

**Interferon- α induced mTOR activation is an anti-hepatitis C virus signal
via the phosphatidylinositol 3-kinase-Akt-independent pathway**

Azusa Matsumoto¹, Tatsuki Ichikawa², Kazuhiko Nakao², Hisamitsu Miyaaki², Kumi Hirano¹,
Masumi Fujimoto², Motohisa Akiyama², Satoshi Miuma², Eisuke Ozawa², Hidetaka Shibata²,
Shigeyuki Takeshita², Hironori Yamasaki³, Masanori Ikeda⁴, Nobuyuki Kato⁴, Katsumi Eguchi¹

1. Department of Clinical Pharmaceutics, 2. The First Department of Internal Medicine,

3. Health Research Center,

Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

4. Department of Molecular Biology, Graduate school of Medicine and Dentistry,

Okayama University, Okayama, Japan

Running title; IFN induced mTOR is anti-HCV signal

Corresponding author;

Tatsuki Ichikawa,

The First Department of Internal Medicine,

Graduate School of Biomedical Sciences, Nagasaki University

1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Phone: +81-95-819-7260, Fax: +81-95-819-7270

E-mail address: ichikawa@net.nagasaki-u.ac.jp

Abbreviations used in this paper:

IFN: interferon

HCV: hepatitis C virus

STAT: signal transducers and activators of transcription

ISGF-3: IFN-stimulated gene factor 3

ISRE: IFN-stimulated regulatory element

PKR: double-stranded RNA-dependent protein kinase

Rapa: Rapamycin

Phosphatidylinositol 3-kinase: PI3-K

mTOR: mammalian target of rapamycin

siRNA: small interfering RNA

Key words:

mTOR

STAT-1

Interferon

HCV

PKR

ABSTRACT

Object. The IFN induced Jak-STAT signal alone is not sufficient to explain all the biological effects of IFN. The PI3-K pathways have emerged as a critical additional component of IFN-induced signaling. This study attempted to clarify that relationship between IFN induced PI3-K-Akt- mTOR activity and anti-viral action. **Result.** When the human normal hepatocyte derived cell line was treated with rapamycin (Rapa), before accretion of IFN- α , tyrosine phosphorylation of STAT-1 was diminished. Pretreatment of Rapa had an inhibitory effect on the IFN- α induced expression of PKR and p48 in a dose dependent manner. Rapa inhibited the IFN- α inducible IFN-stimulated regulatory element luciferase activity in a dose-dependently manner. However, Wortmannin, LY294002 and Akt inhibitor did not influence IFN- α inducible luciferase activity. To examine the effect of PI3-K-Akt-mTOR on the anti-HCV action of IFN- α , the full-length HCV replication system, OR6 cells were used. The pretreatment of Rapa attenuated its anti-HCV replication effect in comparison to IFN- α alone, whereas the pretreatment with PI3-K inhibitors, Wortmannin and LY294002, and Akt inhibitor did not influence IFN induced anti-HCV replication. **Conclusion.** IFN induced mTOR activity, independent of PI3K and Akt, is the critical factor for its anti-HCV activity. Jak independent mTOR activity involved STAT-1 phosphorylation and nuclear location, and then PKR is expressed in hepatocytes.

INTRODUCTION

Currently, a chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide (1). Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. Advancement in the treatment of HCV by a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3, but less than 50% of genotype 1. To ameliorate the salvage rate of HCV infection, new anti-HCV agents have been developed to inhibit the life cycle of HCV and are combined with IFN- α (2). Since IFN- α is the most basic agent for HCV treatment, it is necessary to improvement the salvage rate of HCV infection by clarifying the efficacy of IFN treatment.

The factors associated with a refractory response to IFN treatment are the HCV genotype, viral load, age, sex, fibrosis of the infected liver and metabolic factors such as insulin resistance and steatosis (3). Increased hepatic expression of the suppressor of cytokine signaling (SOCS) family, known as the Jak-STAT signal inhibitors, especially SOCS-3, is associated with non-response to IFN treatment (4,5). It is thought that inflammatory cytokines, such as, interleukin 6, induced by HCV infection can induce SOCS-3 in hepatocyte (5). SOCS-3 inhibits IFN induced tyrosine phosphorylation of Jak, then intra-hepatocyte IFN signal transduction is inhibited. For HCV survival, Jak1, Tyk2 and STAT-1,-2 signaling, which is the essential pathway for type 1 IFN induced anti-viral activity, becomes the attack targets from HCV. The relative lack of a viral response to IFN treatment is associated with blunted IFN signaling (6). HCV coding proteins also inhibit STAT-1

tyrosine phosphorylation (7). The cause of a refractory response to IFN treatment is thought to be HCV induced Jak-STAT signal inhibition.

Type 1 IFN is a pleiotropic cytokine which activates various intra-cellular signal pathways other than the Jak-STAT signal (8). Additional signaling pathways could either collaborate with STATs at the promoter level and contribute to the activation of the STATs plus transcription factor genes or function totally independent of any STAT factors, thus leading to the activation of transcription factor only genes (8). The Jak-STAT signal alone is not sufficient to explain all the biological effects of type 1 IFN. The PI3-K and p38 kinase pathways have emerged as critical additional component of IFN-induced signaling (8.9.10). p38, activated via IL-1 β is enhanced STAT-1 tyrosine phosphorylation and express the anti-viral protein, PKR (9). The IFN induced PI3-K-Akt pathway has Jak independent activation and it is the critical signal for cell survival and insulin action (10), but its relationship with the anti-viral action and PI3-K-Akt pathway is still unclear.

Recently, mTOR, a downstream kinase of PI3-K-Akt pathway, was shown to play a critical role in protein synthesis and anti-viral effects. Kaur and his colleagues reported that the IFN activated mTOR pathway exhibits important regulatory effects in the generation of the IFN responses, including the anti-encephalomyocarditis virus effect (11). The IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE) dependent promoter gene activity. Human cytomegalovirus is inhibited by 5'-AMP-activated protein kinase mediated inhibition of mTOR kinase (12). In contrast, vesicular stomatitis virus is mTOR

dependent (13). A relationship has been reported between the replication of hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70 S6 kinase pathway and regulates the replication of HCV (14). mTOR activation is dependent upon the PI3-K-Akt and ERK pathways. Gao and colleagues reported that HCV-NS5A protein activates the PI3-K-Akt-mTOR pathway and could inhibit HBV RNA transcription and reduce HBV DNA replication in HepG2 cells (15). The activation of the N-Ras-PI3-K-Akt-mTOR pathway by HCV is required for cell survival and HCV replication (16). Therefore, PI3-K, Akt and mTOR activated by HCV are inhibitory signals of HCV replication and survival signals of HCV infected cells. Furthermore, the PI3-K-Akt-mTOR pathway, which is activated by HCV, is thought to be one mechanism for chronic HCV infection (14.15.16). However, type 1 IFN induced PI3-K, Akt and mTOR have not yet been fully evaluated regarding their influence on HCV replication.

This study investigated whether IFN- α induced the PI3-K-Akt-mTOR pathway, whether the Jak-STAT pathway has a relationship with the PI3-K-Akt-mTOR pathway, and, finally, whether IFN induced signal transduction, other than the Jak-STAT pathway, is associated with the anti-HCV activity.

MATERIALS AND METHODS

Reagents and Cell Culture

Recombinant human IFN- α 2b was a generous gift from Schering-Plough KK (Tokyo, Japan). Wortmannin, LY 294002, Akt inhibitor and rapamycin were purchased from Calbiochem (La Jolla, CA). Hc human hepatocyte cells (Applied Cell Biology Research Institute, Kirkland, Washington) and HuH-7 human hepatoma cells (American Type Culture Collection, Rockville, Maryland) were maintained in a chemically defined medium, CS-C completed (Cell Systems, Kirkland, Washington) and RPMI (Invitrogen, Grand Island, New York), respectively, supplemented with 5% fetal bovine serum. In the pretreatment of rapamycin and chemical inhibitors for 3 hr, the cells were cultured in 5% RPMI, and then exchanged the medium and treated the cells with IFN- α 2b at the indicated time.

Cell viability assay

The cells were measured using the colorimetric cell viability assay method. Cell viability was determined by the colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). After two days of 100 IU/mL IFN- α and 1000 nmol/L rapamycin treatment, Cell viability is expressed as a percentage of the viability in standard media without IFN- α and rapamycin. Data were expressed as the mean \pm standard deviation (SD). Statistical significance was assessed using Student's *t* test. Statistical difference was defined as $P < 0.05$. All numerical results were reported as the mean of four independent experiments.

Western Blotting and antibodies

Western blotting with anti-PKR, anti-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-tyrosine-701 phosphorylated STAT-1, anti-serine-727 phosphorylated STAT-1, anti-p48, anti-serine-437 phosphorylated Akt, anti-threonin-308 phosphorylated anti-Akt, anti-Akt, anti-serine-2448 phosphorylated mTOR, anti-serine-2481 phosphorylated mTOR, anti-mTOR, anti-JAK-1 or anti-tyrosine 1022/1023 JAK-1 (Cell Signaling, Beverly, MA) was performed as described previously (9). Briefly, Hc cells were lysed by the addition of a lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 1mmol/L PMSF, 1 µg/ml each of aprotinin, leupeptin, and pepstatin, 1 mmol/L sodium o-vanadate, and 1 mmol/L NaF). The samples were separated by electrophoresis on 8-12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England).

Fluorescence immunohistochemistry

The Hc cells were seeded onto 11-mm glass cover-slips in 24-well plates at 2.4×10^5 cells /well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 10 nmol/L or 100 nmol/L rapamycin, or vehicle, for 3 hr and then stimulated with 100 IU/mL IFN- α for 10 min. Fluorescence immunohistochemistry was performed as described

previously (17). The cells were incubated with anti-tyrosine-701 phosphorylated STAT1 antibody for 1 hr at room temperature, washed three times in PBS, incubated with rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hr, washed in PBS, and mounted in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Nuclear staining was performed using Hoechst 33258 (Invitrogen Japan K.K., Tokyo, Japan). An immunofluorescence analysis was done using an Olympus BX50 microscope (Tokyo, Japan) and the image was captured by a Nikon DXM 1200 digital camera (Tokyo, Japan).

Reporter gene assay

A pISRE-Luc cis-reporter plasmid containing five copies of the ISRE sequence and the firefly luciferase gene and pRL-SV40 containing the SV40 early enhancer/promoter and the renilla luciferase gene were obtained from Clontech (San Diego, CA) and Promega (Madison, WI), respectively. The HuH-7 cells were grown in 24-well multiplates and transfected with 1 µg of pISRE-Luc and 10 ng of pRL-SV40 as a standard by the lipofection method. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers and IFN- α , and the luciferase activities in the cells were determined using a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative ISRE-luciferase activity.

HCV Replicon System

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE (18), were used to examine the influence of the anti-HCV effect of IFN. The cells were cultured in Dulbecco's

modified Eagle's medium (Gibco-BRL, Invitrogen) supplemented with 10% fetal bovine serum, penicillin and streptomycin and maintained in the presence of G418 (300mg/L; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After the treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and then were subjected to a luciferase assay according to the manufacturer's protocol. mTOR gene knock down is used siRNA (Cell Signaling). 100 nmol/L mTOR specific and non-targeted siRNA as a control was transfected to OR6 cells in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 10 IU/mL IFN- α .

RESULTS

IFN- α induced Activity of STAT-1 is inhibited by rapamycin pretreatment

To attempt to clearly identify the influence of mTOR to IFN- α induced anti-viral protein expression rapamycin (Rapa), the specific inhibitor of mTOR, was added prior to treatment with IFN- α . Hc cells have been used as normal hepatocytes in previous reports (19). The Hc cells were incubated in the absence or presence of IFN- α with or without pretreatment with Rapa for 2 hr the cells were then harvested for the Western blot analysis (**Fig. 1**). IFN- α clearly induced tyrosine and serine phosphorylation of STAT-1 at 5 (**Fig. 1A, lane 4**) and 10 min (**Fig. 1A, lane 6**), respectively in the absence of Rapa. However, when the Hc cells were pretreated with Rapa before IFN- α stimulation, the levels of tyrosine and serine phosphorylated STAT-1 were clearly and rapidly lower than those induced by IFN- α alone 5 min after treatment in tyrosine (**Fig. 1A, lane 5**). Jak-1, an upstream protein of STAT-1, was equally phosphorylated by IFN- α with (Fig. 1B lane3) or without (Fig. 1B lane2) pretreatment with Rapa. The viability of the Hc cells was 1 in vehicle, 0.93 ± 0.21 in IFN- α treatment and 0.88 ± 0.34 in rapamycin treatment. No difference in the cell viability the among vehicle, IFN- α and rapamycin treatment was not recognized in our assay. The viability of the HuH-7 and OR6 cells also demonstrated no difference between the presence of IFN- α and rapamycin treatment and the absence thereof.

IFN inducible gene products are diminished by pretreatment of rapamycin

Since pretreatment with Rapa inhibited the IFN- α induced STAT-1 activity, the phosphorylation of tyrosine and serine and nuclear translocation, the effect of pretreated with Rapa

on the IFN- α inducible gene product was examined. The protein levels of PKR, an anti-viral protein that acts as a mRNA translation inhibitor activated by double stranded RNA (20,21), and p48, key component of ISGF-3 with activated STAT-1 and -2 (22), were induced by IFN- α treatment for 3 hr in Hc cells (**Fig. 1C, lane 1 and 2**). However, pretreatment with Rapa had an inhibitory effect on IFN- α induced PKR and p48 in a dose dependent manner (**Fig. 1C, lanes 2, 3 and 4**).

The serine 473 on Akt and serine 2448 on mTOR are phosphorylated by IFN- α

Because pretreatment with Rapa affected the IFN- α signaling (**Fig. 1**), the ability of IFN- α to activate the Akt-mTOR pathway was investigated. The phosphorylation of serine-2448 residues of mTOR and serine-473 residue of Akt by 100 IU/ml of IFN- α was detected at five min and at 60 min after IFN- α treatment, respectively (**Fig. 2A**). The band intensity of serine 2448 phosphorylated mTOR increased at 30 min and decreased at 60 min after IFN- α treatment. In contrast, a slight band intensity of serine phosphorylated 473 Akt was only detected at 60 min after IFN- α treatment. In addition, a Western blot analysis of phosphorylated serine 2481 of mTOR and threonine 308 Akt was conducted under the same conditions as Figure 2A, but no bands were detected (data not shown). In Figure 2D, IFN- α induced Serine 2448 phosphorylated mTOR was not inhibited by PI3-K inhibitors (lanes 2 and 3).

The IFN- α induced nuclear translocation of tyrosine phosphorylated STAT-1 was inhibited by pretreatment with Rapa

The location of tyrosine phosphorylated STAT-1 was evaluated by fluorescence immunohistochemistry of cultured Hc cells (**Fig. 3**). The IFN- α induced nuclear translocation of

tyrosine phosphorylated STAT-1 was observed (**Fig. 3C**), but its translocation was inhibited by pretreatment with Rapa and the inhibition of the translocation of STAT-1 was more definitive at 1000 nmol/L Rapa (**Fig. 3E**) than 100 nmol/L (**Fig. 3G**).

IFN- α induced ISRE-contained promoter activity is inhibited by pretreatment of Rapa, but not by Wortmannin, LY294002 and Akt inhibitor

The influence of pretreatment of PI3-K-Akt-mTOR inhibitors on IFN- α inducible luciferase activity of the ISRE-containing promoter was examined. Since Hc cells were not sufficient for reporter gene transfection, HuH-7 cells were used in the transfection assay. HuH-7 cells were transfected with pISRE-Luc containing five repeats of the ISRE sequence and pRV-SV40 as a standard and then were treated with IFN- α after 3 hr with or without pretreatment with Rapa, Wortmannin, LY294002 or Akt inhibitor. Rapa inhibited IFN- α inducible luciferase activity in a dose-dependent manner (**Fig. 4 lane 2, 3 and 4**). However, Wortmannin and LY294002, PI3-K inhibitor, and Akt inhibitor had no effect on IFN- α inducible luciferase activity (**Fig. 4, lane 2, 5, 6 and 7**).

The expression of IFN- α induced tyrosine phosphorylated STAT-1 was determined after pretreatment with Akt inhibitor and LY294002 to evaluate the result of luciferase assay (**Fig. 4**). The Hc cells were incubated under the same conditions used in **Figure 4**, but phosphorylated STAT-1 was not inhibited by the Akt inhibitor (**Fig. 2B**) and LY294002 (**Fig. 2C**).

Rapamycin and mTOR specific siRNA, but not PI3-K inhibitor and Akt inhibitor, can cancel the IFN- α induced anti-HCV replicon activity.

OR6 cells the full-length HCV replication system was used to examine the anti-viral effect of PI3-K, Akt and mTOR on IFN- α stimulation. The cells were treated with IFN- α after 3 hr in the presence or absence of Rapa, Akt inhibitor or PI3-K inhibitor (**Fig. 5**). Pretreatment with Rapa attenuated its anti-HCV replication effect in comparison to IFN- α alone (**Fig. 5, lanes 1, 2, 3 and 4**), whereas pretreatment with PI3-K inhibitors and Akt inhibitor did not increase the *Renilla* luciferase activity (**Fig. 5, lanes 1, 2 and 5-8**). We performed siRNA transfection for mTOR knock down (**Fig. 6**). Although transfection efficiency of siRNA is barely 10%, IFN- α induced anti-HCV action was clearly inhibited in siRNA against mTOR transfected cells (**lane 5**) in comparison to the control cells (lane 6).

DISCUSSION

Rapa inhibited the IFN- α induced tyrosine and serine phosphorylation and nuclear translocation of STAT-1, the ISRE-promoter activity, the expression of PKR and the replication of HCV replicon. This suggests that the IFN induced mTOR activity, through Jak independent STAT-1 phosphorylation, is a critical signal for IFN induced anti-HCV action. Interestingly, mTOR activated by IFN was PI3-K-Akt independent in this study.

mTOR activity may have inhibitory action for HCV replication through STAT-1 phosphorylation, but not the translation initiation action of mTOR. This study assumed that IFN induced PKR expression and ISRE-luciferase activity were inhibited by Rapa as the result of a suppression effect on IFN inducible STAT-1 activation. IFN inducible PKR contributes the anti-HCV action (20), and anti-HCV action of ribavirin is also attributable to its ability to up-regulate PKR activity (21). Previous reports revealed that the mTOR activity did not influence the HCV-IRES activity because the viral promoter has cap-independent translation (23). Although mTOR is the mRNA translational regulator through phosphorylation of a downstream target such as 4E-BP and S6K (24), we think that the IFN induced mTOR activity influences the phosphorylation of STAT-1 in our study (Fig. 1). In addition, it is thought that the alternation of STAT-1 phosphorylation by the mTOR activity influences the gene expression of anti-virus protein and IFN induced anti-viral action.

In our study, serine-473 on Akt showed a delayed phosphorylation in comparison to that of serine-2448 on mTOR after IFN stimulation (**Fig. 2A**). Since serine-473 on Akt is phosphorylated

by mTOR/Rictor/GβL (25,26) and a PDK-1 independent pathway (25), IFN induced serine-473 phosphorylated Akt may not involve the mTOR activity. Therefore, PI3-K inhibitor and Akt inhibitor had no effect on IFN inducible anti-HCV action. The pathway of mTOR activation is prismatic. PI-3Ks, upstream kinase of Akt and mTOR, are grouped into three classes (I-III) according to their substrate preference and sequence homology (27). PI3-k inhibitor, Wortmannin and LY294002, inhibit class I and III PI3-Ks, and to a lesser extent class II PI3-K, upstream kinase of Akt (27). In our study, neither PI3-K nor Akt inhibitor inhibited IFN induced ISRE luciferase activity and loss of HCV replication (**Fig. 4 and 5**). These results indicate that the IFN induced anti-HCV activity is mTOR dependent, but not PI3-K and Akt dependent. In the current report, the production of IL-1 receptor antagonist in IFN-stimulated monocytes depends on the PI3-K pathway, but not STAT-1 (28), and chronic myelogenous leukemia cells are differentially regulated by the IFN induced PI3-K-Akt-mTOR pathway with no relation to STAT-1 phosphorylation (29). Similar to the findings of those reports, the PI3-K-Akt pathway has been reported to be generally independent of the STAT activity (10). Therefore, the difference in the cell type (8) may explain the discrepancy between these data and our data. We therefore speculate that in hepatocytes, unlike lymphoid cells, IFN induced mTOR activity is not dependent on the PI3-K activity. In addition, the mTOR activity is not related to the STAT activity in lymphoid cells. However, in hepatocytes, the IFN induced mTOR activity was closely linked to the IFN induced STAT activity in our study.

mTOR is a serine and threonine kinase (10). Phosphorylation of STATs by mTOR occurs also on a serine residue, but not tyrosine (10.30). The mTOR pathway is critical for IFN- γ induced suppression of tyrosine phosphorylated STAT-3 in a prostate cancer cell line (31). Although this is not consistent with the results of our study, this also showed mTOR to be associated with tyrosine phosphorylation without reference to SOCS and phosphatase. In addition, in a mouse embryo fibroblast cell line, IFN- γ induced tyrosine and serine phosphorylation of STAT-1 is inhibited by Rapa (32), while in the hepatoma cell line, HLF, IFN- β stimulated STAT-1 tyrosine phosphorylation partially decreases by LY294002, but the effect of Rapa has not yet been studied (33). In the current study (31.32.33), not only STAT-1 serine phosphorylation but also tyrosine was found to be downstream of the IFN induced mTOR activity, however, the mechanism controlling the tyrosine phosphorylation of STAT-1 and the mTOR activity, remains to be elucidated.

In conclusion, IFN induced mTOR activity, independent of PI3-K and Akt, is the critical factor for anti-HCV action. The Jak independent mTOR activity is therefore involved in STAT-1 phosphorylation and nuclear location, thus resulting in the development of IFN induced anti-HCV protein, especially the expression of PKR, in HCV infected hepatocytes.

REFERENCES

- 1 Fattovich G, Stroffolini T, Zagni I, Donato F, Hepatocellular carcinoma in cirrhosis: incidence and risk factors., *Gastroenterology* 2004; 127: S35-50
- 2 Pawlotsky JM, Chevaliez S, McHutchison JG, The hepatitis C virus life cycle as a target for new antiviral therapies., *Gastroenterology* 2007; 132: 1979-98
- 3 Persico M, Capasso M, Persico E, Svelto M, Russo R, Spano D, et al, Suppressor of cytokine signaling 3 (SOCS3) expression and hepatitis C virus-related chronic hepatitis: Insulin resistance and response to antiviral therapy., *Hepatology* 2007; 46: 1009-15
- 4 Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdie DM, Clouston AD, et al, Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signaling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1., *Gut* 2006; 55: 529-35
- 5 Huang Y, Feld JJ, Sapp RK, Nanda S, Lin JH, Blatt LM, et al, Defective hepatic response to interferon and activation of suppressor of cytokine signaling 3 in chronic hepatitis C., *Gastroenterology* 2007; 132: 733-44
- 6 Taylor MW, Tsukahara T, Brodsky L, Schaley J, Sanda C, Stephens MJ, et al, Changes in gene expression during pegylated interferon and ribavirin therapy of chronic hepatitis C virus distinguish responders from nonresponders to antiviral therapy., *J Virol* 2007; 81: 3391-401
- 7 Lan KH, Lan KL, Lee WP, Sheu ML, Chen MY, Lee YL, et al, HCV NS5A inhibits

interferon-alpha signaling through suppression of STAT1 phosphorylation in hepatocyte-derived cell lines., *J Hepatol* 2007; 46: 759-67

8 van Boxel-Dezaire AH, Rani MR, Stark GR, Complex modulation of cell type-specific signaling in response to type I interferons., *Immunity* 2006; 25: 361-72

9 Ichikawa T, Nakao K, Nakata K, Yamashita M, Hamasaki K, Shigeno M, et al, Involvement of IL-1beta and IL-10 in IFN-alpha-mediated antiviral gene induction in human hepatoma cells., *Biochem Biophys Res Commun* 2002; 294: 414-22

10 Kaur S, Uddin S, Platanius LC, The PI3' kinase pathway in interferon signaling., *J Interferon Cytokine Res* 2005; 25: 780-7

11 Kaur S, Lal L, Sassano A, Majchrzak-Kita B, Srikanth M, Baker DP, et al, Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling., *J Biol Chem* 2007; 282: 1757-68

12 Kudchodkar SB, Del Prete GQ, Maguire TG, Alwine JC, AMPK-mediated inhibition of mTOR kinase is circumvented during immediate-early times of human cytomegalovirus infection., *J Virol* 2007; 81: 3649-51

13 Minami K, Tambe Y, Watanabe R, Isono T, Haneda M, Isobe K, et al, Suppression of viral replication by stress-inducible GADD34 protein via the mammalian serine/threonine protein kinase mTOR pathway., *J Virol* 2007; 81: 11106-15

14 Ishida H, Li K, Yi M, Lemon SM, p21-activated kinase 1 is activated through the mammalian target of rapamycin/p70 S6 kinase pathway and regulates the replication of hepatitis C virus in

human hepatoma cells., *J Biol Chem* 2007; 282: 11836-48

15 Guo H, Zhou T, Jiang D, Cuconati A, Xiao GH, Block TM, et al, Regulation of hepatitis B virus replication by the phosphatidylinositol 3-kinase-akt signal transduction pathway., *J Virol* 2007; 81: 10072-80

16 Mannova P, Beretta L, Activation of the N-Ras-PI3K-Akt-mTOR pathway by hepatitis C virus: control of cell survival and viral replication., *J Virol* 2005; 79: 8742-9

17 Nishimura D, Ishikawa H, Matsumoto K, Shibata H, Motoyoshi Y, Fukuta M, et al, DHMEQ, a novel NF-kappaB inhibitor, induces apoptosis and cell-cycle arrest in human hepatoma cells., *Int J Oncol* 2006; 29: 713-9

18 Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N, Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system., *Biochem Biophys Res Commun* 2005; 329: 1350-9

19 Obora A, Shiratori Y, Okuno M, Adachi S, Takano Y, Matsushima-Nishiwaki R, et al, Synergistic induction of apoptosis by acyclic retinoid and interferon-beta in human hepatocellular carcinoma cells., *Hepatology* 2002; 36: 1115-24

20 Wang C, Pflugheber J, Sumpter R Jr, Sodora DL, Hui D, Sen GC, et al, Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication., *J Virol* 2003; 77: 3898-912

21 Liu WL, Su WC, Cheng CW, Hwang LH, Wang CC, Chen HL, et al, Ribavirin up-regulates the activity of double-stranded RNA-activated protein kinase and enhances the action of

interferon-alpha against hepatitis C virus., *J Infect Dis* 2007; 196: 425-34

22 Tamada Y, Nakao K, Nagayama Y, Nakata K, Ichikawa T, Kawamata Y, et al, p48

Overexpression enhances interferon-mediated expression and activity of double-stranded RNA-dependent protein kinase in human hepatoma cells., *J Hepatol* 2002; 37: 493-9

23 Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N, Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells., *Cancer Res* 2007; 67: 10804-12

24 Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N, mTOR, translation initiation and cancer., *Oncogene* 2006; 25: 6416-22

25 Hanada M, Feng J, Hemmings BA, Structure, regulation and function of PKB/AKT--a major therapeutic target., *Biochim Biophys Acta* 2004; 1697: 3-16

26 Hresko RC, Mueckler M, mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes., *J Biol Chem* 2005; 280: 40406-16

27 Engelman JA, Luo J, Cantley LC, The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism., *Nat Rev Genet* 2006; 7: 606-19

28 Molnarfi N, Hyka-Nouspikel N, Gruaz L, Dayer JM, Burger D, The production of IL-1 receptor antagonist in IFN-beta-stimulated human monocytes depends on the activation of phosphatidylinositol 3-kinase but not of STAT1., *J Immunol* 2005; 174: 2974-80

29 Parmar S, Smith J, Sassano A, Uddin S, Katsoulidis E, Majchrzak B, et al, Differential regulation of the p70 S6 kinase pathway by interferon alpha (IFNalpha) and imatinib mesylate

(STI571) in chronic myelogenous leukemia cells., *Blood* 2005; 106: 2436-43

30 Nguyen H, Ramana CV, Bayes J, Stark GR, Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression., *J Biol Chem* 2001; 276: 33361-8

31 Fang P, Hwa V, Rosenfeld RG, Interferon-gamma-induced dephosphorylation of STAT3 and apoptosis are dependent on the mTOR pathway., *Exp Cell Res* 2006; 312: 1229-39

32 El-Hashemite N, Zhang H, Walker V, Hoffmeister KM, Kwiatkowski DJ, Perturbed IFN-gamma-Jak-signal transducers and activators of transcription signaling in tuberous sclerosis mouse models: synergistic effects of rapamycin-IFN-gamma treatment., *Cancer Res* 2004; 64: 3436-43

33 Matsumoto K, Okano J, Murawaki Y, Differential effects of interferon alpha-2b and beta on the signaling pathways in human liver cancer cells., *J Gastroenterol* 2005; 40: 722-32

FIGURE LEGENDS**Figure 1. Alteration in the distribution of IFN- α induced phosphorylated STAT-1 (A) and Jak-1 (B) by rapamycin and Effect of rapamycin on IFN- α -induced PKR and p48 (C)**

Hc cells were pretreated without (lanes 1, 2, 4, 6 and 8) or with 1 μ mol/L Rapa (lanes 3, 5, 7 and 9).

These Hc cells were stimulated by 100 IU/L IFN- α (lane 2-9) for 30 min. Phosphorylated STAT-1 at tyrosine-701 residue (upper panel) and at serine-727 residue (lower panel) were analyzed by

Western blotting. (A). After pretreatment of 1000 nmol/L Rapa (lane 3) for 3 hr, Hc cells were untreated (lane 1) or treated with 100 IU/mL IFN- α (lanes 2-3) for 3 min, then phosphorylated

JAK-1 at tyrosine-1022/1023 residue (first panel), expression of JAK-1 (second panel) were

analyzed by Western blotting (B). Hc cells were treated with 100 IU/mL of IFN- α in the absence

(lane 2) or of the presence of pretreatment (lane 3 and 4). Lane 1 was not treated IFN- α and

calcineurin inhibitors. One day latter, PKR and p48 was determined by Western blotting (C).

Figure 2. Effect of IFN- α on Akt and mTOR (A) and effect of Akt inhibitor (B) and LY294002 (C) on IFN- α -induced tyrosine phosphorylated STAT-1 and Serine phosphorylated mTOR (D).

Hc cells were stimulated by 100 IU/L IFN- α for 60 min. At the indicated time, the cells were

harvested. Phosphorylated Akt at serine-473 residue (first panel), Akt (second panel), mTOR at

serine-2448 residue (third panel) and mTOR (fourth panel) were analyzed by Western blotting.

After pretreatment with 5 or 20 $\mu\text{mol/L}$ Akt inhibitor (lane 3 and 4, respectively) (B) and 1 or 10 nmol/L LY294002 (lane 3 and 4, respectively) (C) for 3 hr, Hc cells were untreated (lane 1) or treated with 100 IU/mL IFN- α (lanes 2-4) for 5 min and phosphorylated STAT-1 at tyrosine-701 residue (first panel), expression of STAT-1 (second panel) were analyzed by Western blotting. (D)

After pretreatment with 100 nmol/L Wortmannin (lane 2) and 1 nmol/L LY294002 (lane 3) for 3 hr, the Hc cells were either untreated (lane 1) or treated with 100 IU/mL IFN- α (lanes 2-4) for 10 min and then were phosphorylated mTOR at Serine-2448 residue (first panel), the expression of mTOR (second panel) was analyzed by Western blotting.

Figure 3. Inhibition of IFN- α induced nuclear translocation of phosphorylated STAT-1 by rapamycin.

The Hc cells were pretreated without (A. B. C. D) or with 1000 nmol/L Rapa (E. F) or 100 nmol/L Rapa (G. H). After pretreatment, the Hc cells were stimulated by 100 IU/L IFN- α (C. D. E. F. G. H) for 30 min. Thereafter, the cells were fixed, permeabilized, processed for immunofluorescence (A. C. E. G) and Hoechst staining (B. D. F. H), and visualized by fluorescence microscopy. The results shown are from one representative experiment from a total of three performed.

Figure 4. Suppression effect of rapamycin, not PI3-k inhibitors and Akt inhibitor, on IFN- α -induced reporter gene assay.

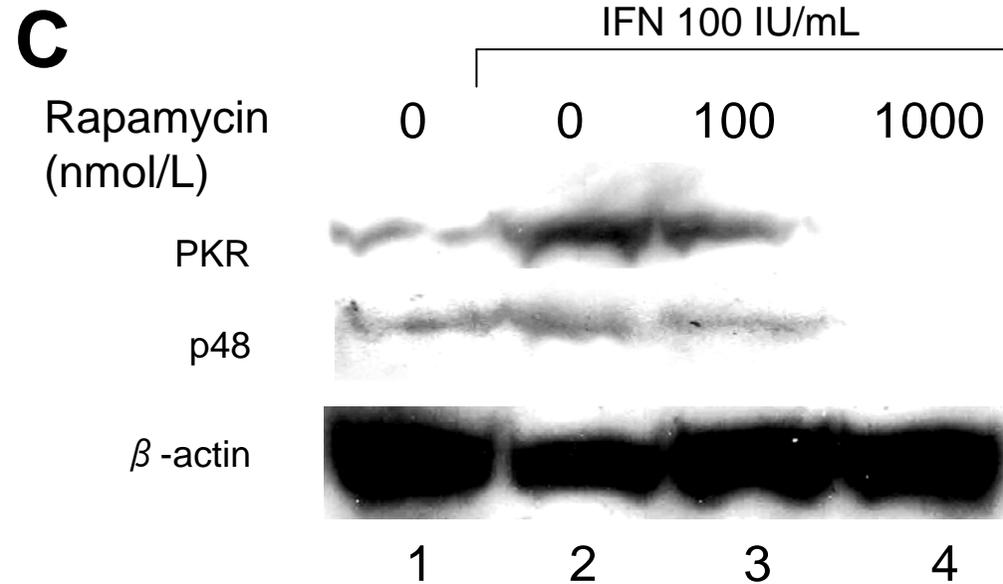
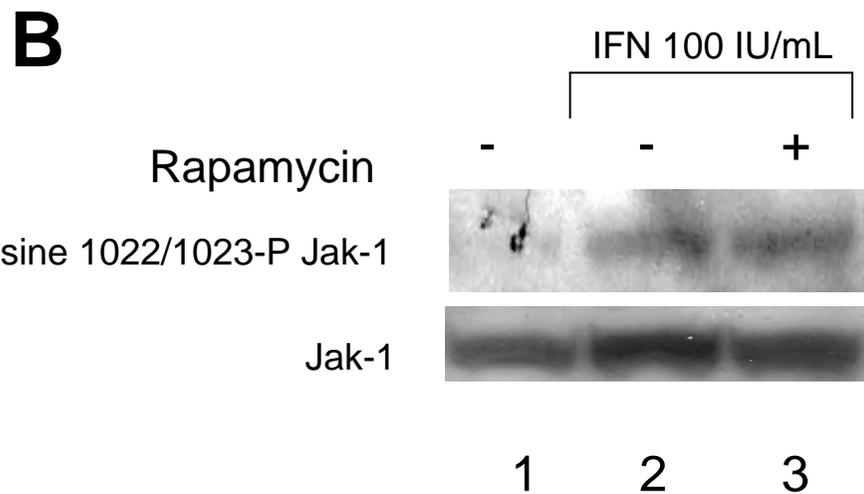
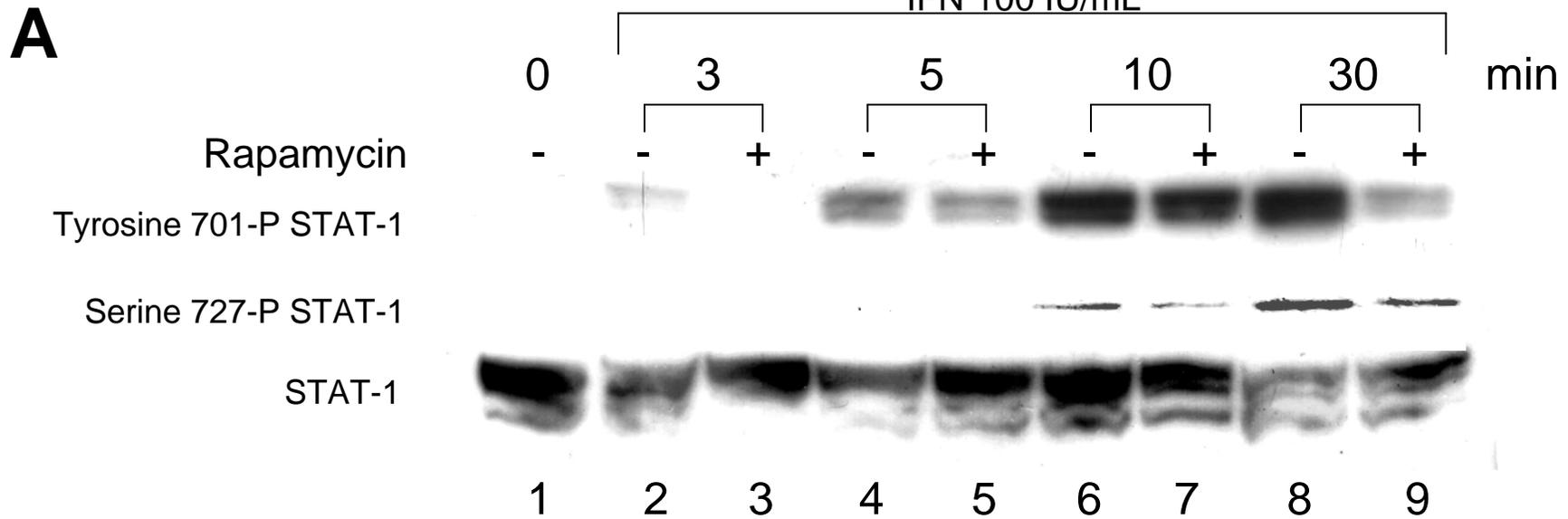
HuH-7 cells transfected with reporter gene (pISRE-Luc and pRL-SV40) were either untreated (lane 1) or pretreated with Rapa (lane 3. 4), Wortmannin (lane 5), LY294002 (lane 6) or Akt inhibitor (lane 7) for 3 hr, followed by IFN- α 100 IU/mL (lane 2 -7). Six hr later, the relative ISRE-luciferase activity (n=4) was determined as described in the MATERIALS AND METHODS. The data are expressed as the mean \pm SD and are representative example of four similar experiments.

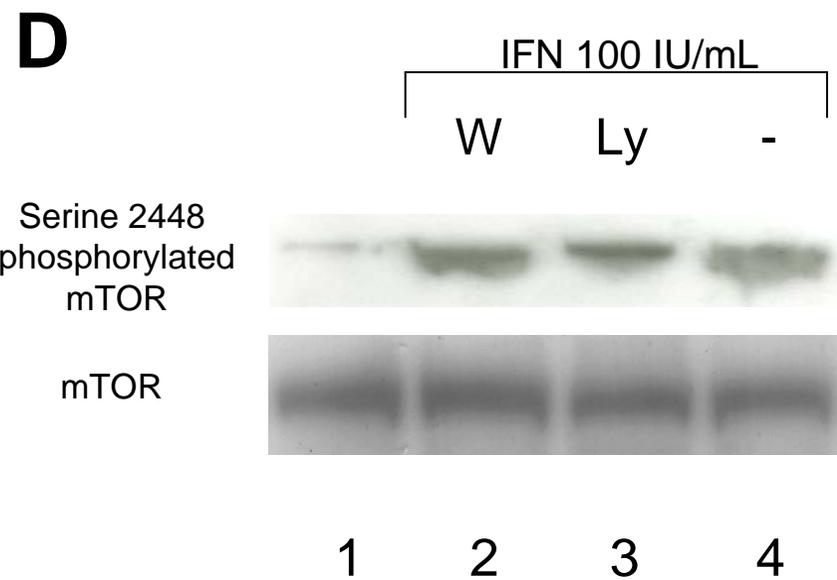
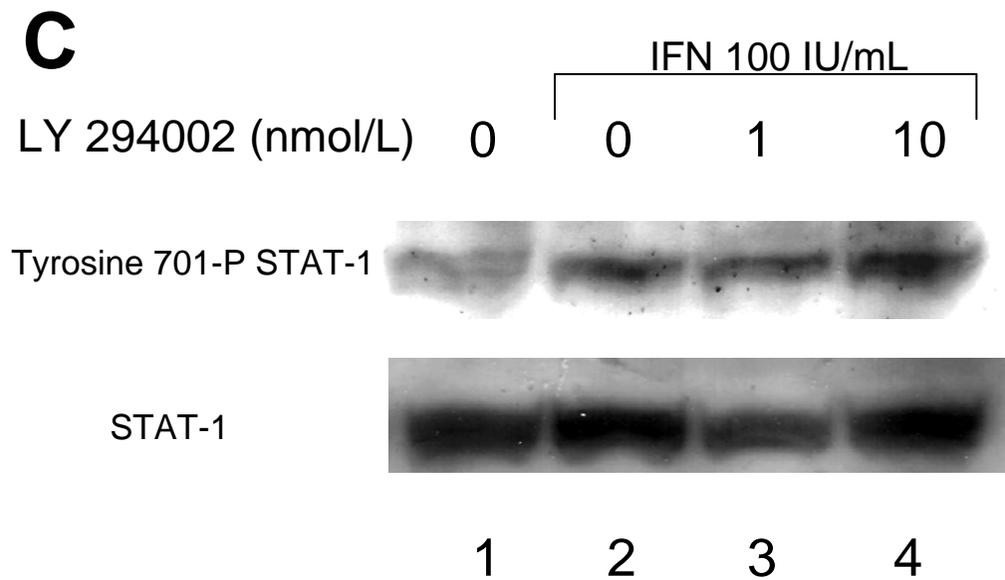
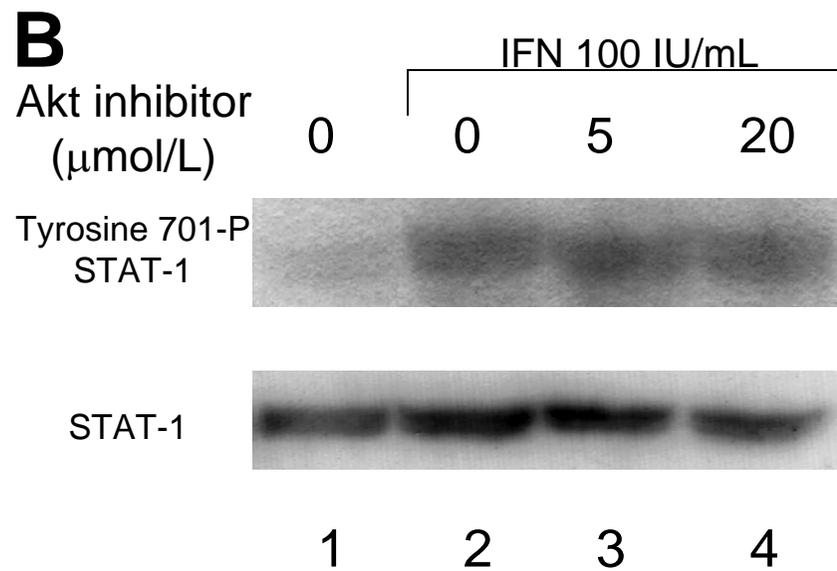
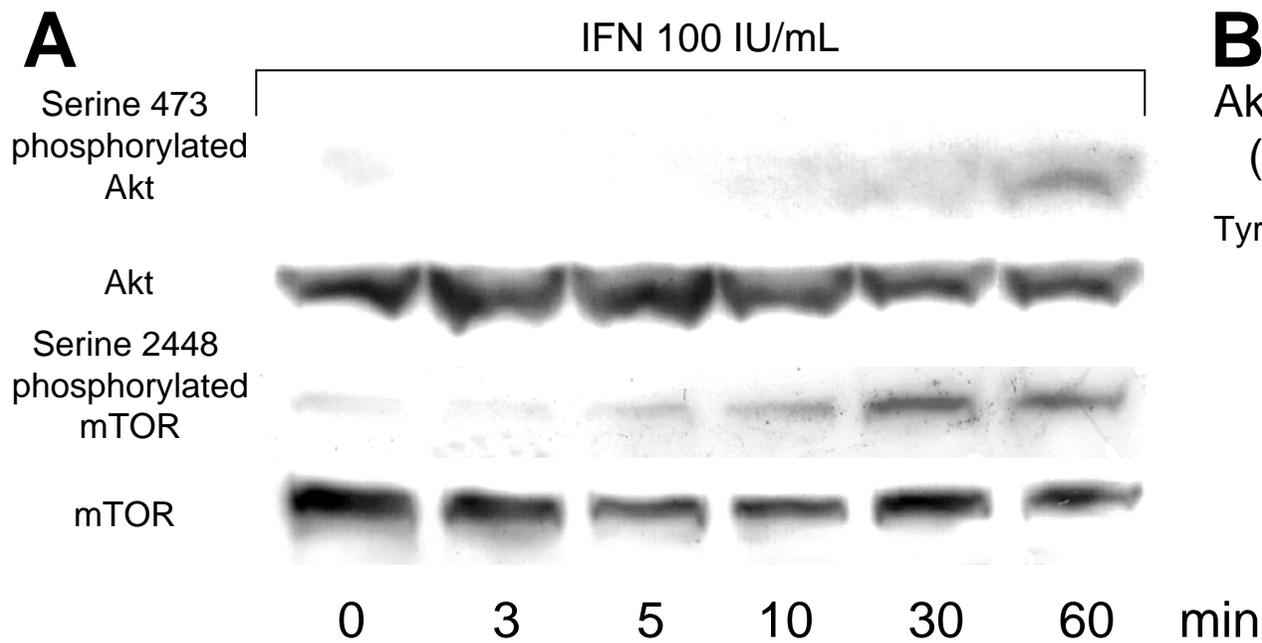
Figure 5. Alternation of IFN- α suppressed HCV replication by rapamycin, but not PI3-K inhibitors and Akt inhibitor.

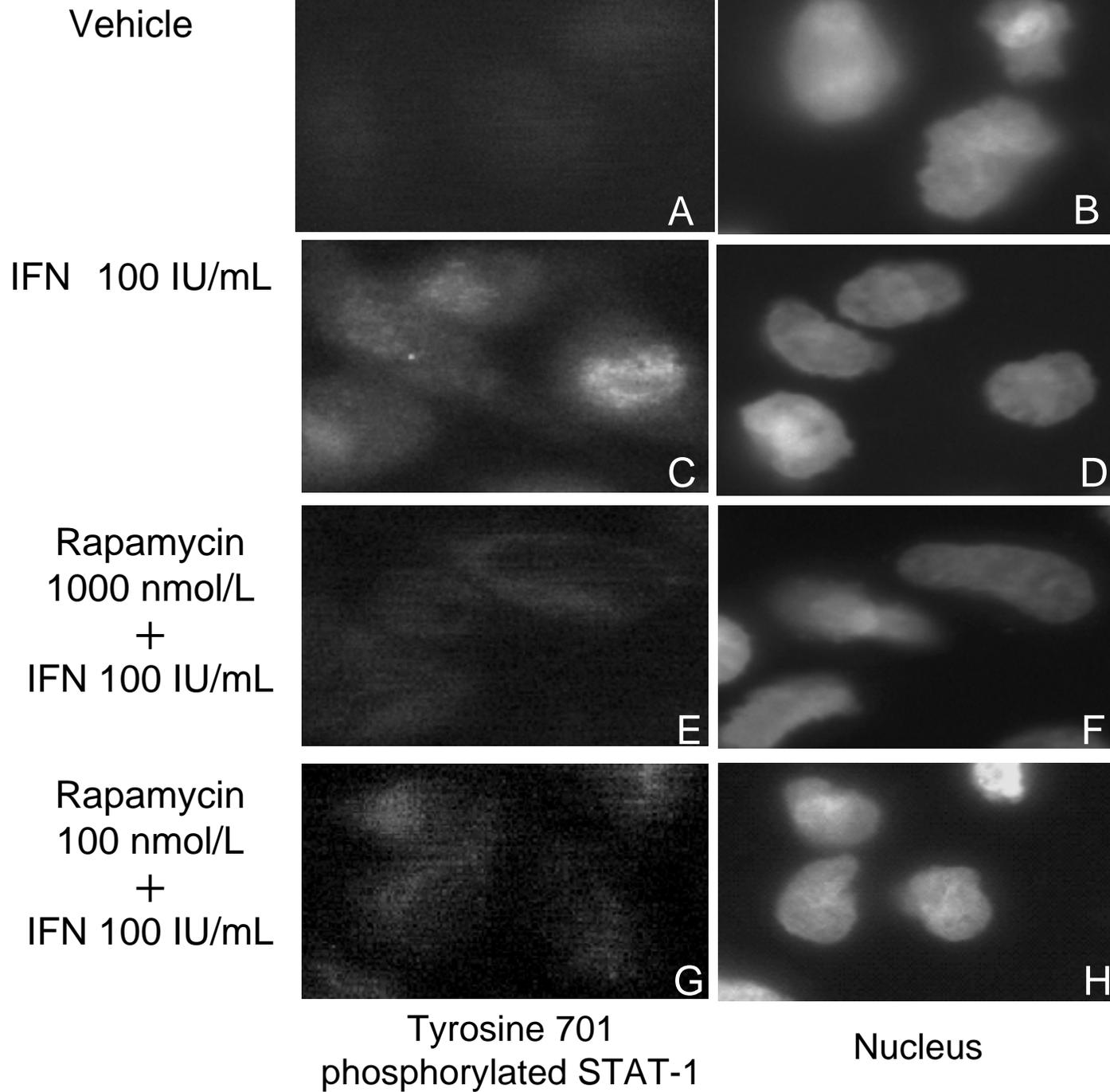
OR6 cells, a full-length replicon system, were treated with 100 IU/mL of IFN- α in the absence (lane 2) or presence of pretreatment (lane 3-8) for 3 hr. Lane 1 was not treated IFN- α alone. One day latter, *Rennila* luciferase activity was determined by luminometer (n=4). The data are expressed as the mean \pm SD and are representative example of four similar experiments.

Figure 6. Alternation of IFN- α suppressed HCV replication by siRNA against mTOR.

The OR6 cells were transfected the siRNA against mTOR (lane 2 and 5) and the non-targeted siRNA (lanes 3 and 6). One day later, the cells were IFN- α treatment (lane 4-6). HCV replicon assay is same as Figure 5. The data are expressed as the mean \pm SD and are representative example of four similar experiments

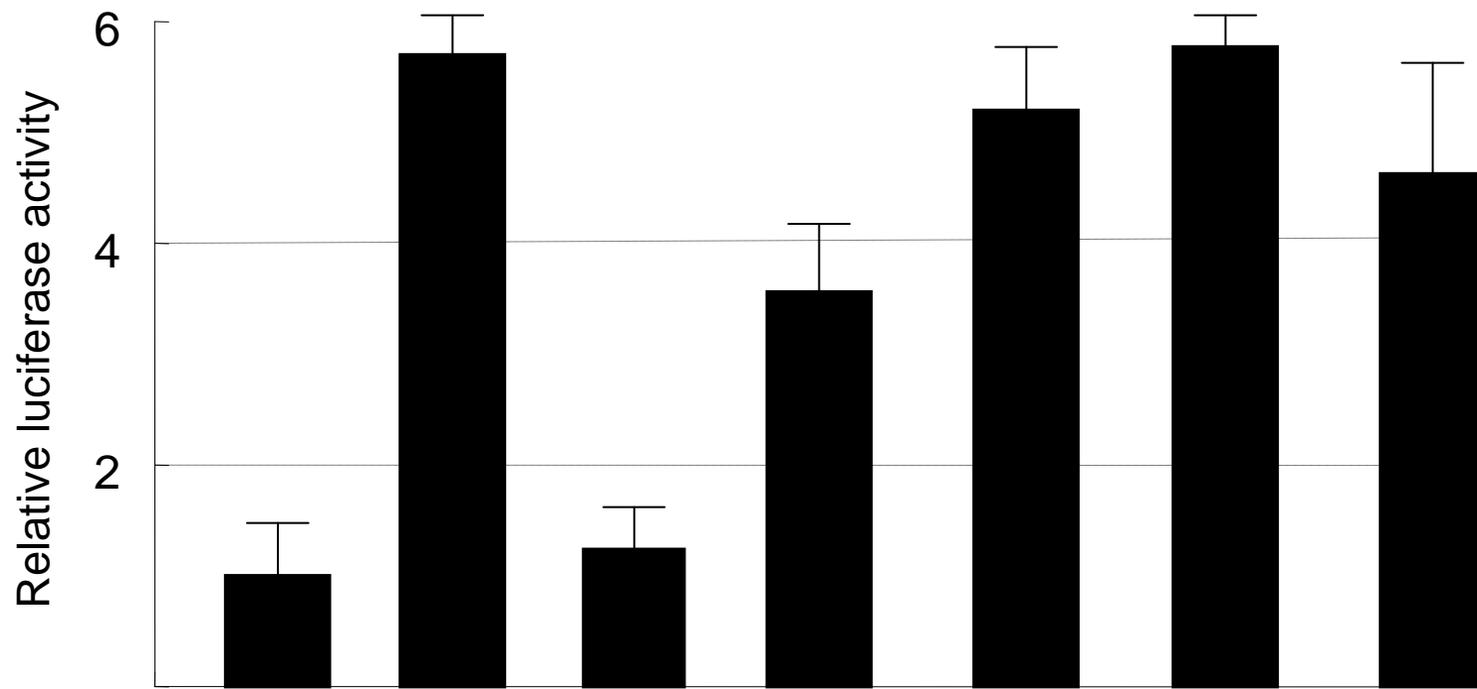




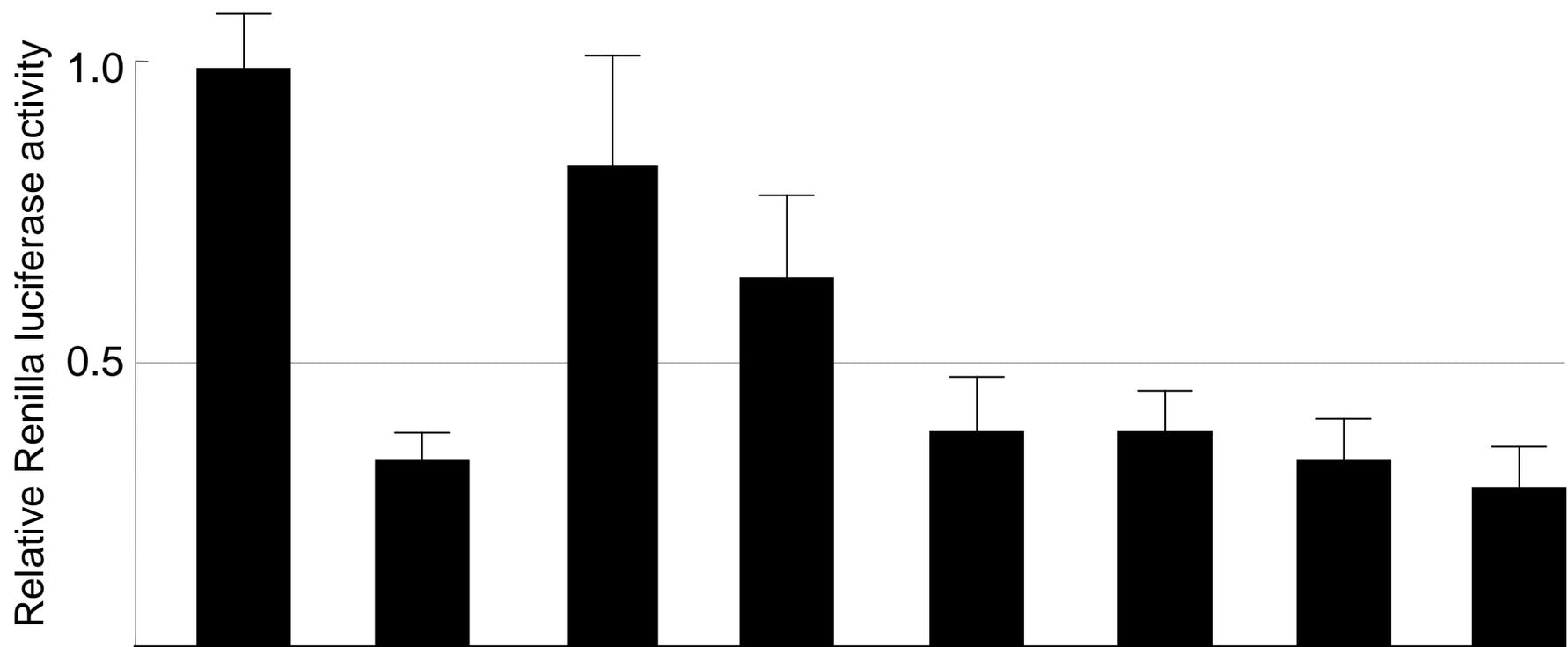


Tyrosine 701
phosphorylated STAT-1

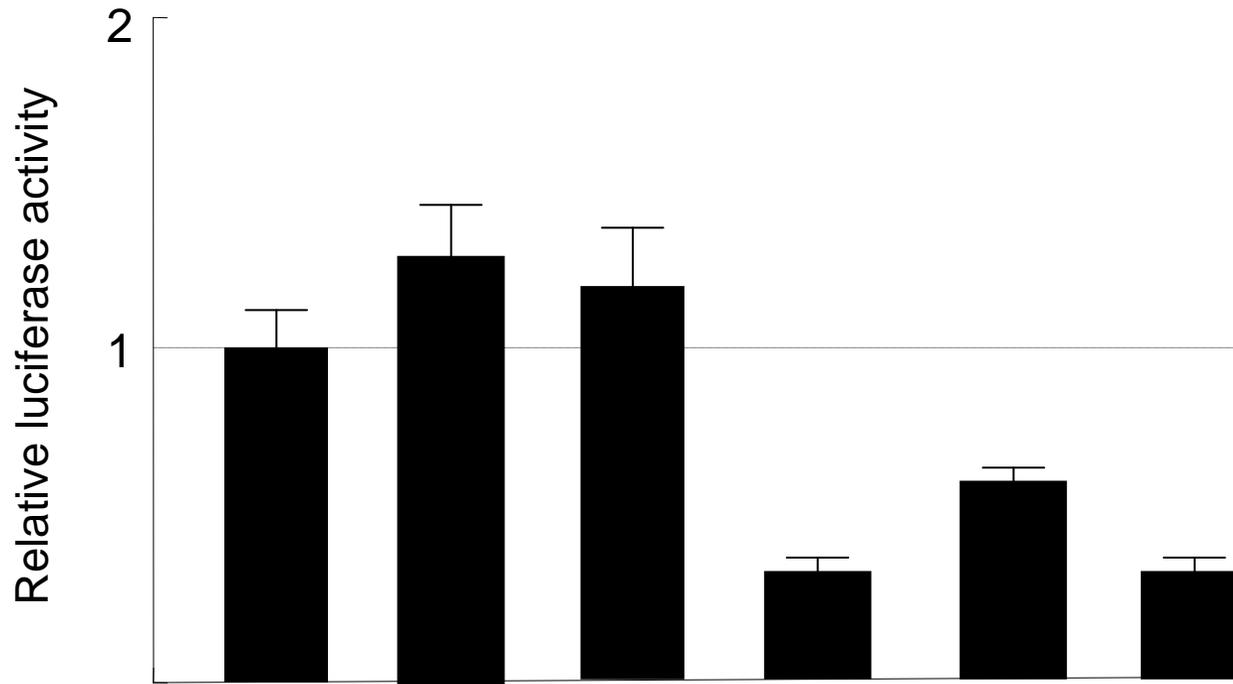
Nucleus



| | | | | | | | |
|-------------------------------------|---|---|------|-----|-----|----|----|
| IFN (100 IU/mL) | - | + | + | + | + | + | + |
| Rapamycin (nmol/L) | - | - | 1000 | 500 | - | - | - |
| Wortmannin (nmol/L) | - | - | - | - | 100 | - | - |
| LY294002 ($\mu\text{mol/L}$) | - | - | - | - | - | 10 | - |
| Akt inhibitor ($\mu\text{mol/L}$) | - | - | - | - | - | - | 10 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |



| | | | | | | | | |
|------------------------------|---|---|------|-----|-----|----|---|----|
| IFN (10 IU/mL) | - | + | + | + | + | + | + | + |
| Rapamycin (nmol/L) | - | - | 1000 | 100 | - | - | - | - |
| Wortmanin (nmol/L) | - | - | - | - | 100 | - | - | - |
| LY294002 (μ mol/L) | - | - | - | - | - | 10 | 1 | - |
| Akt inhibitor (μ mol/L) | - | - | - | - | - | - | - | 10 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |



| | | | | | | |
|----------------|---|---|---|---|---|---|
| IFN (10 IU/mL) | - | - | - | + | + | + |
| mTOR siRNA | - | + | - | - | + | - |
| control siRNA | - | - | + | - | - | + |
| | 1 | 2 | 3 | 4 | 5 | 6 |