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ISG15 over-expression inhibits replication of the Japanese encephalitis virus in human medulloblastoma cells

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ABSTRACT

IFN-stimulated gene 15 (ISG15), an ubiquitin-like protein, is rapidly induced by IFN-α/β, and ISG15 conjugation is associated with the antiviral immune response. Japanese encephalitis virus (JEV), a mosquito-borne neurotropic flavivirus, causes severe central nervous system diseases. We investigated the potential anti-JEV effect of ISG15 over-expression. ISG15 over-expression in human medulloblastoma cells significantly reduced the JEV-induced cytopathic effect and inhibited JEV replication by reducing the viral titers and genomes ($p < 0.05$, Student's *t*-test); it also increased activation of the interferon stimulatory response element (ISRE)-luciferase *cis*-acting reporter in JEV-infected cells ($p < 0.05$, Chi-square test). Furthermore, Western blotting revealed that ISG15 over-expression increased phosphorylation of IRF-3 (Ser396), JAK2 (Tyr1007/1008) and STAT1 (Tyr701 and Ser727) in JEV-infected cells ($P < 0.05$, Chi-square test). Confocal imaging indicated that nucleus translocation of transcription factor STAT1 occurred in ISG15-over-expressing cells but not in vector control cells post-JEV infection. ISG15 over-expression activated the expression of STAT1-dependent genes including IRF-3, IFN-β, IL-8, PKR and OAS before and post-JEV infection ($p = 0.063$, Student's *t*-test). The results enabled elucidation of the molecular mechanism of ISG15 over-expression against JEV, which will be useful for developing a novel treatment to combat JEV infection.

1. Introduction

Japanese encephalitis virus (JEV), a member of the *Flaviviridae* family, causes severe central nervous system diseases such as poliomyelitis-like acute flaccid paralysis, aseptic meningitis and encephalitis, and is prevalent in eastern and southern Asia, including Nepal, Pakistan, Torres Strait, Australia, and Saipan Island (Hanna et al., 1995; Paul et al., 1993; Wakai, 1998). Pigs are the main amplifying host in the JEV transmission cycle, ardeid wading birds are the primary maintenance host, and mosquitoes such as *Culex tritaeniorhynchus* are important vectors. JEV is maintained in a zoonotic cycle between ardeid wading birds and/or pigs and Culex mosquitoes, and is transmitted to humans via the bite of infected mosquitoes (Mackenzie et al., 2004). JEV infection in humans results in widespread involvement of the nervous system, including the thalamus, basal ganglia, brainstem, cerebellum, cerebral cortex and spinal cord (Kalita and Misra, 2000; Liu et al., 2008; Misra et al., 2003). Japanese encephalitis (JE) has a high fatality rate of 30%, and around half of JE survivors have severe neurological sequelae (Solomon et al., 2000). Approximately 50,000 JE cases, 10,000 of which are fatal, are reported annually in the JE endemic region (Burk and Monath, 2001; Tsai, 1997).

Type I interferons (IFNs, IFNα, IFNβ and IFNω) and type II IFN (IFNγ) mediate a wide range of biological activities including antiviral activity, cell growth, differentiation, apoptosis and immune response (Biron, 2001). IFN-α/β binds to a common heterodimeric receptor composed of IFN-α/β receptor 1 (IFNAR1) and IFN-α/β receptor 2 (IFNAR2), and then activates the Janus/just another kinase (JAK) family and signal transducers and activators of the transcription (STATs) family (Tang et al., 2007). For example, phosphorylation of STAT1 at tyrosine 701 by JAK1 is required for the formation of STAT1–STAT2 heterodimers and

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nucleus translocation (Banninger and Reich, 2004). In addition, phosphorylation of STAT1 at serine 727 by ERK1/2 and p38 MAPK facilitates interaction of STAT1 with the basal transcription machinery, enabling full expression of antiviral genes such as protein kinase R (PKR), 2′,5′-oligoadenylate synthetase (OAS) and IFN-stimulated gene 15 (ISG15) (Deb et al., 2003; Uddin et al., 2002). ISG15, a 15-kDa IFN-stimulated protein, contains two domains, and has a 30% structural homology with ubiquitin (Narasimhan et al., 2005). The C-terminal LRLRGG motif of ISG15 conjugates to target proteins by a sequential reaction catalyzed by E1/E2/E3 enzymes (ISGylation) (D'Cunha et al., 1996; Owhashi et al., 2003). ISG15 expression is regulated by transcription factors of the interferon regulatory factor (IRF) family (such as IRF-1, IRF-3, IRF-4, IRF-7 and IRF-8) that bind to the interferon-stimulated response element motif in the regulatory region of ISG15 (Ritchie and Zhang, 2004). Several key regulators of signal transduction (JAK1, STAT1, ERK1 and phospholipase Cγ1) have been shown to be modified by ISG15 conjugation (Malakhov et al., 2003), and recent studies have identified more than 100 ISG15-conjugated proteins that encompass diverse cellular pathways, particularly in terms of antiviral innate immune responses (Giannakopoulos et al., 2005; Zhao et al., 2005). Influenza B virus non-structural NS1B protein binds to ISG15 and prevents its association with ubiquitin-activating enzyme E1-like (UbE1L) (Yuan and Krug, 2001), and hepatitis C virus NS3/4A protease cleaves IFN promoter-stimulator-1 (IPS-1), leading to reduced ISG15 expression and conjugation (Loo et al., 2006). In addition, over-expression of ISG15 reduces replication of the Newcastle disease virus (NDV) and the influenza virus, and inhibits the release of human immunodeficiency virus 1 (HIV-1) virions (Okumura et al., 2006). Importantly, in vivo assays have demonstrated that expression of ISG15 protects alpha/beta-IFN-receptor-deficient mice, ISG15-deficient mice and CD1-deficient mice against lethal infection with the Sindbis virus (Lenschow et al., 2005, 2007; Zhang et al., 2007).

Clinical, neurophysiological and radiological examination of JE patients has indicated the presence of JEV antigens and genomes in the thalamus, basal ganglia, brainstem, cerebellum, cerebral cortex and spinal cord (Kalita and Misra, 2000; Liu et al., 2008; Misra et al., 2003). TE671, human neural cells derived from a medulloblastoma have been used for investigation into the viral pathogenesis of the measles virus (Miller and Carrigan, 1982; Ziegler and Staufferm, 1987) and HIV-1 (Srinivasan et al., 1988). Recently, JEV replication and the JEV NS2B-NS3 protease protein have been demonstrated to induce apoptosis of TE671 human medulloblastoma cells (Yang et al., 2009). In this study, we investigated the antiviral effect of ISG15 over-expression on JEV replication in TE671 human medulloblastoma cells and examined the possible mechanism of the anti-JEV effect of ISG15 over-expression.

2. Materials and methods

2.1. Viruses and cells

JEV strain T1P1 was used in this study, as previously described (Lin et al., 2008). Vero cells for JEV amplification were maintained in Dulbecco's modified Eagle's medium (DMEM), and BHK-21 cells used for the determination of JEV plaques were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). To induce over-expression of ISG15, human medulloblastoma TE-671 cells were grown in MEM with 2 mM L-glutamine, 1 mM sodium pyruvate and 10% FBS.

2.2. Construction and expression of recombinant ISG15 protein

Total RNA was isolated from human promonocyte HL-CZ cells in the presence of 1000 U/ml IFN-β for 4 h using a Total RNA Purification System (Invitrogen). cDNA was synthesized from 1000 ng total RNA with oligo dT primer and a Super-Script III reverse transcriptase kit (Invitrogen). A human ISG15 cDNA fragment was amplified using PCR with forward primer ISG15 5′-ATCGGATATCTATGGGCTGG GACCTGACG-3′ and reverse primer 5′-ATCGGCGGCCGCTTAGCTCCGCCCGCCAGG-3′; the forward primer contained an EcoRV restriction site and the reverse included a NotI restriction site and a stop codon. The PCR product was cloned into pTriEX-4 Neo vector (Novagen), and the resulting plasmid pTriEX-ISG15 was sequenced in order to verify the sequence of human ISG15 using an ABI PRISM BioDye Terminator Cycle Sequencing Ready Reaction Kit. Plasmid pTriEX-ISG15 (5 μg) was transfected into human medulloblastoma TE-671 cells using the Arrest-In transfection reagent (Open Biosystems). According to the manufacturer's instructions, transfected cells were maintained in 2 ml of MEM containing 20% FBS after a 5-h incubation period with the mixture of plasmid DNA and Arrest-In transfection reagent. For transfected cell clone selection, cells were incubated with MEM containing 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS and 800 μg/ml of G418. For the detection of expression of recombinant ISG15 fusion proteins with an N-terminal His tag, the cells stably transfected with pTriEX-ISG15 were harvested and analyzed using Western blotting with anti-His Tag mAb (Serotec).

2.3. Detection of apoptosis by flow cytometry

Vector-empty control cells and ISG15-over-expressing cells were infected with JEV at a multiplicity of infection (MOI) of 1 and harvested 48 h post-infection. Cells were fixed with 70% ethanol at 4 °C overnight and then re-suspended in PBS containing 50 μg/mL propidium iodide (PI), 0.1 mg/mL RNase and 0.1% Triton X-100 in the dark. After 30 min at 37 °C, the cells were analyzed with a flow cytometer (Becton-Dickinson) equipped with an argon ion laser at a wavelength of 488 nm and the apoptotic cell rates were then determined (Yang et al., 2009). In addition, a JEV-induced cytopathic effect (CPE) in the cultures of vector-empty control cells and ISG15-over-expressing cells was observed 4, 12, 24, and 48 h post-infection using light microscopy.

2.4. Plaque-forming unit assay

In order to determine the growth curve of JEV from vector-empty control cells and ISG15-over-expressing cells, both infected with JEV at a MOI of 1, culture supernatant from the JEV-infected cells was harvested 12, 24, 48 and 72 h post-infection for the determination of viral plaque-forming units. BHK-21 cell monolayers were incubated with serial dilutions of the cultured supernatant at 37 °C for 1 h and then overlaid with MEM medium containing 1.1% methylcellulose. Viral plaques were stained with naphthol blue-black dye after 3 days of incubation.

2.5. Real-time RT-PCR for the detection of JEV RNA load in cultured supernatant

Viral RNA genome was extracted from the cultured supernatants of vector-empty control cells and ISG15-over-expressing cells 48 and 72 h post-JEV infection using a QIAamp Viral RNA Mini Kit (Qiagen). As described in our previous report (Huang et al., 2008), real-time RT-PCR was performed to obtain JEV cDNA fragments using Superscript II reverse-transcriptase with a mixture of random primer nucleotides (Invitrogen). For the two-step real-time RT-PCR process, the reaction mixture contained 2 μl of the first-strand cDNA synthesis reaction, 0.3 μM of each primer (JEV D3-F 5′-GGGAGTGATGGCCCCTGCAAAATT-3′ and JEV D3-R 5′-TCCAATGGAGCCAAAGTCCCAGGC-3′) and 20 μl of Smart Quant Green Master Mix (SYBR Green I, HotStart DNA polymerase, $MgCl_2$,

dUTP and ROX passive reference) (Protech Technology). PCR was performed using an amplification protocol consisting of 1 cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 s, and 60 °C for 1 min. Amplification and detection of specific products were carried out using an ABI PRISM 7700 sequence detection system (PE Applied Biosystems). Fluorescence of SYBR Green I was detected at 530 nm.

2.6. Analysis of in vivo signaling transduction pathway using cis-reporter plasmids

The *cis*-reporter plasmid pISRE-Luc was purchased from Stratagene. Vector-empty control cells and ISG15-over-expressing cells were co-transfected with *cis*-reporter plasmid and an internal control reporter pRluc-C1 (BioSignal Packard) in 6-well plates with Arrest-In transfection reagent. After a 1-day incubation period, transfected cells were seeded into 24-well plates with MEM containing 10% FBS, then infected with JEV at a MOI of 1; post-JEV infection, the enzyme activities of firefly and Renilla luciferases in the cells were measured using the dual Luciferase Reporter Assay System (Promega) and a luminometer (TROPIX TR-717; Applied Biosystems). The relative firefly luciferase activity of the *cis*-acting reporter was normalized to Renilla luciferase.

2.7. Western blotting analysis

Cell lysates harvested from vector-empty control cells and ISG15-over-expressing cells with/without JEV infection at a MOI of 1 were dissolved in 2× SDS-PAGE sample buffer without 2-mercaptoethanol and boiled for 10 min. As described in our previous report (Lin et al., 2008), cell lysate proteins were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose paper. The resultant blots were then blocked with 5% skim milk and reacted with appropriately diluted monoclonal antibodies against RIG-1, IRF-3, phospho-IRF-3 (Ser396), phospho-JAK2 (Tyr 1007/1008), phospho-JAK1 (Tyr701), STAT1, phospho-STAT1 (Tyr701), phospho-STAT1 (Ser727), phospho-STAT2 (Tyr690) and β-actin (Cell Signaling Technology) for a 3-h incubation period. Immune complexes were detected using horseradish peroxidase-conjugated goat anti-mouse IgG antibodies, followed by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech).

2.8. Immunocytochemistry

To determine the subcellular localization of STAT1, vector-empty control cells and ISG15-over-expressing cells with/without JEV infection at a MOI of 1 were double-stained with anti-STAT1 monoclonal antibodies followed by FITC-conjugated anti-mouse IgG antibodies and counterstained with DAPI. The cells were fixed on glass coverslips with ice-cold acetone for 4 min and blocked with 1% bovine serum albumin, then incubated with mouse anti-STAT1 monoclonal antibodies at 4 °C overnight and reacted with FITC-conjugated goat anti-mouse immunoglobulin at room temperature for 2 h. After washing, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei staining, and confocal image analysis of the cells was performed using a Leica TCS SP2 AOBS laser-scanning microscope (Leica Microsystems, Heidelberg GmbH, Germany).

2.9. Quantitative real-time RT-PCR assay of interferon-stimulating gene expression

Total RNA was isolated from vector-empty control cells and ISG15-over-expressing cells with/without JEV infection at a MOI of 1 using a Total RNA Purification System (Invitrogen), and cDNA was then synthesized from 1000 ng total RNA with oligo dT primer and a SuperScript III reverse transcriptase kit (Invitrogen). To monitor the expression of interferon-stimulating genes (ISGs) including protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (OAS), quantitative TaqMan real-time PCR analysis was employed. Briefly, the oligonucleotide primers and TaqMan probes for human PKR were 5′-CAACC AGCGG TTGAC TTTTT-3′ (forward primer), 5′-ATCCA GGAAG GCAAA CTGAA-3′ (reverse primer) and probe #50 (Universal ProbeLibrary probe). The forward primer for OAS was 5′-GATGT GGTTA GGTTT ATAGCTG-3′ and the reverse primer was 5′-TTGGG GGTTA GGTTT CTGCCTTT-3′. The TaqMan probe for GAPDH was probe #37 (Universal ProbeLibrary probe). In addition, the forward primer for glyceraldehyd-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was 5′-AGCCA CATCG CTCAG ACAC-3′, and the reverse primer was 5′-GCCCCA ATACG ACCAA ATCC-3′; the TaqMan probe for GAPDH was probe #60 (Universal ProbeLibrary probe). The real-time PCR reaction mixture contained 2.5 μl of cDNA (reverse transcription mixture), 200 nM of each primer and 200 nM of TaqMan hybridization probe in TaqMan universal master mix (LightCycler TaqMan Master, Roche Diagnostics). In addition, the primer pairs used in the SybGreen I real-time PCR were as follows: 5′-GATTCTGGACCCCACCTACA-3′ (RIG-1-F) and 5′-TGGCTTCACAAAGTCCACAG-3′ (RIG-1-R) for RIG-1; 5′-CTGGCTAGAGCATGGAAACC-3′ (IRF-3-F) and 5′- AGCAGCTAACCG-CAACACTT -3′ (IRF-3-R) for IRF-3; 5′-AACTGCAACCTTTCGAAGCC-3′ (IFN-β-F) and 5′-TGTCGCCTACTACCTGTTGTGC-3′ (IFN-β-R) for IFN-β; and 5′-CGATGTCAGTGCATAAAGACA-3′ (IL-8-F) and 5′-TGAATTCTCAGCCCTCTTCAAAAA-3′ (IL-8-R) for IL-8. PCR was performed using an amplification protocol consisting of 1 cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Amplification and detection of specific products were carried out using an ABI PRISM 7700 sequence detection system (PE Applied Biosystems). Relative changes in the mRNA levels of indicated genes were normalized to the housekeeping gene GAPDH.

2.10. Statistical analysis

All data were analyzed with Student's *t*-test or the Chi-square test. Statistical significance between vector-control cells and ISG15-overexpressing cells was indicated when $p < 0.05$.

3. Results

3.1. Human ISG15 over-expression inhibited JEV-induced CPE in TE-671 cells

To examine the effect of ISG15 over-expression on JEV-induced CPE, human ISG15 cDNA was amplified using PCR, cloned into

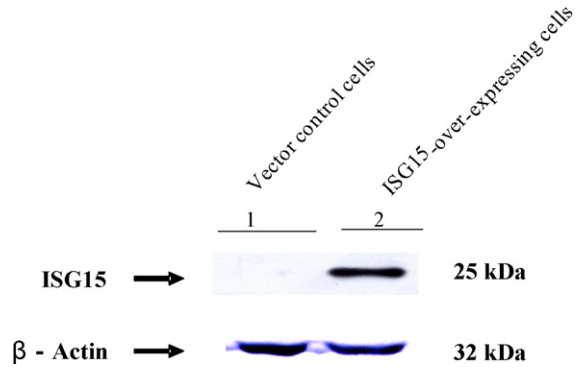

Fig. 1. Expression of human ISG15 in human medulloblastoma TE-671 cells. TE671 cells were transfected with ISG15-pTriEX-4 Neo plasmid or empty vector control cells using Arrest-In™ reagent (OPEN BIOSYSTEMS). After selection with G418, cell lysates were collected and analyzed by Western blotting with anti-His antibodies.

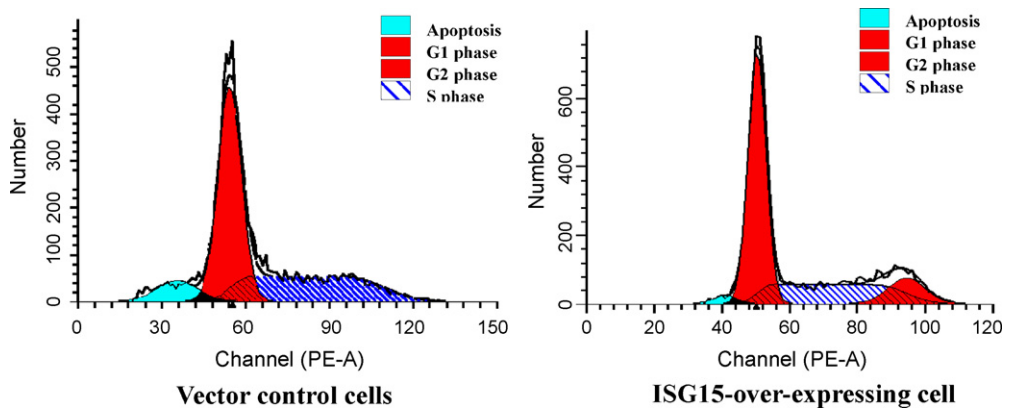

Fig. 2. Cell cycle analysis of empty vector control cells and ISG15-over-expressing cells infected with JEV. Empty vector control cells and ISG15-over-expressing cells were infected with JEV at a MOI of 1. Cells were harvested and examined by flow cytometric analysis of the cell cycle by propidium iodide staining.

pTriEX-4 Neo vector, then expressed as recombinant ISG15 fusion proteins with His-tag, S-tag, thrombin and enterokinase cleavage sites at the N-terminus in human medulloblastoma TE-671 cells. Western blotting assay with anti-His-tag antibody revealed an immunoreactive band near 25 kDa of the recombinant ISG15 fusion protein in ISG15-over-expressing cells (Fig. 1, lane 2), but not in empty vector cells (Fig. 1, lane 1). These results demonstrated over-expression of ISG15 in the transfected TE-671 cells with plasmid pTriEX-ISG15. Subsequently, CPE in cell cultures infected with JEV at a MOI of 1 was observed 4, 12, 24, and 48 h post-infection (data not shown). The activity of JEV-induced CPE in ISG15-overexpressing cells was observably less than in empty-vector cells 4, 12, 24 and 48 post-infection. In addition, flow cytometric analysis of the cell cycle phase indicated that JEV induced G1/S cell cycle arrest and an increase in the percentage of cells in the sub-G1 phase (apoptosis) in empty-vector-transfected cells, but not in ISG15-over-expressing cells (2.3%) at 48 h post-infection at a MOI of 1 (Fig. 2). These results indicated that over-expression of ISG15 had an inhibitory effect on JEV-induced CPE.

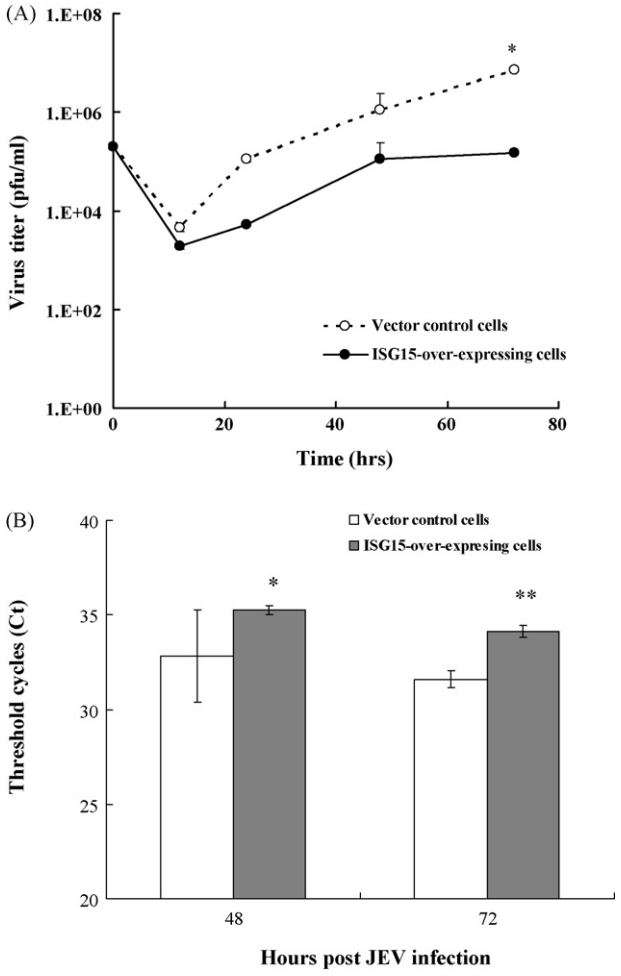

Fig. 3. Inhibition of JEV replication by ISG15 over-expression. Empty vector control cells and ISG15-over-expressing cells were infected with JEV at a MOI of 1, and cultured supernatants were collected 12, 24, 48, and 72 h post-JEV infection. The JEV titers in the cultured supernatants were determined by plaque-forming unit assay (A), and JEV RNA loads were analyzed using real-time RT-PCR (B). $^{*}p = 0.048$ by Student's *t*-test. $^{**}p = 0.003$ by Student's *t*-test.

3.2. Inhibition of JEV replication by ISG15 over-expression

To further test the antiviral activity of ISG15 against JEV, the growth curves of JEV in vector control cells and ISG15-over-

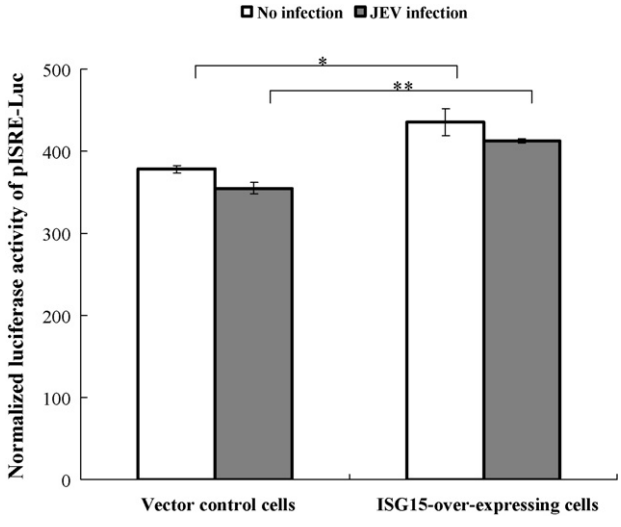

Fig. 4. Effect of ISG15 over-expression on ISRE-Luc *cis*-reporter activity. Empty vector control cells and ISG15-over-expressing cells were transiently co-transfected with pISRE-Luc *cis*-reporter plasmid and an internal control reporter (pRluc-C1), then infected with JEV at a MOI of 1. Firefly and Renilla luciferase enzyme levels were measured 4 h post-JEV infection, and according to the dual Luciferase Reporter Assay System, the relative firefly luciferase activity was normalized to Renilla luciferase. $^{*}p = 0.044$ by the Chi-square test; $^{**}p = 0.036$ by the Chi-square test.

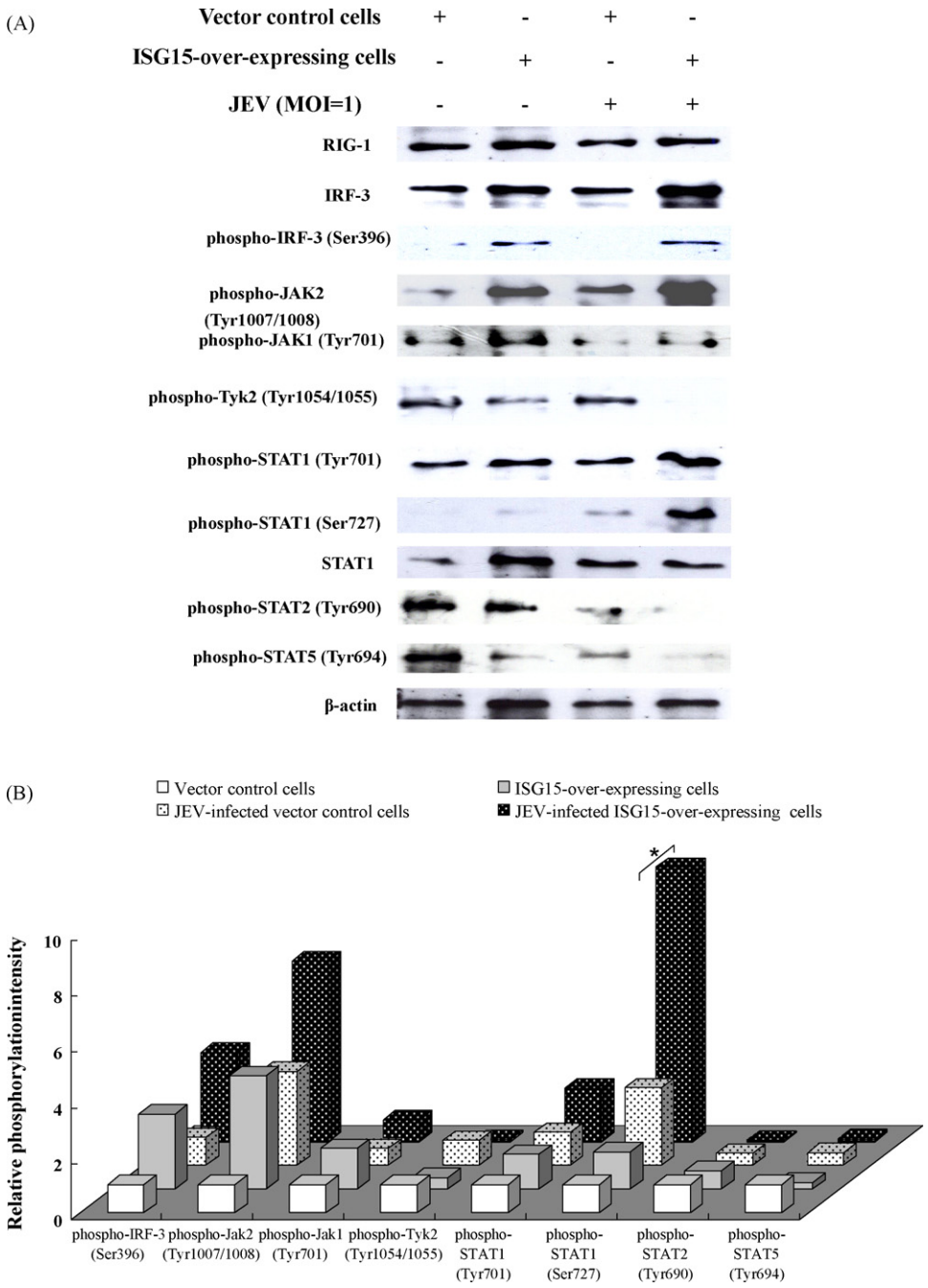

Fig. 5. Effect of ISG15 over-expression on phosphorylation of IRF-3, JAK, Tyk2 and STATs. Cell lysates of empty vector control cells and ISG15-over-expressing cells 6 h post-JEV infection were analyzed by 10% SDS-PAGE and Western blotting. Blots were probed with specific monoclonal antibodies, and immune complexes were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG antibodies and enhanced chemiluminescence (A). Scanning densitometry was performed in order to quantitate specific immunoreactive bands on the Western blots (B). $^{*}p = 0.047$ by the Chi-square test.

expressing cells were determined by plaque-forming unit assay (Fig. 3A). The virus growth curve showed that over-expression of ISG15 in human medulloblastoma cells significantly reduced the JEV plaque titer by 21.4-fold at 24 h post-infection, 11.4-fold at 48 h post-infection, and 48.3-fold at 72 h post-infection ($p = 0.048$, Student's *t*-test). In addition, real-time RT-PCR assay indicated that the JEV RNA level in the supernatant of infected ISG15-over-expressing cells was significantly lower than that in the infected vector control cells ($p = 0.003$, Student's *t*-test) (Fig. 3B). The results demonstrated that over-expression of ISG15 significantly inhibited JEV replication in human medulloblastoma cells.

3.3. Increase of the interferon-stimulated response element (ISRE) activity by ISG15 over-expression

To investigate the role of ISRE-derived genes in the anti-JEV activity of ISG15, cells were further characterized using a luciferase reporter plasmid containing direct repeat elements of ISRE (Fig. 4). ISG15-over-expressing cells and vector control cells were further co-transfected with *cis*-reporter plasmid and an internal control reporter, pRluc-C1. Cells were harvested at 4 h post-JEV infection, and the relative expression of firefly luciferase driven from the indicated *cis*-reporter plasmid was normalized to Renilla luciferase. The ratio of firefly luciferase intensity revealed that ISG15 over-

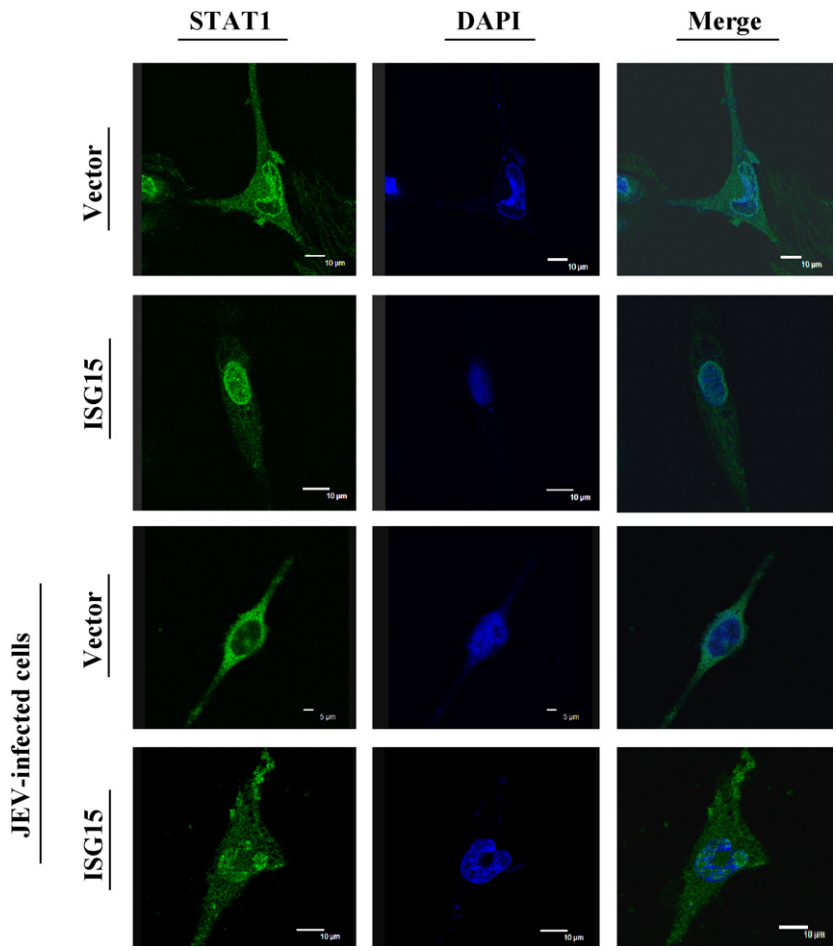

Fig. 6. Subcellular localization of STAT1 by confocal image analysis. Immunofluorescent staining of empty vector control cells and ISG15-over-expressing cells was performed 24 h post-JEV infection. STAT1 was probed by FITC-conjugated secondary antibodies, followed by DAPI staining for fluorescent counterstaining of nuclei. Confocal image analysis of the cells was performed using a Leica TCS SP2 AOBS laser-scanning microscope.

expression in TE-671 cells resulted in a 16.3% increase of ISRE activity in response to JEV infection compared with vector control cells ($p < 0.05$, Chi-square test) (Fig. 4). This result revealed that over-expression of ISG15 led to upregulation of the ISRE response against JEV.

3.4. Activation of JAK–STAT signaling by ISG15 over-expression

To further investigate the phosphorylation levels of components of the JAK–STAT signaling pathway, the phosphorylation levels of IRF-3, JAK1, JAK2, Tyk2, STAT1, STAT2 and STAT5 were analyzed using Western blotting with specific anti-phospho-tyrosine antibodies in ISG15-over-expressing cells and empty-vector control cells 6 h post-JEV infection (Fig. 5A). The densitometric value of each immune reactive band was normalized to β-actin in each lysate, and the relative levels of protein expression and protein phosphorylation were then calculated by normalizing to that in the vector control cells, which were given a value of 100% (Fig. 5B). The levels of phospho-IRF-3 (Ser396), phospho-JAK2 (Tyr1007/1008) and phospho-STAT1 (Ser727) were found to be increased more than 2-fold in ISG15-over-expressing cells and JEV-infected ISG15-over-expressing cells as compared with vector control cells and JEV-infected vector control cells, respectively ($p < 0.05$, Chi-square test) (Fig. 5B). In addition, ISG15 over-expression reduced the phosphorylation levels of JAK1 (Tyr701), Tyk2 (Tyr1054/1055), STAT2 (Tyr690) and STAT5 (Tyr694) in JEV-infected cells (Fig. 5). These results implied that activation of the JAK2–STAT1 pathway by ISG15 over-expression might be responsible for the innate immune response against JEV.

3.5. Confocal image analysis of STAT1 subcellular localization and expression profiling of pro-inflammatory cytokines in ISG15-over-expressing cells

To investigate the subcellular localization of STAT1, immunofluorescent staining of empty-vector control cells and ISG15-over-expressing cells was performed post-JEV infection (Fig. 6). STAT1 labeled with FITC-conjugated secondary antibodies showed green fluorescence (Fig. 6), and localization of the nucleus was visualized by DAPI staining. Confocal imaging of the stained cells revealed nuclear translocation of STAT1 in ISG15-over-expressing cells before and post-JEV infection, but not in vector control cells. Subsequently, STAT1-dependent expression of IRF-3, RIG-1, INF-β, IL-8, PKR and OAS was measured 24 h post-JEV infection (Fig. 7), and quantitative real-time RT-PCR indicated that the mRNA levels of IRF-3, PKR, OAS, IFN-β and IL-8 in ISG15-over-expressing cells and JEV-infected ISG15-over-expressing cells were 2-fold higher than those in vector control cells and JEV-infected vector control cells, respectively ($p = 0.023$, Student's t-test). These results indicated that ISG15 over-expression induced STAT1 nuclear translocation and expression of IRF-3, PKR, OAS, IFN-β and IL-8 genes before and post-JEV infection, showing that ISG15 over-expression could antagonize blocking of STAT1 nuclear translocation and tyrosine phosphorylation of Tyk2 and STAT1 by JEV nonstructural proteins.

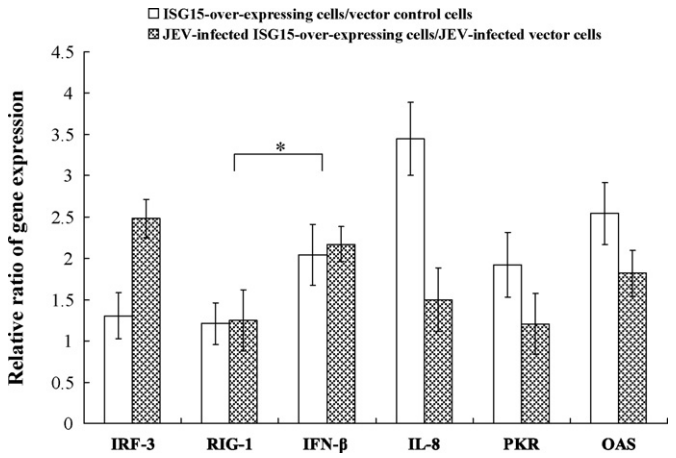

Fig. 7. Effect of ISG15 over-expression on expression of STAT1-dependent genes. The mRNA expression levels of the indicated genes were analyzed by real-time PCR using 2.5 μl reverse-transcribed cDNA. The measured amounts of the indicated gene mRNA were normalized to the amount of GAPDH mRNA, and the relative fold increases in the indicated mRNA levels were calculated as the ratio of mRNA expression level of ISG15-over-expressing cells *vs.* that of vector control cells, and that of JEV-infected ISG15-over-expressing cells *vs.* that of JEV-infected vector control cells. * $p = 0.023$ by Student's *t*-test.

4. Discussion

In this study, we found that ISG15 over-expression in human medulloblastoma cells significantly reduced the JEV-induced cytopathic effect and inhibited JEV-induced apoptosis by flow cytometry (Fig. 2) In addition, ISG15 over-expression suppressed JEV replication, as shown by a significant decrease in virus titers and viral genomes (Fig. 3). These results were in agreement with those reported in studies of the inhibitory effects of ISG15 on the multiplication of NDV, influenza A virus, HIV-1, Sindbis virus, and HSV-1 (Lenschow et al., 2007; Okumura et al., 2006; Osiak et al., 2005; Zhao et al., 2005). ISG15 conjugation targets such as PKR, MxA, HuP56, STAT1, JAK1 and RIG-I have been suggested to play a substantial role in the antiviral state induced by IFN in human cells (Zhao et al., 2005). However, the precise mechanism of the antiviral effect of ISG15, which may be virus-specific, has not yet been elucidated.

The in vivo signal transduction pathway reporting system indicated that ISG15 over-expression induced a significant increase in ISRE-promoter reporter activity as compared with that in vector control cells before and post-JEV infection (Fig. 4). In addition, ISRE-promoter reporter activity correlated well with activation of the JAK2–STAT1 signaling pathway in ISG15-over-expressing cells (Fig. 5). ISG15 over-expression in human medulloblastoma cells stimulated a more than 2-fold increase in the phosphorylation of IRF-3 at serine 396, JAK2 at tyrosine 1007/1008, and STAT1 at serine 727 post-JEV infection (Fig. 5). An earlier report indicated that JEV NS5 protein significantly blocked tyrosine phosphorylation of Tyk2 and STAT1, resulting in inhibition of the type-I IFN-induced JAK–STAT signaling pathway by JEV (Lin et al., 2004; Lin et al., 2006). In this study, we found that JEV infection could suppress tyrosine phosphorylation of Tyk2 and JAK1, as described in the earlier report, but not JAK2 and STAT1, in ISG15-over-expressing cells (Fig. 5). Importantly, Western blotting assay demonstrated a significant increase in the phosphorylation of STAT1 at serine 727 in ISG15-over-expressing cells compared with vector control cells post-JEV infection. Serine phosphorylation of STAT1 is required for maximal transcriptional activity of ISRE promoter, and IFN-γ has been shown to be involved in the phosphorylation of STAT1 at Tyr701 and Ser727 through activation of JAK2 and MAPK (Kovarik et al., 1999). ISG15 over-expression might induce the antiviral activity of IFN-γ-like antiviral responses; therefore, our data could suggest that over-expression of ISG15 via JAK2/STAT1 antiviral responses subjugated the inhibitory effect of JEV on type-I IFN-induced JAK1–STAT1 signaling events.

Confocal imaging analysis of stained cells showed that STAT1 nuclear translocation occurred in ISG15-over-expressing cells but not vector control cells before JEV infection (Fig. 6). STAT1 nuclear translocation in ISG15-over-expressing cells was found to antagonize inhibition of STAT1 nuclear translocation by JEV NS5 protein, as reported in previous studies (Lin et al., 2004, 2006). The NS5 proteins of both LGTV and JEV have been shown to suppress STAT1 nucleus translocation in response to IFN treatment (Best et al., 2005; Lin et al., 2004, 2006; Osiak et al., 2005). However, the antiviral effect of IFNs on flavivirus replication was abrogated if they were used in the late stage of viral infection (Guo et al., 2005; Ho et al., 2005; Lin et al., 2004, 2006; Munoz-Jordan et al., 2005). In this study, it was found that phosphorylation and nuclear translocation of STAT1 in ISG15-over-expressing cells could be responsible for significant increases in the expression of IFN-inducible antiviral genes including IRF-3, IRF-3, PKR, OAS, IFN-β and IL-8 before and post-JEV infection (Fig. 7). Therefore, our results indicated that ISG15 over-expression enhanced nucleus translocation of STAT1, up-regulating the JAK2–STAT1 signaling pathway in JEV-infected cells.

In conclusion, we demonstrated that ISG15 over-expression significantly inhibited JEV-induced CPE and JEV replication in human medulloblastoma cells. ISG15 over-expression activated antiviral responses including increase of IRSE-promoter activity, phosphorylation of JAK2 (Tyr1007/1008), STAT1 (Tyr701 and Ser727), and nuclear translocation of STAT1. Furthermore, ISG15 over-expression induced JAK2/STAT1-dependent production of downstream genes such as PKR, OAS, IL-6 and IL-8. Therefore, the molecular mechanism of ISG15 against JEV correlated with JAK2/STAT1 signaling.

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The authors have no conflicts of interest to disclose.

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