Antiviral Research 171 (2019) 104612

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Construction and characterization of Genotype-3 hepatitis C virus replicon revealed critical genotype-3-specific polymorphism for drug resistance and viral fitness

Mingzhe Guo[a,b,c], Jie Lu[d], Tianyu Gan[a,c], Xiaogang Xiang[d], Yongfen Xu[a], Qing Xie[d], Jin Zhong[a,b,c,*]

[a] CAS Key Laboratory of Molecular Virology and Immunology, Unit of Viral Hepatitis, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, 200031, China
[b] Shanghai Tech University, Shanghai, 201210, China
[c] University of Chinese Academy of Sciences, Beijing, 100049, China
[d] Department of Infectious Disease, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

ARTICLE INFO

Keywords:
Hepatitis C virus
Genotype-3
Replicon
Direct-acting antiviral agents
Antiviral resistance

ABSTRACT

Hepatitis C virus (HCV), a major causative agent of chronic hepatitis, is a positive-stranded RNA virus and has a high degree of genetic diversity due to its error-prone RNA-dependent RNA polymerase. Development of direct-acting antiviral agents (DAAs) has greatly improved the therapeutic outcome of chronic hepatitis C patients. However, naturally existing resistance-associated variants (RAVs) or occurrence of resistance-associated substitutions (RASs) in the HCV genome may impose a challenge to the long-term success of the DAA-based therapies. Genotype-3 HCV is the most difficult genotype to treat by DAAs, but the underlying molecular mechanisms remain to be explored. Here we developed a novel genotype-3a subgenomic replicon PR87A7 by screening a HCV cDNA pool amplified from a patient serum RNA. PR87A7 replicon displayed strong resistance to anti-NS3 DAAs, mainly owing to a genotype-3-specific polymorphism 168Q in NS3. Introduction of NS3 168Q into a genotype-2a JFH1 strain rendered resistance to anti-NS3 DAAs while greatly diminished the viral replication, and yet this fitness defect can be rescued by additional genotype-3-specific polymorphism. In conclusion, we developed a novel genotype-3a subgenomic replicon by a functional screening approach, and revealed genotype-3-specfic amino acid residues that confer resistance to anti-NS3 DAAs while retaining viral fitness.

1. Introduction

HCV is an enveloped, positive-strand RNA virus in the *Flaviviridae* family. Its 9.6-kb single-stranded genome consists of a single ORF flanked by highly conserved 5′- and 3′-untranslated regions (UTRs). Translation of the ORF produces a polyprotein precursor, which is in turn cleaved by cellular and viral proteases into the structural proteins (core, E1 and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Grakoui et al., 1993). The nonstructural proteins from NS3 to NS5B are essential and sufficient for viral genome replication. HCV causes persistent infection in about 3% of the global population, which may develop liver cirrhosis and hepatocellular carcinoma (HCC) (Hajarizadeh et al., 2013). There are seven major HCV genotypes and over 50 subtypes. Genotype-3 represents 22–30% of all infection, trailing behind genotype-1 and is distributed in South and Central Asia (Messina et al., 2015). Genotype-3 HCV infection is typically associated with moderate-to-severe steatosis, more rapid progression to severe liver diseases and higher risk of HCC development (Nkontchou et al., 2011; Probst et al., 2011; Shrivastava et al., 2016).

Development of highly effective direct-acting antiviral agents (DAAs) has vastly improved prognosis of HCV patients (Pawlotsky, 2014). These DAAs target 3 different viral proteins: NS3/4A protease that is responsible for cleavage of HCV polypeptide (Morikawa et al., 2011); NS5A that does not possess any known enzymatic activity, but is essential for HCV genome replication and virion assembly (Gao et al., 2016; Link et al., 2019). NS5B is RNA-dependent RNA polymerase and catalyzes viral replication (Das and Pandya, 2018; Svarovskaia et al., 2014). While current IFN-free DAA regiments are potent and convenient, natural existence of resistance-associated variants (RAV) or emergence of resistance-associated substitutions (RAS) during the DAA

---

[*] Corresponding author. Institut Pasteur of Shanghai, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai, 200031, China.
E-mail address: jzhong@ips.ac.cn (J. Zhong).

https://doi.org/10.1016/j.antiviral.2019.104612
Received 17 August 2019; Received in revised form 17 September 2019; Accepted 18 September 2019

treatment can lead to the failure of therapies (Sarrazin et al., 2016). Sustained virologic response (SVR) rates of genotype-3 patients receiving DAA-based therapies are considerably lower than that of other genotypes (Chan et al., 2017). Sofosbuvir/Voxilaprevir/Velpatasvir cocktail or Glecaprevir/Pibrentasvir are currently approved DAA regimens for genotype 3 HCV infection (Stamm et al., 2019). Glutamine at position 168 of NS3 (168Q) is a genotype-3-specific RAV for anti-NS3 inhibitors, whereas other genotypes have DAA-sensitive aspartic acid at this position (168D) (Soumana et al., 2016). It remains elusive why 168Q only naturally occurs in genotype-3 but not in other HCV genotypes. Furthermore, genotype-3 HCV displays lower SVR to the Sofosbuvir-based therapies, suggesting genotype-3 may have a lower genetic barrier to Sofosbuvir resistance than other genotypes (Ramirez et al., 2016; Wing et al., 2019). All these clinical issues justify more research in genotype-3 HCV.

HCV subgenomic replicon, a self-replicating RNA consisting of HCV nonstructural genes and a selectable marker, is the first cellular model to study HCV replication, and has made significant contributions to anti-HCV drug development (Blight et al., 2000; Lohmann et al., 1999). The first robust *in-vitro* HCV infection culture model (HCVcc), based on a genotype-2a clone JFH1, recapitulates the entire viral life cycle (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Conventionally, construction of these models is mainly dependent on the consensus viral sequences which may not exist naturally and do not reflect the complexity of viral quasispecies, the actual form that HCV circulates in a single patient. In previous studies, we developed a functional screening method to establish HCV cell culture model (Lu et al., 2013, 2014). Instead of using the consensus viral sequences, HCV cDNA clones can be selected functionally from a cDNA pool directly constructed from patient sera HCV RNA, which better recapitulates the complexity of quasispecies in clinical isolates. Here we sought to use this strategy to construct a subgenomic replicon from a genotype-3 patient, and to characterize its response to a spectrum of DAAs.

2. Materials and methods

2.1. Anti-HCV reagents

Asunaprevir, Simeprevir, Grazoprevir, Paritaprevir, Daclatasvir, and Sofosbuvir were purchased from MedChem Express (Monmouth Junction, New Jersey, USA). Telaprevir was purchased from Selleck Chemicals (Houston, TX, USA). All the compounds were dissolved in dimethylsulfoxide (DMSO). Recombinant human IFN-α2b was purchased from Hoffmann-La Roche (Basel, Switzerland).

2.2. HCV patient serum

Genotype-3a PR87 serum (4.3×10^6 IU/ml) was collected from a chronic hepatitis C patient at Department of Infectious Diseases of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. Serum RNA isolation and reverse transcription were performed as previously described (Lu et al., 2013). The PR87 consensus sequence was acquired by bulk sequencing and deposited to the NCBI genebank (Accession number HQ912953). The study protocol was approved by the Ethics Committee of Shanghai Ruijin Hospital in accordance with the Helsinki Declaration, and written informed consent was obtained.

2.3. Cell culture

Huh7.5.1-Sec14L2 cells were described previously (Li et al., 2018). All cells were maintained in complete Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 mg of streptomycin/ml (Invitrogen). All cells ere cultured in humidified air containing 5% CO_2 (v/v) at 37 °C.

2.4. Plasmid and HCV cDNA pool construction

HCV cDNA was amplified with Q5 DNA polymerase (NEB, Ipswich, MA, USA). The 5′UTR of PR87 was amplified from the serum RNA, while a genotype-3 consensus 3′UTR (Accession number: JN652211) was synthesized by GenScript (Nanjing, China). The UTR fragments were ligated into a pUC15 backbone with a T7 promoter at the 5′-end and self-cleaving ribozyme followed by a T7 terminator at the 3′-end (Kato et al., 2007). Non-structural protein-coding region (NS3-NS5B) was amplified from the PR87 serum RNA in three overlapping fragments using the following primer sets by nested PCR. Fragment 1: Outer forward, 5′-TTGGCGACTGTTGGCCCCGATTAC-3’; Outer reverse, 5′-GCAAGGTGGAGAGGCCTGCTAGGTATTG-3’; Inner forward, 5′-GCCCCGATTACAGCATACACCCAGC-3’; Inner reverse, 5′-GGTGGCTACTATGGGCTCAATGACAGC-3’. Fragment 2: Outer forward, 5′-GCGAGCTACCCAGCAGCAAGCTGTC-3’; Outer reverse, 5′-TAGAGCAGCAGACCACGCTCTGCTCC-3’; Inner forward, 5′-ATTGAGCCCATAGTAGCCACCAACTGG-3’; Inner reverse, 5′-CGCTAACAGTGGACCAAGAGTCACAAC-3’. Fragment 3: Outer forward, 5′-CCAGACTTGAGTTGTGACTCTTGGTCC-3’; Outer reverse, 5′-TCACCGTGCTGGCAGGAGAAAGATGC-3’; Inner forward, 5′-TGACTCTTGGTCCACTGTTAGCGATAG-3’; Inner reverse, 5′-GCAGGAGAAAGATGCCTACCCCTAC-3’. The three cDNA fragments were ligated into a HCV replicon backbone by Gibson Assembly® Cloning Kit (NEB). Individual point mutations were introduced by QuikChange II XL site-directed mutagenesis kit (Agilent technologies, Santa Clara, California). All constructs were verified by DNA sequencing. New HCV sequences were deposited to the NCBI Genbank as follows: the 5′UTR of PR87 (MN231293), PR87A (MN231294), PR87A7 (MN231295).

2.5. RNA extraction, in-vitro transcription, RNA electroporation and HCV replicon cell selection

These assays were performed as previously described (Cao et al., 2017; Tao et al., 2009). The antibodies used in Western blot, rabbit anti-HCV NS5A, mouse anti-β-actin, Goat-anti Mouse HRP antibody and Goat-anti Rabbit HRP antibody, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.6. Direct-acting antivirals treatment assay

Serially diluted anti-HCV compounds were added to about 1.5×10^4 HCV replicon cells seeded in 48-well plates for overnight. Three days after treatment, total cellular RNA was extracted from the cells. HCV RNA were determined by reverse transcription-quantitative PCR (RT-qPCR) (Cao et al., 2017; Tao et al., 2009, 2017), expressed as the percentage of mock treatment. The median 50% inhibition concentration (IC50) was calculated as the concentration of inhibitor required for a 50% reduction in the HCV RNA level, and the dose response curves were plotted using GraphPad Prism 7.0.

2.7. Phylogenetic analysis and sequence alignment

The complete viral genomes of various prototype HCV strains were downloaded from NCBI GenBank, and the non-structural protein-coding region of each sequence was aligned in AlignX module of Vector NTI advance 11 (Invitrogen). The phylogenetic tree was constructed using the Neighbor Joining Method. The consensus amino acid sequences of NS3 protease domain of each HCV genotype were generated from 3738 genotype-1, 210 genotype-2, 382 genotype-3, 90 genotype-4, 14 genotype-5 and 106 genotype-6 sequences from the Los Alamos HCV sequence database respectively (www.lanl.gov).

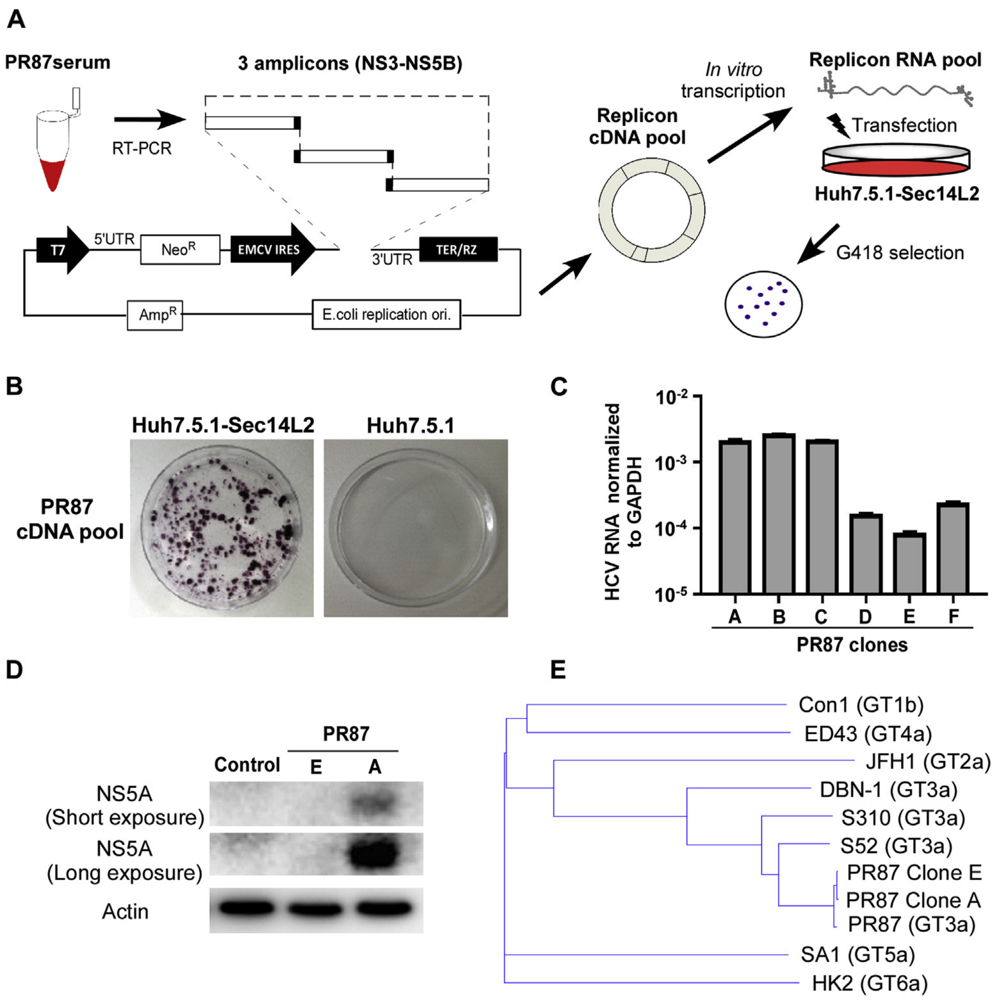


Fig. 1. Development of PR87 subgenomic replicon by screening of a cDNA pool constructed from a genotype-3 clinical isolate.
(A) The protocol to construct the HCV cDNA pool and to select subgenomic replicon cells. **(B)** Crystal violet staining of Huh7.5.1-Sec14L2 and parental Huh7.5.1 cells transfected with PR87 subgenomic replicon RNA pool. **(C)** HCV RNA levels in different PR87 replicon cell clones were determined by RT-qPCR, and presented as values relative to the cellular GAPDH mRNA level. The error bars represented 3 independent experiments. **(D)** Western blot analysis of HCV NS5A protein in isolated PR87 replicon cell clone A and E. Images with longer (10s) and shorter (0.1s) exposure were shown. **(E)** Phylogenetic analysis of PR87 replicon clones together with prototype HCV strains of other genotypes.

3. Results

3.1. Construction of HCV genotype-3a subgenomic replicon

We previously developed a method to construct functional HCVcc cDNA clones by screening a cDNA pool amplified from patient serum HCV RNA (Lu et al., 2013, 2014). In this study we aimed to use the similar approach to construct a genotype-3 HCV subgenomic replicon. The serum specimen (denoted PR87) from a patient chronically infected with genotype-3a HCV, was used for the study. The experimental protocol was illustrated in Fig. 1A. The 5′UTR of PR87 was amplified from the patient serum RNA by RT-PCR and then subcloned. Because the HCV 5′UTR is highly conserved among genotypes, the 5′UTR subclone had an identical sequence to the 5′UTR of PR87 consensus sequence. The 3′UTR of PR87 was chemically synthesized and cloned based on genotype-3 consensus 3′UTR sequence. The 5′ and 3′ UTRs were then cloned into a previously developed HCV subgenomic replicon vector that consists of a neomycin-resistant selectable marker (neo^R), the internal ribosomal entry site (IRES) of encephalomyocarditis virus (EMCV) mediating translation of HCV non-structural proteins and self-cleaving ribozymes flanking the 3′ UTR (Fig. 1A) (Kato et al., 2003, 2007). Non-structural HCV protein-coding region (NS3-NS5B) of PR87 was divided into 3 overlapping fragments (position: fragment 1, 3097–5274; fragment 2, 5254–7262; fragment 3, 7239–9056), and each fragment was amplified by nested RT-PCR from the patient serum RNA. The pool of 3 fragments was ligated into the linearized vector by *in-vitro* homologous recombination with 25 bp-homologous arms. The resultant recombinant plasmid pool was then used as the template to generate a pool of HCV subgenomic RNA by *in-vitro* transcription, which was electroporated into parental Huh7.5.1 cells as well as Huh7.5.1 cells stably expressing Sec14L2 (Li et al., 2018) which is a vitamin E-binding protein and had been shown to support HCV replication without tissue culture-adaptive mutations (Saeed et al., 2015). After 3 weeks of G418 selection, colony formation was observed in Huh7.5.1-Sec14L2 cells but not in Huh7.5.1 cells (Fig. 1B), suggesting that PR87 replication was critically dependent on the Sec14L2 expression. Six individual HCV replicon colonies were isolated, and their HCV RNA levels were quantified by RT-qPCR. As shown in Fig. 1C, clone A, B and C had a much higher HCV RNA level than clone D, E and F. Consistently, the NS5A protein expression can be detected in clone A but not in clone E (Fig. 1D). Phylogenetic analysis indicated that both clone A and E were closely related to the PR87 consensus sequence, and fell into cluster of genotype-3a with previously reported strains S310 (Saeed et al., 2013), S52 (Gottwein et al., 2010) and DBN (Ramirez et al., 2016) (Fig. 1E).

Sequencing results showed that six PR87 clones possessed distinct viral mutations and none of these mutations was shared by the all clones (Fig. 2A). Compared with the consensus sequence obtained from PR87 serum RNA, clone A (referred as PR87A), one of the clones with the highest HCV RNA replication, had 8 amino acid changes, 6 of which (Q1341H, V1729I, F1879L, S1976N, T2042S, A2765T) could be traced as minor quasispecies in the original PR87 serum HCV RNA as they appeared as a minor peak in the bulk-sequencing chromogram of the PR87 clinical isolate (Fig. 2A). Two other changes, T1286I in NS3 and S2861A in NS5B, could not be identified in bulk-sequencing chromogram of the PR87 serum RNA, and may be cell-culture adaptive mutations acquired during the selection and/or subculture of PR87A

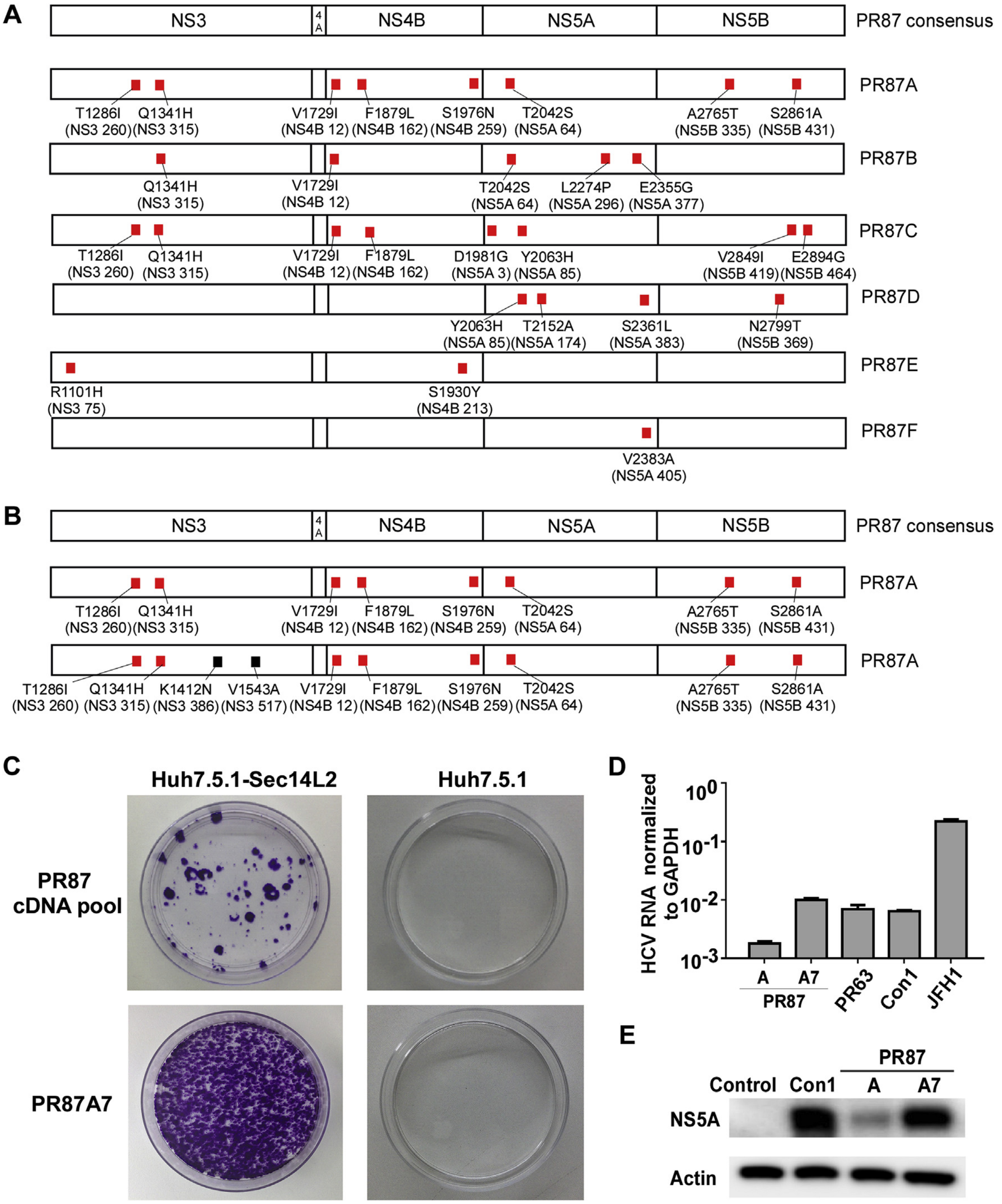


Fig. 2. Subcloning of PR87A resulted in a more actively replicating replicon.
Schematic diagram of sequence comparison of PR87 consensus and six PR87 replicon clones **(A)** and sequence comparison of PR87 consensus, PR87A and PR87A7 **(B)**. The mutations in each PR87 clone or PR87A7 as compared to the PR87 consensus were labeled in red square, and two additional mutations in PR87A7 as compared to PR87A were labeled in black square. The amino acids were numbered according to their positions in the full viral polyprotein, and their relative positions within each viral protein were also indicated in parenthesis. **(C)**Crystal violet staining of Huh7.5.1-Sec14L2 and parental Huh7.5.1 cells transfected with PR87A7 or original PR87 pool replicon RNA. **(D)** HCV RNA levels in PR87A and PR87A7 replicon as well as other subgenomic replicon (PR63, Con1 and JFH1) were determined by RT-qPCR, and presented as values relative to the cellular GAPDH mRNA level. The error bars represented 3 independent experiments. **(E)** Western blot analysis of HCV NS5A protein in PR87A, PR87A7 and Con1 subgenomic replicon cells.

replicon cells. Interestingly, T1286I in NS3 was previously identified as an adaptive mutation that enhances replication of genotype-3 strain S310 (Saeed et al., 2013). It's worth noting that two mutations (Q1341H and V1729I) were also shared by clone B and C, both of which had the high HCV RNA levels, suggesting that these two mutations may be important for HCV replication. Next we sought to obtain the cDNA clone from the PR87A replicon cells. Total cellular RNA from the PR87A replicon cells was extracted, and HCV non-structural protein-coding sequence was amplified in 3 fragments following the same protocol described in Fig. 1A, and ligated into the replicon backbone for subcloning. One PR87A cDNA subclone was obtained and designated as PR87A7. Sequencing analysis showed that the PR87A7 cDNA clone gained two more amino acid changes (K1412N and V1543A both in NS3) compared to the PR87A sequence (Fig. 2B). Interestingly, K1412N was identified as a minor species in the sequencing chromogram of PR87A RNA, suggesting that it may be a culture-adaptive mutation. V1543A could not be identified in the sequencing chromogram of PR87A, and may either be a very rare species in PR87A replicon RNA or have been introduced during subcloning. The *in-vitro* transcribed HCV subgenomic RNAs from the PR87A7 cDNA and the original PR87 cDNA pool were electroporated into Huh7.5.1-Sec14L2 and control Huh7.5.1 cells respectively. As shown in Fig. 2C, PR87A7 RNA transfection formed significantly more colonies in Huh7.5.1-Sec14L2 cells than the original PR87 cDNA pool, and neither formed any colony in Huh7.5.1 cells. The G418-resistant colonies of PR87A7 were pooled and expanded, and these cells were referred as PR87A7 replicon. Sequencing of HCV RNA from PR87A7 replicon cells confirmed that no more additional amino acid change was found as compared to the PR87A7 cDNA subclone. Interestingly, PR87A7 replicon cells had higher HCV RNA (Fig. 2D) and NS5A protein levels (Fig. 2E) than PR87A cells, suggesting that two additional mutations K1412N and V1543A in NS3 of PR87A7 may boost HCV replication. HCV RNA levels in PR87A7 replicon cells were comparable to genotype-1b Con1 (Krieger et al., 2001) and genotype-2a PR63 (Lu et al., 2014), but lower than JFH1 replicon (Kato et al., 2003). Western blot showed that PR87A7 and Con1 had comparable NS5A protein level. Of notes, the NS5A antibody used in Western blot assay does not react with NS5A of genotype-2a strains PR63 and JFH1 (data not shown).

3.2. *Genotype-3-specific 168Q in NS3 confers resistance to anti-NS3 DAAs*

Three classes of anti-HCV DAAs as well as IFNα were tested on PR87A7 replicon along with JFH1 replicon as a control. The HCV RNA levels were measured by RT-qPCR (Fig. 3), and IC50 values were determined and presented in Table 1. As expected, both replicons were sensitive to the IFNα treatment (Fig. 3A) as well as to anti-NS5A DAA Daclatasvir (Fig. 3B) and anti-NS5B DAA Sofosbuvir (Fig. 3C). The IC50 values of Daclatasvir and Sofosbuvir against PR87A7 were slightly higher than those against JFH1 (3.5 and 2.5 folds respectively) (Table 1). In contrast, anti-NS3 DAAs were much less potent against PR87A7 than JFH1 (Fig. 3D–H). The IC50 values of Telaprevir, Simeprevir, Asunaprevir, Paritaprevir and Grazoprevir against PR87A7 were 26.8, 165.0, 181.8, 363.3 and 51.2-fold higher than those against JFH1 (Table 1). This inefficient inhibition on PR87 by anti-NS3 DAAs is consistent with previously reported *in-vitro* (Soumana et al., 2016) and clinical studies (European Association for the Study of the Liver. Electronic address and European Association for the Study of the, 2018) of genotype-3 HCV.

Previous structural analysis revealed three critical amino acid residues (168Q, 123T, 132L) in NS3 of genotype-3 that are in close contact with NS3 protease inhibitors (Soumana et al., 2016). Next we analyzed how these amino acid residues contribute to the sensitivity of PR87A7 replicon to anti-NS3 DAAs. Since PR87A7 shares the same amino acid at position 132 of NS3 (132L) with JFH1 and most genotype-2 strains, we focused on positions of 168 and 123 of NS3. Glutamine at position 168 and threonine at position 123 in PR87A7 were respectively changed to aspartic acid and arginine that were frequently found at these positions in other HCV genotypes. The resultant mutant replicon RNA (Q168D and T123R) was transfected into Huh7.5.1-Sec14L2 cells followed by G418 selection. After the replicon cells were obtained, their sensitivity to anti-NS3 DAAs was determined by RT-qPCR. As shown in Fig. 4 and Table 2, Q168D completely abolished the resistance of PR87A7 against Simeprevir, Asunaprevir, Paritaprevir and Grazoprevir, while T123R barely had any effect, consistent with previous reports that Q168 is the major genotype-3-specific determinant for anti-NS3 DAA resistance.

3.3. *Active replication of HCV containing 168Q in NS3 requires additional genotype-3-specific polymorphisms*

NS3 168Q is highly conserved among genotype-3, but never found naturally in any other HCV genotypes which all have aspartic acid at this position (168D). Upon anti-NS3 DAA treatment, non-genotype-3 HCV can acquire a mutation at the position 168 in NS3 (for example, D168V and D168A), but the mutations always revert to the wild type after the termination of DAA treatment (Lontok et al., 2015), suggesting that the RAS at this position of non-genotype-3 HCV likely reduces viral fitness. However, genotype-3 HCV containing 168Q replicates well, raising a possibility that additional genotype-3-specific polymorphisms may offset the fitness defect of 168Q so that 168Q can be retained stably in the genome of genotype-3. To test whether the known genotype-3-specific polymorphism (NS3 123T) plays any roles in maintaining fitness in the context of 168Q, the D168Q single mutation, D168Q/R123T double mutations or the entire NS3protease/4A domain of PR87A7 were introduced into a JFH1 subgenomic replicon with neomycin resistance and luciferase cassettes (Gottwein et al., 2011; Kato et al., 2003). The *in-vitro* transcribed mutant or wild-type replicon RNA were electroporated into Huh7 cells, and HCV replication was determined by measuring the increase of luciferase activity between 4 and 48 h after transfection. As shown in Fig. 5A, D168Q greatly diminished JFH1 replication, while this defect was partially rescued by the R123T mutation or fully rescued when entire NS3/4A protease domain was swapped. This result was in line with structural evidences that hydrogen bonds between NS3 site 168 and 123 are important for stability of electrostatic network vital for substrate binding (Soumana et al., 2016). To determine which additional GT-3 specific polymorphism(s) in the NS3 protease domain contribute to replication fitness, we compared the consensus sequence of NS3 protease domain from genotypes-1 to −6 (Fig. 5B). Eighteen genotype-3-specific amino acids, defined as the ones appearing only in the genotype-3 consensus sequence but not in the consensus sequence of other genotypes, were selected to further analyze their frequencies among 382 individual genotype-3 sequences obtained from the Los Alamos HCV sequence database. Of notes, 11 of these amino acids were highly conserved among genotype-3 (frequency over 95% as highlighted in red in Fig. 5B). Therefore, next we determined their effects on HCV replication in the context of JFH1-D168Q/R123T. Eight point mutations with highly conserved genotype-3-specific polymorphisms (E28V, A30T, S49G, V71A, T72L, S133A, T134C, V178Q) were introduced into JFH1-D168Q/R123T (S47T was not tested because JFH1 happens to have theronine at this position). Interestingly, none of these individual mutations could further increase HCV replication level (Fig. 5C), suggesting that full restoration of JFH1-D168Q/R123T replication level may require collective contributions of these genotype-3-specific polymorphisms in the NS3/4A protease domain.

Next we obtained JFH1 subgenomic replicon cells that stably replicate HCV RNA harbouring the D168Q single mutation or D168Q/R123T double mutations. Sequencing analysis indicated that the single or double mutations were retained in the replicon cells and no additional mutation was identified in the viral genome, suggesting the introduced mutations can be tolerated by JFH1, an HCV strain with an extraordinary replicating ability. Quantification of HCV RNA levels in these stable JFH1 replicon cells indicated that D168Q significantly diminished viral replication, while this defect can be partially rescued by R123T mutation (Fig. 5D), consistent with the results of transient transfection assay in Fig. 5B.

Finally, we analyzed the sensitivity of JFH1 subgenomic replicon harbouring the D168Q and R123T double mutation to anti-NS3 DAAs. As shown in Fig. 6 and Table 3, JFH1-D168Q/R123T was more resistant to Simeprevir, Asunaprevir, Paritaprevir and Grazoprevir than the wild-type JFH1.

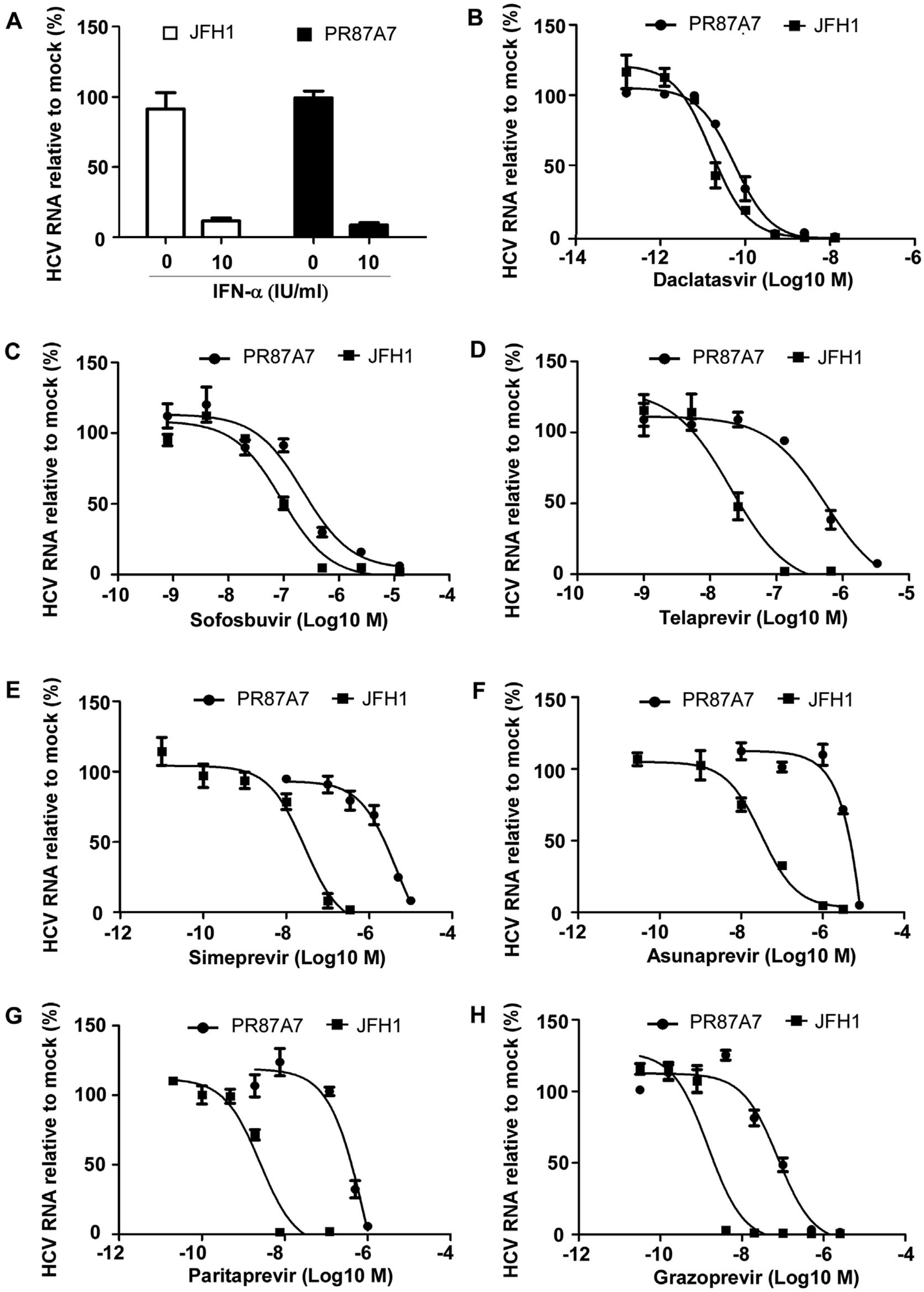


Fig. 3. Antiviral sensitivity analysis of PR87A7 replicon.
PR87A7 and JFH1 replicon cells were treated with 10 IU/ml of IFN-a (**A**), or serially diluted anti-HCV agents Daclatasvir (**B**), Sofosbuvir (**C**), Telaprevir (**D**), Simeprevir (**E**), Asunaprevir (**F**), Paritaprevir (**G**) and Grazoprevir (**H**) for 3 days. The intracellular HCV RNA levels were quantified by RT-qPCR, normalized to cellular GAPDH mRNA levels and expressed as the percentage to the mock-treatment control. Concentration-response curves were plotted in GraphPad Prism 7.0. The error bars were produced from 2 independent experiments.

4. Discussion

The conventional approach to construct HCV cell culture models is based on the consensus viral sequence which is normally compiled from sequencing of patient serum HCV RNA. Although the consensus sequence has the most likely nucleotide at each position of HCV genome, it may not actually exist in nature and does not reflect the complexity of HCV quasispecies within clinical specimen. The cDNA clones built on the consensus sequence does not always produce a viable cell culture model. Here we used a new approach to establish a novel genotype-3a subgenomic replicon by screening a HCV cDNA pool amplified from the patient serum. Our approach not only alleviates the prerequisite of the

Table 1
IC50 values of DAAs against PR87A7 and JFH1.

DAA (antiviral target)	PR87A7 IC50	JFH1 IC50	Fold Change (PR87/JFH1)
Daclatasvir (NS5A)	56.07 ± 8.07 pM	15.91 ± 1.94 pM	3.5
Sofosbusvir (NS5B)	226.82 ± 41.17 nM	92.42 ± 13.53 nM	2.5
Telaprevir (NS3)	549.64 ± 131.08 nM	20.51 ± 7.94 nM	26.8
Simeprevir (NS3)	4520.16 ± 1710.88 nM	27.43 ± 5.92 nM	165.0
Asunaprevir (NS3)	5670.79 ± 1241.73 nM	31.22 ± 3.40 nM	181.8
Paritaprevir (NS3)	922.87 ± 274.37 nM	2.54 ± 0.61 nM	363.3
Grazoprevir (NS3)	76.35 ± 12.81 nM	1.49 ± 0.40 nM	51.2

IC50 values are shown with 95% confidence intervals.

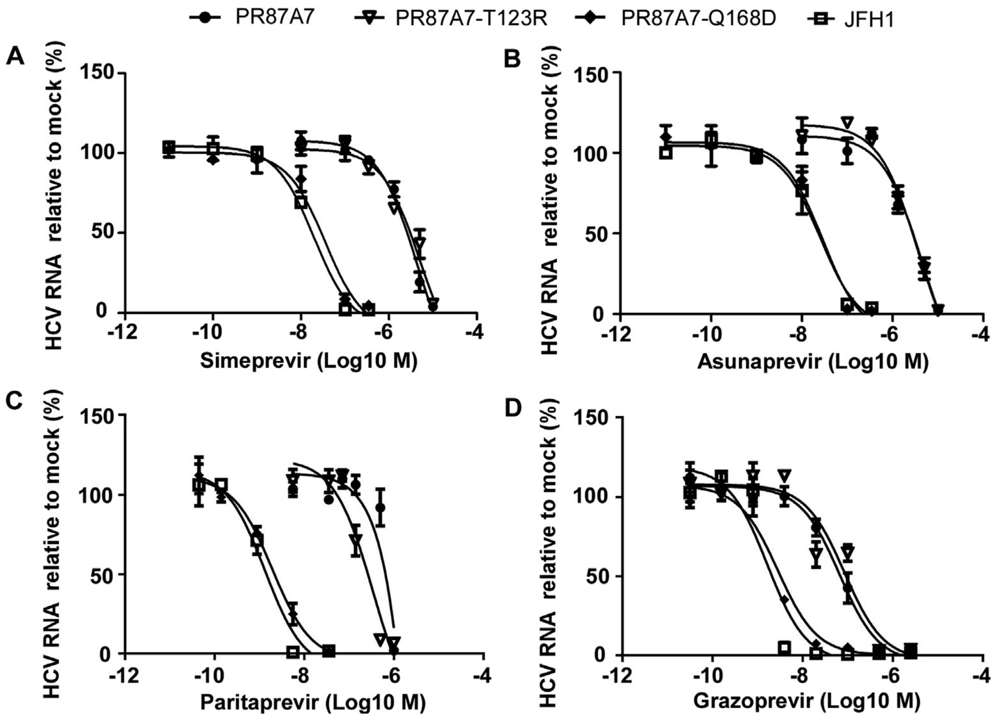


Fig. 4. NS3 168Q is the major determinant of genotype-3 HCV responsible for resistance to anti-NS3 DAA.
The wild-type PR87A7, two mutant PR87A7 (Q168D or T123R) and JFH1 replicon cells were treated with serially diluted Simeprevir (**A**), Asunaprevir (**B**), Paritaprevir (**C**) and Grazoprevir (**D**) for 3 days. The intracellular HCV RNA levels were quantified by RT-qPCR, normalized to cellular GAPDH mRNA levels and expressed as the percentage to the mock-treatment control. Concentration-response curves were plotted in GraphPad Prism 7.0. The error bars were produced from 2 independent experiments.

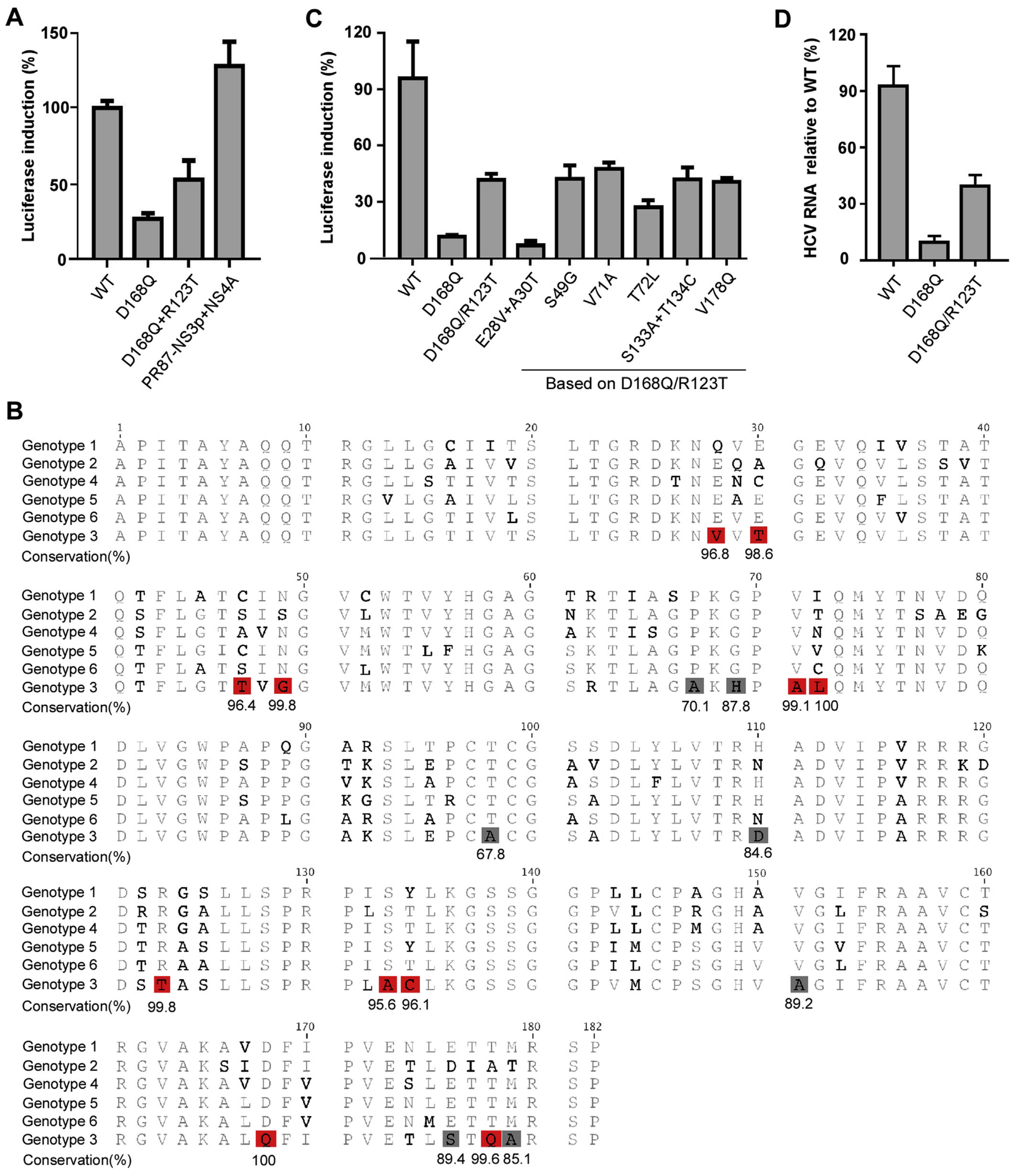
Table 2
IC50 values of anti-NS3 DAAs against PR87A7 and its derivatives.

DAA	PR87A7-T123R IC50	PR87A7-Q168D IC50	PR87A7 IC50	JFH1 IC50
Simeprevir	5028.63 ± 3007.12 nM	35.56 ± 7.22 nM	4289.43 ± 1668.59 nM	19.64 ± 2.34 nM
Asunaprevir	3627.66 ± 1412.97 nM	28.56 ± 6.14 nM	4304.09 ± 2227.32 nM	25.12 ± 4.52 nM
Paritaprevir	351.55 ± 147.65 nM	1.93 ± 0.32 nM	543.98 ± 201.27 nM	1.33 ± 0.34 nM
Grazoprevir	85.14 ± 18.93 nM	2.82 ± 0.36 nM	66.30 ± 9.42 nM	1.65 ± 0.39 nM

IC50 values are shown with 95% confidence intervals.

conventional approach to acquire the consensus sequence which can be time-consuming, but also may increase a chance to succeed by screening a pool of viral quasispecies. Two PR87 replicon clones from the pool, A and E, were compared, and clone A replicates much more efficiently than clone E (Fig. 1C and D). Interestingly, the sequence of clone A is more deviated from the PR87 consensus sequence than that of clone E. While clone A has 8 different amino acid residues from the consensus sequence, clone E only has 2 different amino acid residues (Fig. 2A). Importantly, at least 6 out of the 8 changes in clone A indeed can be traced in the original serum HCV RNA as minor quasispecies. These observations raise a possibility that only a small portion of HCV quasispecies in serum can replicate well in cell culture, which can be identified by our functional screening approach. This hypothesis certainly warrants more investigations. In addition, cell culture-adaptive mutations also contribute to successful establishment of HCV cell culture models. T1286I in NS3, an adaptive mutation previously shown to boost replication of genotype-3 strain S310 (Saeed et al., 2013), was found in clone A but not in clone E, which may explain the enhanced replication capability of clone A. The increased replication in PR87A7 replicon as compared to PR87A replicon is also likely due to two other adaptive mutations K1412N and V1543A in NS3 (Fig. 2B, D and 2E). Therefore, successful development of HCV cell culture model from clinical isolates may rely on screening of *in-vitro* replication-competent viral quasispecies and further adaptation to improve virus replication.

Genotype-3 HCV is difficult to treat by DAAs because of a combination of different genotype-specific features, such as high incidence of severe liver diseases, high baseline frequency of RAVs for anti-NS3 and anti-NS5A DAAs. Similar to other genotype-3 strains, PR87A7 displays strong resistance to anti-NS3 DAAs (Fig. 3). This DAA resistance is mainly dictated by genotype-3-specific 168Q in NS3, as the Q168D



	1–10	11–20	21–30	31–40
Genotype 1	A P I T A Y A Q Q T	R G L L G C I I T S	L T G R D K N Q V E	G E V Q I V S T A T
Genotype 2	A P I T A Y A Q Q T	R G L L G A I V V S	L T G R D K N E Q A	G Q V Q V L S S V T
Genotype 4	A P I T A Y A Q Q T	R G L L S T I V T S	L T G R D T N E N C	G E V Q V L S T A T
Genotype 5	A P I T A Y A Q Q T	R G V L G A I V L S	L T G R D K N E A E	G E V Q F L S T A T
Genotype 6	A P I T A Y A Q Q T	R G L L G T I V L S	L T G R D K N E V E	G E V Q V V S T A T
Genotype 3	A P I T A Y A Q Q T	R G L L G T I V T S	L T G R D K N V V T	G E V Q V L S T A T
Conservation(%)			96.8 98.6	

	41–50	51–60	61–70	71–80
Genotype 1	Q T F L A T C I N G	V C W T V Y H G A G	T R T I A S P K G P	V I Q M Y T N V D Q
Genotype 2	Q S F L G T S I S G	V L W T V Y H G A G	N K T L A G P K G P	V T Q M Y T S A E G
Genotype 4	Q S F L G T A V N G	V M W T V Y H G A G	A K T I S G P K G P	V N Q M Y T N V D Q
Genotype 5	Q T F L G I C I N G	V M W T L F H G A G	S K T L A G P K G P	V V Q M Y T N V D K
Genotype 6	Q T F L A T S I N G	V L W T V Y H G A G	S K T L A G P K G P	V C Q M Y T N V D Q
Genotype 3	Q T F L G T T V G G	V M W T V Y H G A G	S R T L A G A K H P	A L Q M Y T N V D Q
Conservation(%)	96.4 99.8		70.1 87.8	99.1 100

	81–90	91–100	101–110	111–120
Genotype 1	D L V G W P A P Q G	A R S L T P C T C G	S S D L Y L V T R H	A D V I P V R R R G
Genotype 2	D L V G W P S P P G	T K S L E P C T C G	A V D L Y L V T R N	A D V I P V R R K D
Genotype 4	D L V G W P A P P G	V K S L A P C T C G	A S D L F L V T R H	A D V I P V R R R G
Genotype 5	D L V G W P S P P G	K G S L T R C T C G	S A D L Y L V T R H	A D V I P A R R R G
Genotype 6	D L V G W P A P L G	A R S L A P C T C G	A S D L Y L V T R N	A D V I P A R R R G
Genotype 3	D L V G W P A P P G	A K S L E P C A C G	S A D L Y L V T R D	A D V I P A R R R G
Conservation(%)		67.8	84.6	

	121–130	131–140	141–150	151–160
Genotype 1	D S R G S L L S P R	P I S Y L K G S S G	G P L L C P A G H A	V G I F R A A V C T
Genotype 2	D R R G A L L S P R	P L S T L K G S S G	G P V L C P R G H A	V G L F R A A V C S
Genotype 4	D T R G A L L S P R	P I S T L K G S S G	G P L L C P M G H A	V G I F R A A V C T
Genotype 5	D T R A S L L S P R	P I S Y L K G S S G	G P I M C P S G H V	V G V F R A A V C T
Genotype 6	D T R A A L L S P R	P I S T L K G S S G	G P I L C P S G H V	V G L F R A A V C T
Genotype 3	D S T A S L L S P R	P L A C L K G S S G	G P V M C P S G H V	A G I F R A A V C T
Conservation(%)	99.8	95.6 96.1		89.2

	161–170	171–180	181–182
Genotype 1	R G V A K A V D F I	P V E N L E T T M R	S P
Genotype 2	R G V A K S I D F I	P V E T L D I A T R	S P
Genotype 4	R G V A K A V D F V	P V E S L E T T M R	S P
Genotype 5	R G V A K A L D F V	P V E N L E T T M R	S P
Genotype 6	R G V A K A L D F V	P V E N M E T T M R	S P
Genotype 3	R G V A K A L Q F I	P V E T L S T Q A R	S P
Conservation(%)	100	89.4 99.6 85.1	

Fig. 5. Active replication of JFH1 harboring the D168Q mutation requires additional genotype-3-specific polymorphism.
(A) JFH1 subgenomic replicon RNAs containing a luciferase reporter and different NS3 mutations or PR87A7 NS3protease/NS4A were electroporated into Huh7 cells. The increase of luciferase activities between 4 and 48 h post-transfection were measured and expressed as the percentage of the wild-type JFH1 control. The error bars were calculated from two independent experiments. **(B)** Alignment of consensus amino acid sequences of NS3 protease domain from genotype-1 to −6, which were generated from available HCV sequences in the Los Alamos HCV sequence database. The amino acids that appear only in the genotype-3 consensus sequence but not in the consensus sequence of other genotypes were highlighted, and their frequencies among 382 individual genotype-3 sequences were indicated below the residues. Highlighted in red and grey indicate the amino acids with over and below 95% conservation among genotype-3 respectively. **(C)** JFH1 subgenomic replicon RNA containing a luciferase reporter and various mutants containing genotype-3-specific polymorphisms in NS3 protease domain were electroporated into Huh7 cells. The increase of luciferase activities between 4 and 48 h post-transfection were measured and expressed as the percentage of the wild-type JFH1 control. The error bars were calculated from two independent experiments. **(D)** Intracellular HCV RNA levels of wild-type, D168Q or D168Q/R123T JFH1 replicon cells were quantified by RT-qPCR and expressed as the percentage of the wild type control. The error bars represented 2 independent experiments.

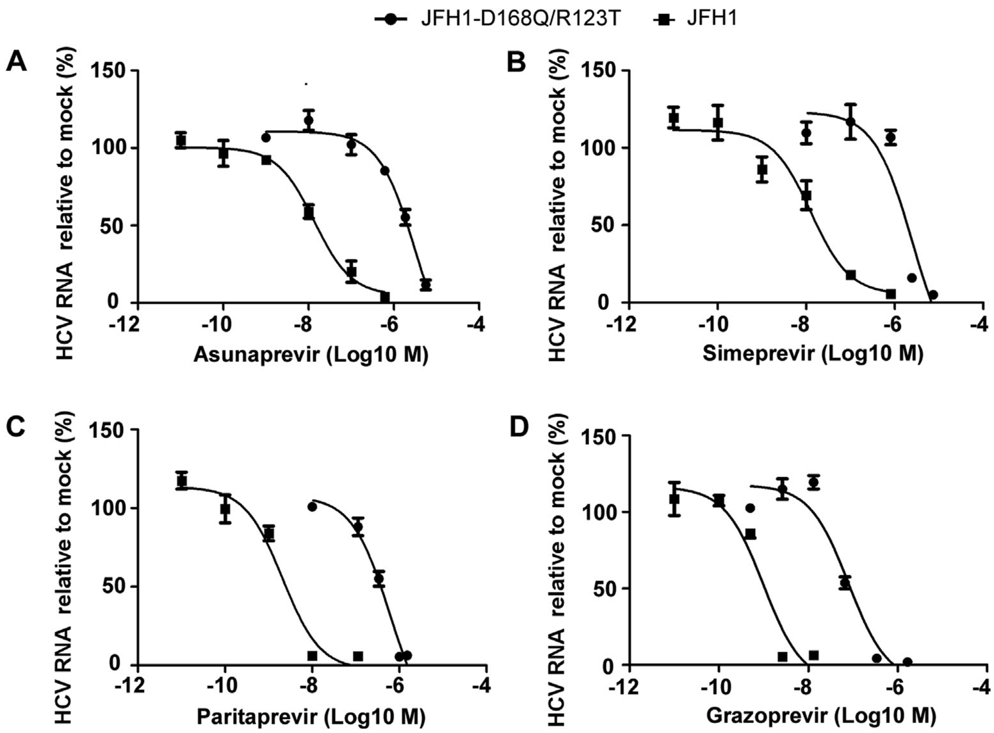


Fig. 6. JFH1 with the D168Q/R123T double mutation displays resistance to anti-NS3 DAAs. The wild-type or D168Q/R123T mutant JFH1 replicon cells were treated with serially diluted Simeprevir (**A**), Asunaprevir (**B**), Paritaprevir (**C**) and Grazoprevir (**D**) for 3 days. The intracellular HCV RNA levels were quantified by RT-qPCR, normalized to cellular GAPDH mRNA levels and expressed as the percentage to the mock-treatment control. Concentration-response curves were plotted in GraphPad Prism 7.0. The error bars were produced from 2 independent experiments.

Table 3
IC50 values of anti-NS3 DAAs against JFH1 and its derivative.

DAA	JFH1-D168Q/R123T IC50	JFH1 IC50	Fold Change (JFH1-D168Q/R123T/ JFH1)
Simeprevir	2529.90 ± 1298.09 nM	12.77 ± 2.74 nM	198.1
Asunaprevir	3307.30 ± 1569.50 nM	13.61 ± 1.64 nM	242.9
Paritaprevir	607.46 ± 191.35 nM	2.08 ± 0.45 nM	292.1
Grazoprevir	79.39 ± 15.31 nM	0.99 ± 0.32 nM	80.2

IC50 values are shown with 95% confidence intervals.

mutation in PR87A7 abolished the DAA resistance (Fig. 4) while D168Q mutation in JFH1 rendered the DAA resistance (Fig. 6). This conclusion is consistent with clinical observations that a D to A/E/H/N/V mutation at this position is frequently detected in non-genotype-3 HCV patients receiving DAA therapies involving anti-NS3 inhibitors (Dietz et al., 2018; Jensen et al., 2015). Interestingly, the D168Q mutation is rarely detected in non-genotype-3 HCV, most likely because the change from aspartic acid to glutamine (D168Q) involves 2 nucleotide changes (from GAY to CAR) while those frequently detected RAS (168 A/E/H/N/V) requires only one nucleotide change. D81, R123, D168, R155 in NS3 form an electrostatic surface within the catalytic active site which is required for tight ligand binding (Romano et al., 2012; Soumana et al., 2016). A recent study showed that a mutation at 156 (A156L) of NS3 of genotype-3 HCV can confer strong resistance to new-generation NS3 protease inhibitors (Jensen et al., 2019). While it may not be directly responsible, 168Q may work with 156L to render this resistance since viral variants containing Y56H/A156G/Q168R can emerge in genotype-3 HCV patients treated with Glecaprevir-Pibrentasvir (Krishnan et al., 2018). It would be interesting to determine whether 168Q contributes to the A156L-mediated DAA resistance in genotype-3 HCV.

While introduction of 168Q in non-genotype-3 HCV renders the DAA resistance, it also reduces viral replication fitness. Conversely, introduction of Q168D also reduces replication fitness in PR87A7 (data not shown). The replication defect induced by D168Q mutation in JFH1 can be partially rescued by introducing an additional genotype-3-specific amino acid (123T) or fully rescued by swapping the entire NS3/4A protease domain (Fig. 5), suggesting that 168Q co-evolves with other genotype-3-specific polymorphisms during the evolution of genotype-3 HCV. Interestingly, except for 123T, all other genotype-3-specific amino acid residues are not located near 168Q in the 3D structure of NS3 (data not shown). Therefore, it is important to figure out how these genotype-3-specific polymorphisms collectively contribute to the restoration of replication fitness of 168Q. A recent study found that NS3 RAS A156L can be maintained in genotype-3 HCV without other substitutions in non-structural proteins (Jensen et al., 2019), suggesting that fitness loss from A156L is minimal in the contexts of genotype-3 NS3 protease. It would be interesting to determine whether retention of A156L is also dependent on other genotype-3-specific polymorphisms in the NS3/4A protease.

In conclusion, we developed a new genotype-3a subgenomic replicon by a novel functional screening approach. Characterization of this replicon revealed critical genotype-3-specfic amino acid residues that confer the DAA resistance while retaining viral fitness.

Acknowledgments

We thank Yisha Liang for the technical assistances and Dr. Lanfeng Wang for the insightful discussion. This study was supported by the grants from Strategic Priority Research Program of the Chinese Academy of Sciences (XDB29010205), the National Natural Science Foundation of China (31670172) and the Chinese National 973 Program (2015CB554300) to JZ, and the National Research Council of Science and Technology Major Projects for "Major New Drugs Innovation and Development" (2017ZX09101-005-001-005) to YX. We declare no conflict of interest.

References

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974.

Cao, R.Y., Xu, Y.F., Zhang, T.H., Yang, J.J., Yuan, Y., Hao, P., Shi, Y., Zhong, J., Zhong, W., 2017. Pediatric drug nitazoxanide: a potential choice for control of zika. Open Forum Infect. Dis. 4 ofx009.

Chan, A., Patel, K., Naggie, S., 2017. Genotype 3 infection: the last stand of hepatitis C virus. Drugs 77, 131–144.

Das, D., Pandya, M., 2018. Recent advancement of direct-acting antiviral agents (DAAs) in hepatitis C therapy. Mini Rev. Med. Chem. 18, 584–596.

Dietz, J., Susser, S., Vermehren, J., Peiffer, K.H., Grammatikos, G., Berger, A., Ferenci, P., Buti, M., Mullhaupt, B., Hunyady, B., Hinrichsen, H., Mauss, S., Petersen, J., Buggisch, P., Felten, G., Huppe, D., Knecht, G., Lutz, T., Schott, E., Berg, C., Spengler, U., von Hahn, T., Berg, T., Zeuzem, S., Sarrazin, C., European H.C.V.R.S.G, 2018. Patterns of resistance-associated substitutions in patients with chronic HCV infection following treatment with direct-acting antivirals. Gastroenterology 154, 976–988 e974.

European Association for the Study of the Liver, 2018. EASL recommendations on treatment of hepatitis C 2018. J. Hepatol. 69, 461–511 Electronic address, e.e.e., European Association for the Study of the, L.

Gao, M., O'Boyle 2nd, D.R., Roberts, S., 2016. HCV NS5A replication complex inhibitors. Curr. Opin. Pharmacol. 30, 151–157.

Gottwein, J.M., Scheel, T.K., Callendret, B., Li, Y.P., Eccleston, H.B., Engle, R.E., Govindarajan, S., Satterfield, W., Purcell, R.H., Walker, C.M., Bukh, J., 2010. Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and in vivo pathogenesis studies. J. Virol. 84, 5277–5293.

Gottwein, J.M., Scheel, T.K., Jensen, T.B., Ghanem, L., Bukh, J., 2011. Differential efficacy of protease inhibitors against HCV genotypes 2a, 3a, 5a, and 6a NS3/4A protease recombinant viruses. Gastroenterology 141, 1067–1079.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., Rice, C.M., 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67, 1385–1395.

Hajarizadeh, B., Grebely, J., Dore, G.J., 2013. Epidemiology and natural history of HCV infection. Nat. Rev. Gastroenterol. Hepatol. 10, 553–562.

Jensen, S.B., Fahnoe, U., Pham, L.V., Serre, S.B.N., Tang, Q., Ghanem, L., Pedersen, M.S., Ramirez, S., Humes, D., Pihl, A.F., Filskov, J., Solund, C.S., Dietz, J., Fourati, S., Pawlotsky, J.M., Sarrazin, C., Weis, N., Schonning, K., Krarup, H., Bukh, J., Gottwein, J.M., 2019. Evolutionary pathways to persistence of highly fit and resistant hepatitis C virus protease inhibitor escape variants. Hepatology 70, 771–787.

Jensen, S.B., Serre, S.B., Humes, D.G., Ramirez, S., Li, Y.P., Bukh, J., Gottwein, J.M., 2015. Substitutions at NS3 residue 155, 156, or 168 of hepatitis C virus genotypes 2 to 6 induce complex patterns of protease inhibitor resistance. Antimicrob. Agents Chemother. 59, 7426–7436.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125, 1808–1817.

Kato, T., Matsumura, T., Heller, T., Saito, S., Sapp, R.K., Murthy, K., Wakita, T., Liang, T.J., 2007. Production of infectious hepatitis C virus of various genotypes in cell cultures. J. Virol. 81, 4405–4411.

Krieger, N., Lohmann, V., Bartenschlager, R., 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J. Virol. 75, 4614–4624.

Krishnan, P., Pilot-Matias, T., Schnell, G., Tripathi, R., Ng, T.I., Reisch, T., Beyer, J., Dekhtyar, T., Irvin, M., Xie, W., Larsen, L., Mensa, F.J., Collins, C., 2018. Pooled resistance analysis in patients with hepatitis C virus genotype 1 to 6 infection treated with glecaprevir-pibrentasvir in phase 2 and 3 clinical trials. Antimicrob. Agents Chemother. 62.

Li, J., Feng, S., Liu, X., Guo, M., Chen, M., Chen, Y., Rong, L., Xia, J., Zhou, Y., Zhong, J., Li, Y.P., 2018. Identification of nucleotides in the 5'UTR and amino acids substitutions that are essential for the infectivity of 5'UTR-NS5A recombinant of hepatitis C virus genotype 1b (strain Con1). Virology 518, 253–263.

Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.

Link, J.O., Taylor, J.G., Trejo-Martin, A., Kato, D., Katana, A.A., Krygowski, E.S., Yang, Z.Y., Zipfel, S., Cottell, J.J., Bacon, E.M., Tran, C.V., Yang, C.Y., Wang, Y., Wang, K.W., Zhao, G., Cheng, G., Tian, Y., Gong, R., Lee, Y.J., Yu, M., Gorman, E., Mogalian, E., Perry, J.K., 2019. Discovery of velpatasvir (GS-5816): a potent pan-genotypic HCV NS5A inhibitor in the single-tablet regimens Vosevi((R)) and Epclusa((R)). Bioorg. Med. Chem. Lett 29, 2415–2427.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Lontok, E., Harrington, P., Howe, A., Kieffer, T., Lennerstrand, J., Lenz, O., McPhee, F., Mo, H., Parkin, N., Pilot-Matias, T., Miller, V., 2015. Hepatitis C virus drug resistance-associated substitutions: state of the art summary. Hepatology 62, 1623–1632.

Lu, J., Tao, W., Li, R., Xiang, Y., Zhang, N., Xiang, X., Xie, Q., Zhong, J., 2013. Construction and characterization of infectious hepatitis C virus chimera containing structural proteins directly from genotype 1b clinical isolates. Virology 443, 80–88.

Lu, J., Xiang, Y., Tao, W., Li, Q., Wang, N., Gao, Y., Xiang, X., Xie, Q., Zhong, J., 2014. A novel strategy to develop a robust infectious hepatitis C virus cell culture system directly from a clinical isolate. J. Virol. 88, 1484–1491.

Messina, J.P., Humphreys, I., Flaxman, A., Brown, A., Cooke, G.S., Pybus, O.G., Barnes, E., 2015. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology 61, 77–87.

Morikawa, K., Lange, C.M., Gouttenoire, J., Meylan, E., Brass, V., Penin, F., Moradpour, D., 2011. Nonstructural protein 3-4A: the Swiss army knife of hepatitis C virus. J. Viral Hepat. 18, 305–315.

Nkontchou, G., Ziol, M., Aout, M., Lhabadie, M., Baazia, Y., Mahmoudi, A., Roulot, D., Ganne-Carrie, N., Grando-Lemaire, V., Trinchet, J.C., Gordien, E., Vicaut, E., Baghad, I., Beaugrand, M., 2011. HCV genotype 3 is associated with a higher hepatocellular carcinoma incidence in patients with ongoing viral C cirrhosis. J. Viral Hepat. 18, e516–522.

Pawlotsky, J.M., 2014. New hepatitis C therapies: the toolbox, strategies, and challenges. Gastroenterology 146, 1176–1192.

Probst, A., Dang, T., Bochud, M., Egger, M., Negro, F., Bochud, P.Y., 2011. Role of hepatitis C virus genotype 3 in liver fibrosis progression–a systematic review and meta-analysis. J. Viral Hepat. 18, 745–759.

Ramirez, S., Mikkelsen, L.S., Gottwein, J.M., Bukh, J., 2016. Robust HCV genotype 3a infectious cell culture system permits identification of escape variants with resistance to sofosbuvir. Gastroenterology 151, 973–985 e972.

Romano, K.P., Ali, A., Aydin, C., Soumana, D., Ozen, A., Deveau, L.M., Silver, C., Cao, H., Newton, A., Petropoulos, C.J., Huang, W., Schiffer, C.A., 2012. The molecular basis of drug resistance against hepatitis C virus NS3/4A protease inhibitors. PLoS Pathog. 8, e1002832.

Saeed, M., Andreo, U., Chung, H.Y., Espiritu, C., Branch, A.D., Silva, J.M., Rice, C.M., 2015. SEC14L2 enables pan-genotype HCV replication in cell culture. Nature 524, 471–475.

Saeed, M., Gondeau, C., Hmwe, S., Yokokawa, H., Date, T., Suzuki, T., Kato, T., Maurel, P., Wakita, T., 2013. Replication of hepatitis C virus genotype 3a in cultured cells. Gastroenterology 144, 56–58 e57.

Sarrazin, C., Dvory-Sobol, H., Svarovskaia, E.S., Doehle, B.P., Pang, P.S., Chuang, S.M., Ma, J., Ding, X., Afdhal, N.H., Kowdley, K.V., Gane, E.J., Lawitz, E., Brainard, D.M., McHutchison, J.G., Miller, M.D., Mo, H., 2016. Prevalence of resistance-associated substitutions in HCV NS5A, NS5B, or NS3 and outcomes of treatment with ledipasvir and sofosbuvir. Gastroenterology 151, 501–512 e501.

Shrivastava, S., Meissner, E.G., Funk, E., Poonia, S., Shokeen, V., Thakur, A., Poonia, B., Sarin, S.K., Trehanpati, N., Kottilil, S., 2016. Elevated hepatic lipid and interferon stimulated gene expression in HCV GT3 patients relative to non-alcoholic steatohepatitis. Hepatol. Int. 10, 937–946.

Soumana, D.I., Kurt Yilmaz, N., Ali, A., Prachanronarong, K.L., Schiffer, C.A., 2016. Molecular and dynamic mechanism underlying drug resistance in genotype 3 hepatitis C NS3/4a protease. J. Am. Chem. Soc. 138, 11850–11859.

Stamm, L.M., Brainard, D.M., McHutchison, J.G., 2019. Sofosbuvir/velpatasvir for patients with chronic genotype 3 HCV infection with compensated cirrhosis: response to EASL Recommendations on Treatment of Hepatitis C 2018. J. Hepatol. 70, 561–562.

Svarovskaia, E.S., Dvory-Sobol, H., Parkin, N., Hebner, C., Gontcharova, V., Martin, R., Ouyang, W., Han, B., Xu, S., Ku, K., Chiu, S., Gane, E., Jacobson, I.M., Nelson, D.R., Lawitz, E., Wyles, D.L., Bekele, N., Brainard, D., Symonds, W.T., McHutchison, J.G., Miller, M.D., Mo, H., 2014. Infrequent development of resistance in genotype 1-6 hepatitis C virus-infected subjects treated with sofosbuvir in phase 2 and 3 clinical trials. Clin. Infect. Dis. 59, 1666–1674.

Tao, W., Gan, T., Lu, J., Zhong, J., 2017. A profiling study of a newly developed HCVcc strain PR63cc's sensitivity to direct-acting antivirals. Antivir. Res. 139, 18–24.

Tao, W., Xu, C., Ding, Q., Li, R., Xiang, Y., Chung, J., Zhong, J., 2009. A single point mutation in E2 enhances hepatitis C virus infectivity and alters lipoprotein association of viral particles. Virology 395, 67–76.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

Wing, P.A.C., Jones, M., Cheung, M., DaSilva, S., Bamford, C., Jason Lee, W.Y., Aranday-Cortes, E., Da Silva Filipe, A., McLauchlan, J., Smith, D., Irving, W., Cunningham, M., Ansari, A., Barnes, E., Foster, G.R., 2019. Amino acid substitutions in genotype 3a hepatitis C virus polymerase protein affect responses to sofosbuvir. Gastroenterology 157, 692–704 e699.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. 102, 9294–9299.