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# Human immunodeficiency virus type 1 Vpr increases hepatitis C virus RNA replication in cell culture

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ABSTRACT

Human immunodeficiency virus (HIV) coinfection with hepatitis C virus (HCV) is associated with an increased HCV RNA level, as well as a more rapid progression to cirrhosis and end-stage liver disease. However, the mechanism underlying this effect is largely unknown. Here, we investigated the role of HIV-1 Vpr in HCV infection and clearly demonstrated that Vpr increased the replication of both the infectious HCV full-length genome and the subgenomic replicon. We also demonstrated that Vpr increased HCV infection by enhancing RNA replication but not viral entry or translation. Further, we showed that Vpr could partially overcome the anti-HCV effect of PEG-IFN. Our findings not only partially explain the clinical observation that patients coinfected with HIV and HCV have higher levels of HCV RNA and viral load than HCV mono-infected patients but also provide important information for HCV treatment in HIV/HCV coinfected patients.



## 1. Introduction

Due to shared routes of transmission, coinfection with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) is common and prevalent among injection drug users (IDUs), with coinfection rates of approximately 82–93%, and in men who have sex with men (MSMs) (Monga et al., 2001; Rotman and Liang, 2009; Torriani et al., 2003; Zhang et al., 2002). With the introduction of highly active antiretroviral therapy (HAART), AIDS-related morbidity and mortality in HIV-infected patients have dramatically decreased (Sherman et al., 2007; Soriano et al., 2002). HCV-related liver diseases have emerged as a major cause of death in HIV-1-infected patients (Monga et al., 2001; Sulkowski and Thomas, 2003). HIV/HCV coinfection has attracted global scientific attention because HIV infection adversely affects the course of HCV-induced liver damage; patients with HIV/HCV coinfection have higher HCV RNA levels, faster fibrosis progression and earlier development of end-stage liver disease compared to HCV mono-infected patients (Di Martino et al., 2001; Monga et al., 2001; Telfer et al., 1994). However, the mechanisms by which HIV accelerates HCV infection and the progression of liver diseases in HIV/HCV coinfected patients are not fully understood.

It is well established that HAART-associated hepatotoxicity is more frequent in patients with chronic hepatitis C (Martin-Carbonero et al., 2001; Sulkowski et al., 2000). Moreover, it has been reported that HIV-related immunodeficiency may be the reason HCV progresses more rapidly (Di Martino et al., 2001; Rockstroh et al., 1996). Nonetheless, these mechanisms do not fully explain the faster progression of liver-related disease. The liver injury in coinfected patients could be explained at the cellular and molecular levels, mainly in terms of direct or indirect interactions among HIV, HCV and the liver. There is a discussion of the induction of apoptosis in hepatocytes by signaling effects produced by the binding of HIV gp120 to the CCR5 and CXCR4 receptors expressed on the surface of hepatocytes, without infecting the cell (Algeciras-Schimnich et al., 2002; Vlahakis et al., 2003). The main cell targets for HIV are $CD4^+$ lymphocytes and macrophages, while HCV was thought to be strictly hepatotropic. It has been suggested that HIV infection increases HCV replication, thus altering the course of HCV-related disease in co-infected patients (Eyster et al., 1994; Rey et al., 2001a). Furthermore, several studies have demonstrated that HIV-1 protein plays an important role in the enhanced HCV replication (Qu et al., 2011, 2012).

Vpr is a 96-amino-acid, 14-kDa nonstructural protein of HIV-1, and is highly conserved in HIV-1, HIV-2 and SIV (Tristem et al.,

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1992). A number of biological functions *in vitro* that are necessary for viral replication and may be important for AIDS pathogenesis have been attributed to Vpr, including transactivation of the HIV-1 long terminal repeat (LTR) (Felzien et al., 1998; Kino et al., 2002), nuclear import of the preintegration complex in newly infected terminally differentiated macrophages (Heinzinger et al., 1994), induction of cell cycle $G_2$ arrest (He et al., 1995; Re et al., 1995) and apoptosis (Stewart et al., 1997). In addition, Vpr is found in virions (Cohen et al., 1990), exists as free molecules present in the plasma of HIV-infected patients (Hoshino et al., 2007), and effectively transduces a variety of cells that cannot be infected directly by HIV-1 (Sherman et al., 2002), suggesting that it may exert its biological functions through different manners.

In this study, we investigated the roles of HIV-1 Vpr in the regulation of HCV replication during the co-infection of the two viruses. We demonstrated that HIV-1 Vpr increased the HCV infection by enhancing RNA replication but not viral entry or translation. We also demonstrated that HIV-1 Vpr was able to partially overcome the anti-HCV effect of PEG-IFN.

## 2. Materials and methods

### 2.1. Cell cultures and antibodies

Huh7.5.1 and 293T cells were grown in complete Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, nonessential amino acids, penicillin, and streptomycin at 37 °C in 5% $CO_2$. Con1 cells are Huh7-Lunet cells that harbor the genotype 1b Con1 subgenomic replicon (Chen et al., 2012, 2013).

The primary antibodies were mouse monoclonal antibodies against HCV-Core (Abcam), HCV-NS3 (Abcam), HCV-NS4B (Virogene), HIV-1 p24 (Abcam) and β-actin (Cell Signaling Technology). The mouse monoclonal antibody against HIV-1 Vpr (8D1) was generated by immunization with a full-length Vpr peptide (Hoshino et al., 2007). HRP-linked anti-mouse immunoglobulin G antibody (Cell Signaling Technology) was used as the secondary antibody. The rabbit polyclonal antibody against CD81 (GeneTex) was used in HCV infection blocking experiments.

### 2.2. Plasmids

The plasmids pNL4-3.Luc.R+E- and pNL4-3.Luc.R−E- contain the firefly luciferase gene in the *nef* position and the Env-inactivating mutation (Connor et al., 1995). The former contains the full-length *vpr* gene, while the latter has a frameshift in *vpr*. The pNL4-3.GFP.R+E- and pNL4-3.GFP.R−E- plasmids were derived from the pNL4-3.Luc.R+E- and pNL4-3.Luc.R−E- plasmids, respectively, by replacing the firefly luciferase gene with the EGFP gene. We used three plasmids encoding different HCV strains. The plasmid pJFH1 containing the full-length genomic cDNA sequence of the HCV genotype 2a strain JFH1 was kindly provided by Prof. T. Wakita. The plasmid pJ399EM was derived from the pJFH1 genome by inserting the EGFP gene into the NS5A region, and this plasmid produces infectious viral particles as robustly as the pJFH1 (Han et al., 2009). The plasmid pJ399LM was derived from the pJ399EM genome by replacing the EGFP gene with the *Renilla* luciferase gene. The HCV reporter subgenomic replicons pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND were constructed according to the method described previously (Kato et al., 2005). The pSGR2-JFH1/Luc-GND construct is a replication-deficient replicon that carries a point mutation in the GDD motif of the RNA-dependent RNA polymerase.

### 2.3. HIV-1 reporter pseudotypes

The HIV-1 pNL4-3.Luc.R+E- or pNL4-3.Luc.R−E- constructs and the pNL4-3.GFP.R+E- or pNL4-3.GFP.R−E- constructs were pseudotyped with vesicular stomatitis virus (VSV-G) envelope and denoted as HIV-1 LucVpr+ or LucVpr− and GFPVpr+ or GFPVpr−, respectively. Briefly, 293T cells ($2 \times 10^6$) were seeded in 10-cm culture dishes; the following day, the cells were cotransfected with 7.5 μg of HIV-1 proviral constructs and 2.5 μg of VSV-glycoprotein-envelope expression plasmids using the Lipofectamine™2000 Transfection Regent (Invitrogen). The culture medium was changed 4–6 h post-transfection, and the virus-containing supernatants were collected 72 h later. The virus supernatants were filtered through a 0.45-μm filter to remove cellular debris and titered by measuring $p24^{gag}$ with a HIV-1 P24 Antigen Capture Assay (Advanced BioScience Laboratories, Inc.). The virus supernatants were stored at −80 °C for the infection assays.

### 2.4. Production of HCVcc

HCV transcripts were generated and transfected using the previously described *in vitro* protocol (Kato et al., 2006). In brief, the pJFH1, pJ399EM and pJ399LM plasmids were digested with *XbaI*, and the linearized plasmids were then treated with Mung bean nuclease and proteinase K. *In vitro* transcription was performed using a MEGAscript kit (Promega). For the electroporation, Huh7.5.1 cells ($7.5 \times 10^6$) were resuspended in 400 μl Cytomix buffer, and 10 μg RNA was mixed with the cell suspension in a 4-mm cuvette. A Gene Pulser system (Bio-Rad) was used to deliver a single pulse at 950 μF and 260 V, and the cells were transferred immediately into complete medium. The culture medium containing infectious HCV particles was collected, and transfected cells were passaged until the virus production decreased. The titer of the virus was determined as previously described (Han et al., 2009).

### 2.5. Production of HCVpp

Murine leukemia virus (MLV)-based pseudotyped particles bearing HCV E1E2 glycoproteins were generated as previously described (Chen et al., 2012, 2013). Briefly, 293T cells were co-transfected with the pVPack-GP, pFB-luc, and HCV E1E2 expression plasmids. The virus-containing culture medium was collected at 48 h post-transfection, filtered through a 0.45 μM filter and was used in the viral entry assay.

### 2.6. Cell cycle analysis

Huh7.5.1 or Con1 cells were seeded in 24-well plates at a density of $2 \times 10^5$ cells per well. Twenty-four hours after seeding, the cells were infected with HIV-1 LucVpr+ or LucVpr−, and the cells were collected at 72 h post-infection. The cells were washed with PBS and fixed in 70% ethanol at 4 °C overnight. After fixation, the cells were washed twice with PBS before being resuspended in staining buffer (50 μg/ml propidium iodide (PI), 100 μg/ml RNase A) at room temperature in the dark for 1 h. PI-stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

### 2.7. Luciferase reporter gene assay

Luciferase activity was assayed using the Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, the cells were harvested and washed once with PBS, and 100 μl of lysis buffer was added to each well of a 24-well plate. A 10 μl sample was mixed with the luciferase assay substrate. For the single luciferase reporter assay, the firefly or *Renilla* luciferase activity was normalized by the protein OD of each sample. For

the dual-luciferase reporter assay, firefly luciferase activity was normalized based on the activity of the *Renilla* luciferase. Assays were performed in triplicate, and the data are expressed as the mean ± standard deviation (SD) of luciferase activity.

### 2.8. Quantification of HCV RNA by real time RT-PCR

Real-time RT-PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) and an Applied Biosystems 7500 Real-Time PCR system. We detected the RNA of the HCV and the primers we used were complementary to the sequences located in the 5′ UTR. The primers sequence are as follows: HCV JFH1 positive-strand RNA, 5′-TCT GCG GAA CCG GTG AGT A-3′ (nucleotides 146–164) and 5′-TCA GGC AGT ACC ACA AGG C-3′ (nucleotides 295–277) (Chen et al., 2012; Zhong et al., 2005); HCV JFH1 negative-strand RNA, 5′-TGA CTA GGG CTA AGA TGG AG-3′ (nucleotides 74–93) and 5′-TAG GTA CAC TCC ATA GCT AA-3′ (nucleotides 224–205); HCV Con1 positive-strand RNA, 5′-ATC ACT CCC CTG TGA GGA ACT-3′ (nucleotides 36–56) and 5′-GCG GGT TGA TCC AAG AAA GG-3′ (nucleotides 211–192) (Chen et al., 2012; Rey et al., 2001b); and GAPDH, 5′-GAA GGT GAA GGT CGG AGT C-3′ and 5′-GAA GAT GGT GAT GGG ATT TC-3′. Quantification of HCV genomic RNA was performed using the comparative $C_T$ ($\Delta\Delta C_T$) method. The GAPDH gene was used as the reference gene.

### 2.9. Statistical analyses

The data were presented as the mean ± SD of three independent experiments performed in triplicate, and all analyses were performed using Student's *t*-test. $P < 0.05$ (*) was considered statistically significant.

## 3. Results

### 3.1. HIV-1 Vpr increases HCV reporter virus infection

We first measured the effect of HIV-1 Vpr on HCV GFP reporter virus J399EM and HCV *Renilla* luciferase reporter virus J399LM infection. Twenty-four hours after infection with HIV-1 LucVpr− or LucVpr+ at concentrations ranging from 25 to 100 ng/ml $p24^{gag}$, Huh7.5.1 cells were inoculated with J399EM at an MOI of 0.1. The cells were harvested, and the GFP-expressing cells were analyzed by flow cytometry at 48 and 72 h post-HCV infection respectively. The results at 48 h were shown in Fig. 1A and B. From these figures we can see that there was no significant difference in the number of GFP positive cells between the cells infected with LucVpr− and LucVpr+ at the concentration of 25 ng/ml $p24^{gag}$, and the number of GFP-expressing cells increased by 58% (29.3% *vs.* 46.3%) and 65% (32.6% *vs.* 53.7%) in the cells infected with 50 and 75 ng/ml $p24^{gag}$ LucVpr+, respectively, compared to the cells infected with LucVpr−. However, there was a tiny increase between the cells infected with 100 ng/ml $p24^{gag}$ LucVpr+ and LucVpr−. The results at 72 h (Fig. 1C) were similar to the results at 48 h.

Next, Huh7.5.1 cells infected with HIV-1 GFPVpr− or GFPVpr+ for 24 h were inoculated with the J399LM virus at an MOI of 0.1 and analyzed for *Renilla* luciferase activity at 48 and 72 h post-HCV infection respectively. The results at 48 h (Fig. 1D) were also similar to the results at 72 h (Fig. 1E). The results showed that the luciferase activity in the cells infected with 50 and 75 ng/ml $p24^{gag}$ GFPVpr+ was dramatically increased compared to the cells infected with GFPVpr− and that there was no significant increase between the cells infected with GFPVpr+ and GFPVpr− at the concentration of 100 ng/ml $p24^{gag}$.

Taken together, these results indicated that the infection of HCV reporter viruses J399EM and J399LM increased when lower levels of HIV-1 Vpr were present.

### 3.2. HIV-1 Vpr increases wild type HCV infection

Next, we measured the effect of HIV-1 Vpr on wild type HCV JFH1 infection using different methods. One important function of HIV-1 Vpr is to arrest infected cells at the $G_2$ stage of the cell cycle. In order to determine an appropriate concentration of virus for infection, Huh7.5.1 cells were infected with 0, 25, 50, 75, or 100 ng/ml $p24^{gag}$ HIV-1 LucVpr− or LucVpr+ and analyzed for their cell cycle profile. Representative cell cycle profiles are shown in Fig. 2A and B. The results indicated that less than 75 ng/ml $p24^{gag}$ LucVpr+ virus infection did not induce a $G_2$ cell cycle arrest in Huh7.5.1 cells. Thus, to minimize the impact of Vpr on cells, 50 ng/ml $p24^{gag}$ was considered optimal for the infection. The firefly luciferase activity of infected cells was assayed to confirm the infection levels (Fig. 2C). Huh7.5.1 cells were infected with 50 ng/ml $p24^{gag}$ HIV-1 LucVpr− or LucVpr+ for 24 h and were then inoculated with JFH1 at an MOI of 0.1. We assessed the HCV positive-strand (Fig. 2D) and negative-strand (Fig. 2E) RNA levels using real-time RT-PCR at 24 and 48 h post-HCV infection. The results showed that HCV RNA levels were almost unaffected at 24 h in the cells infected with LucVpr+ but increased significantly at 48 h post-HCV infection, compared with the cells infected with LucVpr−. The expression of the HCV proteins NS3 and Core were also examined in the infected cells at 72 h post-HCV infection (Fig. 2F). Consistent with the RNA levels, the expression of the HCV proteins were also significantly enhanced in the cells infected with LucVpr+ compared to the cells infected with LucVpr−. The bands representing Vpr and Gag indicated the validity of the HIV-1 pseudotypes. We also detected the Core protein in the culture supernatant at 72 h post-HCV infection (Fig. 2G). When compared to the cells infected with LucVpr−, the amount of Core protein released from the cells infected with LucVpr+ was increased by 56%. Overall, these results indicated that HIV-1 Vpr increased the wild type HCV JFH1 infection.

### 3.3. HIV-1 Vpr increases HCV subgenomic replicon (SGR) replication

We further assessed the effect of HIV-1 Vpr on Con1 cells that were derived from Huh7-Lunet cells and stably expressed the Con1-based selectable subgenomic replicon. The cell cycle profile of Con1 cells under the influence of Vpr was also examined (Fig. 3A and B). The results indicated that more than 50 ng/ml $p24^{gag}$ LucVpr+ virus infection could induce a $G_2$ cell cycle arrest in Con1 cells and that Vpr arrested Con1 cells in a dose-dependent manner, because the G2+M:G1 value of the cells infected with LucVpr+ increased from 0.30 to 0.61 when the level of $p24^{gag}$ increased from 50 to 150 ng/ml. The firefly luciferase activity of infected cells was assayed to confirm the infection levels (Fig. 3C). In addition to the cell cycle analysis, Con1 cells were also subjected to the real-time RT-PCR and western blot analyses. As shown in Fig. 3D, the expression of HCV Con1-SGR RNA was severely enhanced in the cells infected with 75, 100 and 150 ng/ml $p24^{gag}$ LucVpr+ compared to the cells infected with LucVpr−. This result was confirmed by western blot analysis, as the expression levels of HCV NS3 and NS4B proteins significantly increased in these cells (Fig. 3E). To our surprise, HCV Con1-SGR replication increased in the cells that were cell cycle arrested. Overall, these results demonstrated that HIV-1 Vpr increased the replication of HCV Con1-SGR despite the $G_2$ cell cycle arrest in cells.

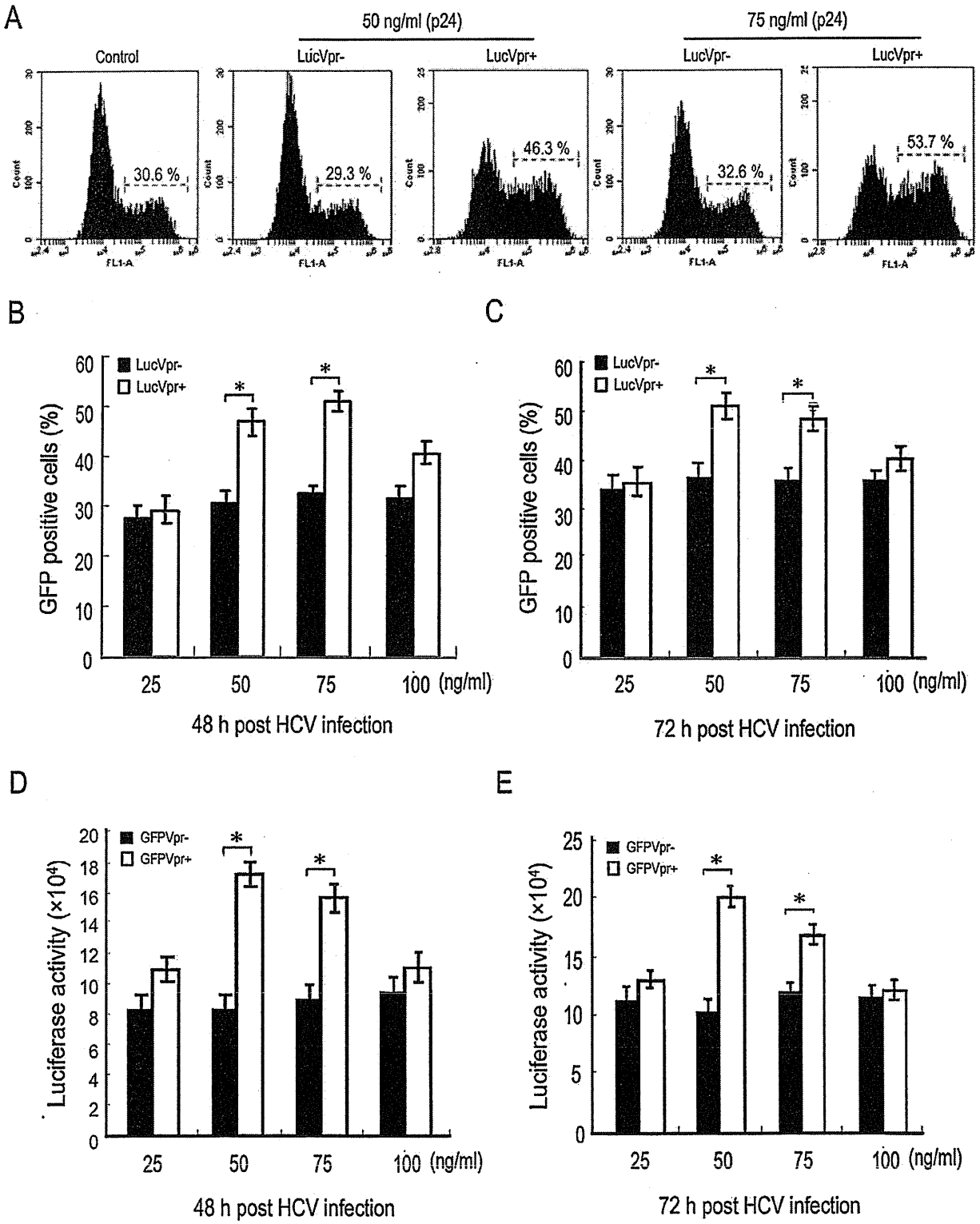


**Fig. 1.** The effects of HIV-1 Vpr on HCV reporter virus infection. (A–C) Huh7.5.1 cells were infected with 25, 50, 75, or 100 ng/ml $p24^{gag}$ HIV-1 LucVpr– or LucVpr+ for 24 h and were then inoculated with J399EM at an MOI of 0.1. The cells were harvested and analyzed by flow cytometry at 48 and 72 h post-HCV infection to determine the number of GFP-expressing cells. Cells infected only with HCV were used as a positive control. Representative cells expressing GFP (A), the statistical analysis of the GFP-expressing cells at 48 h (B) and 72 h (C) are presented. (D and E) Huh7.5.1 cells were infected with 25, 50, 75, or 100 ng/ml $p24^{gag}$ HIV-1 GFPVpr– or GFPVpr+ for 24 h and were then inoculated with J399LM at an MOI of 0.1. The cells were analyzed for *Renilla* luciferase activity at 48 h (D) and 72 h (E) post-HCV infection.

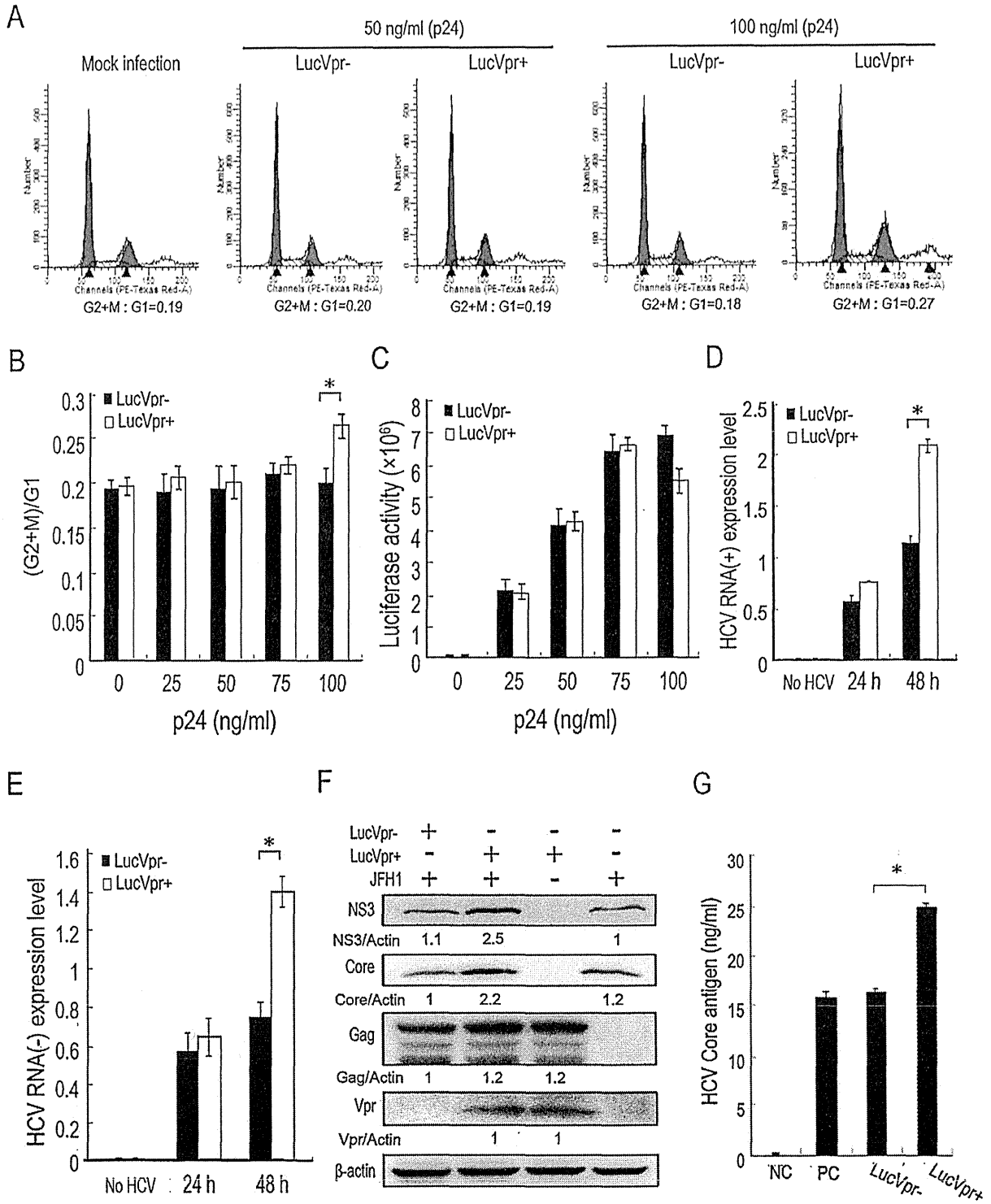


**Fig. 2.** The effects of HIV-1 Vpr on HCV JFH1 infection. (A and B) Huh7.5.1 cells were infected with 0, 25, 50, 75, or 100 ng/ml $p24^{gag}$ of HIV-1 LucVpr− or LucVpr+ for 72 h and were then analyzed for cell cycle profile by flow cytometry. The percentages of G1 and G2+M cell populations were determined using the ModFit software. Representative cell cycle profiles (A) and statistical analysis of the G2+M:G1 ratios (B) are presented. (C) The firefly luciferase activity was analyzed in infected cells 72 h post-infection. (D–G) Huh7.5.1 cells were infected with 50 ng/ml $p24^{gag}$ HIV-1 LucVpr− or LucVpr+ for 24 h and were then inoculated with JFH1 at an MOI of 0.1. The levels of HCV positive-strand RNA (D) and negative-strand RNA (E) were examined using real-time qPCR at 24 and 48 h post-HCV infection. Protein expression of HCV and HIV-1 was monitored by western blot analysis at 72 h post-HCV infection, and the level of β-actin served as an internal control (F). Levels of released HCV particles were assayed by measuring the Core protein in the culture supernatant using a commercial Elisa Kit (CELL BIOLABS INC.) at 72 h post-HCV infection. NC: negative control, cells with pseudotyped HIV-1 monoinfection; PC: positive control, cells with HCV monoinfection (G).

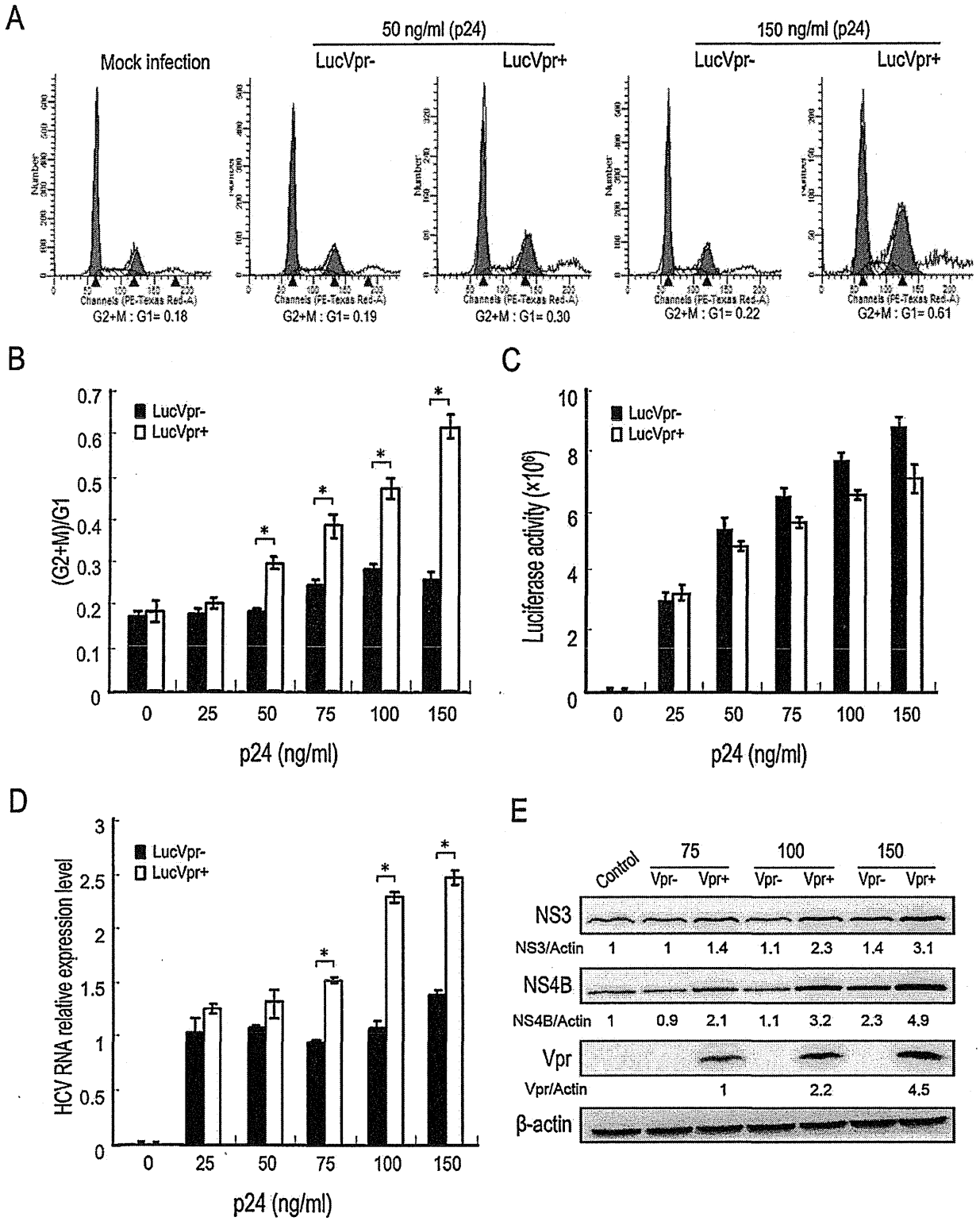


**Fig. 3.** The effects of HIV-1 Vpr on HCV Con1-SGR replication. (A–E) Con1 cells were infected with 0, 25, 50, 75, 100, or 150 ng/ml $p24^{gag}$ HIV-1 LucVpr− or LucVpr+ for 72 h and were then performed for different analyses. The cell cycle profiles were analyzed by flow cytometry (A and B). The firefly luciferase activity was analyzed (C). The HCV RNA expression was analyzed using real-time qPCR (D). The HCV protein expression was analyzed by western blot analysis (E).

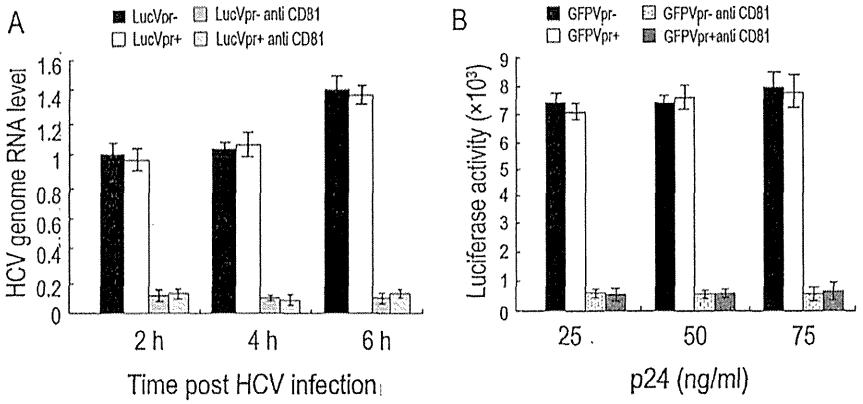


**Fig. 4.** The effects of HIV-1 Vpr on HCV viral entry. (A) Huh7.5.1 cells were infected with 50 ng/ml $p24^{gag}$ HIV-1 LucVpr− or LucVpr+ for 24 h and were then inoculated with JFH1 at an MOI of 0.1. HIV-infected cells were incubated with 10 μg/ml anti-CD81 antibody at 37 °C for 1 h before JFH1 infection. The cells were collected, and the HCV genomic RNA was analyzed using real-time qPCR at 2, 4, and 6 h post-HCV infection. (B) Huh7.5.1 cells were infected with 25, 50, or 75 ng/ml $p24^{gag}$ HIV-1 GFPVpr− or GFPVpr+ for 24 h and were then infected with HCVpp for 48 h. HIV-infected cells were also treated with anti-CD81 antibody to block HCVpp infection. The cells were collected and subjected to a firefly luciferase activity assay.

### 3.4. HIV-1 Vpr does not affect HCV viral entry

The enhancement of HCV infection in the presence of HIV-1 Vpr indicated that the Vpr protein may increase the process of viral entry, translation, or RNA replication. To define which step(s) was affected, we first analyzed the viral entry process in the presence of Vpr by determining the amount of HCV genomic RNA that entered into the cells. Briefly, Huh7.5.1 cells were infected with 50 ng/ml $p24^{gag}$ HIV-1 LucVpr− or LucVpr+ and were inoculated with JFH1 at an MOI of 0.1 the following day. To confirm the JFH1 infection, HIV-infected cells were incubated with anti-CD81 antibody at 37 °C for 1 h before JFH1 infection. The cells were collected for real-time RT-PCR analysis at 2, 4, and 6 h post-HCV infection. To remove the virions that did not penetrate the cell surface, the cells were treated with RPMI containing 0.1 mg/ml trypsin for 5 min at 4 °C before collection (Tanaka et al., 2010). As shown in Fig. 4A, there was no significant difference in the levels of HCV genomic RNA between the cells infected with LucVpr− and LucVpr+ at each indicated time point. We also observed that, in all of the infected cells, the level of HCV genomic RNA remained almost unchanged from 2 to 4 h. However, the level increased at 6 h post-HCV infection, suggesting that the RNA level assayed at 2 and 4 h represented the amount of viral RNA that had entered the cells and that the increase in RNA level at 6 h post-HCV infection was due to the RNA replication. The results indicated that HIV-1 Vpr did not affect HCV viral entry.

To further confirm this finding, we also used HCVpp to assay the viral entry in the presence of HIV-1 Vpr. To eliminate the impact of the Vpr-induced $G_2$ arrest, Huh7.5.1 cells were infected with 25, 50, or 75 ng/ml $p24^{gag}$ HIV-1 GFPVpr− or GFPVpr+ for 24 h and were then infected with HCVpp for 48 h. To confirm the HCVpp infection anti-CD81 antibody was also used. The cells were collected and subjected to a luciferase activity assay. As shown in Fig. 4B, no significant difference in HCVpp infection was observed between the cells infected with GFPVpr− and GFPVpr+ at each indicated concentration of $p24^{gag}$.

Overall, the results demonstrated that HIV-1 Vpr did not affect HCV viral entry but did affect the post-entry steps of the HCV life cycle.

### 3.5. HIV-1 Vpr does not affect HCV IRES-mediated translation

We further investigated whether HCV IRES-directed translation was affected by HIV-1 Vpr. A transient transfection assay was conducted to evaluate the level of HCV IRES-directed translation using a dual-luciferase reporter gene construct. In this construct, the translation of upstream *Renilla* luciferase gene (Rluc) was controlled by the 5′ cap structure, and the downstream firefly luciferase gene (Fluc) was mediated by an HCV IRES element (Fig. 5A) (Chen et al., 2013). In brief, Huh7.5.1 cells were infected with 25, 50, or 75 ng/ml $p24^{gag}$ HIV-1 GFPVpr− or GFPVpr+ for 24 h in 24-well plates and were then transfected with 0.5 μg of the HCV IRES dual-luciferase reporter construct. The cells were lysed in passive lysis buffer and then subjected to the dual-luciferase assay. As shown in Fig. 5B, no significant difference was observed in the luciferase activity between the cells infected with GFPVpr− and GFPVpr+ at

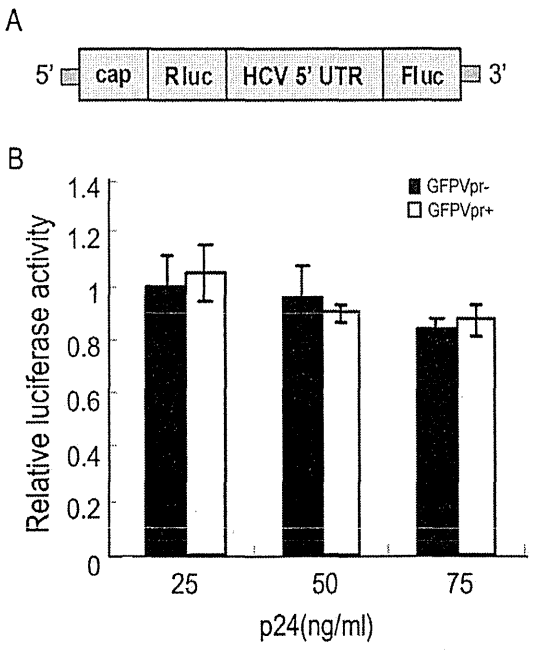


**Fig. 5.** The effects of HIV-1 Vpr on HCV IRES-mediated translation. (A) A dual-luciferase reporter gene construct containing an HCV IRES element was used. (B) Huh7.5.1 cells were infected with 25, 50, or 75 ng/ml $p24^{gag}$ of HIV-1 GFPVpr− or GFPVpr+ for 24 h and were then transiently transfected with the dual-luciferase reporter gene. Luciferase assays were performed at 48 h post-transfection. The relative luciferase activity is expressed as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

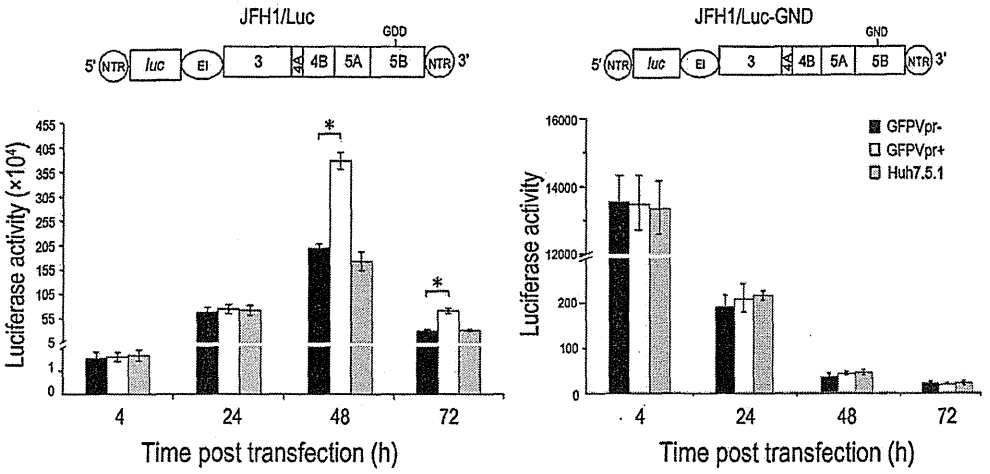


**Fig. 6.** The effects of HIV-1 Vpr on HCV RNA replication. Huh7.5.1 cells were infected with 50 ng/ml $p24^{gag}$ of HIV-1 GFPVpr– or GFPVpr+ for 24 h and were then transfected by electroporation with the firefly luciferase reporter replicon RNA transcribed from pSGR-JFH1/Luc (left panel) or pSGR-JFH1/Luc-GND (right panel). The transfected cells were seeded into 24-well culture plates and harvested for luciferase activity analysis at the given time points.

each indicated concentration of $p24^{gag}$. These results suggested that HIV-1 Vpr did not affect HCV IRES-mediated translation.

### 3.6. HIV-1 Vpr increases HCV RNA replication

Having excluded the possibilities that HIV-1 Vpr increases HCV viral entry or translation, we speculated that HIV-1 Vpr might have a profound impact on HCV RNA replication. To examine this, Huh7.5.1 cells were infected with 50 ng/ml $p24^{gag}$ HIV-1 GFPVpr– or GFPVpr+ and, the following day, were transfected by electroporation with 5 μg of the firefly luciferase reporter replicon RNA transcribed from pSGR-JFH1/Luc or pSGR-JFH1/Luc-GND. The transfected cells were seeded into 24-well culture plates and harvested for luciferase activity analysis at 4, 24, 48 and 72 h post-transfection. Because JFH1/Luc-GND carries an inactivating mutation in the active center of the viral RNA-dependent RNA polymerase NS5B, the transfection of this variant was used to determine whether viral RNA replication was affected by HIV-1 Vpr. As shown in Fig. 6, in the case of the JFH1/Luc replicon, the luciferase activity increased and reached its maximum level 48 h post-transfection. However, in the case of the replication-deficient replicon JFH1/Luc-GND, the luciferase activity decreased over time during the course of the experiment. We also observed that, in the case of the JFH1/Luc replicon, luciferase activity in the cells infected with GFPVpr+ was significantly enhanced compared to the cells infected with GFPVpr– and the uninfected cells at 48 and 72 h post-transfection. In the case of the JFH1/Luc-GND replicon, there was no significant difference in luciferase activity between the cells infected with GFPVpr– and GFPVpr+ at any indicated time after transfection. Because HIV-1 Vpr had no impact on the luciferase expression from the JFH1/Luc-GND replicon but selectively enhanced the expression of JFH1/Luc replicon at later time points, we concluded that HIV-1 Vpr enhanced HCV RNA replication.

### 3.7. HIV-1 Vpr partially overcomes the anti-HCV effect of PEG-IFN

Finally, we examined the effect of HIV-1 Vpr on HCV replication under PEG-IFN treatment. We first assayed the anti-HCV effect of PEG-IFN with various concentrations in cell culture. Huh7.5.1 cells were infected with JFH1 at an MOI of 0.1 for 4 h and then treated with PEG-IFN for 48 h. The cells were collected and subjected to HCV RNA analysis. As shown in Fig. 7A, PEG-IFN potently inhibited JFH1 infection of Huh7.5.1 cells in a dose-dependent manner. Next, we examined the impact of Vpr on JFH1 infection under PEG-IFN treatment. Twenty-four hours after infection with 50 ng/ml $p24^{gag}$ HIV-1 LucVpr– or LucVpr+, Huh7.5.1 cells were inoculated with JFH1 at an MOI of 0.1. Four hours post-HCV infection, the cells were treated with PEG-IFN for 48 h and then were harvested for HCV RNA analysis. As shown in Fig. 7B, HCV RNA levels in both cells decreased as PEG-IFN concentration increased, but at each concentration of PEG-IFN, HCV RNA levels in cells infected with LucVpr+ were enhanced significantly compared with cells infected with LucVpr–. These results demonstrated that HIV-1 Vpr was able to partially overcome the anti-HCV effect of PEG-IFN.

## 4. Discussion

In the present study, we demonstrated that HIV-1 Vpr increased the infection of HCV, including the infectious full-length HCV genome (JFH1, J399EM and J399LM), which belongs to the GT2a family, and the HCV subgenomic replicon (Con1), which belongs to the GT1b family. We also demonstrated that HIV-1 Vpr increased HCV infection by enhancing viral RNA replication but did not affect viral entry or translation. Further, we showed that HIV-1 Vpr can partially overcome the anti-HCV effect of PEG-IFN.

To investigate the role of HIV-1 Vpr in HCV viral entry, we assayed the level of HCV genomic RNA using real-time RT-PCR, and we used HCV pseudoparticles (HCVpp). HCVpp are the first robust *in vitro* assay of HCV glycoprotein-mediated entry, as its entry is strictly E1/E2-dependent and is easily detected by measuring the reporter gene activity (von Hahn and Rice, 2008). The results from the real-time RT-PCR and HCVpp assays demonstrated that HIV-1 Vpr did not affect HCV viral entry, and these results were also supported by the observation that there was no significant difference in luciferase activity between cells infected with HIV-1 GFPVpr– and GFPVpr+ at 4 h post-transfection (Fig. 6).

By using a dual-luciferase reporter construct containing the HCV IRES element, we demonstrated that HIV-1 Vpr had little impact on HCV IRES-mediated translation. However, the expression of HCV proteins was enhanced when HIV-1 Vpr was present. We speculated that the increase in HCV protein expression may be due to the enhancement of HCV RNA replication, as it has been reported that the translation of HCV RNA is coupled to RNA replication and preferentially depends on active RNA replication (Liu et al., 2012). To determine whether HCV RNA replication was affected

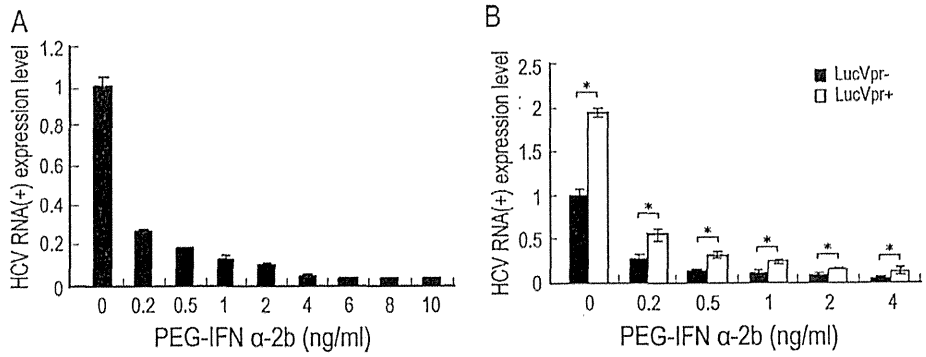


**Fig. 7.** The effects of HIV-1 Vpr on HCV replication under PEG-IFN treatment. (A) Huh7.5.1 cells were infected with JFH1 at an MOI of 0.1 for 4 h and then were treated with PEG-IFN for 48 h. The cells were collected and subjected to HCV RNA analysis. (B) Twenty-four hours after infection with 50 ng/ml $p24^{gag}$ HIV-1 LucVpr− or LucVpr+, Huh7.5.1 cells were inoculated with JFH1 at an MOI of 0.1. Four hours post-HCV infection, the cells were treated with PEG-IFN for 48 h and then were harvested for HCV RNA analysis.

by HIV-1 Vpr, the HCV replication-competent replicon JFH1/Luc and the replication-inactive replicon JFH1/Luc-GND were used. As expected, HIV-1 Vpr selectively enhanced the luciferase expression from the JFH1/Luc but had no impact on the expression from the JFH1/Luc-GND. Thus, we concluded that HIV-1 Vpr enhanced HCV infection by increasing HCV RNA replication and that the increase in HCV RNA replication led to the enhancement of RNA translation.

A very interesting phenomenon was observed in our experiment. We found that the order of inoculation with HIV and HCV determined the effects that Vpr had on HCV infection. Taking HCV JFH1 as example, when cells were infected with pseudotyped HIV-1 for 24 h prior to HCV inoculation, Vpr increased HCV infection almost 100% at 48 h post-HCV inoculation. However, when cells were infected with pseudotyped HIV-1 and HCV at the same time, the HCV infection increased only 40% at 48 h post-HCV inoculation, and when cells were infected with HCV for 24 h prior to pseudotyped HIV-1 infection, Vpr had no impact on HCV infection at 48 h post-HCV inoculation (data not shown). These results suggested that the increase in HCV infection depended on the presence of the Vpr protein that is expressed late in the HIV-1 life cycle and that Vpr might act at an early stage of the HCV life cycle.

The results from Huh7.5.1 cell cycle analysis (Fig. 2B) and the results from J399EM and J399LM infection (Fig. 1B and C) indicated that for infectious HCV infection, HIV-1 Vpr had two potential effects. Vpr enhanced the infection of infectious HCV when present in levels that were not sufficient to induce a cell cycle arrest; conversely, when the level of Vpr was high enough to induce a cell cycle arrest, its ability to enhance infectious HCV infection was weakened (Fig. 1). These results suggested that Vpr's activity strictly depended on its concentration and that different concentrations of the Vpr may lead to different functions. This finding was also supported by other research that showed Vpr augmented UNG2 degradation at low concentrations but antagonized it at high concentrations (Wen et al., 2012). We ascribed the slight increase in HCV infection in the arrested cells to the cessation of cell proliferation as virus replication strictly depends on the internal environment of the host cells. To our surprise, despite the induction of a $G_2$ arrest in cells, the replication of HCV Con1-SGR was still enhanced by Vpr. This problem was hard to explain theoretically and should be explored further.

Interestingly, in addition to its roles as an intracellular protein, Vpr has been reported to act as an extracellular protein independent of intact HIV-1 and is involved in many activities, such as the regulation of productive infection and latency of HIV-1 (Levy et al., 1994, 1995), the induction of L1-RTP (Iijima et al., 2013), and reducing the levels of intracellular ATP and GSH during the course of HIV-1-associated neurological disease (Ferrucci et al., 2012, 2013). Obviously, there is a defect in our study as we did not demonstrate that extracellular Vpr could increase HCV replication. The data reported in our manuscript were obtained by using an *in vitro* HIV/HCV coinfection system. We concede that it appears to be irrelevant to the situation *in vivo* that HIV-1 is not likely to infect hepatocytes. Indeed, HIV-1 infects mononuclear leukocytes bearing CD4 and the chemokine receptors CCR5 and CXCR4, while HCV attacks hepatocytes.

In conclusion, we demonstrated that HIV-1 Vpr increased HCV infection by enhancing RNA replication and that the increase in HCV infection depended on the appropriate Vpr level. Our *in vitro* findings are consistent with the clinical observation that serum HCV RNA levels and viral loads are significantly increased in HIV/HCV co-infected persons when compared with HCV mono-infected persons. Although the relationship between high levels of HCV RNA and faster progression of HCV-related disease has not been fully clarified, our findings contribute new information concerning the role of HIV-1 Vpr in the HCV life cycle and provide additional insight into the interactions between HIV and HCV during HIV/HCV coinfection. In addition, our study provide important information for HCV treatment in HIV/HCV coinfected patients as we demonstrated that HIV-1 Vpr could partially overcome the anti-HCV effect of PEG-IFN. In the future, specific Vpr inhibitors should be identified and could potentially be used as a treatment option for HIV/HCV coinfection. Next, we will prove our data by using exogenous Vpr protein and study further to uncover the precise mechanism by which HIV-1 Vpr increases HCV RNA replication.

## Conflict of interest

There are no actual or potential conflicts of interests.

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