times 10^{-5}$ )	functions as a tumor suppressor in HCC and negatively regulates hepatic insulin action. <sup>111,112</sup>	increased PTPN11 levels may be associated with the absence of HCC progression in PA28 $\gamma^{-/-}$ CoreTG.
RABEP1	III (-)	Endocytosis ( $p = 2.08 \times 10^{-22}$ )	interacts with NS3; <sup>45</sup> functions in early endocytic events and regulates mast cell activation. <sup>113,114</sup>	may possibly function in HCV propagation.
RB1	III (+)	Cell cycle ( $p = 4.04 \times 10^{-20}$ )	tumour suppressor, downregulated in HCC. <sup>115,116</sup>	increased RB1 levels in III consistent with the lack of HCC progression in the PA28 $\gamma^{-/-}$ CoreTG mice.
TRADD	III (-)	Apoptosis ( $p = 1.84 \times 10^{-15}$ )	forms a complex with Core and TNFR1, implicated in HCV-induced chronic liver disease. <sup>117</sup>	decreased TRADD levels may contribute to the lack of IR and liver disease in PA28 $\gamma^{-/-}$ CoreTG

<sup>a</sup>Host factors that were previously known to be associated with HCV pathogenesis and HCC and were mapped to various enriched KEGG pathways associated with the CoreTGvsWT, PA28 $\gamma^{-/-}$ CoreTGvsWT, and PA28 $\gamma^{-/-}$ CoreTGvsCoreTG PPI networks. <sup>b</sup>Data set I: CoreTGvsWT; Data set II: PA28 $\gamma^{-/-}$ CoreTGvsWT; Data set III: PA28 $\gamma^{-/-}$ CoreTGvsCoreTG; +: upregulated; -: downregulated.



**Figure 2.** CoreTGsWT SNARE network. Network illustration of the interactions between the PowerBlot identified differentially expressed proteins in CoreTGsWT and human proteins mapped to the enriched KEGG pathway “SNARE interactions in vesicular transport”. The node sizes differ for better clarity and do not reflect any topological attributes.

mechanism for the involvement of PA28 $\gamma$  in HCV propagation. Potentially, the suppression of PA28 $\gamma$  activity in PA28 $\gamma^{-/-}$ CoreTG mice may contribute to the overexpression of STX11 (and downregulation of USO1), thereby impairing the trafficking to the cell surface and consequently the release of the infectious HCV particles.

HCV has also been detected in the macrophages of certain infected patients,<sup>64</sup> suggesting that HCV may possibly infect the macrophages *in vivo* and regulate the STX11 (and USO1) expression to modulate the viral release and cytokine secretion.

EPS15 and RABEP1 (KEGG pathway “Endocytosis”,  $p = 2.08 \times 10^{-22}$ ) were decreased 2.11- and 2.74-fold, respectively, in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG (Table 1, Table S5). EPS15 is an adaptor protein associated with the epidermal growth factor (EGF) signaling; it is localized to the clathrin-coated pits and functions in receptor-mediated endocytosis<sup>65,66</sup> and may play an important role in HCV pathogenesis (Table 2).

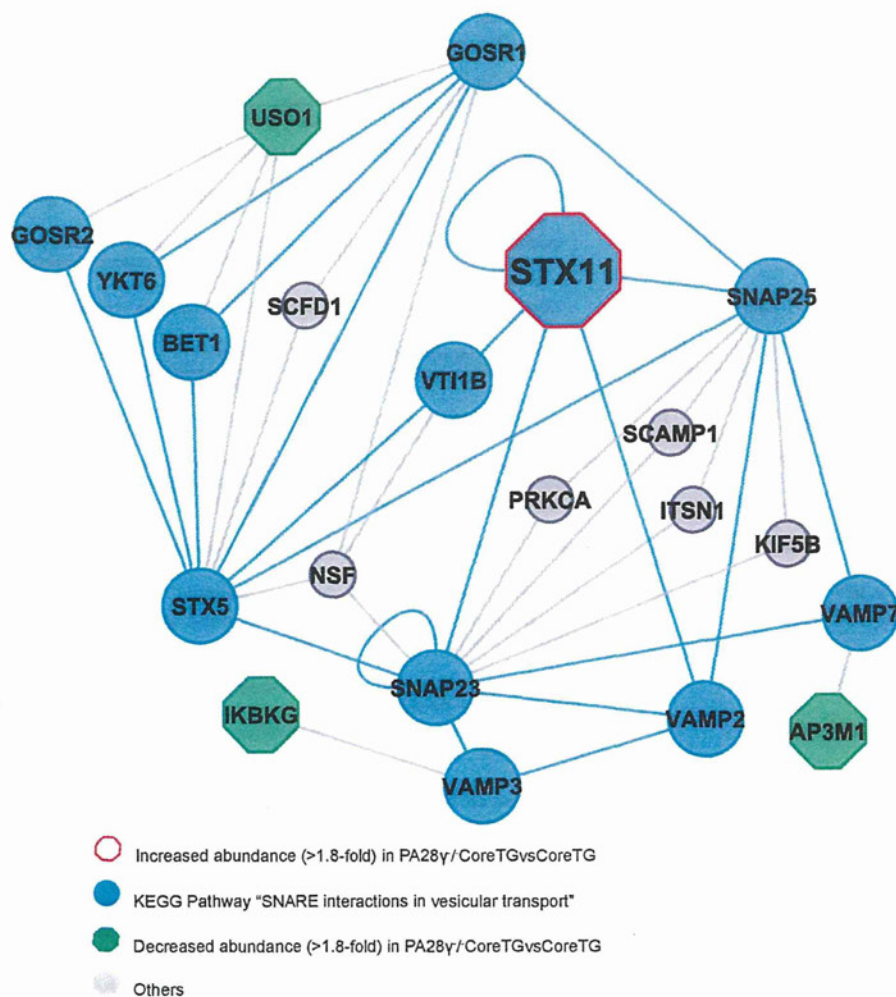
### Immune System and Signal Transduction

HCV infection induces varied active and passive host immune responses such as the recognition of the infecting HCV RNA and proteins by the macrophages and the dendritic cells expressing Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). These events trigger the production of Type I interferons (IFN- $\alpha/\beta$ ) and inflammatory cytokines in the

infected hepatocytes, thereby initiating viral clearance. The ability to impair host immune responses contributes to the HCV persistence in the host.<sup>67–72</sup>

The PowerBlot analysis showed differentially expressed host proteins (IKBKG, MAP2K2, PPP3CA, SHC3, STAT1, TRADD) in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG and their interacting partners that were mapped to one or more enriched KEGG pathways associated with the immune system (Table S5). IKBKG (IKK Gamma) is an antiapoptotic protein that is essential for NF $\kappa$ B activation and modulates TNF-mediated apoptosis.<sup>73</sup> IKBKG mutations are associated with immune deficiency phenotype (Table S7) and IKBKG may contribute to the activity of the hepatic carcinoma associated protein MAFIP in suppressing the proliferation of the cancer cells.<sup>74</sup> Additionally, specific deletion of IKBKG in the hepatocytes promotes NK-cell dependent liver damage.<sup>75</sup> Taken together, the elevated IKBKG abundance as observed in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG (1.97-fold; Table 1) may contribute to the lack of HCC progression and reduced liver damage in the PA28 $\gamma^{-/-}$ CoreTG mice.

PPP3CA, MAP2K2, and SHC3 were mapped to the KEGG pathway “Natural killer cell mediated cytotoxicity” ( $p = 1.67 \times 10^{-5}$ ; Table S5), the components of which function in the host immune response against the cancer cells and cells beset with pathogen infection.<sup>76</sup> PPP3CA levels were increased 12.94-fold,



**Figure 3.** PA28 $\gamma$ <sup>-/-</sup>CoreTGvsCoreTG SNARE network. Network illustration of the interactions between the PowerBlot identified differentially expressed proteins in PA28 $\gamma$ <sup>-/-</sup>CoreTGvsCoreTG and human proteins mapped to the enriched KEGG pathway "SNARE interactions in vesicular transport". The node sizes differ for better clarity and do not reflect any topological attributes.

while MAP2K2 and SHC3 levels were suppressed 2.35-fold and 1.9-fold, respectively, in PA28 $\gamma$ <sup>-/-</sup>CoreTGvsCoreTG (Table 1). These observations together with the increased STX11 abundance are consistent with the enhanced NK-cell mediated cytotoxicity that accompanies the STX11 overexpression.

PPP3CA is a tumor suppressor that negatively modulates the vascular endothelial growth factor (VEGF)-stimulated cell proliferation<sup>77</sup> and is downregulated in some cancerous cells.<sup>78,79</sup> PPP3CA was also mapped to the enriched KEGG pathways "VEGF signaling pathway" ( $p = 2.59 \times 10^{-5}$ ), "MAPK signaling pathway" ( $p = 2.37 \times 10^{-16}$ ) and "Wnt signaling pathway" ( $p = 1.048 \times 10^{-10}$ ; Table S5), which are implicated in the HCV infection and HCC. The 7.55-fold increase in PPP3CA abundance in PA28 $\gamma$ <sup>-/-</sup>CoreTGvsCoreTG (Table 1) suggests that increased PPP3CA activity may significantly contribute to the lack of tumorigenesis and HCC progression in PA28 $\gamma$ <sup>-/-</sup>CoreTG mice.

MAP2PK2 is a dual specificity MAP kinase that plays a critical role in the mitogen growth factor signal transduction. It is a key regulator of the TNF- $\alpha$  signaling and plays an important role in the tumor progression in certain cancers.<sup>80</sup>

Reduced MAP2K2 levels in the hepatocytes are implicated in enhanced apoptosis.<sup>81</sup> Therefore, the 2.66-fold decrease in MAP2K2 protein levels in PA28 $\gamma$ <sup>-/-</sup>CoreTGvsCoreTG (Table 1) suggests that decreased MAP2K2 activity may contribute to the lack of HCC progression in PA28 $\gamma$ <sup>-/-</sup>CoreTG mice. Our analysis thus identified potentially significant PA28 $\gamma$ -dependent roles of MAP2K2 and PPP3CA in a probable STX11-mediated regulation of NK cell activity in HCV pathogenesis.

MAP2K2, PPP3CA and SHC3 were also associated with the "Insulin signaling pathway" ( $p = 5.88 \times 10^{-9}$ ; Table S5), the disruption of which may contribute to insulin resistance (IR). IR is linked with steatosis, fibrosis progression and poor interferon- $\alpha$  response in HCV infection.<sup>82,83</sup> PA28 $\gamma$  contributes to hyperinsulinemia and IR in the CoreTG mice by impairing the insulin-signaling pathway through the suppression of Insulin receptor substrate 1 (IRS1) phosphorylation and increased tumor necrosis factor alpha (TNF- $\alpha$ ) secretion.<sup>12,84</sup> The Powerblot analysis revealed that TRADD, which regulates TNF- $\alpha$  signaling as an antiapoptotic factor<sup>85,86</sup> and possibly functions in HCV pathogenesis (Table 2), was suppressed 2-fold in PA28 $\gamma$ <sup>-/-</sup>CoreTGvsWT (Table 1).

Table 3. Summary of Proteins and Pathways Prioritized with TargetMine and Adjusted with the Help of Knowledge-Based Inputs for Experimental Investigation and/or Biomarker Discovery

(a) HCV replication and release					
KEGG pathways	p-value	proteins <sup>a</sup>	data set <sup>b</sup>	knowledge-based evidence	refs
SNARE interactions in vesicular transport	0.023	VT11A (+), STX8 (+)	I	regulation of the endosome-based membrane trafficking pathway implicated in HCV release	50, 52, 55
Steroid hormone biosynthesis	0.002	COMT (+)	I	COMT siRNA impairs HCV genotype 1b replication; functions in genotype 2a replication not known	50, 103
SNARE interactions in vesicular transport	0.003	STX11 (+)	III	modulation of specific components of the endosome-based membrane trafficking pathway implicated in HCV release; modulation of cytokine secretion in immune cells	50, 55, 57
Endocytosis	$1.03 \times 10^{-18}$	CAV1 (+)	I	close homologue CAV2 associated with HCV replication complex; possible role in HCV replication	103
(b) steatosis, fibrosis, and hepatocarcinogenesis in HCV infection					
KEGG pathways	p-value	proteins <sup>a</sup>	data set <sup>b</sup>	knowledge-based evidence	refs
Insulin signaling pathway	$5.72 \times 10^{-12}$	AKT1 (-)	I	reduced AKT1 levels and phosphorylation associated with insulin resistance, which contributes to steatosis, fibrosis and HCC	118
Natural killer cell mediated cytotoxicity	$1.67 \times 10^{-5}$	PPP3CA (+), MAP2K2 (-)	III	PPP3CA is a tumor suppressor with decreased levels in some cancers; PTPN1 is a tumor suppressor and regulates hepatic insulin signaling; decreased MAP2K2 levels in hepatocytes associated with enhanced apoptosis	78, 79, 81
Adherens junction	$1.45 \times 10^{-20}$	CDH1 (-)	I	decreased CDH1 abundance associated with hepatocarcinogenesis and various cancers	106
Focal adhesion	$5.57 \times 10^{-12}$	SHC1 (+)	II	loss of SHC1 function associated with tumor metastasis	119
Apoptosis	$1.58 \times 10^{-18}$	BAX (+), CASP8 (+)	I	BAX interacts with NS5A and contributes to abnormal cytochrome c release in HCV infection; CASP8 activated in HCV infection	45, 90, 92, 93
Apoptosis	$1.84 \times 10^{-15}$	TRADD (-)	III	antiapoptotic factor that forms a ternary complex containing Core, with likely functions in HCV-induced chronic liver disease	117
Chemokine signaling pathway	$1.18 \times 10^{-13}$	ARRB1 (-)	I	interacts with PKM2, a key enzyme in glycolytic metabolism and cell growth and death in tumor cells	120

<sup>a</sup>+: upregulated. -: downregulated. <sup>b</sup>Data set I: CoreTGvsWT; Data set II: PA28 $\gamma^{-/-}$ /CoreTGvsWT; Data set III: PA28 $\gamma^{-/-}$ /CoreTGvsCoreTG.

### Cell Adhesion

The Powerblot analysis revealed that host protein CSNK2B, the regulatory (beta) subunit of Casein Kinase II (CK2), was increased 1.88-fold in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG (1.9-fold in PA28 $\gamma^{-/-}$ CoreTGvsWT). CK2 phosphorylates NS5A and regulates the production of infectious viral particles<sup>87</sup> and thus HCV pathogenesis (Table 2). CSNK2B was mapped to the enriched KEGG pathways "Adherens junction" ( $p = 1.4 \times 10^{-13}$ ) and "Tight junction" ( $p = 4.46 \times 10^{-7}$ ), some components of which are implicated in HCV entry and infection.<sup>88</sup>

SHC3 and TNR were decreased 1.9- and 4.62-fold, respectively, in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG and were mapped to the enriched KEGG pathway "Focal adhesion" ( $p = 3.56 \times 10^{-12}$ ; Table 1; Table S5), which regulates cell migration and adhesion to the extracellular matrix. Its deregulation is linked with tumor progression and possibly HCV propagation.<sup>45</sup> Previously, a Core interacting protein ENO1, associated with the focal adhesion, was identified as a novel regulator of HCV replication and release,<sup>22</sup> suggesting that SHC3 and TNR may play important roles in HCV pathogenesis.

### Cell Growth and Death

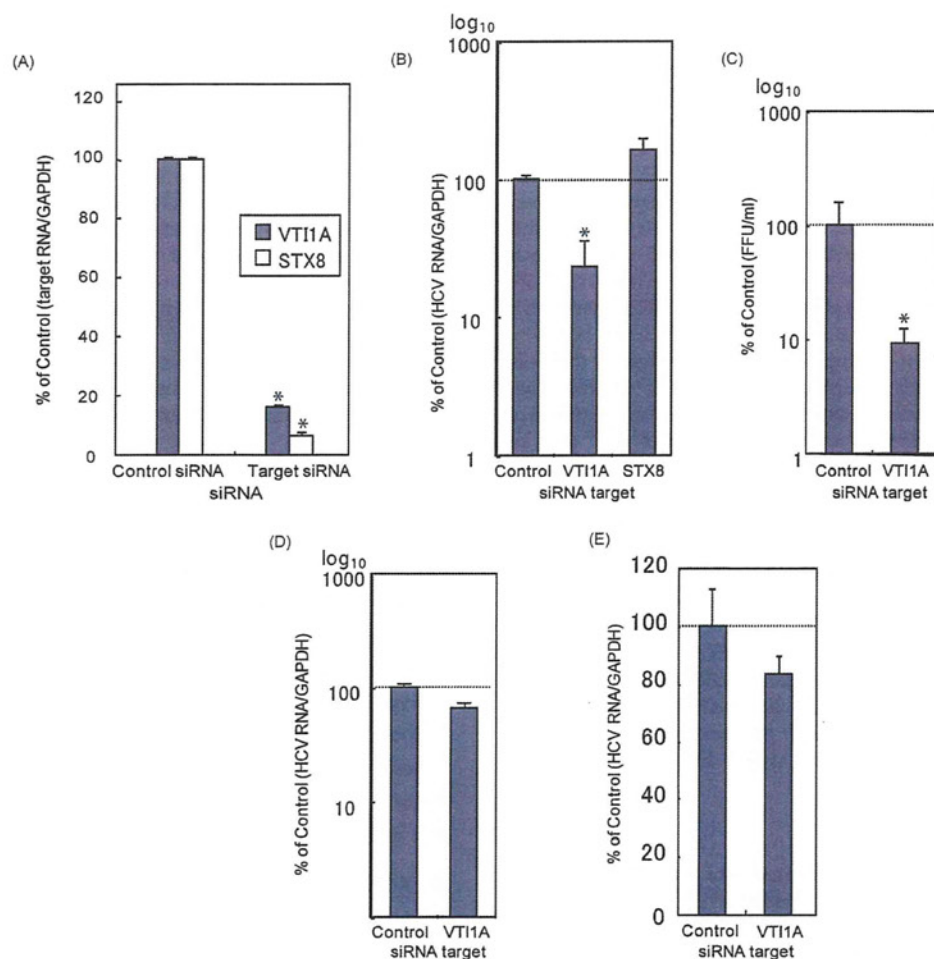
Host cell apoptosis plays a critical role in HCV pathogenesis. The induction of apoptosis in the hepatocytes contributes to cell damage and fibrosis, whereas the induction of apoptosis in the peripheral blood mononuclear cells (PMBC), such as the T-cells, contributes significantly to the impaired immune response and HCV persistence in the host.<sup>89-94</sup> PA28 $\gamma$  is implicated in the cell cycle regulation, cell proliferation, and apoptosis<sup>95-98</sup> and likely plays a critical role in the manipulation of the cell cycle and apoptosis in HCV pathogenesis.

PPP3CA, TRADD, PRKAR2A, and IKBKG, with increased or decreased abundances in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG, were mapped to KEGG pathway "Apoptosis" ( $p = 1.84 \times 10^{-15}$ ; Table S5). PPP3CA was also mapped to "Oocyte meiosis" ( $p = 9.84 \times 10^{-10}$ ; Table S5), associated with cell division. PPP3CA levels were highly elevated (12.94-fold) in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG (Table 1), which may contribute to the accelerated cell death and the lack of tumor progression in PA28 $\gamma^{-/-}$ CoreTG mice.

PRKAR2A levels were decreased 1.88-fold in PA28 $\gamma^{-/-}$ CoreTGvsCoreTG (Table 1). PRKAR2A codes for a regulatory subunit of the cAMP dependent protein kinase (PKA), an important mediator of the cAMP signal transduction and elevated PRKAR2A expression is associated with an increased proliferation of the rat alveolar cells.<sup>99</sup> The suppression of PRKAR2A activity may therefore contribute to the lack of tumor proliferation in the PA28 $\gamma^{-/-}$ CoreTG mice.

### Prioritization and Validation of the Novel Candidates for Their Role in HCV Replication and Release

Target prioritization using TargetMine is a simple process that involves uploading an initial list of candidates (in this instance the proteins in the CoreTGvsWT, PA28 $\gamma^{-/-}$ CoreTGvsWT, and PA28 $\gamma^{-/-}$ CoreTGvsCoreTG extended PPI networks) and estimating enriched biological themes associated with the input list.<sup>16</sup> Knowledge-based inputs may then be employed to further screen the proteins mapped to the top ranking significant associations to infer a manageable set of candidates. With the help of TargetMine, we previously investigated the significance of interactions between HCV Core and NS4B proteins and host factors in HCV infection and identified three novel regulators of HCV replication and propagation.<sup>22</sup>



**Figure 4.** Effects of knockdown of candidate proteins on HCV propagation and replication. Host proteins VT11A and STX8 were suppressed by RNAi (A) in Huh7OK1 cells infected with HCV JFH1 strain (genotype 2a; B, C, D) and in cells including subgenomic JFH1 replicon (E). The amounts of mRNA of the intracellular host proteins (A) and the supernatant viral RNA (B), viral titer (C), and intracellular viral RNA were estimated (D). The amount of the subgenomic viral RNA was also estimated (E). Each value was represented as percentage of the cells transfected with control siRNA; FFU: focus-forming units; \*  $p < 0.01$ .

Table 3 summarizes the prioritized candidates and pathways, all of which have been discussed above. Traditionally, viral and host proteins associated with the HCV lifecycle (internalization, replication, assembly, and release) have been preferred targets in the anti-HCV studies. The prioritized candidates and pathways in Table 3a fall within this category. In particular, our analysis suggested novel and potentially crucial roles of the host proteins VT11A and STX8, which were elevated in CoreTG but not in PA28 $\gamma^{-/-}$ CoreTG, in the replication and/or the release stages of the HCV lifecycle, therefore making these host proteins attractive targets for further investigation.

Because of 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 identify potential anti-HCV drug targets.<sup>5,7-9</sup> To further explore the roles of selected candidates in the HCV life cycle, we performed cellular assays to assess the impact of VT11A and STX8 siRNA knockdowns on HCV replication and release. Since the HCV-production systems using the HCV JFH1 infectious strain (genotype 2a) isolates alone are capable of both efficient replication and the production of the infectious

HCV particles,<sup>33,100</sup> 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 (Figure 4A). Supernatant viral RNA and the viral titer were significantly decreased by the knockdown of VT11A but were unaffected by the STX8 knockdown (Figure 4B,C). However, VT11A and STX8A knockdowns had no effect on the intracellular viral RNA levels in the HCV infected cells (Figure 4D) or replicon cells derived from JFH1 strain (Figure 4E) or replicon cells derived from the Con1 (genotype 1b) strain (data not shown). These observations suggest that VT11A regulates HCV propagation but not HCV replication.

The standard therapy of PEGylated interferon- $\alpha$  plus rebavirin treatment often results in severe side effects such as depression, flu-like symptoms, anemia, and fatigue that force the treatment to discontinue in affected patients, thus necessitating improved and combinatorial treatment strategies.<sup>101,102</sup> The genetic variability of HCV has led to increasing drug resistance. Thus, antivirals that target host proteins critical to viral pathogenesis, with a lower rate of mutation and

preferably with minimal adverse side effects, may provide attractive alternatives to HCV protein targets. VTI1A-deficient (knockout) mice are viable and fertile,<sup>55</sup> suggesting that the suppression of VTI1A activity may not have significantly undesirable side effects.

Inhibition of COMT (which was increased 2.71- and 2.96-fold in CoreTGvsWT and PA28 $\gamma^{-/-}$ CoreTG, respectively; Table 1) activity via siRNA knockdown was previously shown to result in a decreased HCV replication in cells infected with the Con1 strain.<sup>103</sup> To investigate other possible aspects (such as genotype specificity) of COMT function in the HCV life cycle, we assessed the impact of the COMT siRNA knockdown on HCV replication and release. COMT knockdown, however, had no effect on HCV propagation or replication in the cells including full length or subgenomic replicons derived from JFH1 or Con1 strains (data not shown). The discrepancy between our observations and those of Chan et al.<sup>103</sup> may be explained by the differences in the methodologies. We employed a transient transfection method to knockdown the selected targets to assess their roles in HCV replication and release, whereas Chan et al. employed a lentiviral expressing system for their experiments. Lentiviral mediated siRNA delivery is known to result in a persistent knockdown of gene expression,<sup>104</sup> and a persistent knockdown of COMT expression may be necessary to inhibit HCV replication *in vitro*.

That we were able to experimentally validate one of the three genes selected for experimental characterization reinforces the strengths of the elaborate PPI network-based approach to identify and prioritize suitable targets for experimental and therapeutic investigation.

## CONCLUSIONS

By analyzing high-throughput proteomics data from transgenic mice expressing HCV Core protein in the liver (an *in vivo* model of HCV pathogenesis) with or without the knockout of the proteasome activator PA28 $\gamma$ , we highlighted the cellular responses to HCV infection *in vivo* and obtained further insights into the role of PA28 $\gamma$  in HCV infection.

We investigated the network context of the changes in the protein abundances by mapping them onto the human interactome with the help of the TargetMine data warehouse. The differentially expressed proteins that were integrated with the human interactome were observed to participate in compact and well connected cellular networks reflecting the ability of HCV to rapidly and efficiently react to the host responses to HCV infection. A functional analysis of the PPI networks highlighted the cellular pathways associated with vesicular transport, immune system, cellular adhesion, cell growth, and cell death among others that were most prominently influenced by Core and PA28 $\gamma$  in HCV infection. We also confirmed the previous observations that host factors such as AKT1, BAX, CASP8 CDH1, COMT, MCM2, PTPN11, and RB1 showed increased or decreased abundances in HCV infection. However, to the best of our knowledge, the precise molecular mechanisms of these factors' involvement in HCV pathogenesis and HCC were unknown, and our analysis suggests novel contributions of Core and PA28 $\gamma$  to the functions of these proteins.

Our observations were then used to prioritize potential candidates for the follow-up experimental investigations. Cellular assays based on siRNA knockdowns of selected candidates in the HCV infected and replicon cells validated VTI1A, a SNARE protein associated with vesicular transport,

which was upregulated in CoreTG but not in PA28 $\gamma^{-/-}$ CoreTG, as a novel regulator of HCV propagation but not replication. VTI1A-deficient mice are largely indistinguishable from the normal mice except for minor growth retardation in a few instances; therefore, VTI1A is a promising novel candidate for anti-HCV therapy.

Our analysis not only builds on the present understanding of the Core-PA28 $\gamma$  interplay in HCV infection but also provides novel insights that would facilitate the clinical evaluation of proteomic changes associated with HCV pathogenesis. Our analysis also provides a generic framework for investigating large scale proteomic data. Such investigation may help identify common themes associated with different physiological conditions, especially pathogen (such as viral) infection and disease, and help develop effective broad spectrum strategies aimed at ameliorating pathogen infection and diseases.

## ASSOCIATED CONTENT

### Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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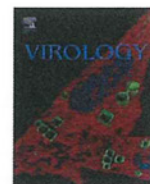
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## Trans-complemented hepatitis C virus particles as a versatile tool for study of virus assembly and infection

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### ABSTRACT

In this study, we compared the entry processes of *trans*-complemented hepatitis C virus particles (HCVtcp), cell culture-produced HCV (HCVcc) and HCV pseudoparticles (HCVpp). Anti-CD81 antibody reduced the entry of HCVtcp and HCVcc to almost background levels, and that of HCVpp by approximately 50%. Apolipoprotein E-dependent infection was observed with HCVtcp and HCVcc, but not with HCVpp, suggesting that the HCVtcp system is more relevant as a model of HCV infection than HCVpp. We improved the productivity of HCVtcp by introducing adapted mutations and by deleting sequences not required for replication from the subgenomic replicon construct. Furthermore, blind passage of the HCVtcp in packaging cells resulted in a novel mutation in the NS3 region, N1586D, which contributed to assembly of infectious virus. These results demonstrate that our plasmid-based system for efficient production of HCVtcp is beneficial for studying HCV life cycles, particularly in viral assembly and infection.

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### Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic liver diseases (Hoofnagle, 2002). HCV is an enveloped virus of the family *Flaviviridae*, and its genome is a positive-strand RNA consisting of the 5'-untranslated region (UTR), an open reading frame encoding viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and the 3'-UTR (Suzuki et al., 2007).

Host–virus interactions are required during the initial steps of viral infection. It was previously reported that CD81 (Bartosch et al., 2003a, b; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (Bartosch et al., 2003a, b; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. CD81 interacts with HCV E2 via a second extracellular loop (Bartosch et al., 2003a, b; Hsu et al., 2003) and its role in the internalization process was confirmed (Cormier et al., 2004; Flint et al., 2006). It has also been shown that infectious

HCV particles produced in cell cultures (HCVcc) exist as apolipoprotein E (ApoE)-enriched lipoprotein particles (Chang et al., 2007) and that ApoE is important for HCV infectivity (Owen et al., 2009).

Investigation of HCV had been hampered by difficulties in amplifying the virus *in vitro* before development of robust cell culture systems based on JFH-1 isolates (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Retrovirus-based HCV pseudoparticles (HCVpp), in which cell entry is dependent on HCV glycoproteins, have been used to study virus entry (Bartosch et al., 2003a; Hsu et al., 2003). Vesicular stomatitis virus (VSV)-based pseudotypic viruses bearing HCV E1 and E2 and replication-competent recombinant VSV encoding HCV envelopes have also been available as surrogate models for studies of HCV infection (Mazumdar et al., 2011; Tani et al., 2007).

It was recently shown that HCV subgenomic replicons can be packaged when structural proteins are supplied *in trans* (Adair et al., 2009; Ishii et al., 2008; Masaki et al., 2010; Steinmann et al., 2008). These *trans*-complemented HCV particles (HCVtcp) are infectious, but support only single-round infection and are unable to spread. Establishment of flexible systems to efficiently produce HCVtcp should contribute to studying HCV assembly, in particular encapsidation of the viral genome, and entry to cells with less stringent biosafety and biosecurity measures. Although single-round infection can be achieved by using the HCVcc system with receptor knock-out

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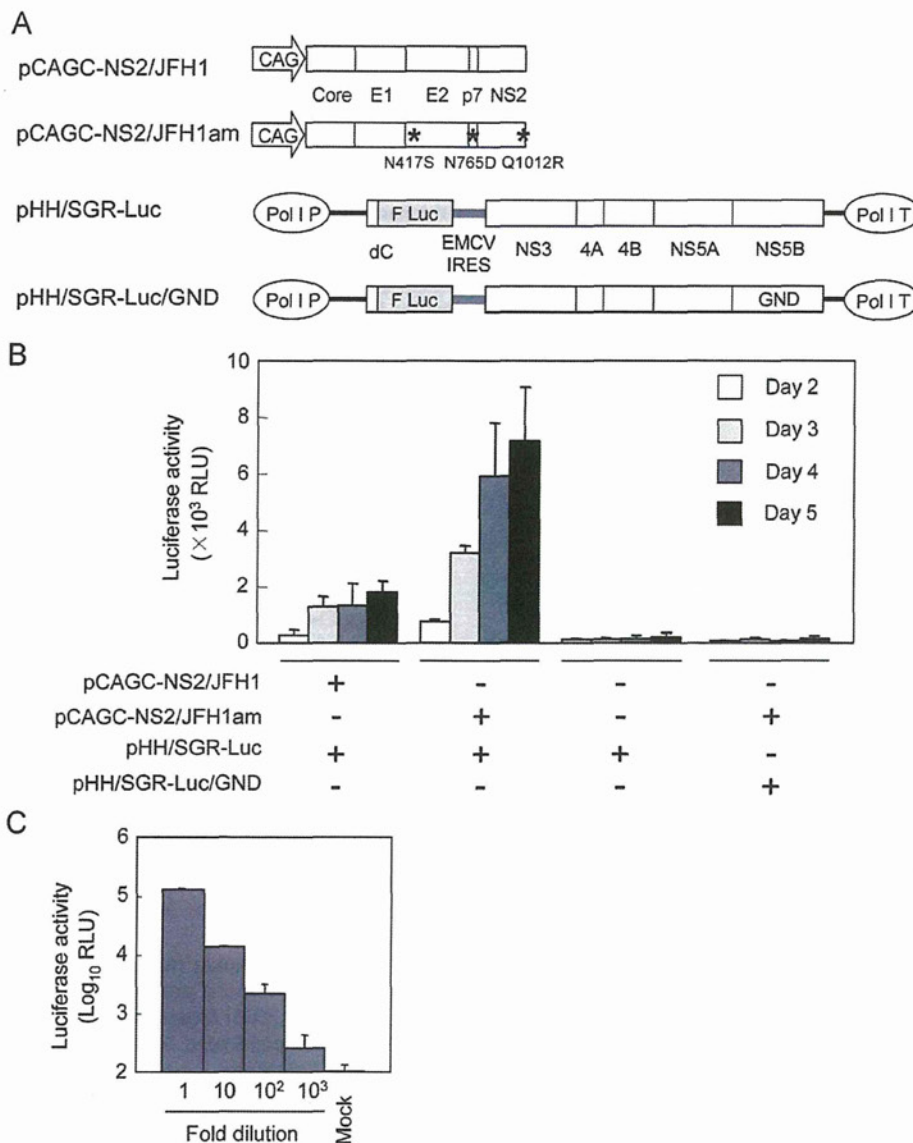
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cells, the single-round HCVcc system is not suitable for studying virus entry. We previously described plasmid-based production of HCVcc and HCVtcp (Masaki et al., 2010). Here, we demonstrated that HCVtcp production can be enhanced by introducing the previously reported cell-culture adaptive mutations and by deleting sequences not essential for replication in the subgenomic replicon construct. By providing genotype 1b-derived core-to-p7 in addition to intragenotypic viral proteins, chimeric HCVtcp were generated. Furthermore, blind passage of HCVtcp in the packaging cells resulted in the identification of a novel cell culture-adaptive mutation in NS3 that enables us to establish the efficient production of HCVtcp with structural proteins from various strains. Taken together, our system for producing single-cycle infectious HCV particles should be useful in the study of entry and assembly steps of the HCV life cycles. This technology may also have potential to be the basis for the safer vaccine development.

**Results**

*Enhancement of HCVtcp production by adaptive mutations in E2, p7 and NS2 and by deleting sequences not essential for replication from replicon construct*

In our HCVtcp system, the RNA polymerase I (Pol I)-driven replicon plasmid, which carries a dicistronic subgenomic luciferase reporter replicon of JFH-1 strain with a Pol I promoter and terminator (pHH/SGR-Luc), as well as a plasmid containing core-NS2 cDNA under the CAG promoter (pCAGC-NS2) were used (Masaki et al., 2010). In an effort to improve the yield of HCVtcp production, cell culture-adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) which were previously selected from serial passage of HCVcc (Russell et al., 2008) were introduced into the core-NS2 expression plasmid (Fig. 1A) (residues are numbered



**Fig. 1.** HCVtcp production by two-plasmid transfection. (A) Schematic representation of plasmids is shown. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. The internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) is denoted as gray lines. Adaptive mutations are indicated as asterisks. F Luc: firefly luciferase gene; CAG: CAG promoter; Pol I P: RNA polymerase I promoter; Pol I T: RNA polymerase I terminator; GND: replication-deficient GND mutation. (B) Luciferase activity in Huh7.5.1 cells inoculated with supernatant from cells transfected with indicated plasmids at the indicated time points. Data are averages of triplicate values with error bars showing standard deviations. (C) Luciferase activity in cells inoculated with serially diluted HCVtcp.

according to positions within the JFH-1 polyprotein). Supernatants of cells transfected with plasmids (Fig. 1A) were collected and were used to infect Huh7.5.1 cells, which were analyzed by luciferase assay. Introduction of adaptive mutations (pCAGC-NS2/JFH1am) resulted in more than 4-fold higher production of HCVtcp at 5 day post-transfection, as compared to wild-type (WT) (pCAGC-NS2/JFH1) (Fig. 1B), indicating that the adaptive mutations contribute to enhancing HCVtcp production. To confirm that luciferase activity levels in HCVtcp-infected cells are correlated with the number of infectious particles, Huh7.5.1 cells were inoculated with serial dilutions of HCVtcp. Luciferase activity was well correlated with viral load (Fig. 1C), indicating that luciferase assay in HCVtcp-infected cells can be used to quantify HCV infection.

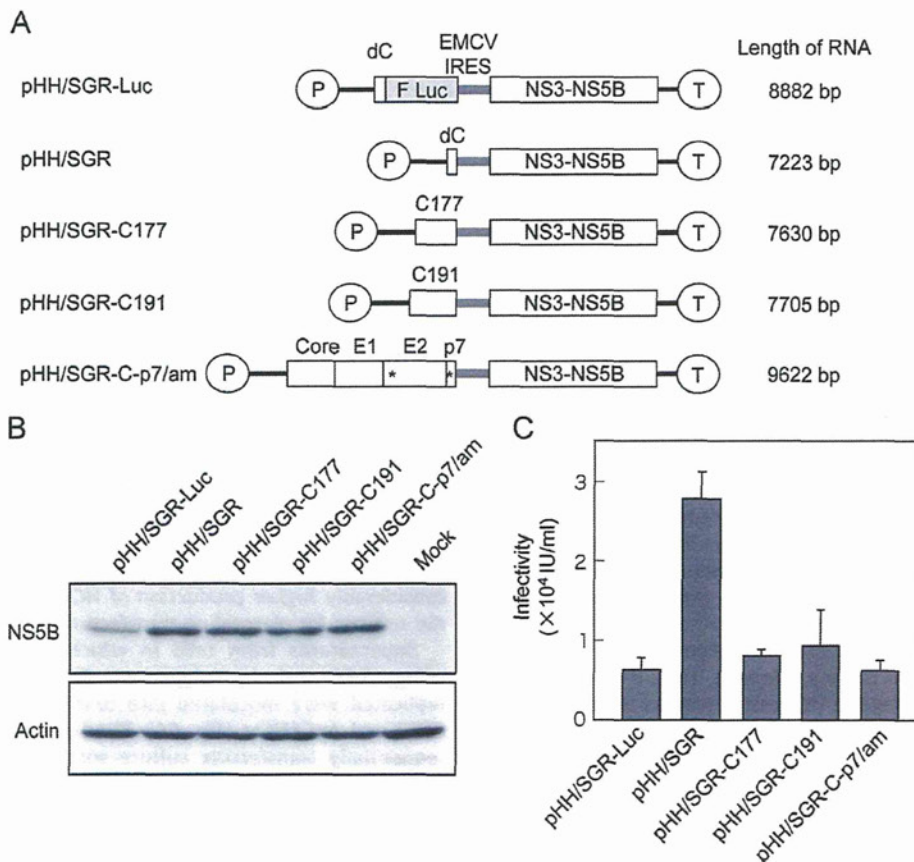
In order to further explore the efficient production of HCVtcp, we generated replicon constructs that lack the luciferase gene or include the partial coding sequences for structural proteins instead of reporter (Fig. 2A). Replication of each replicon in plasmid-transfected cells was then assessed by Western blotting (Fig. 2B). Among the constructs tested, NS5B levels were lowest in cells expressing pHH/SGR-Luc. NS5B levels in cells replicating other replicons appeared to be comparable. Cells were infected with supernatants of cells transfected with each replicon plasmid, along with pCAGC-NS2/JFH1am, followed by infectious unit assay (Fig. 2C). The highest production of HCVtcp was obtained from cells transfected with pHH/SGR, where the luciferase sequence was deleted from pHH/SGR-Luc, thus suggesting that deletion of the sequence not essential for RNA replication in the replicon may contribute to enhancing HCVtcp production.

#### Production of chimeric HCVtcp by providing heterologous core-p7

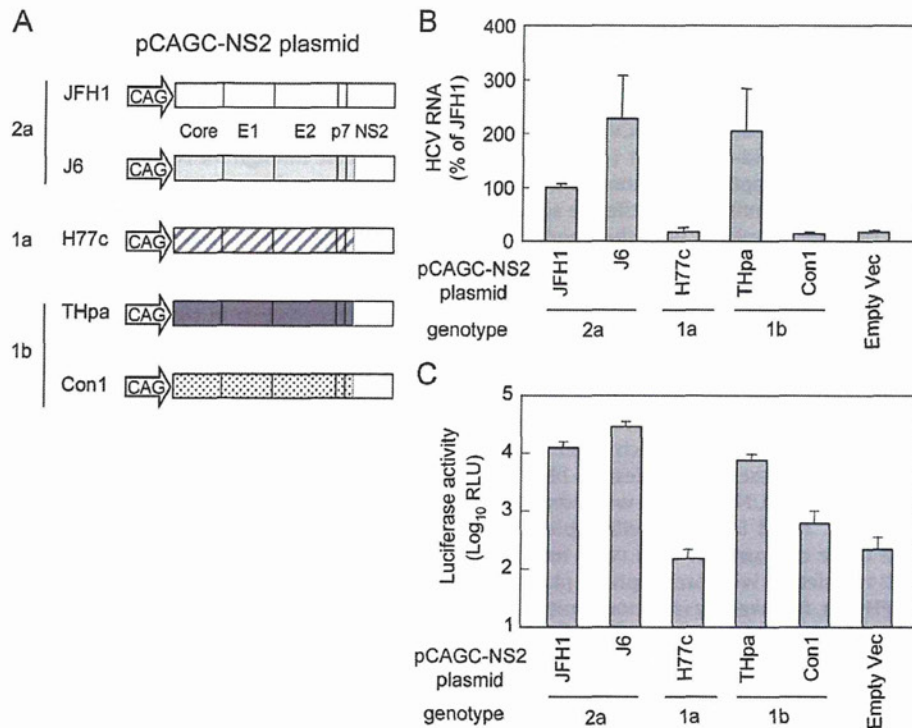
In order to elucidate whether *trans*-encapsidation of JFH-1 replicon can be achieved by providing core-p7 from other HCV strains, core-NS2 plasmids were constructed (Fig. 3A). In these plasmids, core through the N-terminal 33 aa of NS2, which contains transmembrane domain 1 of NS2, was derived from either H77c (genotype 1a), THpa (genotype 1b), Con1 (genotype 1b) or J6 (genotype 2a) strain. Residual NS2 was derived from JFH-1, as described previously (Pietschmann et al., 2006). HCVtcp was efficiently produced by core-p7 of J6 and THpa strains, but its production was less efficient in the case of Con1 strain. *Trans*-packaging was not detectable when core-p7 of H77c strain was used (Fig. 3C). Among HCV strains tested, difference in luciferase activity levels in HCVtcp-infected cells (Fig. 3C) were in agreement with that in the viral RNA levels in the culture supernatants of the transfected cells (Fig. 3B). Although the efficacy of *trans*-complementation was variable among strains, chimeric HCVtcp can be generated by providing genotype 1b-derived core-p7 in addition to intragenotypic viral proteins, and was used in subsequent studies.

#### ApoE- and CD81-dependent infection by HCVtcp

There is accumulating evidence that apolipoproteins, particularly ApoE, contribute to HCV production and infectivity (Chang et al., 2007; Owen et al., 2009). To determine whether ApoE is involved in infection of target cells by HCVtcp, we infected cells in the presence of increasing concentrations of anti-ApoE antibody.



**Fig. 2.** Production of HCVtcp with different replicon constructs. (A) Schematic representation of plasmids used for production of HCVtcp. Deduced length of transcribed RNA from each construct is shown on the right. HCV polyproteins from JFH-1 strain are indicated by open boxes. HCV UTRs are indicated by bold lines. The EMCV IRES is denoted by gray bars. Adaptive mutations are indicated by asterisks. F Luc: firefly luciferase gene; P: RNA polymerase I promoter; T: RNA polymerase I terminator. (B) Detection of NS5B and actin in Huh7.5.1 cells transfected with indicated plasmids at 4 day post-transfection. (C) Infectivity of culture supernatants from cells transfected with indicated replicon plasmids along with pCAGC-NS2/JFH1am at 4 day post-transfection.



**Fig. 3.** HCVtcp production with structural proteins from various strains. (A) Schematic representation of plasmids used. HCV polyproteins of JFH-1, J6, H77c, THpa and Con1 strain are shown in the open box, bright gray box, box with diagonal lines, dark gray box and dotted box, respectively. (B) Relative levels of HCV RNA in the supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc. (C) Luciferase activity in cells inoculated with supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc at 4 day post-transfection.

pCAGC-NS2/THpa and pCAGC-NS2/JFH1am were used as core-NS2 plasmids for HCVtcp production carrying core-p7 derived from genotypes 1b and 2a (HCVtcp-1b and HCVtcp-2a, respectively). HCVpp derived from JFH-1 and VSVpp were generated and used for comparison. Infection with HCVtcp-1b or HCVtcp-2a was blocked by anti-ApoE antibody in a dose-dependent manner. In contrast, anti-ApoE antibody did not affect infection with HCVpp and VSVpp (Fig. 4A).

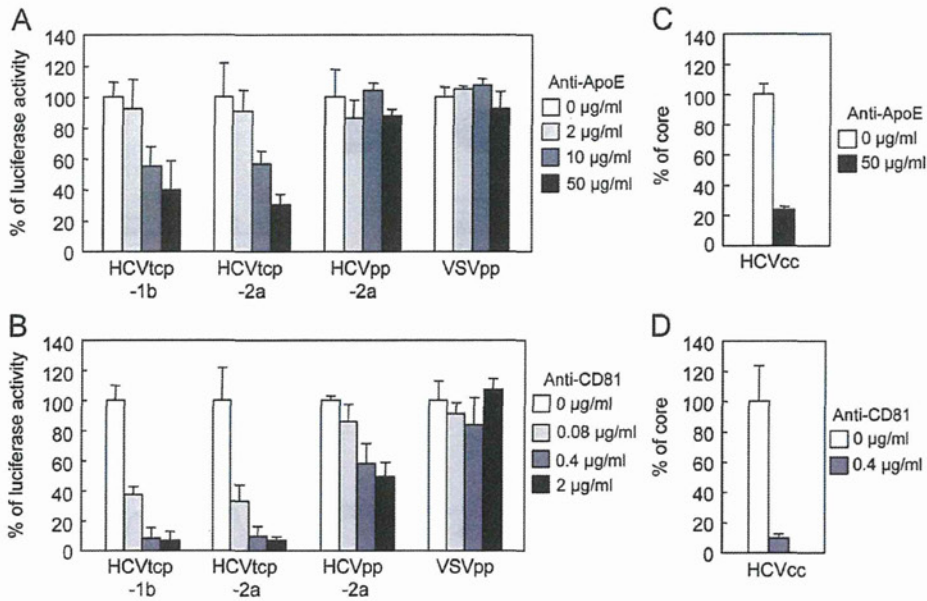
The CD81 dependence of infection was also compared between HCVtcp and HCVpp (Fig. 4B). Anti-CD81 antibody inhibited the entry of HCVtcp-1b, HCVtcp-2a, and HCVpp in a dose-dependent manner. The antibody had no effect on VSVpp infection. HCVtcp infection appears to be more sensitive to anti-CD81 antibody when compared with HCVpp infection; more than 60% inhibition was observed at 0.08  $\mu\text{g}/\text{mL}$  anti-CD81 antibody for HCVtcp-1b and HCVtcp-2a, whereas approximately 50% inhibition was observed for HCVpp at 2  $\mu\text{g}/\text{mL}$  antibody. Neutralization of HCVcc by anti-ApoE and anti-CD81 antibodies was also determined. Antibodies blocked HCVcc infection (Fig. 4C and D), as observed with HCVtcp. These results suggest that ApoE, as well as CD81, play an important role in HCVtcp infection. Thus, HCVtcp may be more useful for evaluating the HCV entry process than HCVpp.

#### Identification of novel culture-adaptive mutation in NS3 by serial passage of HCVtcp in packaging cells

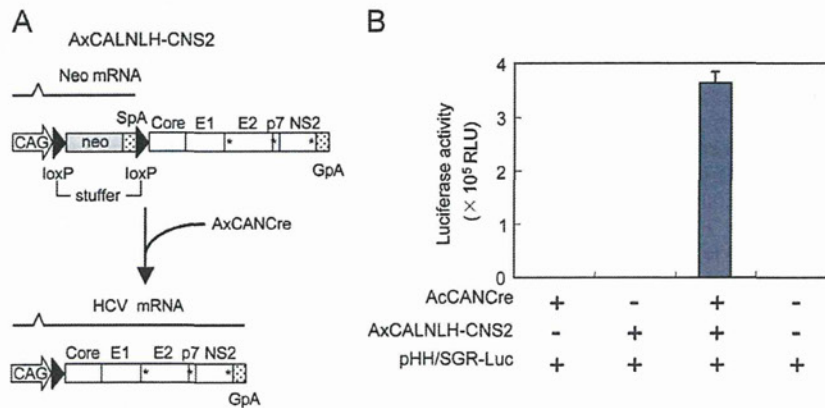
The HCVtcp system was further applied to analyses of genetic changes during serial passages in target cells. As an initial attempt, supernatants of cells co-transfected with pCAGC-NS2/JFH1am and pHH/SGR were inoculated into Huh7.5.1 cells transiently transfected with pCAGC-NS2/JFH1am. However, infectious titer was lost after repeated inoculation, likely due to low HCVtcp titers and

low efficiency of plasmid transduction (data not shown). To overcome this, we utilized recombinant adenovirus vectors (rAdVs) to provide core-NS2. As we were not able to obtain rAdV directly expressing core-NS2, conditional transgene expression based on a Cre-loxP strategy was employed (Kanegae et al., 1995). We constructed an rAdV containing core-NS2 gene downstream of a stuffer DNA flanked by a pair of loxP sites (AxCALNLH-CNS2). When cells were doubly infected with AxCALNLH-CNS2 and the Cre-expressing rAdV, AxCANCre (Kanegae et al., 1995), the Cre-mediated excisional deletion removed the stuffer DNA, resulting in core-NS2 expression under control of the CAG promoter (Fig. 5A). As expected, tightly regulated production of HCVtcp was observed. The cells infected with AxCANCre and AxCALNLH-CNS2 along with transduction of pHH/SGR-Luc produced HCVtcp at high levels. Production of HCVtcp was undetectable when either AxCANCre or AxCALNLH-CNS2 was not infected (Fig. 5B). The Cre-mediated rAdV expression system appears to have yielded considerably higher production of HCVtcp when compared with the settings for plasmid co-transfection.

Supernatants from cells in which core-NS2 was expressed using rAdVs and the subgenomic RNA derived from pHH/SGR replicated were inoculated into cells infected with AxCALNLH-CNS2 and AxCANCre (Fig. 6A). Blind passage was performed by sequentially transferring culture supernatants to cells infected with the above rAdVs. The two independent 10 blind passages (p10) showed virus titers of  $> 1 \times 10^6$  IU/mL, which were markedly higher than those of the passage 0 (p0) stock cultures ( $4 \times 10^4$  IU/mL). Side-by-side infection analysis revealed that the HCVtcp p10 #1 achieved a virus titer approximately 36 times higher than that of HCVtcp p0 on the packaging cells at 6 day post-infection (Fig. 6B). Sequencing of the entire replicon in the supernatants at p10 in two independent experiments revealed



**Fig. 4.** Effects of anti-ApoE and anti-CD81 antibodies on HCV entry. (A) Aliquots of virus sample were incubated with increasing concentrations of anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells. Luciferase activity was determined at 72 h post-infection and is expressed relative to activity without antibodies (white bar). (B) Huh7.5.1 cells were preincubated for 1 h with increasing concentrations of anti-CD81 antibodies, followed by inoculating virus samples. Luciferase activity was determined and expressed as shown in (A). (C) Aliquots of HCVcc were incubated with anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells at an MOI of 0.05. Intracellular core levels were quantitated at 24 h post-infection and are expressed relative to levels without antibodies (white bar). (D) Huh7.5.1 cells were preincubated for 1 h with anti-CD81 antibodies. HCVcc infection and measurement of core proteins were performed as indicated in (C).



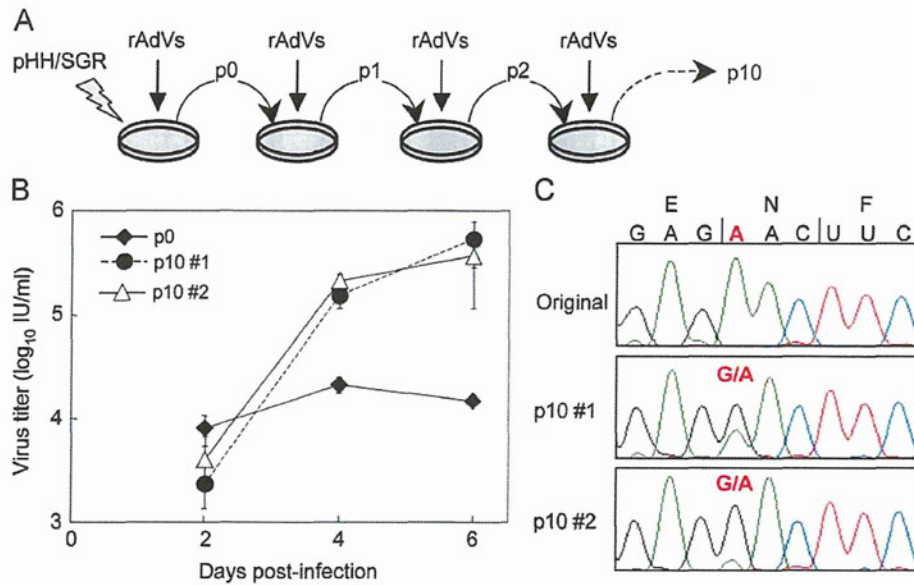
**Fig. 5.** Transgene activation mediated by rAdVs expressing Cre recombinase under control of CAG promoter. (A) Cre recombinase expressed by AxCANCre recognizes a pair of its target sequences loxP in AxCALNLH-CNS2, and removes the stuffer region resulting in expression of HCV core-NS2 polyprotein by CAG promoter. CAG: CAG promoter; SpA: SV40 early polyA signal; GpA: rabbit b-globin poly(A) signal. (B) Luciferase activity in Huh7.5.1 cells inoculated with 4-day post-transfection culture supernatant from cells transfected with pHH/SGR-Luc, and then infected with indicated rAdVs.

that both passaged HCVtcp had an identical nonsynonymous mutation in the NS3 region (N1586D) (Fig. 6C).

In order to examine the role of NS3 mutation identified on HCV RNA replication and on HCVtcp production, the N1586D mutation was introduced into pHH/SGR-Luc. Luciferase activities of the N1586D-mutated replicon were apparently lower than those of the WT-replicon, thus suggesting that the NS3 mutation reduced viral RNA replication (Fig. 7A). HCV RNA levels in the supernatants of cells transfected with WT- or mutant replicon plasmid along with pCAGC-NS2/JFH1am and luciferase activity in cells inoculated with supernatants from the transfected cells were then determined (Fig. 7B). The viral RNA level secreted from cells replicating the N1586D-mutated replicon was lower than that from cells replicating WT replicon (Fig. 7B, left). By contrast, a significantly higher infectivity of HCVtcp produced from the mutant replicon-cells was observed, as compared to WT replicon-cells (Fig. 7B, right),

suggesting that the adaptive mutation increased the specific infectivity (almost 9-fold) of the virus particles. To further determine whether the N1586D mutation affects infectious viral assembly and/or virus release, we used the CD81-negative Huh-7 subclone, Huh7-25 (Akazawa et al., 2007), which may produce infectious particles, but is not susceptible to HCV entry due to a lack of CD81 expression, therefore allowing us to examine viral assembly and release without the influence of reinfection by produced HCVtcp. Measurement of intracellular and extracellular HCVtcp indicated that Huh7-25 cells replicating the N1586D-mutated replicon produced more infectious virus than WT in both supernatants and cell lysates (Fig. 7C). Thus, it can be concluded that the N1586D mutation contributes to enhanced infectious viral assembly, not RNA replication. We could not exclude the possibility that N1586D mutation affects virus release, since the mutation enhanced extracellular virus titers more than did the intracellular titer.





**Fig. 6.** Genotypic changes in HCVtcp following blind passage. (A) Experimental procedure for blind passage of HCVtcp. Huh7.5.1 cells were transfected with pHH/SGR and were doubly infected with AxCANCre and AxCALNLH-CNS2. Culture fluids were collected and were inoculated into cells infected with AxCANCre and AxCALNLH-CNS2. These procedures were repeated 10 times with two independent samples (#1 and #2). (B) Growth curves of HCVtcp p0 and p10 on Huh7.5.1 cells expressing core-NS2. Cells were infected with HCVtcp at an MOI of 0.05, and medium was collected at the indicated time points and subjected to titration. (C) Nucleotide sequences of original and blind-passaged replicons from HCVtcp. Nucleotides of mutated position are shown in red and bold.

The impact of the N1586D mutation on production of intra- and intergenotypic HCVtcp chimeras was also investigated. The N1586D mutation in the replicon enhanced the production of chimeric HCVtcp by providing core-p7 from all strains examined, although not statistically significant in THpa, and Con1 strains (Fig. 7D). Finally, to determine whether the N1586D mutation was responsible for enhancing HCVcc production, this mutation was introduced into pHHJFH1, which carries the full-length wild-type JFH-1 cDNA (Masaki et al., 2010), yielding pHHJFH1N1586D. The virus titer obtained from cells transfected with the pHHJFH1N1586D was significantly higher than that of WT (Fig. 7E), thus demonstrating that the N1586D mutation enhances yields of HCVcc, in addition to HCVtcp.

## Discussion

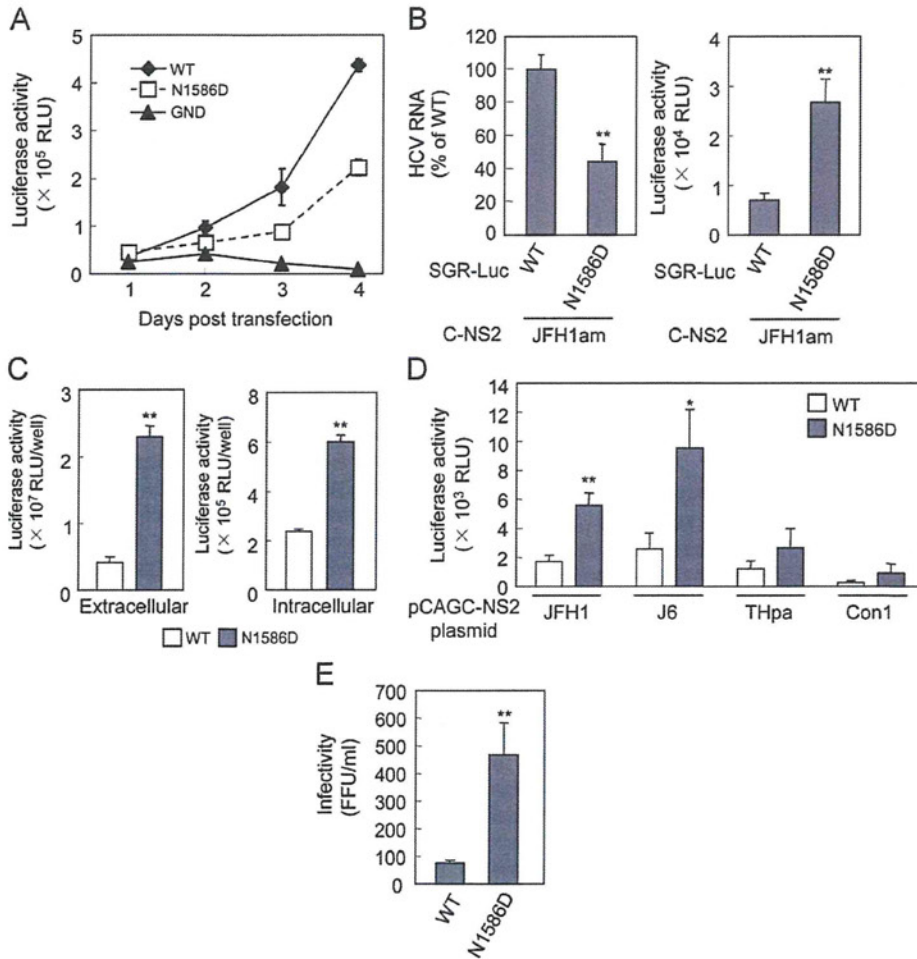
Single-round infectious viral particles generated by *trans*-packaging systems are considered to be valuable tools for studying virus life cycles, particularly the steps related to entry into target cells, assembly and release of infectious particles. However, limited HCV strains have been applied for the efficient production of HCVtcp to date. In this study, we improved the HCVtcp system in order to enhance the productivity of infectious particles. Production of chimeric HCVtcp by providing genotype 1b-derived core-p7, in addition to intragenotypic viral proteins, was also confirmed. Furthermore, we exploited the system to investigate genetic changes during serial passage of target cells and identified a novel cell culture-adaptive mutation in NS3, which also contributes to enhance the productivity of HCVtcp.

HCVpp (Bartosch et al., 2003a; Hsu et al., 2003) has proven to be a valuable surrogate system by which the study of viral and cellular determinants of the viral entry pathway is possible. Early steps of HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp (Lavie et al., 2007). However, as HCVpp is generated in non-hepatic cells such as the human embryo kidney cells 293T, it

is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc. Hepatocytes play a role in maintaining lipid homeostasis in the body by assembling and secreting lipoproteins, including VLDL. It is highly likely that HCV exploits lipid synthesis pathways, as there is a tight link between virion formation and VLDL synthesis. Down-regulation of ApoE considerably reduces HCV production (Benga et al., 2010; Chang et al., 2007; Hishiki et al., 2010; Jiang and Luo, 2009; Owen et al., 2009). Infectivity of HCVcc is also neutralized by anti-ApoE antibodies (Chang et al., 2007). These data suggest that ApoE is important for HCV infectivity. Furthermore, Niemann-Pick C1-like 1 (NPC1L1), involving cholesterol uptake receptor, was recently identified as a host factor for HCV entry (Sainz et al., 2012). Knockdown of NPC1L1 had no effect on the entry of HCVpp whereas HCVcc entry was impaired, possibly due to different cholesterol content of these particles. Here, we found that the anti-ApoE antibody neutralized infection by HCVtcp and HCVcc, but not by HCVpp (Fig. 4A and C), thus suggesting that biogenesis and/or secretion pathways of VLDL are involved in HCVtcp similarly to HCVcc, but not in HCVpp.

We also observed that infectivity of HCVtcp and HCVcc is more efficiently neutralized by the anti-CD81 antibody, as compared to that of HCVpp (Fig. 4B and D). It has recently been reported that E2 of HCVcc contained both high-mannose-type and complex-type glycans, whereas most of the glycans on HCVpp-associated E2 were complex-type, which is matured by Golgi enzymes (Vieyres et al., 2010). Mutational analysis of the N-linked glycosylation sites in E1/E2 demonstrated that several glycans on E2 may affect the sensitivity of HCVpp against antibody neutralization, as well as access of CD81 to its binding site on E2 (Helle et al., 2010). The differences in sensitivity between HCVtcp and HCVpp to neutralization by anti-CD81 antibody observed here may be due to differences in carbohydrate composition of HCV glycoproteins during expression and processing of E1/E2 in cells and morphogenesis of HCVtcp and HCVpp.

By analyzing the various replicons for *trans*-packaging, we observed the highest production of HCVtcp with replicons from pHH/SGR, which lacked sequences not essential for RNA



**Fig. 7.** Effects of N1586D mutation on RNA replication and production of HCVtvp or HCVcc. (A) RNA replication of replicons in cells transfected with pHH/SGR-Luc (WT) or N1586D mutant. Luciferase activities at 1 to 4 day post-transfection were determined. (B) Relative levels of HCV RNA in the supernatants from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am were shown in the left panel. Luciferase activities in cells inoculated with supernatants from cells transfected with indicated plasmids at 4 day post-transfection were shown in the right panel. (C) Luciferase activity in cells inoculated with supernatant and cell lysates from Huh7-25 cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am at 5 day post-transfection. (D) Luciferase activity in cells inoculated with culture supernatant from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with indicated core-NS2 plasmids at 4 day post-transfection. (E) Infectivity of supernatant from cells transfected with pHH/JFH1 (WT) or its derivative plasmid containing N1586D mutation at 6 day post-transfection. Statistical differences between WT and N1586D were evaluated using Student's *t*-test. \**p* < 0.05, \*\**p* < 0.005 vs. WT.

replication, while less efficient productivity was observed from pHH/SGR-Luc, pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am (Fig. 2C). Differences in the replication efficiency of the replicon do not appear to be a major determinant for HCVtvp productivity, at least in the present settings, as all replicon constructs except pHH/SGR-Luc replicated at similar levels, as confirmed by Western blotting (Fig. 2B). Although the shorter viral genome sequence may offer advantages over the longer sequence, further investigation is required in order to understand the molecular mechanisms underlying viral genome packaging. By comparing pHH/SGR vs. pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, it is likely that the expression of the structural protein in *cis* does not increase HCVtvp production when sufficient amounts of structural proteins are supplied in *trans*.

Blind passage of HCVtvp in packaging cells infected with rAdVs providing core-NS2 enabled us to identify a novel culture-adaptive mutation in NS3. The N-terminal third of NS3 forms a serine protease, together with NS4A, and its C-terminal two-thirds exhibits RNA helicase and RNA-stimulated NTPase activities. In addition, similarly to flaviviruses (Kummerer and Rice, 2002; Liu et al., 2002), it is now apparent that HCV NS3 is also involved in viral

morphogenesis (Han et al., 2009; Ma et al., 2008), although its precise role and underlying molecular mechanism(s) have not fully been elucidated. Two cell-culture adaptive NS3 mutations which are involved in HCV assembly have been identified. The Q1251L mutation in helicase subdomain 1 resulted in approximately 30-fold higher production of HCV without affecting NS3 enzymatic activities (Ma et al., 2008). The M1290K adaptive mutation was also located in subdomain 1 of the NS3 helicase (Han et al., 2009). The N1586D mutation identified here was located in subdomain 3 of helicase. Analogous to Q1251L and M1290K, the N1586D mutation enhanced the infectious viral assembly by increasing specific infectivity without affecting the efficiency of viral RNA replication. Considering the possibility that NS3 plays a role in linking between the viral replicase and assembly sites (Jones et al., 2011), it is likely that NS3 helicase is one of the determinants for interaction with the structural proteins. Our results, together with earlier studies, suggest that chimeric and defective mutations as well as supplying the viral components in *trans*, function as selective pressures in virion assembly.

In summary, we have established a plasmid-based reverse genetics for efficient production of HCVtvp with structural

proteins from various strains. Single-round infectious HCVtcp can complement the HCVcc and HCVpp systems as a valuable tool for the study of HCV life cycles.

## Materials and methods

### Cells

Huh7 derivative cell line Huh7.5.1 and Huh7-25 were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 10% fetal bovine serum at 37 °C in a 5% CO<sub>2</sub> incubator.

### Plasmids

Plasmids pHHJFH1, pHH/SGR-Luc, pHH/SGR-Luc/GND and pCAG/C-NS2 were as described previously (Masaki et al., 2010). In this study, plasmid pCAG/C-NS2 was designated as pCAGC-NS2/JFH. The plasmid pCAGC-NS2/JFHam having adaptive mutations in E2 (N417S), p7 (N765D), and NS2 (Q1012R) in pCAGC-NS2/JFH was constructed by oligonucleotide-directed mutagenesis. These mutations were also introduced in pHHJFH1, resulting in pHHJFH1am. To generate core-NS2 expression plasmids with different strains of HCV, the cDNA coding core to the first transmembrane region of NS2 (33 amino acids) in pCAGC-NS2/JFH was replaced with the corresponding sequence of the J6 (Lindenbach et al., 2005), H77c (Yanagi et al., 1997), THpa (Shirakura et al., personal communication) and Con1 (Koch and Bartenschlager, 1999) strains. The THpa sequence contained the P to A mutation at 328 aa at E1 in the original TH strain. To generate pHH/SGR, pHH/SGR-Luc was digested with MluI and PmeI, followed by Klenow enzyme treatment and self-ligation to delete the luciferase coding sequence. To generate pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, cDNA coding the partial core and luciferase in pHH/SGR-Luc were replaced with coding sequences for mature core (177aa), full-length core (191aa) or core-p7 polyprotein containing adaptive mutations in E2 and p7, respectively. The selected NS3 mutation (N1586D) was introduced into pHH/SGR-Luc and pHHJFH1 by oligonucleotide-directed mutagenesis.

### Generation of viruses

HCVcc and HCVtcp were generated as described previously (Masaki et al., 2010). For the production of HCVpp-2a, plasmid pcDNAdeltaC-E1-E2(JFH1)am having adaptive mutations in E2 (N417S) in pcDNAdeltaC-E1-E2(JFH1) (Akazawa et al., 2007) was constructed by oligonucleotide-directed mutagenesis. Murine leukemia virus pseudotypes with VSV G glycoprotein expressing luciferase reporter (VSVpp) were generated in accordance with previously described methods (Akazawa et al., 2007; Bartosch et al., 2003a).

### Luciferase assay

Huh7.5.1 cells were seeded onto a 24-well plate at a density of  $3 \times 10^4$  cells/well 24 h prior to inoculation with reporter viruses. Cells were incubated for 72 h, followed by lysis with 100 µL of lysis buffer. Luciferase activity of the cells was determined using a luciferase assay system (Promega, Madison, WI). All luciferase assays were performed in triplicate.

### Quantification of HCV infectivity and HCV RNA

To determine the titers of HCVtcp and HCVcc, Huh7.5.1 cell monolayers prepared in multi-well plates were incubated with dilutions of samples and then replaced with media containing 10% FBS and 0.8% carboxymethyl cellulose. Following incubation for 72 h, monolayers were fixed and immunostained with rabbit polyclonal anti-NS5A antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen), and stained foci or individual cells were counted and used to calculate a titer of focus-forming units (FFU)/mL for spreading infections or infectious units (IU)/mL for non-spreading infections. For intracellular infectivity, the cell pellet was resuspended in culture media, and cells were lysed by four freeze–thaw cycles. Cell debris was pelleted by centrifugation for 5 min at 4000 rpm. Supernatant was collected and used for titration. To determine the amount of HCV RNA in culture supernatants, RNA was extracted from 140 µL of culture medium by QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37 °C for 1 h. Extracted RNA was further purified by using an RNeasy Mini Kit, which includes RNase-free DNase digestion (QIAGEN). Copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously (Wakita et al., 2005).

### Antibodies

Mouse monoclonal antibodies against actin (AC-15) and CD81 (JS-81) were obtained from Sigma (St. Louis, MO) and BD Biosciences (Franklin Lakes, NJ), respectively. Goat polyclonal antibody to ApoE (LV1479433) was obtained from Millipore (Tokyo, Japan). Anti-NS5A and anti-NS5B antibodies were rabbit polyclonal antibody against synthetic peptides.

### Neutralization assay

For neutralization experiments with anti-CD81 antibody, Huh7.5.1 cells were incubated with dilutions of anti-CD81 antibody for 1 h at 37 °C. Cells were then infected with viruses for 5 h at 37 °C. For neutralization experiments with anti-ApoE antibody, viruses were incubated with various concentrations of anti-ApoE antibody at room temperature for 1 h and cells were infected with viruses for 5 h at 37 °C. Following infection, supernatant was removed and cells were incubated with culture medium, and luciferase activity was determined at 3 day post-infection for HCVtcp and pseudotyped viruses. For neutralization experiments with HCVcc generated with pHHJFH1am, a multiplicity of infection (MOI) of 0.05 was used for inoculation, and intracellular core protein levels were monitored by ELISA (Ortho Clinical Diagnostics) at 24 h post-infection.

### Immunoblotting

Transfected cells were washed with PBS and incubated with lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% Triton X-100). Lysates were then sonicated for 5 min and were added to the same volume of SDS sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE, and transferred to PVDF membrane. After blocking, membranes were probed with first antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; PIERCE, Rockford, IL), in accordance with the manufacturer's protocols.

### Generation of recombinant adenoviruses

rAdV, AxCANCre, expressing Cre recombinase tagged with nuclear localization signal under CAG promoter was prepared as described previously (Baba et al., 2005). The target rAdV AxCALNLH-CNS2 expressing HCV core-NS2 polyprotein with adaptive mutations in E2, p7 and NS2 was generated as follows. Cosmid pAxCALNLwit2 is identical to pAxCALNLw (Sato et al., 1998), except that both the terminal sequences of the rAdV genome are derived from pAxCAwit2 (Fukuda et al., 2006). The core-NS2 fragment obtained from pCAGC-NS2/JFH1am by StuI-EcoRI digestion and subsequent Klenow treatment was inserted into the Swal site of pAxCALNLwit2. The resultant cosmid pAx-CALNLH-CN2it2 was digested with PacI and transfected into 293 cells to generate rAdV AxCALNLH-CNS2.

### Preparation of packaging cells for HCVtcp

Huh7.5.1 cells were coinfectd with AxCANCre at an MOI of 1 and AxCALNLH-CNS2 at an MOI of 3 for expression of JFH-1 core-NS2 polyprotein containing the adaptive mutations in E2, p7 and NS2.

### RNA preparation, RT-PCR and sequencing

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription with random hexamer and Superscript III reverse transcriptase (Invitrogen). Three fragments of HCV cDNAs that cover the entire HCV subgenomic replicon genome, were amplified by nested PCR with TaKaRa Ex Taq polymerase (Takara, Shiga, Japan). Amplified products were separated by agarose gel electrophoresis, and were used for direct DNA sequencing.

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