VIROLOGICA SINICA, October 2012, 27 (5):278-285
DOI 10.1007/s12250-012-3257-6

Research Article

Regulation of Hepatitis C Virus Replication and Gene Expression by the MAPK-ERK Pathway*

Rongjuan Pei[1,3], Xiaoyong Zhang[2], Song Xu[1,3], Zhongji Meng[2], Michael Roggendorf[2], Mengji Lu[2] and Xinwen Chen[1]✉

1. Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China;
2. Institute of Virology, University Hospital of Essen, University of Duisburg-Essen, Essen 45122, Germany;
3. Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

The mitogen activated protein kinases-extracellular signal regulated kinases (MAPK-ERK) pathway is involved in regulation of multiple cellular processes including the cell cycle. In the present study using a Huh7 cell line Con1 with an HCV replicon, we have shown that the MAPK-ERK pathway plays a significant role in the modulation of HCV replication and protein expression and might influence IFN-α signalling. Epithelial growth factor (EGF) was able to stimulate ERK activation and decreased HCV RNA load while a MAPK-ERK pathway inhibitor U0126 led to an elevated HCV RNA load and higher NS5A protein amounts in Con1 cells. It could be further demonstrated that the inhibition of the MAPK-ERK pathway facilitated the translation directed by the HCV internal ribosome entry site. Consistently, a U0126 treatment enhanced activity of the HCV reporter replicon in transient transfection assays. Thus, the MAPK-ERK pathway plays an important role in the regulation of HCV gene expression and replication. In addition, cyclin-dependent kinases (CDKs) downstream of ERK may also be involved in the modulation of HCV replication since roscovitine, an inhibitor of CDKs had a similar effect to that of U0126. Modulation of the cell cycle progression by cell cycle inhibitor or RNAi resulted consistently in changes of HCV RNA levels. Further, the replication of HCV replicon in Con1 cells was inhibited by IFN-α. The inhibitory effect of IFN-α could be partly reversed by pre-incubation of Con-1 cells with inhibitors of the MAPK-ERK pathway and CDKs. It could be shown that the MAPK-ERK inhibitors are able to partially modulate the expression of interferon-stimulated genes.

Hepatitis C Virus (HCV); Mitogen activated protein kinases-extracellular signal regulated kinase (MAPK-ERK); Cell cycle progression

Hepatitis C virus (HCV) is an enveloped positive RNA virus and belongs to the family *Flaviviridae*[8]. HCV causes persistent infection in 50 %-80 % of infected persons and may lead to the development of fibrosis, cirrhosis, and hepatocellular carcinoma[2,33,37]. The establishment of a HCV subgenomic replicon system[21] was a major breakthrough in research on HCV. Furthermore, an HCV infection system was established based on the HCV JFH-1 molecular clone and Huh-7 derived cell lines[3,20,39,41]. These systems facilitate studies on the HCV life cycle and HCV-host cell interaction.

Since the viral replication is dependent on the host cell machinery, activation or suppression of cell signalling pathways may modulate virus replication. In the case of HCV, a number of intracellular signalling pathways, such as PI3K/Akt, IRF3, and the JAK-STAT pathway, have been shown to influence HCV replication[4,7,10,11,13,15,26,34]. HCV has developed different strategies to interfere with host cell signalling pathways. It has been demonstrated that HCV NS3/4A blocks retinoic acid-inducible I gene (RIG-I) mediated signalling by cleaving the signalling adaptor Cardif and TLR3 mediated IRF3 activation through cleavage of the adaptor Toll/interleukin receptor domain-containing adapter-inducing interferon β (TRIF), thereby interfering with the induction of innate responses[12,19,28,35]. The blockage of the RIG-I mediated activation of innate responses and subsequent interferon (IFN) production is essential for the maintenance of HCV replication, as HCV is highly sensitive to the antiviral action of IFN.

Received: 2012-04-26, Accepted: 2012-08-17
* Foundation items: Partly supported by a joint grant of Chinese Academy of Science and Deutsche Akademische Austausch Dienst, and the the National Basic Research Priorities Program of China (2009CB522501, 2005CB522901, 2007CB512901).
✉ Corresponding author.
Phone: +86-27-87199106, Fax: +86-27-87199106,
Email: chenxw@wh.iov.cn

The mitogen activated protein kinases (MAPKs) are widely expressed serine/threonine kinases and mediate signals for the regulation of important cellular functions such as gene transcription, post transcriptional regulation, and cell cycle progression[32]. There are three main groups of MAPKs: extracellular signal regulated kinases (ERK), the p38 family kinases, and the JUN amino terminal kinases. The activation of the MAPK-ERK pathway is pivotal for the cell cycle progression[22,27]. Mitogenic stimulation of cells causes phosphorylation of ERK and translocation of active ERK to the nucleus[9]. This translocation is necessary for ERK-dependent activation of DNA synthesis and progression from G1 into S phase[6]. It has been shown that the HCV NS5A protein may interfere with the activation of the MAPK-ERK pathway by altering the trafficking of epithelial growth factor (EGF) receptor[23,25], thus attenuating the cellular response to EGF.

However, the significance of the MAPK pathway activation in HCV replication is not clear. In a previous study, HCV translation mediated by the internal ribosome binding site (IRES) was shown to be enhanced by inhibitors of the MAPK pathway[29]. However, this study did not clarify the influence of the MAPK pathway on HCV replication. Huang *et al.* examined the role of the MAPK pathway in the antiviral action of interferon-γ against HCV[16]. In a luciferase-based reporter replicon system, a blockage of the MAPK pathway activation could partially interfere with the inhibition of the reporter replicon by IFN-γ. It was suggested that the phosphorylation of HCV NS5A plays an important role in the HCV replication and is influenced by the MAPK activity.

In the current study, we examined the influence of the MAPK-ERK pathway on HCV replication. Using the HCV replicon system, we examined whether HCV replication and HCV IRES-dependent translation could be modulated by the inhibition or activation of the MAPK-ERK pathway. Furthermore, we investigated how the modulation of the MAPK-ERK pathway influences the IFN signalling and the inhibition of HCV replication by IFN. As the MAPK-ERK pathway is able to directly influence cell progression by activation of cyclin-dependent kinase 2 (CDK2), an important key protein of cell cycle control, we tested whether a blockage of cdk2 by an inhibitor roscovitine would also modulate HCV replication. A cell cycle inhibitor aphidicolin and an siRNA of a cellular negative regulator CDKN2B were used to modulate the cell cycles and to test the influence on HCV replication.

MATERIALS AND METHODS

Cell culture

Huh7-lunet cells (kindly provided by Dr. Ralf Bartenschlager) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 2 mmol/L of glutamine, 100 U/mL of penicillin and 100 U/mL of streptomycin at 37 °C in a 5 % CO_2 atmosphere. The subgenomic replicon system based on Huh7-lunet cells and the subgenomic HCV replicon pFKI389neo/NS3-3′ was kindly provided by Dr. Ralf Bartenschlager. Con1 cells with subgenomic HCV replicon were maintained in the same medium supplemented with 0.5 mg/mL G418 (Cytogen).

Reagents

The inhibitor of MAPK-ERK pathway U0126 and the CDK inhibitor roscovitine were purchased from Sigma and InvivoGene, respectively. Epidermal growth factor (EGF) was purchased from BD Biosciences. Aphidicolin was purchased from Sigma. Polyclonal antibodies to HCV NS5A were kindly provided by Dr. Ralf Bartenschlager. Antibodies to ERK1 (K-23) and pERK (E-4) were purchased from Santa Cruz Biotechnology, Inc. A monoclonal mouse anti-actin (α-Sarcomeric) antibody was provided by Sigma-Aldrich and the secondary antibodies peroxidase affinipure goat anti-mouse IgG (H+L) and peroxidase affinipure goat anti-rabbit IgG (H+L) by Jackson ImmunoResearch Inc.

Electroporation and transient HCV replication assay

1×10^7 lunet cells were transfected by electroporation using 5 μg of a luciferase replicon pFKI389luc/NS3-3' as described[18]. 12 mL of complete DMEM were then added to cells. 1 mL aliquots of the cell suspension were seeded in each well of a 24-well-plate and harvested at indicated time points for luciferase assay. Luciferase activity was expressed as relative light units (RLU). These values represent the percentage of luciferase activity determined at a given time point relative to the one measured 4 h after transfection.

RNA extraction and quantification of HCV RNA by real time RT-PCR

Total RNAs from cultured cells were extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The quantification of HCV RNA was performed by real-time RT-PCR using a QuantiFast SYBR Green RT-PCR kit (Qiagen). The copy numbers of human beta-actin mRNAs were determined and used for normalization of real-time RT-PCR detection of other RNAs. The following primers were used: 5'-atcactcccctgtgagga act-3' (nt 36-56) and 5'- gcgggttgatccaagaaagg-3' (nt 192-172) for HCV (Numbering of the sequence is according to GenBank entry AJ242652), A1 5′-tccctggagaagagctacga-3′ (nt 879-905) and A2 5′- agcactgtgttggcgtacag-3′ (nt 1224-1200) for β-actin (Numbering of the sequence is according to GenBank entry NM_001101.2). The primers for real time RT-PCR of CDKN2B were validated and provided by Qiagen (Order number QT00203147).

The HCV RNA levels in untreated Con1 cells ranged between 0.5 to 2 copies/beta-actin RNAs under normal culture conditions, as determined by real time RT-PCR. For real time RT-PCR, HCV RNA copy numbers per reaction were around 10^6 in the majority of the samples.

Treatment of cells with EGF, inhibitors, and IFNs

Cells were seeded at a density of 7×10^4 per well in 24-well plates in DMEM omitting G418. After 18 h incubation, cells

reached a confluence of 80%. Different compounds at appropriate concentrations were then added to the wells at indicated time points.

Detection of HCV NS5A and cellular proteins by Western blotting

The detection of HCV NS5A protein was performed as described previously[5]. Cells were lysed in 50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 0.5 % (v/v) Triton-X100, 1 mmol/L EDTA, and 1 mmol/L PMSF or directly in Laemmli sample buffer. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5 % non-fat milk in Phosphate Buffered Saline with Tween 20 (PBST), membranes were incubated with specific primary antibodies. Peroxidase-conjugated secondary antibodies matched to the primary antibodies were used for detection. Detected proteins were visualized using ECL Western blot detection reagents (GE Healthcare).

Reporter assays based on the CoA-acetyl transferase (CAT) and luciferase systems

Huh7-lunet or Con-1 cells were transfected with reporter plasmids (pISRE-Luc (Clontech) or a dicistronic vector pHCV-IRES_D128) using lipofectamine 2000 (Invitrogen). Plasmid pHCV-IRES_D128 was constructed based on pD128 (kindly provided by Dr. M Niepmann) and consisted of a chloramphenicol acetyltransferase (CAT) reporter gene directed by the cap-dependent CMV promoter and a luciferase gene directed by HCV IRES. The HCV 5' noncoding region (nt 1-362) was amplified with suitable primers and cut with restriction enzymes, and cloned into pre-digested pD128 vector. Luciferase reporter assays were performed using the luminescence reporter gene assay system (PerkinElmer) according to the manufacturer's instructions. The levels of CAT in transfected cells were determined by using the Roche CAT ELISA Kit.

Treatment of cells with siRNA

Cells were grown for 24 h to a confluence of 80 % and then transfected with siRNAs. Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions. Twenty pmol siRNA and 1 μL of Lipofectamine 2000 per well were applied in a final volume of 0.5 mL Opti-MEM. After 5 h, the medium was replaced by fresh culture medium. The siRNAs were purchased from Qiagen. The siRNA siHCV 5′-ggucucgua gaccgugcacTT-3′ targeted the HCV sequence nt 331 to 351 (numbered according to the sequence with GenBank accession number AJ242654)[40]. Another siRNA to CDKN2B is a validated siRNA from Qiagen (Ordering number SI00288281).

RESULTS

Inhibition of MAPK-ERK pathway led to an increased HCV replication and protein expression

To investigate the role of MAPK-ERK pathway in HCV replication, we first examined whether HCV replication and protein expression could be modulated by inhibiting the MAPK-ERK pathway. Con1 cells were treated with U0126, an inhibitor of MAPK-ERK pathways, at different concentrations for 48 h. The U0126 treatment prevented the phosphorylation of ERK upon stimulation with EGF (data not shown). HCV RNA and protein levels in cells were determined by real time RT-PCR and Western blot (Fig 1. A and B). U0126, at a high concentration of 10 μmol/L, led to a more than twofold increase of HCV RNA load. The abundance of NS5A protein also increased at the lower concentration of 2.5 μmol/L. The HCV RNA levels in U0126-treated Con1 cells increased gradually up to 72 h (Fig. 1C). These results indicated that inhibition of the MAPK-ERK pathway leads to an enhancement of HCV replication and protein expression.

The influence of the MAPK-ERK pathway on HCV replication was also tested in a transient transfection system using a HCV replicon based on the luciferase reporter gene [18]. U0126 was added to cells 4 h after electroporation and HCV replication was assessed by measuring the luciferase activity in transfected cells. Consistently, U0126 was able to enhance the luciferase expression in transfected cells in a dose-dependent manner, indicating an enhancement of HCV replication activity (Fig. 1D). However, a high concentration of 10 μmol/L of U0126 abolished HCV replication completely, probably due to a strong

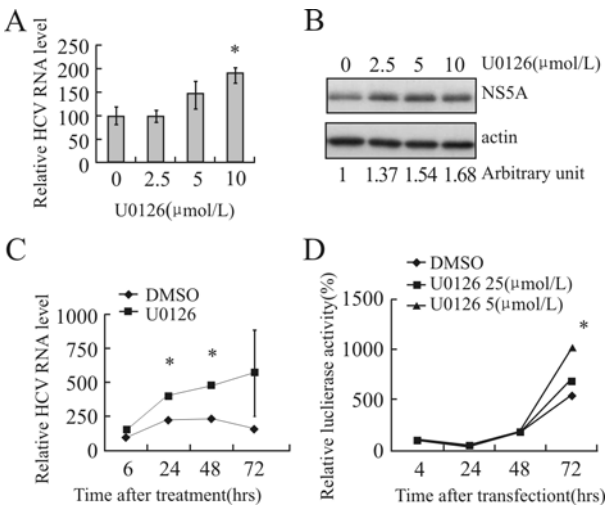

Fig. 1. The ERK inhibitor U0126 increases HCV RNA and protein level in Con1 cells. Con1 cells were treated with U0126 at concentrations of 2.5, 5, and 10 μmol/L for 48 h. HCV RNA (A) and NS5A protein (B) levels in cells were detected by real time RT-PCR and Western blot, respectively. HCV RNA levels in cells were determined as the ratio of HCV RNA/beta-actin RNA (see Material and methods section for details). Relative HCV RNA levels were calculated as the untreated control was set as 100%. Beta-actin was detected by using specific antibodies for normalization. The NS5A protein levels were quantified by densitometry, normalized against beta-actin and expressed in arbitory units. C: Con1 cells were treated with U0126 and harvested for RNA extraction at indicated time points. HCV RNA levels in cells were detected by real time RT-PCR. D: Transfection of HCV reporter replicon in the presence of U0126 at concentrations of 0, 2.5, and 5 μmol/L. The transfected cells were harvested for determination of luciferase expression at indicated time points. The significant differences of the different groups are shown as *(p<0.05).

inhibition of recovery of lunet after electroporation (data not shown).

Activation of MAPK-ERK pathway by EGF modulates the HCV replication

Next, we tested whether activation of the MAPK-ERK pathway influences HCV replication. First, we confirmed that EGF was able to activate the MAPK-ERK pathway in our system. The steady-state level of phosphorylated ERK and the activation of ERK by EGF were determined in naïve Lunet cells and Con1 cells under normal culture conditions. As shown in Fig. 2A, the amounts of ERK1/2 and the phosphorylated form of ERK1/2 in both cell lines were comparable. Furthermore, similar kinetics of EGF-directed ERK1/2 phosphorylation were observed in both Lunet and Con1 cells. The phosphorylation of ERK1/2 occurred rapidly and reached a peak level 5 min after EGF stimulation. The dephosphorylation of ERK1/2 followed and led to a gradual decrease of phosphorylated ERK1/2. Thus, the presence of HCV replicon did not change the steady-state level of phosphorylated ERK1/2 and the response to EGF in our system. Previously, activation by short term stimulation by EGF of the MAPK pathway in Huh7 cells harboring HCV replicon was shown to be suppressed[23]. Interestingly, the HCV RNA level in cells declined transiently with a maximum reduction of 35% at 24 h and returned to a normal level after 48 h (Fig. 2B). A slight treatment and returned to the normal level after 48 h if EGF change in HCV protein abundance occurred shortly after the with was not added after 24 h (Fig. 2C). An additional treatment EGF after 24 h did not reduce further the HCV RNA level but prevented its return to the pre-treatment level (data not shown), consistent with published data[16]. It could be concluded that activation of the MAPK-ERK pathway by EGF leads to a slight and transient decrease in HCV replication in Con1 cells.

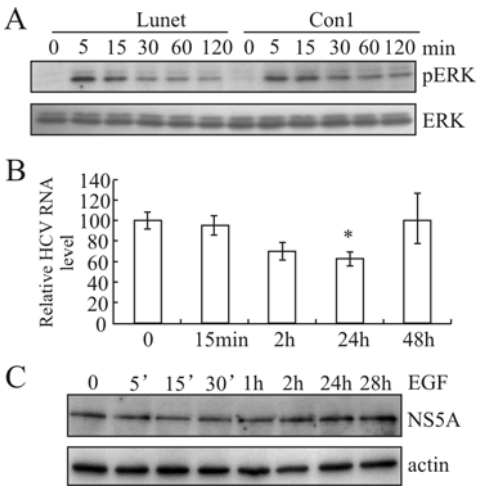

Fig. 2. EGF modulates HCV replication and gene expression. Con1 cells were treated with 50 units of EGF for indicated time and harvested for RNA extraction and Western blotting analysis. Phosphorylated and unphosphorylated ERK were detected using specific antibodies (A). HCV RNA (B) and NS5A protein (C) were detected by real time RT-PCR and Western blot, respectively. HCV RNA levels in cells were determined as the ratio of HCV RNA/beta-actin RNA. Relative HCV RNA levels were calculated as the untreated control was set as 100%. Beta-actin was detected by using specific antibody for normalization. The significant differences of the different groups are shown as * (p<0.05).

Modulation of the HCV IRES-dependent gene expression

The inhibition of the MAPK-ERK pathway led to changes in the HCV RNA and protein abundance. Thus, it is possible that HCV translation is influenced by the MAPK-ERK pathway. HCV translation is specifically mediated by an internal ribosome entry site (IRES) at the 5' non-coding region of HCV. To study the IRES-dependent translation, a dicistronic vector was constructed. The dicistronic vector consisted of a CAT reporter gene directed by the CMV promoter and a firefly luciferase gene directed by HCV IRES (Fig. 3A). Lunet cells were transfected with the dicistronic vector and then treated with U0126 at indicated concentrations for 24 h. Luciferase and CAT activities were detected separately. The results showed that the rate of the Cap-dependent translation decreased in cells treated with U0126 (Fig. 3B). However, the ratio of IRES-dependent translation to Cap-dependent translation was raised in the presence of U0126 (Fig. 3C). Therefore, the IRES-dependent translation was preferred in comparison with the cellular cap-dependent translation by the inhibition of the MAPK-ERK pathway. This result indicated that the inhibition of the MAPK-ERK pathway facilitates the IRES-directed HCV translation and contributes to enhanced HCV replication.

Inhibition of MAPK-ERK pathway modulates the interferon-stimulated response element (ISRE)-dependent gene expression and influences the inhibitory effect of IFN on HCV

Previously, Huang *et al.*[16] showed that a modulation of MAPK-ERK pathway influenced the anti-HCV action of IFN-γ,

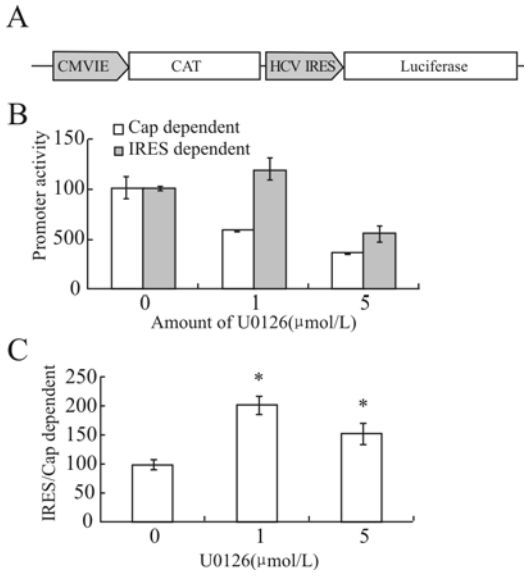

Fig. 3. Modulation of the HCV IRES-dependent gene expression by U0126. A: The schema of dicistronic vector with HCV IRES. B: The dicistronic vector were transfected into Con1 cells. The cells were treated with U0126 at concentrations of 1 and 5 μmol/L. The activity of luciferase and CAT enzyme were determined by luciferase assay and CAT ELISA, respectively. The untreated controls were set as 100%. C: The ratio of the HCV IRES-dependent to Cap-dependent gene expression. The significant differences of the different groups are shown as * (p<0.05).

indicating that a cross-talk between the MAPK-ERK pathway andthe IFN signalling pathway may exist. Therefore, we investigated whether the modulation of MAPK-ERK pathway influences IFN-α signalling and the antiviral action of IFN-α in Con1 cells. First, the effect of inhibitors of the MAPK-ERK pathway on the ISRE-dependent gene expression was examined. Con1 cells were transfected with a reporter plasmid pISRE-Luc vector. The transfected cells were treated with U0126 for 2 h and then stimulated with IFN-α for 6 h. Clearly, U0126 was able to inhibit the IFN-α-stimulated reporter gene expression in a dose-dependent manner (Fig. 4A). These results indicate that 1000 units per mL) for 48 h. Both HCV RNA and protein levels decreased in Con1 cells after the treatment with IFN-α (Fig. 4B and C). In cells treated with U0126, the levels of HCV RNA and HCV NS5A protein were significantly higher compared with the control. However, IFN-α was still effective in U0126 an inhibition of MAPK-ERK pathway influences IFN signalling.

We addressed the question whether an inhibition of MAPK-ERK pathway reduces the ability of IFN-α to suppress HCV replication in Con1 cells. Con1 cells were treated with U0126 and then incubated with IFN-α at different concentrations(0 to treated cells and was able to reduce the HCV RNA and protein levels in a dose-dependent manner (Fig. 4B and C). These results

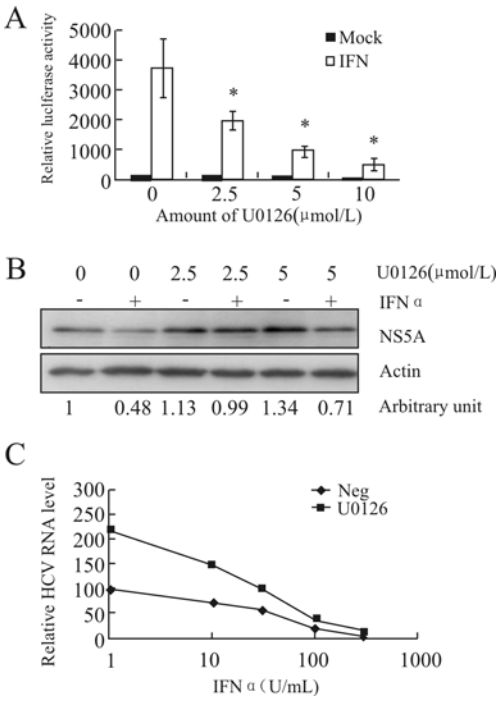

Fig. 4. U0126 inhi5A protein (C) were detected by real time RT-PCR and Western blobited the activation of ISRE reporter gene by IFN-α (A) and attenuated the anti-HCV effect of IFN-α (B and C). Con1 cells were pre-incubated with different inhibitors before IFN-α treatment. HCV RNA (B) and NSt, respectively, 48 h later. HCV RNA levels in cells were determined as the ratio of HCV RNA/beta-actin RNA. Relative HCV RNA levels were calculated as the untreated control was set as 100%. Beta-actin was detected by using specific antibody for normalization. The NS5A protein levels were quantified by densitometry, normalized against beta-actin and expressed in arbitory units. The significant differences of the different groups are shown as * (p<0.05).

imply that IFN and MAPK-ERK pathways may synergistically regulate HCV replication.

Inhibition of CDK2 enhanced HCV replication in the replicon system

One important function of activated ERK 1/2 is to activate the cell cycle regulator CDK2 by inducing degradation of p27[1,17,36]. Thus, a blockage of MAPK-ERK activation by U0126 may prevent cell cycle progression. The question has been raised as to whether the modulation of the cell cycle progression by prevention of CDK2 activation causes changes of the HCV replication levels. Therefore, HCV replication and protein expression was tested during the inhibition of CDK2 activity by roscovitine. An incubation of Con-1 cells with roscovitine led to a significant increase of the HCV RNA levels and HCV NS5A expression (Fig. 5A and B). Similarly to U0126, a roscovitine treatment modulated the effect of IFN-α on HCV replication in lunet cells (Fig. 5C, D and E). These data suggested that MAKP-ERK pathway may modulate HCV replication and protein expression by influencing the cell cycle progression.

To verify the influence of the cell cycle progression on HCV replication, two different approaches were applied. First, the cell cycle progression was arrested by aphidicolin, an inhibitor of cellular DNA polymerase (Fig. 6A). A treatment of Con1 cell with this compound for 24 h led to an increase of HCV RNA levels compared to in those untreated cells. Second, CDKN2B, a negative regulator of cell cycle progression, was targeted by RNAi. A transfection of Con-1 cells with siRNA to CDKN2B resulted in a decrease of the level of the specific mRNAs to less than 50% of the controls within 72 h (Fig. 6B). At the same time, the HCV RNA level was reduced to about 40% of that measured in control cells (Fig. 6C). This result is consistent with the previous findings that a treatment with EGF transiently reduced HCV RNA and protein levels (Fig. 1B). Either a treatment with EGF or knock down of CDKN2B facilitated the cell cycle progression.

DISCUSSION

In this study, we demonstrate that HCV replication could be modulated by activation or inhibition of MAPK-ERK pathway. While an inhibition of MAPK-ERK pathway enhanced HCV translation and replication, the activation of this pathway by EGF led to a reduction of HCV replication. Trujillo-Murillo *et al.*[38] showed that treatment of cells with acetylsalicylic acid led to an inhibition of HCV replication and protein expression through cyclooxygenase 2 signalling pathways with involvement of ERK1/2. Thus, different mediators leading to the activation or inhibition of ERK1/2 may possess the ability to modulate HCV replication. The blockage of MAPK-ERK pathway facilitates the HCV IRES-dependent over the cap-dependent translation. Thus, HCV protein levels were elevated in Con1 cells treated with the MAPK-ERK pathway inhibitors. It is also possible that the increased viral translation contributed to HCV

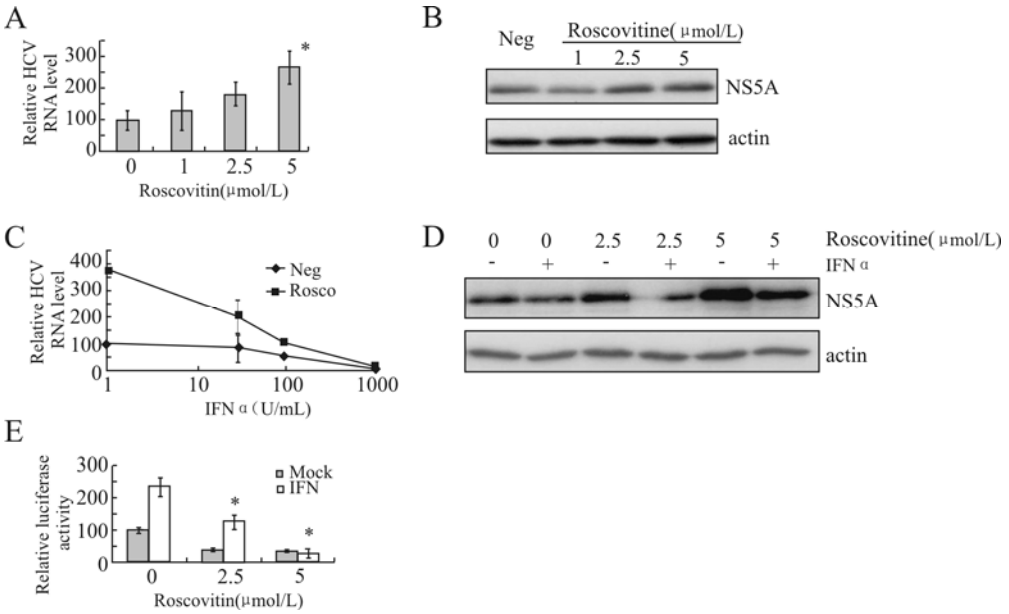

Fig. 5. Roscovitine modulated HCV replication and attenuated the anti-HCV effect of IFN-α. Con1 cells were treated with roscovitine for 48 h at the concentrations indicated in the absence (A, B) or presence (C, D) of IFN-α. HCV RNA (A, C) and NS5A protein (B, D) were detected by real time RT-PCR and western blot, respectively. HCV RNA levels in cells were determined as the ratio of HCV RNA/beta-actin RNA. Relative HCV RNA levels were calculated as the untreated control was set as 100%. E: Con1 cells were transfected with pISRE-Luc and maintained in culture medium in the presence of roscovitine for 48 h with the concentrations indicated. The relative luciferase activities were determined by luciferase assay. The untreated controls were set as 100%. The significant differences of the different groups are shown as * (p<0.05).

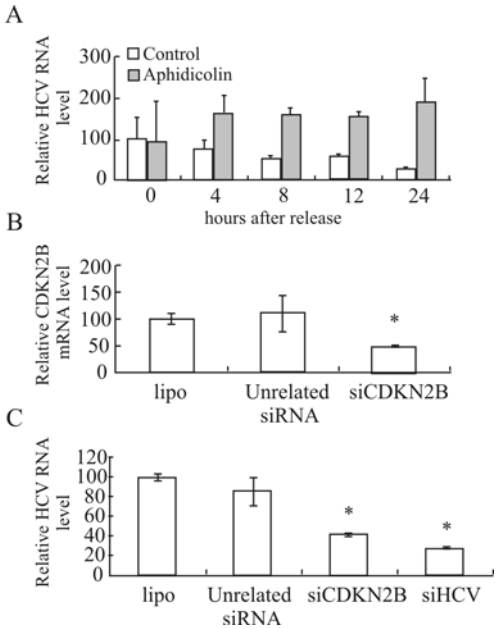

Fig. 6. Modulation of the cell cycle progression led to changes of HCV RNA levels. A: Treatment with aphidicolin. Con1 cells were incubated with aphidicolin at a concentration of 10 μg/mL for 24 h. Aphidicolin was washed away to avoid the possible toxicity. Cells were then harvested for RNA extraction at the indicated time points. B and C: Silencing of CDKN2B. ConI cells were transfected with siRNAs targeting CDKN2B and HCV or unrelated siRNA and harvested after 72 h later for RNA extraction. CDKN2 (B) and HCV RNA levels (C) were determined by real time RT-PCR and expressed as the ratio to beta-actin RNA respectively. Relative HCV RNA levels were calculated as the untreated control was set as 100%. The significant differences of the different groups are shown as * (p<0.05).

replication, resulting in a higher level of HCV RNA. However, this hypothesis needs to be experimentally proven.

The activation of the MAPK-ERK pathway has a multiple effect on cellular processes[32]. A critical function of ERK is to promote the cell cycle progression by degradation of p27 and release of CDK2/cyclin E[17]. In the current study, roscovitine, the inhibitor of CDKs, exerted a similar or even a stronger effect on HCV replication and protein expression than U0126. Two different approaches with aphidicolin and siRNA to CDKN2B demonstrated consistently the correlation between the cell cycle progression and HCV replication. This phenomenon could be explained by the fact that HCV usually replicates in non-proliferating hepatocytes. It could be hypothesized that the growth arrest of hepatoma cells may lead to a cellular status that is more favourable for HCV replication. An inhibitory effect of cell confluence on HCV replication was observed. However, this inhibitory effect was a result of the reduced intracellular pools of nucleosides in confluent cells, possibly through the shutoff of the de novo nucleoside biosynthetic pathway when cells become confluent[31]. Adding exogenous uridine and cytidine to the culture medium restored HCV replication and expression in confluent cells.

An interesting finding was that the inhibition of the MAPK-ERK pathway reduced the ISRE-dependent gene expression. These results suggested that the MAPK-ERK and IFN signalling pathways may crosstalk over some yet unknown links. Thus, understanding of the mechanisms of interaction of different cellular signalling events will be helpful for design of

new strategies directed at modulation of the cellular gene expression related to antiviral activities. The modulation of MAPK-ERK pathway led to a change of effectiveness of the antiviral action of IFN but did not block the inhibition of HCV replication by IFN signalling. MAPK-ERK and IFN pathways may act synergistically to regulate HCV replication.

Controversial reports have been published on this topic. While some reports claimed an enhanced replication of HCV by inhibition of MEK-ERK pathway[16,29,30], others indicated that the inhibition of this pathway could reduce HCV replication[14]. Here we demonstrated that MAPK-ERK pathway and the downstream cell cycle regulators play a significant role in the regulation of HCV replication and protein expression in subgenomic containing cell lines. However, when we applied the MEK inhibitor, U0126, in the HCVcc infectious system, a reduction of infectious virus produced in the supernatant was observed. This may indicate a complex role of the MEK-ERK pathway in the HCV life cycle.

Acknowledgements

The authors would like to thank Dr. Ralf Bartenschlager and Dr. M Niepmann for providing cell lines and other reagents. The authors would also like to thank Dr. Simon Rayner for kindly reading and criticizing the manuscript.

References

1. **Aktas H, Cai H, Cooper G M.** 1997**.** Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin d1 and the cdk inhibitor p27kip1. **Mol Cell Biol**, 17(7): 3850–3857.
2. **Alter M J.** 1997**.** Epidemiology of hepatitis c. **Hepatology**, 26(S3): 62S–65S.
3. **Bartenschlager R.** 2006**.** Hepatitis c virus molecular clones: From cdna to infectious virus particles in cell culture. **Current Opinion in Microbiology**, 9(4): 416–422.
4. **Binder M, Kochs G, Bartenschlager R, *et al.*** 2007**.** Hepatitis c virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. **Hepatology**, 46(5): 1365–1374.
5. **Broering R, Wu J, Meng Z, *et al.*** 2008**.** Toll-like receptor-stimulated non-parenchymal liver cells can regulate hepatitis c virus replication. **J Hepatol**, 48(6): 914–922.
6. **Brunet A, Danie` Le Roux, Lenorm P, *et al.*** 1999**.** Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. **The EMBO Journal**, 18(3): 664–674.
7. **Chang Y, Lei Y, Wu W, *et al.*** 2007. Effect of hepatitis c virus core protein on interferon-induced antiviral genes expression and its mechanisms. **Virologica Sinica**, 22(5): 274-379.
8. **Choo Q L, Kuo G, Weiner a J, *et al.*** 1989**.** Isolation of a cdna clone derived from a blood-borne non-a, non-b viral hepatitis genome. **Science**, 244(4902): 359–362.
9. **Cyert M S.** 2001**.** Regulation of nuclear localization during signaling. **J Biol Chem**, 276(24): 20805–20808.
10. **Fimia G M, Evangelisti C, Alonzi T, *et al.*** 2004**.** Conventional protein kinase c inhibition prevents alpha interferon-mediated hepatitis c virus replicon clearance by impairing stat activation. **J Virol**, 78(23): 12809–12816.
11. **Foy E, Li K, Wang C, *et al.*** 2003**.** Regulation of interferon regulatory factor-3 by the hepatitis c virus serine protease. **Science**, 300(5622): 1145–1148.
12. **Foy E, Li K, Sumpter R, Jr, *et al.*** 2005**.** Control of antiviral defenses through hepatitis c virus disruption of retinoic acid-inducible gene-i signaling. **Proc Natl Acad Sci U S A**, 102(8): 2986–2991.
13. **Frese M, Schwarzle V, Barth K, *et al.*** 2002**.** Interferon-gamma inhibits replication of subgenomic and genomic hepatitis c virus rnas. **Hepatology**, 35(3): 694–703.
14. **Gretton S, Hughes M,Harris M.** 2010**.** Hepatitis c virus rna replication is regulated by ras-erk signalling. **J Gen Virol**, 91(Pt 3): 671–680.
15. **Guo J-T, Zhu Q, Seeger C.** 2003**.** Cytopathic and noncytopathic interferon responses in cells expressing hepatitis c virus subgenomic replicons. **J Virol**, 77(20): 10769–10779.
16. **Huang Y, Chen X C, Konduri M, *et al.*** 2006**.** Mechanistic link between the anti-hcv effect of interferon gamma and control of viral replication by a ras-mapk signaling cascade. **Hepatology**, 43(1): 81–90.
17. **Kawada M, Yamagoe S, Murakami Y, *et al.*** 1997**.** Induction of p27kip1 degradation and anchorage independence by ras through the map kinase signaling pathway. **Oncogene**, 15(6): 629–637.
18. **Krieger N, Lohmann V, Bartenschlager R.** 2001**.** Enhancement of hepatitis c virus rna replication by cell culture-adaptive mutations. **J Virol**, 75(10): 4614–4624.
19. **Li K, Foy E, Ferreon J C, *et al.*** 2005**.** Immune evasion by hepatitis c virus ns3/4a protease-mediated cleavage of the toll-like receptor 3 adaptor protein trif. **Proc Natl Acad Sci U S A**, 102(8): 2992–2997.
20. **Lindenbach B D, Evans M J, Syder a J, *et al.*** 2005**.** Complete replication of hepatitis c virus in cell culture. **Science**, 309(5734): 623–626.
21. **Lohmann V, Ouml, Rner F, *et al.*** 1999**.** Replication of subgenomic hepatitis c virus rnas in a hepatoma cell line. **Science**, 285(5424): 110–113.
22. **Maccorkle R, Tan T-H.** 2005**.** Mitogen-activated protein kinases in cell-cycle control. **Cell Biochemistry and Biophysics**, 43(3): 451–461.
23. **Macdonald A, Chan J K Y, Harris M.** 2005**.** Perturbation of epidermal growth factor receptor complex formation and ras signalling in cells harbouring the hepatitis c virus subgenomic replicon. **J Gen Virol**, 86(4): 1027–1033.
24. **Macdonald A, Crowder K, Street A, *et al.*** 2003**.** The hepatitis c virus non-structural ns5a protein inhibits activating protein-1 function by perturbing ras-erk pathway signaling. **J Biol Chem**, 278(20): 17775–17784.
25. **Mankouri J, Griffin S, Harris M.** 2008**.** The hepatitis c virus non-structural protein ns5a alters the trafficking profile of the

epidermal growth factor receptor. **Traffic**, 9(9): 1497–1509.

26. **Mannova P, Beretta L.** 2005**.** Activation of the n-ras-pi3k-akt-mtor pathway by hepatitis c virus: Control of cell survival and viral replication. **J Virol**, 79(14): 8742–8749.
27. **Meloche S, Pouyssegur J.** 2007**.** The erk1//2 mitogen-activated protein kinase pathway as a master regulator of the g1- to s-phase transition. **Oncogene**, 26(22): 3227–3239.
28. **Meylan E, Curran J, Hofmann K, *et al.*** 2005**.** Cardif is an adaptor protein in the rig-i antiviral pathway and is targeted by hepatitis c virus. **Nature**, 437(7062): 1167–1172.
29. **Murata T, Hijikata M, Shimotohno K.** 2005**.** Enhancement of internal ribosome entry site-mediated translation and replication of hepatitis c virus by pd98059. **Virology**, 340(1): 105–115.
30. **Ndjomou J, Park I W, Liu Y, *et al.*** 2009**.** Up-regulation of hepatitis c virus replication and production by inhibition of mek/erk signaling. **PLoS One**, 4(10): e7498.
31. **Nelson H B, Tang H.** 2006**.** Effect of cell growth on hepatitis c virus (hcv) replication and a mechanism of cell confluence-based inhibition of hcv rna and protein expression. **J Virol**, 80(3): 1181–1190.
32. **Schaeffer H J, Weber M J.** 1999**.** Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. **Mol Cell Biol**, 19(4): 2435–2444.
33. **Seeff L B.** 1997**.** Natural history of hepatitis c. **Hepatology**, 26(S3): 21S–28S.
34. **Street A, Macdonald A, Crowder K, *et al.*** 2004**.** The hepatitis c virus ns5a protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. **J Biol Chem**, 279(13): 12232–12241.
35. **Sumpter R, Jr, Loo Y M, Foy E, *et al.*** 2005**.** Regulating intracellular antiviral defense and permissiveness to hepatitis c virus rna replication through a cellular rna helicase, rig-i. **J Virol**, 79(5): 2689–2699.
36. **Takuwa N, Takuwa Y.** 1997**.** Ras activity late in g1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into s phase in growth factor-stimulated nih 3t3 fibroblasts. **Mol Cell Biol**, 17(9): 5348–5358.
37. **Tong M J, El-Farra N S, Reikes a R, *et al.*** 1995**.** Clinical outcomes after transfusion-associated hepatitis c. **N Engl J Med**, 332(22): 1463–1466.
38. **Trujillo-Murillo K, Rincon-Sanchez a R, Martinez-Rodriguez H, *et al.*** 2008**.** Acetylsalicylic acid inhibits hepatitis c virus rna and protein expression through cyclooxygenase 2 signaling pathways. **Hepatology**, 47(5): 1462–1472.
39. **Wakita T, Pietschmann T, Kato T, *et al.*** 2005**.** Production of infectious hepatitis c virus in tissue culture from a cloned viral genome. **Nat Med**, 11(7): 791–796.
40. **Yokota T, Sakamoto N, Enomoto N, *et al.*** 2003**.** Inhibition of intracellular hepatitis c virus replication by synthetic and vector-derived small interfering rnas. **EMBO Rep**, 4(6): 602–608.
41. **Zhong J, Gastaminza P, Cheng G, *et al.*** 2005**.** Robust hepatitis c virus infection *in vitro*. **Proc Natl Acad Sci USA**, 102(26): 9294–9299.