

Antiviral Effects of IFIT1 in Human Cytomegalovirus-Infected Fetal Astrocytes

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The prominent feature of human cytomegalovirus (HCMV) is cell tropism specificity for human fetal nervous system, which leads to severe fetal nervous system damage especially in first-trimester gestation. In this study, human astrocytes isolated from fetal brain were infected with HCMV AD169 and whole genome transcriptome profile was performed. The results showed that the gene expression of interferon stimulated genes (ISGs), chemokine and chemokine receptors were significantly up-regulated ($P < 0.01$). The antiviral replication effects of IFIT1 (Interferon-induced protein with tetratricopeptide repeats 1, Fc = 148.17) was investigated. Lentivirus with IFIT1 overexpression or knockdown was transduced into astrocytes, respectively. The viral mRNA, protein expression and HCMV titers were determined. The results showed that IE1, IE2, pp65, and viral titers were significantly decreased in IFIT1 overexpression group and enhanced in the knock-down group compared with control one ($P < 0.01$). Taken together, this study revealed IFIT1 played an important antiviral role in HCMV infected fetal astrocytes. The prominent feature of human cytomegalovirus (HCMV) is cellular tropism specificity for human fetal brain nervous system leading to severe fetal nervous damage especially in first-trimester gestation. In this study, human astrocytes isolated from first-trimester fetal brain were infected with HCMV AD169 and IFIT1 was studied for its antiviral replication effects. The results provided insights into the function of IFIT1 as a key factor in antiviral defense contributing to development of targeted therapeutics to fetal brain with HCMV infection. *J. Med. Virol.* 89:672–684, 2017.

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KEY WORDS: human cytomegalovirus; interferon; microarrays; nervous system; antiviral agents; disease control

INTRODUCTION

Human cytomegalovirus (HCMV; Human herpesvirus, a member of β -herpes virus subfamily) is a ubiquitous pathogen and the most common infectious cause of congenital diseases [Stagno, 1985; Boppana et al., 2005; Colugnati et al., 2007]. Primary infection during pregnancy poses a 30–40% risk of intrauterine transmission, with severe adverse outcomes more likely if the infection occurs within the first trimester gestation. Due to strict viral host specificity, so far there is no animal model of HCMV infection available. Current state of knowledge of CMV pathogenesis and host defense against HCMV has come largely from clinical observation and in vitro studies with cell lines or primary human cells [Sinzger and Jahn, 1996].

Previous study in our laboratory has identified human fetal astrocytes permissive to HCMV infection [Zhang et al., 2013]. It was reported that astrocytes constitute important parts of host defense mechanism, and play important roles in preserving CNS (central nervous system) function and protecting CNS against infectious agents [Carson and Sutcliffe,

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1999]. That HCMV or its proteins modulate gene expression in astrocytes has also been investigated. After being infected by HCMV, astrocytes produce as well as respond to a number of immune mediators, including interferon, ISG (interferon-stimulated gene) proteins, cytokines, and chemokines [Grandvaux et al., 2002] to control the spread of invading HCMV.

Type I IFN and ISG proteins have been shown to possess antiviral properties in CNS. Early expression of cytokines in CNS has been demonstrated to correlate with the control of viral replication [Carson and Sutcliffe, 1999]. When virus contact with host cells, viral proteins and nucleic acids are detected by pathogen recognition receptors (PRRs) as pathogen-associated molecular patterns (PAMPs). Subsequently, the signal transduction pathways are activated. Type I IFN and production of other cytokines are triggered. Type I IFN then augments the expression of several ISGs and subverts virus replication by a variety of mechanisms [Poland et al., 1994; Peisley and Hur, 2013]. However, not all ISGs are induced in IFN-dependent manner. Some are induced in IRF3-dependent manner [Grandvaux et al., 2002]. Moreover, not all ISG proteins confer direct antiviral effects, and the anti-HCMV effects of ISG proteins have not been attributed to every ISG family members so far characterized. Inhibitory phenotypes directed at specific ISG proteins need to be studied for HCMV. Given the observed replication of the virus in the presence of multiple antiviral host proteins, it is probable that a number of ISG-inhibitory phenotypes are to be uncovered for HCMV [Munir, 2013; Peisley and Hur, 2013]; studies on ISG protein against HCMV infection were carried out using either cell lines or mature astrocytes, which do not mimic the natural course of HCMV infection and there are many gaps to be filled in the understanding of host defense against HCMV infection in CNS. To overcome these limitations, we used human fetal astrocytes of the first trimester gestation to investigate the antiviral effects of ISG proteins, with expectation of developing a targeting therapy without therapy-limiting side effects of IFNs.

IFIT1 (Interferon-induced protein with tetratricopeptide repeats 1) is one of the identified ISGs [Wathelet et al., 1986; Levy et al., 2004]. All members in IFIT family contain multiple tetratricopeptide motifs that are known to mediate protein-protein interactions through scaffolds formed among tandem TPR repeats [Lamb et al., 1995]. IFIT1 is induced in response to type I IFNs stimulation, presence of double-stranded RNAs, and virus infection [Guo et al., 2000; Terenzi et al., 2006]. IFIT1 interacts with the translation initiation factor eIF-3, leading to the inhibition of protein synthesis. IFIT1 has been implicated to have similar antiviral effects as IFNs in fighting against West Nile virus and LCMV [Wacher et al., 2007]. It is also reported to inhibit human papillomavirus DNA replication by binding to the viral protein E1 [Terenzi et al., 2008]; studies demonstrated

that the 2'-O methylation of the 5' cap of viral RNA functions to subvert innate host antiviral responses through escape of IFIT-mediated suppression [Daffis et al., 2010]; It was discovered that IFIT proteins selectively recognize viral RNA via a 5'-triphosphate group (PPP-RNA), and lend insight into their downstream effector function [Pichlmair et al., 2011; Abbas et al., 2013]. Recently, it was reported that ISG54 and ISG56 may contribute to immune and inflammatory reactions elicited by TLR3/IFN- β signaling pathway in astrocytes, and may play an important role both in antiviral immunity and in neuroinflammatory diseases [Imaizumi et al., 2014]. However, little has been reported to date on the protective mechanism of IFIT1 against HCMV infection.

In present study, human fetal astrocytes were infected with HCMV AD169 and whole genome transcriptome profile microarrays were performed. The results showed that HCMV infection regulated multiple gene expression, including up-regulation of interferon-stimulated genes, and genes of chemokine and chemokine receptors, indicating HCMV infection in astrocytes profoundly altered host cell biology. The expression of ISGs were up-regulated dramatically in HCMV infected group with IFIT1 expression most significantly increased (change folds=148.17). IFIT1 was further studied for its antiviral effects using overexpression and knockdown lentivirus transduction.

MATERIALS AND METHODS

Cell Culture and Virus Infection

Human astrocytes from three 65- to 75-day-old fetal brains and HELF's (human embryonic lung fibroblast cells) were obtained from aborted fetuses in the Department of Obstetrics and Gynecology of the Affiliated Hospital, Qingdao University Medical College. The use of aborted fetal brain was approved by Qingdao University Human Investigation Committee. The pregnant women who voluntarily underwent abortion showed negative peripheral blood HCMV antibody detection. Astrocytes were isolated and purified as previous studies [Luo et al., 2010; Zhang et al., 2013, 2014], cells were seeded onto different culture dishes respectively in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin. After cells grew to about 70–80% confluence, the medium was replaced with DMEM/F12 medium supplemented with 2% FBS. Cells were infected with HCMV AD169 (m.o.i=2.5) for 2 hr, then the medium was replaced with fresh DMEM/F12 medium supplemented with 2% FBS. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ for 2, 4, 6, 72 hr.

Whole Genome Profile Microarrays

After 6 or 72 hr HCMV infection, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and reversed to cDNA. Four micrograms of

dscDNA were sent to the NimbleGen expression array platform. The NimbleGen platform proposes high-density expression arrays in which every human gene is represented by several independent probes. These probes consist of 60-mers isothermal oligonucleotides, which show more robust hybridization than shorter oligonucleotides. The NimbleGen human expression array (Format: 12 × 135k; Source: NCBI; Build: HG18, Build 36; Probe Length: 60 mer; Genes: 45033; Probes/Target: 3; Replicates: 1) involves 45,033 gene transcripts (i.e., different accession numbers corresponding to the same or different isoforms). DNA end-labeling, hybridization, scanning, and data normalization were performed at NimbleGen, which provided the final data file. Array hybridization using the NimbleGen Hybridization System followed by washing with the NimbleGen wash buffer kit and Array scanning using the Axon GenePix 4000B microarray scanner (Molecular Devices Corporation, Sunnyvale, CA) were conducted. Scanned images were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level files and Gene level files were generated after normalization. All gene level files were imported into Agilent GeneSpring GX software (version 11.5.1) for further analysis. Genes that at least nine out of nine samples greater than or equal to lower cut-off: 50.0 were chosen for data analysis. Differentially expressed genes between two samples were identified through Fold Change filtering. Differentially expressed genes between two groups were identified through Volcano Plot filtering. A set of genes presenting an average of at least twofold induction/repression calculated from the different probes specific for each gene spotted on the NimbleGen chip were selected for promoter analysis. GO analysis were applied to determine the roles of these differentially expressed genes played in these biological GO terms.

qRT-PCR

QRT-PCR was performed in 96-well optical plates (Sorenson Bioscience, Inc., Salt Lake City, UT) with cDNA using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) in 20 µl reaction volumes. A master mix was made according to manufacturer's instructions using SYBR green supermix (Bio-Rad Laboratories). Forward and reverse primers were used at a concentration of 250 nM per well, made in RNase/DNase-free H₂O. Primer sequences for qPCR were as follows: IFIT1: Forward 5'-TTGCTGGATGTATTACCAC-3', reverse 5'-GCTTCTTGCAAATGTTCTCC-3'; MXA: 5'-AGGACCATCGGAATCTTGAC-3', reverse 5'-TCAGGTGGAACACGAGGTT-3'; CCL5: Forward 5'-ATCCCTCACCGTCATCCTCGTTG-3', reverse 5'-TTGGCACACACTTGGCGGTTTC-3'; CXCL2:

Forward 5'-GAAGTCATAGCCACTCTCAAGG-3', reverse 5'-TTCCGTTGAGGGACAGCA-3'; IL-8: Forward 5'-GGCACAACTTTTCAGAGACAG-3', reverse 5'-ACACAGAGCTGCAGAAATAGG-3'; IL-11: Forward 5'-CCCAGT-TACCCAAGCATCCA-3', reverse 5'-AGACAGAGAACAGGGAATTAATGTGT-3'; OAS1: Forward 5'-AGGTGGTA AAGGGTGGCT-3', reverse 5'-TGCTTGACTAGGCGGATG-3'; Vimentin: Forward 5'-CGTACGTGCAATATGA AAGTGTG-3'; TCAGAGAGGTCAGCAAACCTTGGA-3'; GFAP: Forward 5'-ACTACATCGCCCTCCACATC-3', reverse 5'-CAAAGGCACAGTTCCCAGAT-3'; IE2: Forward 5'-ATGAACCACCTCCTCTTCC-3', reverse 5'-GATATTGCGCCTTCTCGT-3'; IE1: Forward 5'-GAGTCTGCCAGGACATCTTT-3'; β-Actin: Forward 5'-TGGAACGGTGAAGGTGACAG-3', reverse 5'-GGCTTTTATTAGGATGGCAAGGG. β-actin and GAPDH primer sets were included for normalization. Data analysis were performed using Bio-Rad iQ5 optical system software version 2.1.

Western Blot Analysis

Astrocytes were harvested at different time points. Cells were trypsinized, pelleted, and extracted for cell protein. Protein lysates were electrophoresed using SDS-PAGE. Proteins were transferred to PVDF membrane (Millipore, Billerica, MA), and the membrane with transferred proteins were incubated with anti-IFIT1, 2, 3, 5 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IE1 (Santa Cruz Biotechnology), anti-IE2 (Beijing Biosynthesis Biotechnology Co. Ltd, Beijing, China), and anti-pp65 antibodies (Beijing Biosynthesis Biotechnology Co. Ltd), respectively, (all antibodies diluted at 1:100 in TBST) for 60 min at 37°C and then with horseradish peroxidase-labeled secondary antibody for 45 min at 37°C. After washing in TBST, the membrane was developed using ECL reagents and photos were taken. Protein bands were quantified using the Quantity One software (Bio-Rad Laboratories). Paired mean comparisons were performed to assess concentration dependent change in the intensity of native protein bands. All western blot analyses were performed three times, with representative images shown.

Lentivirus Package and Lentivirus-Mediated Transduction of Astrocytes and HELFs

Twenty microgram pGC-LV-IFIT1 vector (IFIT1 empty pGC-LV was for negative control vector), 15 µg pHelper 1.0 vector and 10 µg pHelper 2.0 vector DNA solution were transfected to 393T cells with lipofectamine 2000 (11668-500, Invitrogen). After 48 hr transfection, cell supernatant was collected, centrifuged, and filtered with 0.45 µm filter for crude extract, then purified for lentivirus concentration with Plus-20 centrifugal ultrafiltration device (UFC910024, Millipore) at 4000 rpm centrifugation.

Human fetal astrocytes and HELFs were seeded in 6-well tissue culture plates at 3 × 10⁵ cells/well and incubated at 37°C. The cells were transduced with lv/

IFIT1/EGFP (accession number of IFIT1: NM_001548), lv/ctl/EGFP (GV208, Genechem, Shanghai, China), lv/shIFIT1/EGFP (accession number of IFIT1: NM_001548) or lv/shIFIT1/EGFP (GV115, Shanghai Genechem) at m.o.i of 5.0 in a fresh complete culture medium. The medium containing lentivirus was discarded and replaced with fresh complete medium at 6 hr post transfection. The cells were observed under flow cytometry (FCM) and confocal microscope at 48 hr post transfection to evaluate the efficiency of EGFP-expressing cells and then infected or mock infected with HCMV AD169 (m.o.i = 2.5) for 24 and 48 hr.

Immunocytochemistry

Astrocytes were immersed in 4% paraformaldehyde for 0.5 hr at room temperature, rinsed in PBS, permeabilized in 0.1% Triton X-100 for 30 min at room temperature. Ten percent normal goat serum was used for blocking for 0.5 hr at room temperature. The cells were incubated with primary antibody against IE1 (Santa Cruz Biotechnology), IFIT1 (Santa Cruz Biotechnology), IE2 (Beijing Biosynthesis Biotechnology Co. Ltd), and pp65 antibody (Beijing Biosynthesis Biotechnology Co. Ltd), (all antibodies diluted 1:100 in PBS), for 60 min at 37°C. Then the cells were rinsed and incubated with the FITC-conjugated goat anti-mouse or FITC/Cy3-conjugated goat anti-rabbit IgG (diluted 1:100) for 30 min at 37°C. DAPI or PI was used to stain nuclear. Cells were viewed under laser confocal microscopy.

Virus Plaque Titration

Various dilutions (10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9}) of virus with DMEM/F12 medium were added to 75% confluent astrocytes or HELFs and incubated at 37°C for 2 hr. After virus adsorption, HCMV AD169 was tittered by plaque titration and expressed as the number of plaque-forming units per milliliter. The culture medium was removed from infected cultures and centrifuged for determining virus titer of supernatant, while attached cells were washed with PBS and scraped into culture medium for intracellular virus titer determination at 24, 48 hr p.i.

Viability, Proliferation, and Apoptosis Detected by Colorimetric MTT and FCM

Cell viability and proliferation assay was performed by the colorimetric [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay according to the kit instruction (Sigma-Aldrich). Cell morphism was observed under an inverted Olympus microscope. Yellow MTT is reduced to purple formazan, due to the metabolic activity of living cells. MTT (5 mg/ml) was added to astrocytes allowing to metabolize for 30 min at 37°C. Blue crystals were dissolved in DMSO. Absorbance values were measured at 492 nm

(DG3022) at 0, 48, 72, 96, 120 hr p.i. The experiment was repeated in triplicate. Infected and mock infected astrocytes were collected, centrifuged, and 100 μ l DMEM/F12 containing 2% FBS was added to resuspend cells and transferred to 1.5 ml Eppendorf tubes; 100 μ l of Guava Nexin Reagent (annexin V-PE vs. 7-AAD; EMD Millipore) was added to each Eppendorf tube, incubated for 20 min at room temperature in the dark, and then analyzed using FCM (0500-1430, Guava Easy-Cyte Mini; Guava Technologies Inc., Hayward, CA) as soon as possible. The experiment was repeated in triplicate.

Statistical Analysis

Student's *t*-test using the two-tailed distribution was used to determine significant differences in virus replication and gene expression. For comparison the difference between multiple groups, statistical significance was analyzed using a one-way analysis of variance (ANOVA) followed by post Newman-Keul's test. *P* values of <0.05 were considered statistically significant.

RESULTS

Whole Genome Transcriptome Profile Analysis and Verification

Human fetal astrocytes were purified and identified by immunocytochemistry (A1-A3). When infected with HCMV AD169 and obtained at 6 hr post infection (p.i), astrocytes were examined for mRNA expression with transcriptome microarray using the platform developed by NimbleGen (Madison, WI). As one gene is often represented by several transcripts, we focused only on those genes represented by two or more transcripts and displaying a mean fold difference ≥ 2.0 and *t*-test *P*-value <0.05. With these criteria, we found that about 154 genes, including 138 up-regulated and 16 down-regulated, were modulated at 6 hr p.i. Gene expressions with more than 10-fold change were shown in Table I. To verify the results, the mRNA expression of up-regulated genes IFIT1, MXA, MXB, CCL5, CXCL2, BST2, IL8, IL11 at 6 hr p.i vs con and astrocyte genes GFAP and Vim were confirmed by qRT-PCR (Fig. 1B). A good correlation was found between the two analyses, although almost all genes had higher FC values in qRT-PCR than in microarray analysis. Gene Ontology (GO) analysis showed that the top five categories in biological process all belonged to cell response to stimuli, indicating the role of astrocytes against virus infection at 6 hr p.i (Table II).

The expression levels of ISGs were up-regulated dramatically (Table I), including IFIT1 (fold change, $F_c = 148.17$), IFIT2 ($F_c = 91.12$), IFIT3 ($F_c = 69.62$), IFITM1 ($F_c = 18.35$), MXA ($F_c = 52.56$), MXB ($F_c = 32.80$), OAS1 ($F_c = 46.65$), OAS2 ($F_c = 24.61$), OASL ($F_c = 37.92$), USP18 ($F_c = 26.03$), USP41 ($F_c = 14.64$), IFI44 ($F_c = 17.90$), and BST2

TABLE I. Genes (Fold Change More Than 10) in HCMV AD169 Infected Astrocytes

SEQ_ID	P-value	Fold change	Up/down	Gene name	Description
NM_001001887	4.61E-04	148.17294	Up	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
NM_001547	6.57E-05	91.12055	Up	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2
NM_001031683	0.001913484	69.62959	Up	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3
NM_002462	0.006052201	52.563366	Up	MXA	MXA myxovirus (influenza virus) resistance 1, interferon inducible protein p78
NM_016816	0.002228843	46.65933	Up	OAS1	2',5'-oligoadenylate synthetase 1
NM_198213	0.009020009	37.92200	Up	OASL	2'-5'-oligoadenylate synthetase-like
NM_002463	0.012798179	32.80558	Up	MXB	Myxovirus (influenza virus) resistance 2
NM_002985	0.016673766	31.12011	Up	CCL5	Chemokine (C-C motif) ligand 5
NM_017414	0.008110123	26.03400	Up	USP18	Ubiquitin specific peptidase 18
NM_002535	0.008042933	24.61983	Up	OAS2	2'-5'-oligoadenylate synthetase 2
NM_003641	0.003658616	18.35694	Up	IFITM1	Interferon induced transmembrane protein 1
NM_006417	0.011669416	17.90868	Up	IFI44	Interferon-induced protein 44
AJ634912	0.019668868	16.89670	Up	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
NM_006187	0.013115374	15.88929	Up	OAS3	2'-5'-oligoadenylate synthetase 3
XM_036729	0.006409143	14.64420	Up	USP41	Ubiquitin specific peptidase 41
NM_004335	0.003946278	14.48724	Up	BST2	Bone marrow stromal cell antigen 2
NM_017654	0.009343174	13.81734	Up	SAMD9	Sterile alpha motif domain containing 9
NM_002089	0.04555019	12.10514	Up	CXCL2	Chemokine (C-X-C motif) ligand 2
NM_004848	0.005747211	12.00902	Up	C1orf38	Chromosome 1 open reading frame 38
NM_001037335	0.006599061	11.18215	Up	PRIC285	Peroxisomal proliferator-activated receptor A interacting complex 285
NM_003745	0.006988464	10.714319	Up	SOCS1	Suppressor of cytokine signaling 1
NM_014314	0.010410038	10.233637	Up	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58

Fold Change cut-off, 2.0; Condition pairs, 6 hr p.i vs con; hr p.i, hour post infection; con, mock infected control.

($F_c = 14.48$). Chemokines were also up-regulated, including IL-11 ($F_c = 3.50$), CXCL2 ($F_c = 12.10$) and CCL5 ($F_c = 31.12$). IFIT1, showing most significant changes in gene expressions, was chosen for further investigation of its antiviral effect. IFIT1 protein expression was determined by immunocytochemistry (Fig. 1D1–D6) and western blot (Fig. 1C). The results of both assays showed significant expression of IFIT1 in HCMV infected astrocytes compared with mock infected control.

Morphological Observation of Astrocytes and HELFs Transduced With IFIT1 Overexpression Lentivirus

IFIT1 overexpression lentivirus (lv/IFIT1/EGFP) was transduced into human fetal astrocytes and HELFs. After 48 hr transfection, the efficiency of transfection was observed under laser confocal microscope and analyzed by FCM, which showed more than 98.5% cells were positive (Fig. 2A1–A4). These cells were infected with HCMV AD169 (m.o.i = 2.5), and cell morphology changes were monitored under laser confocal microscope at different time points. There were no morphological changes in lv/IFIT1/EGFP transduced astrocytes at 72 hr p.i (Fig. 2B1 and B2) and mock infected groups (Fig. 2B5 and B6) compared with lv/ctl/EGFP transduced control group, the later exhibited classical morphological changes at 72 hr p.i (Fig. 2B3 and B4). On the contrary, HELFs exhibited classical pathogenic effects at 72 hr p.i in both lv/IFIT1/EGFP (Fig. 2B7 and B8) and lv/ctl/EGFP transduced groups (Fig. 2B9 and B10),

while the mock infected group had no morphological changes (Fig. 2B11 and B12). The results indicated a different antiviral role of IFIT1 in astrocytes and HELFs.

Overexpression of IFIT1 and Its Effect on Viral Replication

When lv/IFIT1/EGFP was transduced into HELFs, viral mRNA expression (immediate-early [IE1, IE2] and late [pp65] stages) and virus titer were detected in HCMV infected HELFs. The results showed that there was no statistical difference in both viral mRNA expression and virus titer between lv/IFIT1/EGFP transduced group and control one (Fig. 2C1–C5). When lv/IFIT1/EGFP was transduced into astrocytes, the expression levels of viral mRNA and protein at different stages (IE1, IE2, and pp65) were detected with qRT-PCR or western blot. The results showed significant reduction in the expression of viral mRNA (Fig. 3A1–A3) and protein (Fig. 3B) at all stages (IE1, IE2, and pp65) compared with their corresponding control group lv/ctl/EGFP ($P < 0.01$).

To further identify the effect of IFIT1 on HCMV infected human fetal astrocytes, the release of infectious HCMV particles in cell culture medium and virus titer of intracellular astrocytes were determined with plaque titration method on a monolayer astrocytes at 72 hr p.i. The results showed that HCMV titer both inside and outside cells were significantly reduced in lv/IFIT1/EGFP transduced astrocytes compared with lv/ctl/EGFP-transduced control ($P < 0.001$)

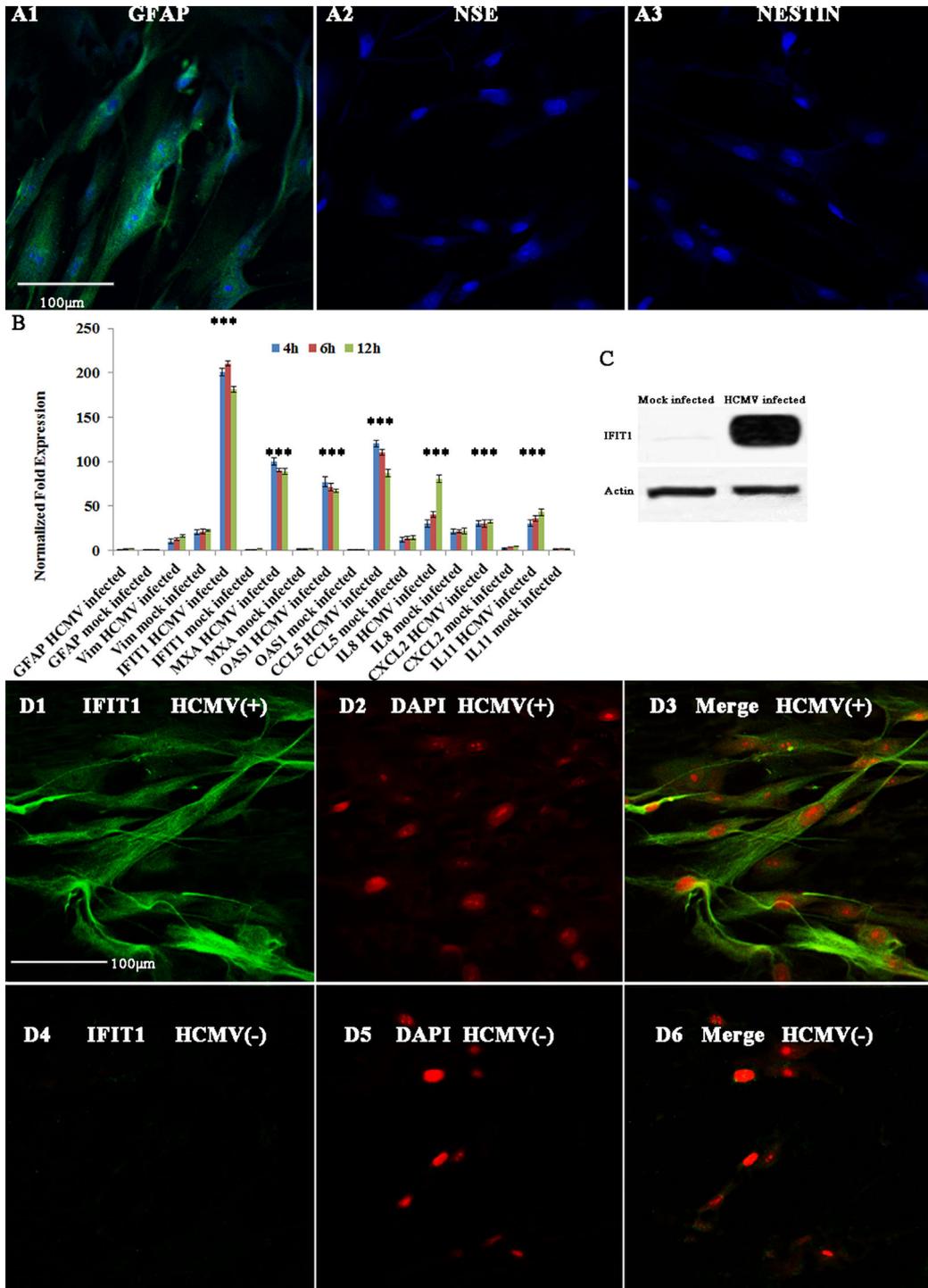


Fig. 1. Identification of purified astrocytes and expression of IFIT1 upon HCMV infection by RT-qPCR, immunocytochemistry and western blot. **A**, Identification of purified astrocytes; **A1**, GFAP; **A2**, NSE positive neurons; **A3**, NESTIN positive neural stem cells. **B**, RT-qPCR confirmation of microarray analysis. The β -actin and GAPDH primer sets were included for normalization. GFAP, glial fibrillary acidic protein; Vim, vimentin; IFIT1, Interferon-induced protein with tetratricopeptide repeats 1; MxA, myxovirus (influenza virus) resistance A; OAS1, 2',5'-oligoadenylate synthetase 1;

CCL5, chemokine (C-C motif) ligand 5; IL8, interleukin 8; CXCL2, chemokine (C-X-C motif) ligand 2; IL11, interleukin 11. **D1–D6**, IFIT1 protein expression at 24 hr p.i by immunocytochemistry. **D1**, IFIT1 stained astrocytes; **D2**, DAPI stained astrocytes; **D3**, merge of IFIT1 and DAPI; **D4**, IFIT1 stained astrocytes; **D5**, DAPI stained astrocytes; **D6**, merge of IFIT1 and DAPI. **C**, IFIT1 protein expression by western blot. HCMV(+), HCMV infected (m.o.i = 2.5); HCMV(-), mock infected; H p.i, hours post infection; *, statistical significance $P < 0.05$.

TABLE II. Categories of Up-Regulated Genes in HCMV Infected Astrocytes

Gene ontology term	P-value	Genes
GO:0009615—response to virus	6.8E-16	DDX58//PML//SAMHD1//BST2//ISGF3G//IFI44//IRF7//MX1//MX2//PLSCR1//EIF2AK2//ZC3HAV1//CCL5//TRIM25//IFITM1//G1P2
GO:0051707—response to other organism	3.7E-13	DDX58//PML//CARD4//ISGF3G//IFI44//IRF7//MX1//MX2//PLSCR1//EIF2AK2 //ZC3HAV1//CCL5//TRIM25//IFITM1//G1P2//PTGS2//SOCS1//SAMHD1//BST2
GO:0051704—multi-organism process	1.9E-12	DDX58//PML//CARD4//ISGF3G//IFI44//IRF7//MX1//MX2//PLSCR1//EIF2AK2//ZC3HAV1//CCL5//TRIM25//IFITM1//G1P2//STAT5A //PTGS2//KLF9//TAP1//SOCS1//ICAM1//SP110//TFRC//SAMHD1//BST2
GO:0009607—response to biotic stimulus	1.6E-11	DDX58//PML//CARD4//ISGF3G//IFI44//IRF7//MX1//MX2//PLSCR1//EIF2AK2//ZC3HAV1//CCL5//TRIM25//IFITM1//G1P2 //PTGS2//SOCS1//SAMHD1//BST2
GO:0006952—defense response	4E-10	DDX58//PML//CCL5//TFRC//CARD4//CFH//TMEM23//CXCL2//NFKBIZ//BMP2//C1ORF38//PTGS2//TAP1//IL1R1//TRIM25//IFITM1//SAMHD1//STAT5A//BST2//MX1//MX2
GO:0002376—immune system process	1.1E-09	ICAM1//IL7R//DDX58//PML//SFRP1//CFH//G1P3//SAMHD1//CD274//CXCL2//IL1R1//OAS1//OAS2//OAS3//CCL5//OASL//BST2//TAP1//CSF1//SOD2//IL11//STAT5A//LMO2//TRIM25//IFITM1
GO:0006955—immune response	4.5E-09	ICAM1//IL7R//DDX58//PML//CFH//BST2//TAP1//IL1R1//TRIM25//IFITM1//SAMHD1//STAT5A//G1P3//CD274//CXCL2//OAS1//OAS2//OAS3//CCL5//OASL
GO:0001816—cytokine production	0.01009	PTGS2//DDX58//CARD4//STAT5A//ISGF3G

Fold Change cut-off, 2.0; Condition pairs, 6 hr p.i vs con; hr p.i, hour post infection; con, mock infected control.

(Fig. 3C1 and C2). Additionally, MTT and FCM for cell proliferation and apoptosis were analyzed, which showed no significant difference at different time points in lv/IFIT1/EGFP or lv/ctl/EGFP transduced group ($P < 0.05$) (Fig. 3D1–D7), suggesting that it was IFIT1 that mediated antiviral effects in human fetal astrocytes. Additionally, transduced astrocytes over-expressed IFIT1 protein was determined by western blot analysis after infected with HCMV AD169 for 24 hr. Very high level increase in IFIT1 expression was observed in astrocytes transduced with lv/IFIT1/EGFP, while medium level increase was detected in non-transduced astrocytes (Fig. 3D8)

Knockdown of IFIT1 and Its Effect on Viral Replication

To investigate whether knockdown of IFIT1 expression modulates HCMV replication, three small interference RNAs (shRNAs) for knocking down the expression of IFIT1 were synthesized. Astrocytes were transduced with control (scrambled) shRNA (lv/ctl/EGFP) or IFIT1 shRNA lentivirus (lv/shIFIT1/EGFP) for 48 hr, and then the cells were infected with HCMV AD169 at m.o.i = 2.5 for an additional 24 or 48 hr to determine antiviral replication effects of IFIT1. The results suggested that neither shRNA-1 nor shRNA-2 affected the expression of other ifit (ifit2, 3, 5) genes (Fig. 4A1). The inhibitory rate of shRNA-1 was higher than that of shRNA-2. Therefore, shRNA-1 was chosen as an interference tool for all subsequent studies and the inhibitory property of shRNA-1 was determined by western blot. The results showed significant inhibition in lv/shRNA/EGFP transduced group compared with lv/ctl/EGFP transduced group (Fig. 4A2).

Total RNA and protein were isolated at 6 or 72 hr p.i and analyzed for HCMV replication by qRT-PCR or western blot. The results showed that mRNA and protein expression of IE1, IE2, and pp65 were all markedly enhanced in lv/shRNA/EGFP transduced cells compared with control group (Fig. 4B1–B3 and C). Then HCMV infectivity of viral titers was detected. Results showed titer of inside and outside of cells increased in cells deficient in IFIT1 compared with control groups (Fig. 4D1 and D2). Finally, MTT analysis for cell viability and proliferation was also performed, which showed no significant difference either in lv/shRNA/EGFP transduced or lv/ctl/EGFP transduced groups at 0, 48, 72, 96, 120 hr p.i ($P < 0.05$) (Fig. 4E), which evidenced it was lv/shRNA/EGFP that mediated inhibition of IFIT1 antiviral effects. Taken together, the results demonstrated that HCMV replication increased in IFIT1 knockdown astrocytes compared with control.

DISCUSSION

HCMV is a widespread human pathogen. The symptoms of primary HCMV infection in healthy adults are generally mild, but infection during first trimester gestation can lead to serious neurodevelopment sequelae in infants. Fetal CNS is especially vulnerable to CMV-induced injury [Poland et al., 1994; McCarthy et al., 2000]. Astrocytes are the most abundant cell type found in the CNS, outnumbering neurons 10-1. These glial cells have the capacity to react to a variety of CNS disturbances such as viral infection through the production of immune mediators and it is well established that primary human astrocytes are fully permissive for CMV replication [Ho et al., 1991;

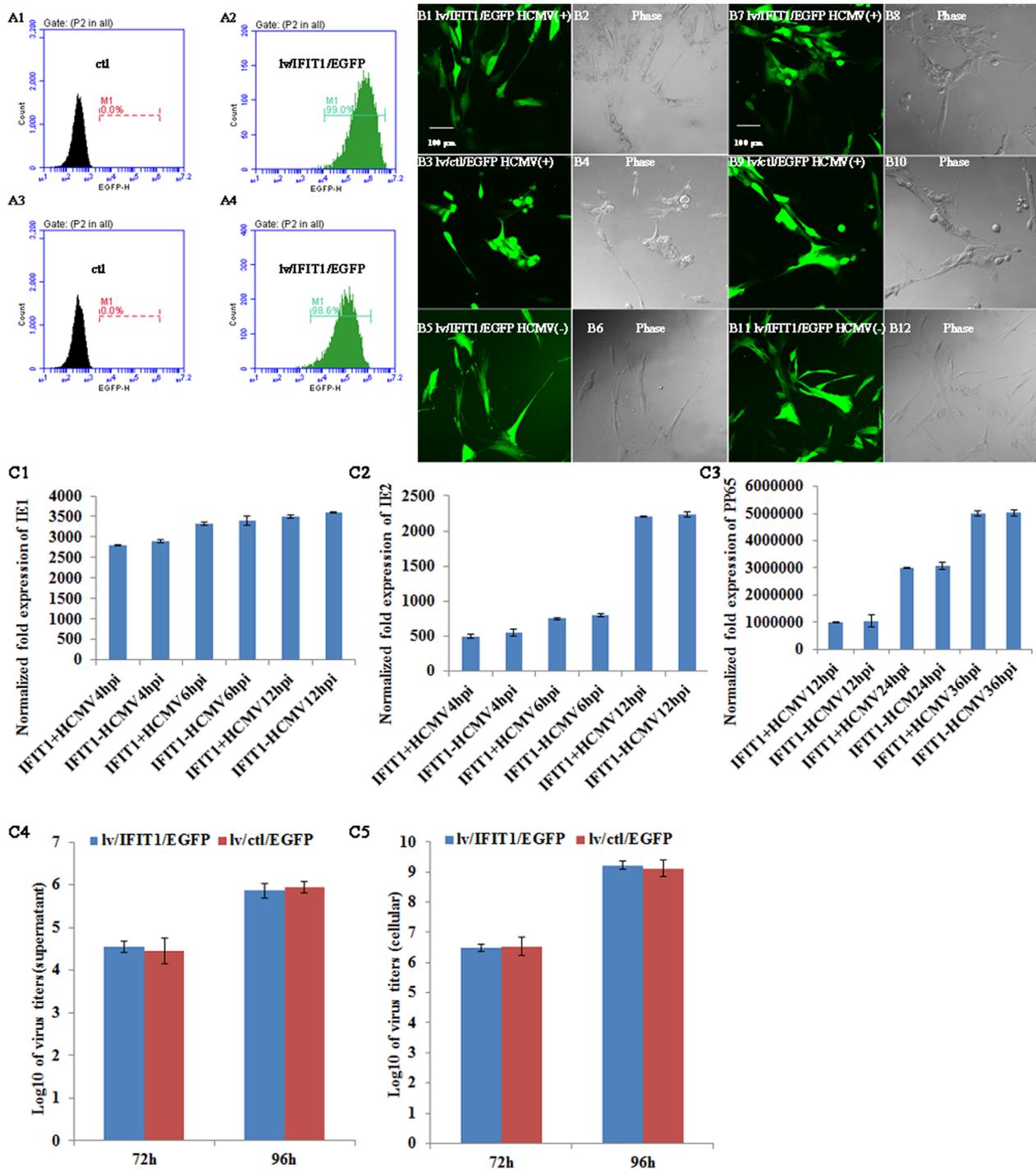


Fig. 2. Overexpression of IFIT1 did not regulate HCMV AD169 replication in HELFs. **A1** and **A2**, transduction efficiency by FCM. **A1**, non-transduced astrocytes; **A2**, lv/IFIT1/EGFP transduced astrocytes after 48 hr; **A3**, non-transduced HELFs; **A4**, lv/IFIT1/EGFP transduced HELFs after 48 hr. **B1–B12**, cell morphology observation under con-focal microscope. **B1**, **B2**, **B5**, and **B6**, lv/IFIT1/EGFP transduced astrocytes; **B3** and **B4**, lv/ctl/EGFP transduced

astrocytes; **B7**, **B8**, **B11**, and **B12**, lv/IFIT1/EGFP transduced HELFs; **B9** and **B10**, lv/ctl/EGFP transduced HELFs. **C1–C3**, IE1, IE2, and pp65 by RT-qPCR **C4**, Virus titer of supernatant at 72 and 96 hr p.i.; **C5**, intracellular virus titer at 72 and 96 hr p.i. Lv/IFIT1/EGFP, IFIT1 lentivirus; lv/ctl/EGFP, lentivirus negative control; HCMV(+), HCMV infected; HCMV(-), mock infected; H p.i, hours post infection.

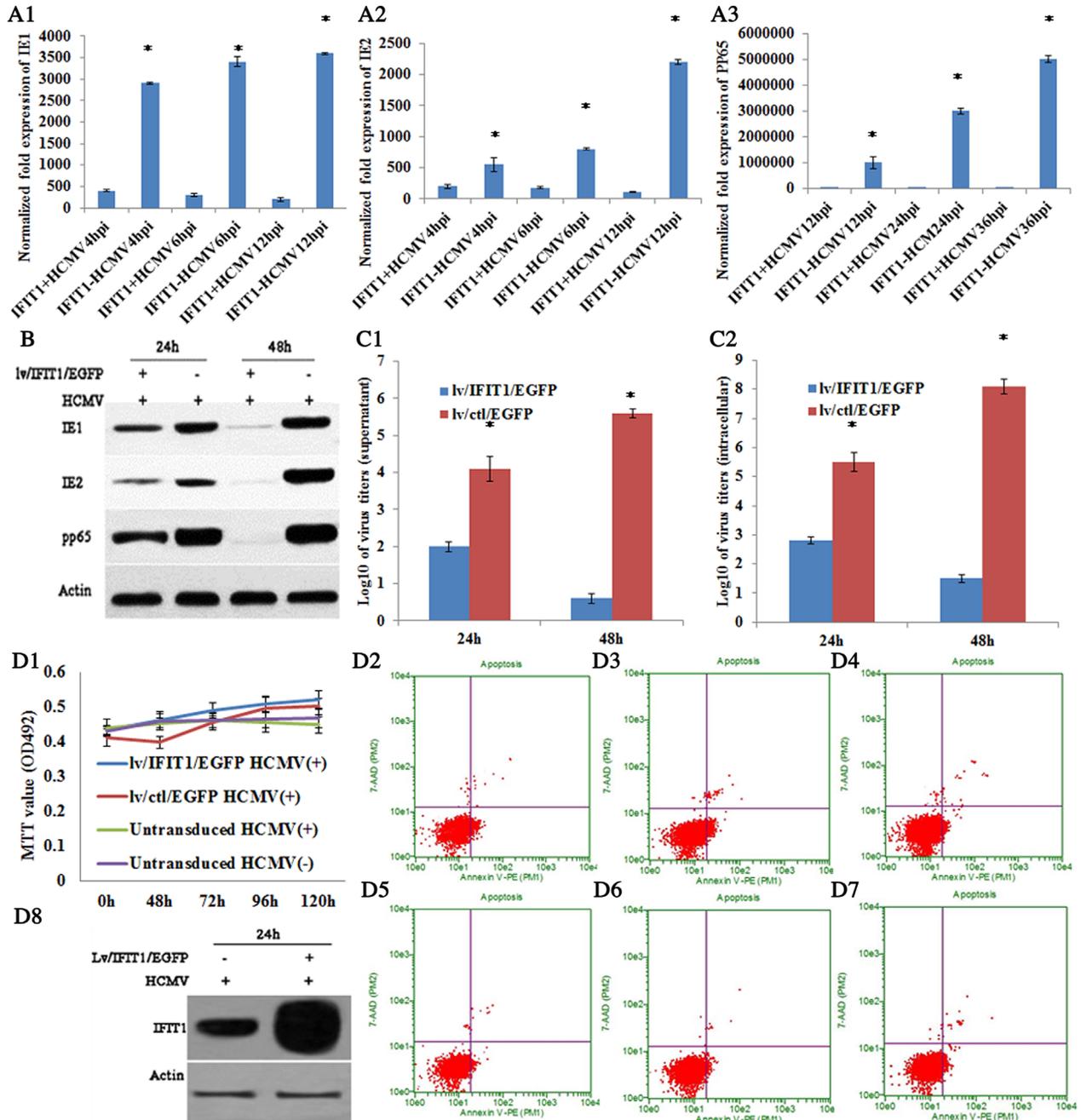


Fig. 3. Overexpression of IFIT1 restricted HCMV AD169 replication. **A1–A3**, mRNA expression of IE1, IE2, and pp65 by RT-qPCR. **B**, protein expression of IE1, IE2, and pp65 by western blot at 24 and 48 hr p.i. **C1**, Virus titer of supernatant; **C2**, intracellular virus titer. **D1**, MTT analysis; **D2–D4**, Cell

apoptosis for lv/IFIT1/EGFP transduced astrocytes; **D2**, 0 hr p.i.; **D3**, 72 hr p.i.; **D4**, 120 hr p.i.; **D5–D7**, lv/ctl/EGFP transduced astrocytes; **D5**, 0 hr p.i.; **D6**, 72 hr p.i.; **D7**, 120 hr p.i. **D8**, Protein expression of IFIT1 at 24 hr p.i. HCMV–, mock infected, HCMV+, HCMV infected; *, statistical significance $P < 0.05$.

Lokensgard et al., 1999]. Host antiviral responses to HCMV begin quickly after virus-cell contact involving interaction with a growing list of pattern recognition receptors that initiate intracellular signaling and consequent expression and secretion of numerous immunologically active cytokines.

In this study, HCMV infected human fetal astrocytes were studied for the effects HCMV on genome

transcription of astrocytes and antiviral activity of astrocytes. Firstly, we performed genome wide gene expression of HCMV infected human fetal astrocytes in vitro. With a 2.0-fold change as a cutoff, it showed HCMV infection led to extensive dysregulation of cellular gene expression in astrocytes. One hundred and fifty-four gene expressions (138 up-regulated, 16 down-regulated) were modulated in HCMV infected

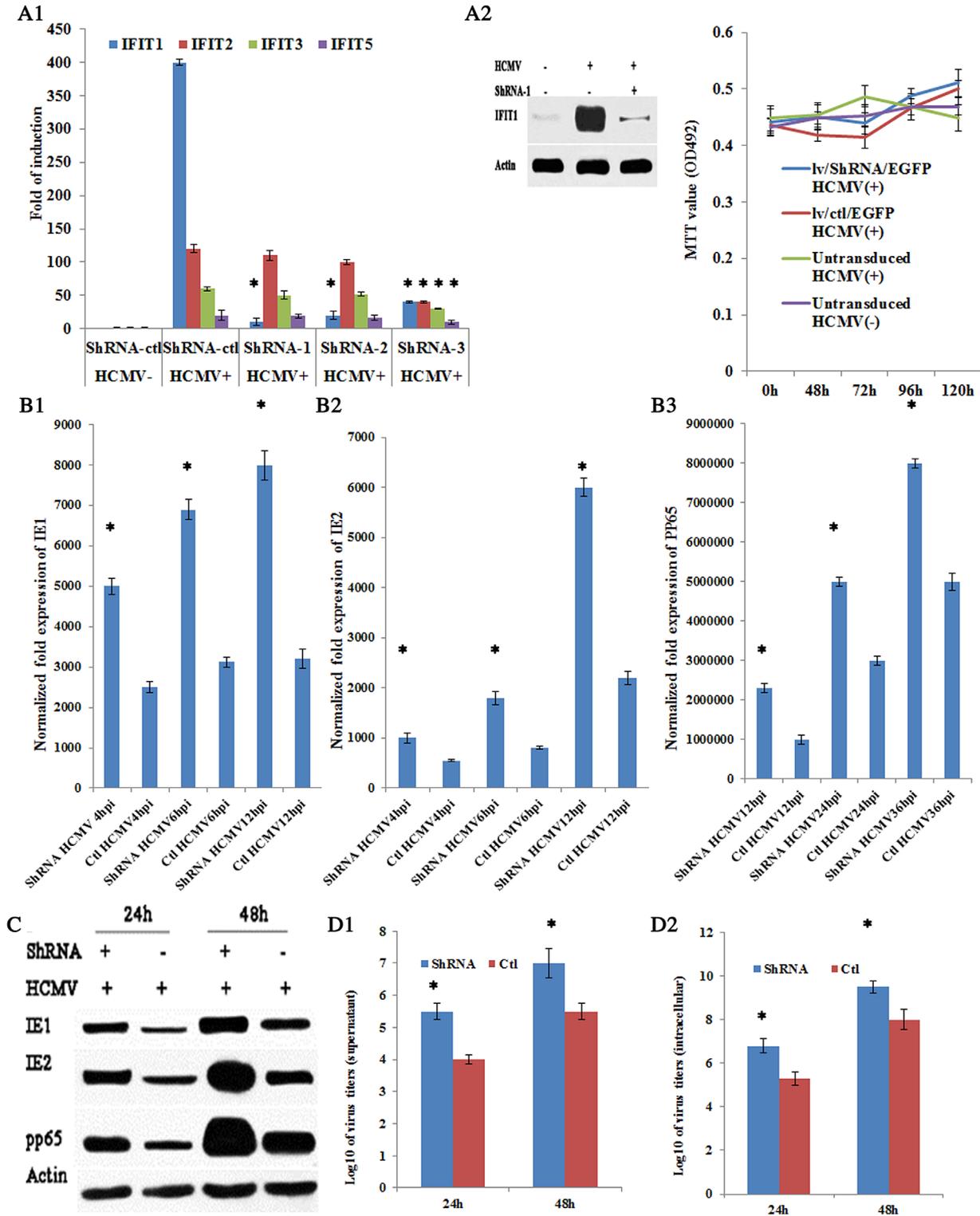


Fig. 4. Knockdown of IFIT1 increased HCMV replication. **A1**, mRNA of ifit genes was determined by RT-qPCR with treatment of shIFIT1-1, shIFIT1-2, or shIFIT1-3; **A2**, protein expression of IFIT1 after treatment with lv/shRNA-1/EGFP; **B1–B3**, mRNA expression of IE1, IE2, and pp65 by RT-qPCR.

C, protein expression of IE1, IE2, and pp65 after treatment with lv/shIFIT-1/EGFP; **D1**, virus titer of supernatant; **D2**, intracellular virus titer. **E**, MTT analysis. HCMV–, mock infected astrocytes, HCMV+, HCMV infected astrocytes; *, statistical significance $P < 0.05$.

astrocytes compared with mock infected group at 6 hr p.i. GO analysis showed the top five categories in biological process and all belonged to cell response to stimuli (Table II). ISGs involved in antiviral response process in early infection time [Ho and van den Pol, 2007] exhibited the highest fold changes in HCMV infected astrocytes compared with mock infected group at 6 hr p.i. However, this result was not consistent with the finding that microglia not astrocytes could produce antiviral cytokines in response to CMV infection [Lokensgard et al., 2002]. The reason could be that the response to HCMV infection in fetal astrocytes was different from mature astrocytes or cell lines. These up-regulated ISGs included IFIT1, IFIT2, IFIT3, IFITM1, MXA, MXB, OAS1, OAS2, OASL, USP18, USP41, IFI44, and BST2. It also showed that gene expression levels of chemokine and chemokine receptors (IL-11, MCP-1, IL-8, RANTES, IP-10, and MIP-1 α , Table III) were up-regulated compared to mock infected group at 6 hr p.i. This result was consistent with the report that CMV-infected human brain vascular pericytes and astrocytes released substantial amounts of the chemokines MCP-1, MIP-1 α , and IL-8 [Lokensgard et al., 2002; Alcendor et al., 2012]. Several studies have demonstrated that chemokine play important roles in regulating brain inflammation during viral encephalitis because of their chemotactic properties, and the importance of CCL5, CCL3, CCL4, IL-11, IL-8, and MCP-1 in regulating immune responses in the CNS

have been emphasized [Weiss et al., 1998; Kutsch et al., 2000; Zlotnik, 2000]. Our results showed different fold changes of these cytokines and chemokines in HCMV infected astrocytes (Table III). Additionally, it has also been reported that early local expression of cytokines with antiviral properties in the CNS such as TNF α , IFN α , IFN β , and IFN γ correlates with control of viral replication. IFN treatment induces the expression of a large set of IFN-stimulated genes (ISGs), whose protein products prevent infection with many pathogens, including HCMV [Samuel, 2001]. In present study, the results showed different fold changes with IL-1 β , IL-8, IL-6 in HCMV infected astrocytes compared with mock infected group at 6 hr p.i, while TNF α , IFN α , IFN β , or IFN γ exhibited little gene expression level in HCMV or mock infected astrocytes and fold changes were no statistical significance in HCMV infected astrocytes compared with mock infected control. The results suggested that HCMV infected astrocytes defended themselves against HCMV infection through expressing of ISGs, cytokines, and chemokines which either exert direct antiviral activities, or recruit antiviral immune cells such as microglia to the sites of infection [Peterson et al., 1997] and ISGs exerted important role in antiviral action. However, it is also plausible that only a subset of ISGs is essential for protection against a specific pathogen or groups of related pathogens. Thus, identification of pathogen-specific ISGs might lead to development of targeted therapeutics.

TABLE III. Astrocytes Responsiveness With HCMV AD169 Infection

Gene name	Up/down regulation	Fold change	P-value
RIG-I	Up	10.2	<0.05
IPS-1	Up	3.2	<0.05
MyD88	Up	3.2	<0.05
TRIF	Up	2.2	<0.05
IRF3	Up	2.3	<0.05
IRF7	Up	11.3	<0.05
IRF9	Up	5.2	<0.05
STAT1	Up	2.2	<0.05
STAT2	Up	3.2	<0.05
NF- κ B	Up	2.1	<0.05
IFN β	—	—	>0.05
IFN α 1	—	—	>0.05
IFN λ 3	—	—	>0.05
IFN γ	—	—	>0.05
TNF α	—	—	>0.05
IL-1 β	Up	4.3	<0.05
IL-6	Up	10.1	<0.05
IL-10	—	—	—
RANTES	Up	31.1	<0.05
IL-8	Up	7.1	<0.05
MIP-1 α	Up	7.1	<0.05
MCP-1	Up	5.3	<0.05
CXCL2	Up	12.1	<0.05
IL-11	Up	3.5	<0.05
IL-4	—	—	—

Fold Change cut-off, 2.0; Condition pairs, 6hr p.i vs con; IFN, interferon; IL, interleukin; IP, IFN-inducible protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; —, no/very low expression; TNF, tumor necrosis factor. hr p.i, hour post infection; con, mock infected control.

Secondly, IFIT1 with the highest Fc among all up-regulated genes (6 hr p.i vs con) was studied for its antiviral effects. Overexpression Lentivirus lv/IFIT1/EGFP gene was transduced into astrocytes or HELFs, respectively. Cells were infected with HCMV AD169 for 72 hr. Cell appearance showed no change in lv/IFIT1/EGFP transduced astrocytes compared with lv/ctl/EGFP transduced control group at 72 hr p.i, while morphological changes were observed in lv/IFIT1/EGFP and lv/ctl/EGFP transduced HELFs. The different antiviral effects of IFIT1 on astrocytes and HELFs might be caused different expression of cellular factors, which may be responsible for the modulation of viral gene expression [Towler et al., 2012], and reinforce the reason to investigate the specific antiviral replication role of IFIT1 in fetal astrocytes. Furthermore, mRNA expression of IE1, IE2, and pp65 were detected by QRT-PCR at 4, 6, 12 hr p.i and protein expression were detected by western blot at 24, 48 hr p.i, due to the delayed expression of protein compared to mRNA. The results showed that both mRNA and protein expressions were significantly reduced compared with lv/ctl/EGFP transduced control ($P < 0.001$). HCMV replication cycle in fetal astrocytes was 48–72 hr (showed in our previous study). Therefore, the release of HCMV infectious particles in cell culture medium and HCMV titer within astrocytes were determined at 48 and 72 hr p.i. The results showed significant decreases in the

release of HCMV infectious particles in cell culture medium and in HCMV titer within astrocytes in lv/IFIT1/EGFP transduced astrocytes compared with the control ($P < 0.001$).

Finally, to further verify the antiviral effects of IFIT1, shIFIT1-1 was used for knocking down IFIT1 gene. After shIFIT1-1 lentivirus was transduced into astrocytes for 48 hr, cells were infected with HCMV AD169. Viral mRNA, protein expression levels and HCMV titers were determined, which showed significant increases in mRNA expressions of IE1, IE2, pp65, and viral titers in knockdown group compared with the control ($P < 0.01$), suggesting the antiviral replication effects of IFIT1 in fetal astrocytes. This is the first report on antiviral effects of IFIT1 in human fetal astrocytes to date and these findings also pointed to the potential importance of studying human primary first-trimester gestation astrocytes because the antiviral effects of particular cytokines depend on specific cell surface receptors that may not be present or functional on established glial cell lines.

Functional studies have shown that IFITs inhibit the translation of viral proteins via different mechanisms. In early studies, IFITs were found to bind eukaryotic initiation factor 3 (eIF3) and interfere with viral protein translation [Hui et al., 2003]. Recent studies showed that IFITs can distinguish the different features between host and viral RNAs, block West Nile virus, Japanese encephalitis virus, and coronavirus mutants that lack 2'-O-methyltransferase activity. Inhibition by IFIT1 occurs by preferentially sequestering capped RNA lacking a 2'-O-methyl group and preventing eukaryotic translation initiation factors from binding to the RNA template specifically [Daffis et al., 2010; Habjan et al., 2013; Kimura et al., 2013]. IFIT1 was also identified as an eIF3-associated protein fractionating with hepatitis C virus (HCV) translation complexes in IFN-treated cells [Wang et al., 2003]. IFIT1 blocks translation driven by the HCV internal ribosome entry site (IRES) and the inhibition depends on eIF3 binding by IFIT1. However, another study suggested IFIT1 preferentially inhibits cap-dependent translation but not translation of the EMCV IRES [Hui et al., 2003], since the EMCV IRES is different from that of HCV and functions by a different mechanism. IFIT1 also inhibits human papillomavirus DNA replication by binding to the viral protein E1 [Terenzi et al., 2008]. Nevertheless, several important questions still remain to be addressed. Our data verified that IFIT1 possessed anti-HCMV function by inhibiting virus replication in human fetal astrocytes. However, the underlying mechanism remains unclear. There are possibilities that IFIT1 inhibits DNA replication by binding to the viral protein, or by suppressing HCMV protein translation by binding eIF3. It will be interesting to ponder new information on the uncovered the processes described here and on how to exploit for novel clinical antiviral or therapeutic strategies.

Further studies are necessary to look into the precise role of this effector molecule.

In conclusion, this study provided insights into the function of IFIT1 as a key factor in antiviral defending, and regulation of IFIT1 expression level is effective for modulating viral replication in HCMV infected human fetal astrocytes. The findings of present study might contribute to the development of targeted therapeutics towards fetal brain HCMV infection.

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ETHICAL STANDARD

The authors ensured that all procedures were performed in compliance with relevant laws and institutional guidelines and that the Qingdao University Human Investigation Committee has approved them. There was informed consent prior to obtaining the fetus.

AUTHORS' CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Bin Wang. Acquisition of data: Li Zhang. Analysis and interpretation of data: Li Zhang, Ling Li, Dong-meng Qian. Drafting of the manuscript: Li Zhang, Hong Yu, Mei-lan Xue. Critical revision of the manuscript for important intellectual content: Ming Hu, Xu-xia Song. Statistical analysis: Mei-lan Xue, Dong-meng Qian. Obtained funding: Bin Wang, Li Zhang. Administrative, technical, and material support: Xu-xia Song. Study supervision: Bin Wang.

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