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Hepatitis C virus-induced innate immune responses in human iPS cell-derived hepatocyte-like cells

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ABSTRACT

Hepatitis C virus (HCV) infection is a major cause of liver-related morbidity and mortality. In order to develop effective remedies for hepatitis C, it is important to understand the HCV infection profile and host-HCV interaction. HCV-induced innate immune responses play a crucial role in spontaneous HCV clearance; however, HCV-induced innate immune responses have not been fully evaluated in hepatocytes, partly because there are few *in vitro* models of HCV-induced innate immunity. Recently, human induced pluripotent stem (iPS) cells have received much attention as an *in vitro* model of infection with various pathogens, including HCV. We previously established highly functional hepatocyte-like cells differentiated from human iPS cells (iPS-HLCs). Here, we examined the potential of iPS-HLCs as an *in vitro* HCV infection model, especially for evaluation of the relationship between HCV infection levels and HCV-induced innate immunity. Significant expressions of type I and III interferons (IFNs) and IFN-stimulated genes (ISGs) were induced following transfection with HCV genomic replicon RNA in iPS-HLCs. Following inoculation with the HCV JFH-1 strain in iPS-HLCs, peaks of HCV genome replication and HCV protein expression were observed on day 2, and then both the HCV genome and protein levels gradually declined, while the mRNA levels of type III IFNs and ISGs peaked at day 2 following inoculation. These results suggest that the HCV genome efficiently replicates in iPS-HLCs, resulting in HCV genome-induced up-regulation of IFNs and ISGs, and thereafter, HCV genome-induced up-regulation of IFNs and ISGs mediates a reduction in the HCV genome and protein levels in iPS-HLCs.

1. Introduction

Hepatitis C virus (HCV) infects approximately 170 million people worldwide and is a major cause of serious liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (McHutchison, 2004; Shepard et al., 2005). In addition to the standard treatments for chronic hepatitis C—that is, interferon (IFN) and ribavirin (RBV) combination therapy—direct-acting antivirals (DAAs) have recently shown a high rate of success. However, our knowledge of the pathogenesis of chronic hepatitis C remains incomplete. The most important aspect of treating HCV infection is understanding the mechanism of chronic HCV infection. Although HCV infection becomes chronic in about 70–80% of those who exhibit acute infection, approximately 20–30% of the patients with acute HCV infection will resolve and spontaneously clear the HCV without any treatment (Bowen and Walker, 2005). HCV-induced innate immune responses, including interferon (IFN) responses, are known to play a major role in such spontaneous HCV clearance (Horner and Gale, 2013). Moreover not only immune cells, including dendritic cells and liver Kupffer cells, but also hepatocytes play a crucial role in HCV-induced innate immunity. In addition, host and viral factors which regulate intracellular innate immune responses against HCV are responsible for the outcome of HCV infection.

The HCV virion contains a single-stranded positive-sense RNA

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genome. The virion binds to cellular receptors, including claudin-1 and occludin, and subsequently enters the cells via an endocytic pathway. After pH-dependent fusion and uncoating, the incoming HCV genome replicates by a virus-derived polymerase in the cytoplasm. During this process, the HCV genome recognized by host intracellular and extracellular pattern recognition receptors (PRRs), including the retinoic acid inducible gene-I (RIG-I) and toll-like receptor 3 (TLR3) (Cao et al., 2015; Li et al., 2012; Sumpter et al., 2005). RIG-I is a cytoplasmic PRR that senses intracellular HCV genomes and activates downstream effector molecules *via* its interaction with IFN-β promoter stimulation protein 1 (IPS-1). TLR3 is mainly localized on the endosomal and lysosomal membrane. The signals of TLR3 are transmitted *via* TIR-domain-containing adaptor-inducing interferon-β (TRIF). These signals through RIG-I and TLR3 lead to activation of transcription factors, including IFN regulatory factor-3 (IRF-3) and nuclear factor-κb (NF-κβ), which induce expression of type I and III IFNs and subsequent expression of IFN-stimulated genes (ISGs) through activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Although direct antiviral ISG action is important to control HCV replication and spread, the details of its function remain unclear.

In order to examine host cell-HCV interactions, *in vitro* HCV infection models have been developed, including HCV replicon systems and the genotype 2a JFH-1 strain (cell culture-adapted HCV (HCVcc)). HCV replicon and HCVcc efficiently replicate in human hepatoma cell line Huh7-derived Huh7.5.1 cells. These models are highly useful tools to understand host-HCV interactions; however, HCV-induced innate immune responses cannot be evaluated in Huh7.5.1 cells because RIG-I-mediated innate immune responses do not occur in Huh7.5.1 cells due to a point mutation within the RIG-I gene (Sumpter et al., 2005). In addition, TLR3 is not functional in Huh7.5.1 cells (Li et al., 2005). Primary human hepatocytes (PHHs) are a suitable model for the evaluation of HCV-induced innate immune responses (Thomas et al., 2012; Yang et al., 2011); however, the scarcity and expense of PHHs hamper their use as an *in vitro* model for the evaluation of HCV-induced innate immune responses.

Recently, much attention has been focused on human induced pluripotent stem (iPS) cells as potential *in vitro* model of pathogen infection. Several studies have demonstrated that human iPS cell-derived differentiated cells, including hepatocytes and cardiomyocytes, are available to evaluate host cell-pathogen interactions (Sakurai et al., 2017; Sharma et al., 2014; Trevisan et al., 2015). Human iPS cells have several advantages as a source of an *in vitro* model of pathogen infection. For example, human iPS cells can replicate indefinitely and differentiate into various types of cells. In addition human iPS cells possessing various genetic backgrounds can be developed. We have already established an efficient protocol for differentiating human iPS cells into hepatocyte-like cells. The resulting, iPS-derived hepatocyte-like cells (iPS-HLCs) show gene expression profiles highly similar to PHHs, and may be useful not only to predict interindividual differences in drug metabolism capacity and drug responses but also as a permissive host for replication of hepatitis B virus (HBV) and the HCV replicon genome (Sakurai et al., 2017; Takayama et al., 2012a; Takayama et al., 2012b; Takayama et al., 2014; Yoshida et al., 2011); however, the relationship between HCV infection levels and HCV-induced innate immune responses in iPS-HLCs has not been evaluated.

In this study, we examined the permissiveness of iPS-HLCs to HCV and HCV-induced innate immune responses in iPS-HLCs. Our results showed that iPS-HLCs support HCV replication and translation. Furthermore, innate immune responses, including induction of type III IFNs and ISGs, occurred in iPS-HLCs following inoculation with HCV, leading to viral clearance. These results indicate that iPS-HLCs are a useful *in vitro* model for the evaluation of host-HCV interactions.

2. Materials and methods

2.1. Cell culture

Huh7.5.1 cells, which are a subclone of Huh7 cells and more permissive to HCV infection than Huh7 cells, were cultured with Dulbecco's Modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. The human iPS cell line, Dotcom (JCRB1327, obtained from the JCRB Cell Bank), was maintained as previously described (Takayama et al., 2012a; Takayama et al., 2011). Cryopreserved primary human hepatocytes (PHHs) were obtained from CellzDirect (lot; Hu8072) (Durham, NC) and Xenotech (lots HC2-14 and HC10-101) (Lenexa, KS) and cultured as previously described (Takayama et al., 2012a).

2.2. Differentiation of human iPS cells to hepatocyte-like cells

iPS-HLCs were differentiated from the human iPS cell line, Dotcom, as previously described (Takayama et al., 2014). For the definitive endoderm differentiation, the human iPS cells were cultured for 4 days in L-Wnt3A-expressing cell (ATCC, CRL2647)-conditioned RPMI1640 medium, which contains 100 ng/ml activin A (R & D Systems, Minneapolis, MN), 4 mM L-glutamine, 0.2% FBS, and 1xB27 Supplement Minus Vitamin A (Life Technologies, Carlsbad, CA). For the induction of hepatoblast-like cells (HBCs), the definitive endoderm cells were cultured for 5 days in RPMI1640 medium containing 30 ng/ml bone morphogenetic protein 4 (BMP4) (R & D Systems), 20 ng/ml fibroblast growth factor-4 (FGF4) (R & D Systems), 4 mM L-glutamine, and 1xB27 Supplement Minus Vitamin A. For the differentiation to hepatocyte-like cells, the HBCs were cultured for 5 days in RPMI1640 medium containing 20 ng/ml hepatocyte growth factor (HGF) (R & D Systems), 4 mM-L-glutamine, and 1xB27 Supplement Minus Vitamin A. Finally, the cells were cultured for 11 days in Hepatocyte Culture Medium (HCM) (Lonza, Basel, Switzerland) with 20 ng/ml oncostatin M.

2.3. Propagation of HCVcc and infection

Cell culture-grown HCV (HCVcc, genotype 2a JFH-1 strain) was propagated in Huh7.5.1 cells as follows. Briefly, Huh7.5.1 cells were seeded at 4×10^5 cells in a 100 mm dish. On the following day, cells were infected with HCVcc at a multiplicity of infection (MOI) of 1–2 for 4 days. The supernatants were collected and condensed with Amicon Ultra filter units (Millipore, Billerica, MA). HCVcc titers were determined by infection of Huh7.5.1 cells with serial dilutions of the viral supernatants, followed by indirect immunofluorescence using anti-HCV NS5A antibody (9E10; kindly provided by Dr. Charles Rice, Rockefeller University, NY), and expressed as focus forming units (FFU).

2.4. Infection of iPS-HLCs with HCVcc

iPS-HLCs cultured on a 12-well plate were treated with 1 ml of 1×10^6 FFU/ml of HCVcc. This titer approximately corresponded to an MOI of 1. The medium containing HCVcc was replaced with fresh medium 6 h after addition of HCVcc. Evaluation of HCVcc genomic RNA levels and HCV protein levels was carried out at the indicated time points as described below. For evaluation of the inhibition of HCVcc infection by anti-HCV agents in iPS-HLCs, iPS-HLCs were inoculated with HCVcc at an MOI of 0.1. Following a 24-h incubation, cyclosporine A (Wako) and recombinant human IFNα2a (R & D Systems) were added to the cells at 1 μg/ml and 100 pg/ml, respectively. Total RNA was isolated from the cells 48 h after addition of anti-HCV agents, followed

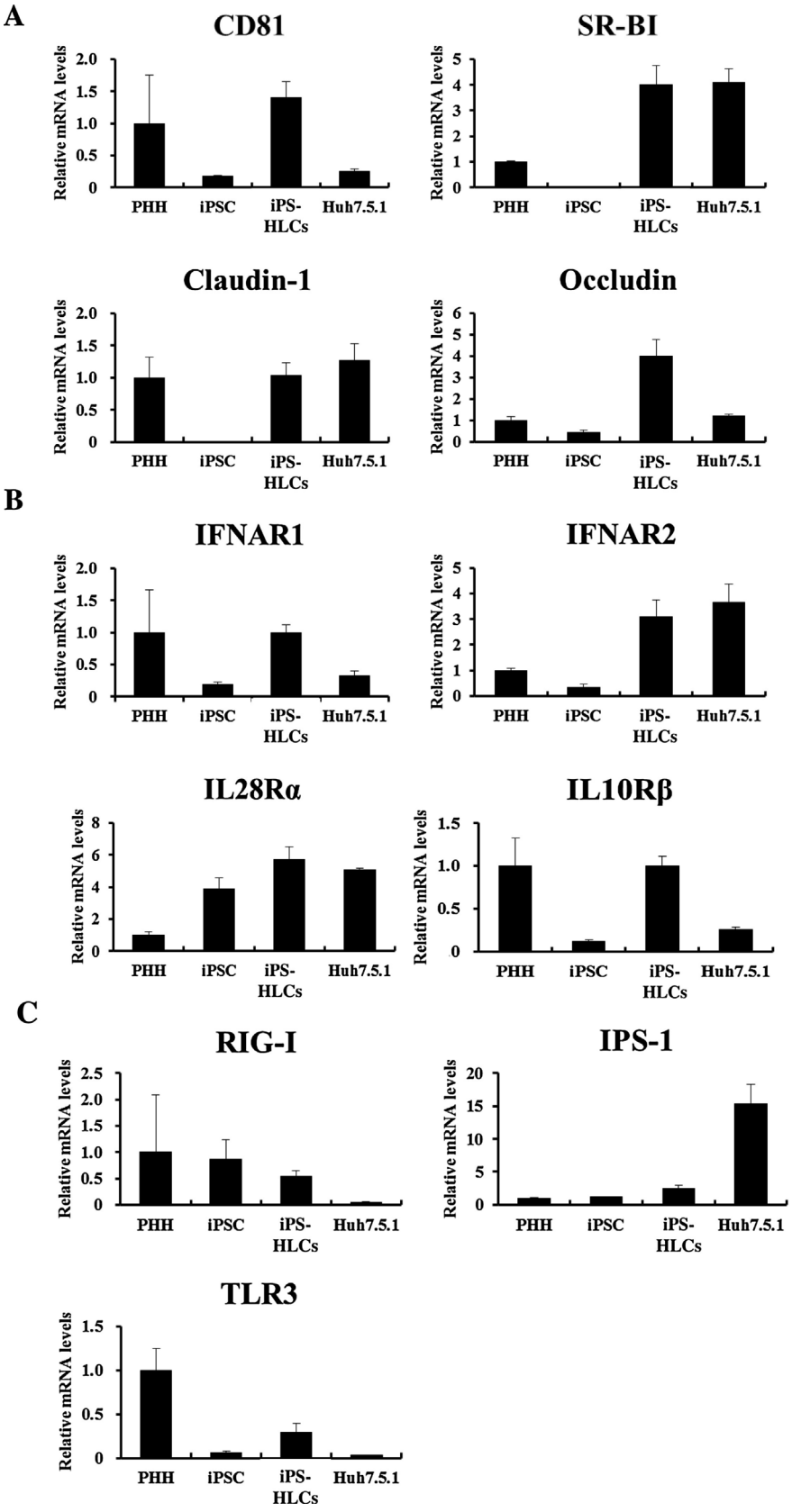

Fig. 1. Expression levels of HCV receptors and HCV-induced innate immunity-related molecules in iPS-HLCs.
The mRNA levels of (A) HCV entry receptors (CD81, SR-B1, Claudin-1, Occludin), (B) type I and III IFN receptors (IFNAR1, IFNAR2, IL28Rα, IL10Rβ), and (C) PRRs and related molecules (RIG-I, IPS-1, TLR3) in PHHs, human undifferentiated iPS cells, iPS-HLCs, and Huh7.5.1 cells were determined by real-time RT-PCR analysis. The data on PHHs was normalized to 1. The data are expressed as the mean ± S.D. (n = 3–4)

by real-time RT-PCR analysis as described below. For infection of Huh7.5.1 cells, Huh7.5.1 cells were seeded on a 24-well plate at 4×10^4 cells/well. On the following day, Huh7.5.1 cells were treated with 1 ml of 4×10^4 FFU/ml of HCVcc for 6 h. Evaluation of HCVcc genomic RNA levels and HCV protein levels was carried out in a similar way.

2.5. Real-time RT-PCR analysis

Intracellular HCV RNA genome levels and mRNA levels of IFNs, ISGs, HCV receptors, IFN receptors, and innate-immunity-related molecules were determined by real-time RT-PCR analysis as follows. Briefly, total RNA was isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. For determination of the mRNA levels of HCV receptors, IFN receptors, and innate-immunity-related molecules in PHHs, total RNA of PHHs was isolated following a 48-h culture after thawing. The total RNA of PHHs was a mixture of the RNA of PHHs from 3 different donors. cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Life Technologies). Real-time RT-PCR analysis was performed using Fast SYBR Master Mix (Applied Biosystems, Foster City, CA) or TaqMan 2xFast Universal PCR Master Mix (Applied Biosystems) and a StepOnePlus System (Applied Biosystems). The values were normalized against the input determined for the mRNA levels of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The sequences of primers and probes used in this study are described in the Supplemental Table 1. For evaluation of the type I IFN responses in iPS-HLCs, iPS-HLCs and Huh7.5.1 cells were treated with 100 pg/ml recombinant human IFNα2a (R & D Systems). Total RNA was recovered from the cells 6 h and 24 h after addition of IFNα2a, followed by real-time RT-PCR analysis as described above.

2.6. Transfection with HCV subgenomic replicon RNA

iPS-HLCs and Huh7.5.1 cells were transfected with 1 μg of total RNA extracted from Huh7.5.1 1b Feo cells (Yokota et al., 2003) and Huh7.5.1 cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The medium was replaced with fresh medium 4 h after transfection. Total RNA was recovered from the cells 6 and 24 h after transfection, followed by real-time RT-PCR analysis as described above.

2.7. Immunofluorescence staining of HCV NS5A protein

Following infection with HCVcc, cells were washed with PBS and fixed with ice-cold 4% paraformaldehyde (Wako) for 10 min. After blocking with phosphate-buffered saline containing 2% bovine serum albumin (BSA) (Sigma, St. Louis, MO) and 0.3% Triton X-100 (Sigma), the cells were incubated with mouse anti-HCV NS5A antibody (9E10) at 4 °C for 16 h, followed by incubation with goat anti-mouse IgG that was labeled with Alexa Fluor 488 (Life Technologies) at room temperature for 1 h. Immunofluorescence was observed under a fluorescence microscope.

3. Results

3.1. Expression levels of cellular factors involved in HCV infection in iPS-HLCs

In order to examine whether iPS-HLCs expressed cellular factors involved in HCV infection, including HCV receptors, IFN receptors, and pattern recognition receptors (PRRs), real-time RT-PCR analysis was performed. We previously demonstrated that iPS-HLCs efficiently expressed hepatocyte-specific markers, including albumin, hepatocyte nuclear factor 4 alpha (HNF4α) and asialoglycoprotein receptor-1 (ASGR1) at levels comparable to PHHs (Sakurai et al., 2017; Takayama et al., 2012a; Takayama et al., 2012b; Takayama et al., 2014). While undifferentiated human iPS cells expressed negligible or much lower levels of the HCV receptors, including CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin, iPS-HLCs expressed comparable or higher levels of the HCV receptors than PHHs (Fig. 1A). mRNA levels of SR-BI and claudin-1 in iPS-HLCs were comparable to those in Huh7.5.1 cells; on the other hand, significantly higher mRNA levels of CD81 and occludin were found in iPS-HLCs than in Huh7.5.1 cells. These results indicate that iPS-HLCs expressed the HCV receptors at levels sufficient for HCV infection.

mRNA levels of type I IFN receptor subunits, IFN-α/β receptor-1 (IFNAR1) and −2 (IFNAR2), in iPS-HLCs were comparable or higher than those in PHHs and Huh7.5.1 cells (Fig. 1B). iPS-HLCs also expressed almost identical or higher levels of type III IFN receptor subunits, interleukin (IL)-28 receptor-α (IL28Rα) and IL10 receptor-β (IL10Rβ), compared with PHHs and Huh7.5.1 cells. The expression level of retinoic acid-inducible gene-I (RIG-I), which is a pattern recognition receptor recognizing the HCV RNA genome (Sumpter et al., 2005), in iPS-HLCs was similar to that in PHHs and higher than that in Huh7.5.1 cells (Fig. 1C). mRNA levels of IFN-β promoter stimulator-1 (IPS-1) in iPS-HLCs were slightly higher than those in PHHs but significantly lower than those in Huh7.5.1 cells. On the other hand, another pattern recognition receptor recognizing the HCV RNA genome, TLR3 (Li et al., 2012), was expressed at a lower level in iPS-HLCs than PHHs, although the TLR3 mRNA level in iPS-HLCs was higher than that in Huh7.5.1 cells. These data suggest that iPS-HLCs are responsive to type I and III IFNs and mediate innate immune responses to the HCV RNA genome.

3.2. Type 1 IFN responses in iPS-iHLCs

In order to examine whether iPS-HLCs were responsive to type I IFNs, we incubated iPS-HLCs with recombinant IFNα2a, followed by real-time RT-PCR analysis of ISG expression. At 6 h and 24 h post-treatment, mRNA levels of the ISGs, including ISG15, ISG56, and MxA, were significantly up-regulated in both iPS-iHLCs and Huh7.5.1 cells (Fig. 2A). ISG15, ISG56, and MxA have been shown to suppress HCV infection (Itsui et al., 2006; Jones et al., 2010; Raychoudhuri et al., 2011). In the present study, more than 100-fold increases in ISG expression were found 6 h after IFNα2a treatment in iPS-HLCs. The ISG mRNA levels were slightly reduced 24 h after addition of IFNα2a, compared with those at the peak. The mRNA levels of these ISGs were almost comparable in iPS-HLCs and Huh7.5.1 cells. These data indicate that iPS-HLCs are able to respond to type I IFNs.

3.3. Innate immune responses following introduction of HCV subgenomic replicon RNA in iPS-iHLCs

In order to examine whether iPS-HCLs exhibited innate immune responses against the HCV genome, total RNA isolated from Huh7.5.1 1b Feo cells (Yokota et al., 2003), which expressed the HCV subgenomic replicon RNA, was transfected into iPS-HLCs. Both the HCV genomic RNA and HCV subgenomic replicon RNA possess the 5′-triphosphate group at the 5′ end and poly-U/UC ribonucleotides in the 3′ untranslated region, which are crucial for HCV genome-induced innate immunity (Saito et al., 2008; Schnell et al., 2012). The HCV subgenomic replicon RNA rapidly induced up-regulation of the mRNA levels of type I and III IFNs, and ISGs in iPS-HLCs (Fig. 3). Total RNA isolated from Huh7.5.1 cells did not significantly induce expression of either type of IFNs or of ISGs. Huh7.5.1 cells exhibited negligible levels of innate immune responses following transfection with HCV subgenomic replicon RNA, probably due to dysfunction of RIG-I and extremely low expression of TLR3 in Huh7.5.1 cells. The levels of up-regulation of the type I IFNs were lower than those of the type III IFNs. Transfection with synthetic double-stranded RNA polyI:C also induced efficient expression of ISGs and type I IFNs in iPS-HLCs (Supplementary Fig. 1). These results indicate that iPS-HLCs are responsive to HCV genomic RNA, and can induce the expression of type I and III IFNs *via* innate immunity.

3.4. HCV infection levels and innate immune responses following inoculation with HCVcc in iPS-HLCs

In order to examine HCV infection profiles and HCV-induced innate

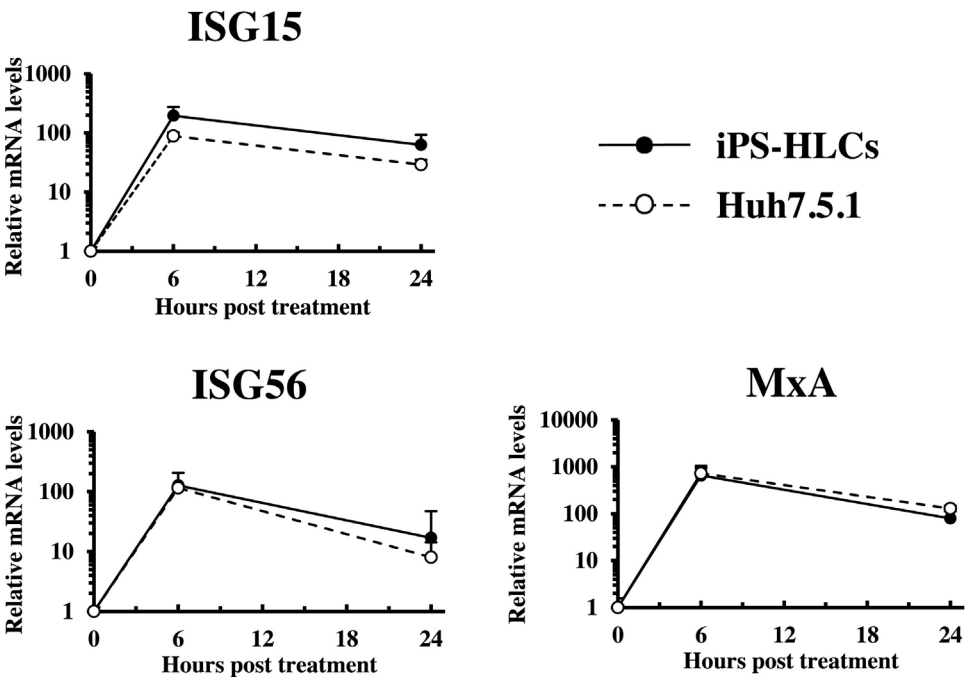

Fig. 2. Expression levels of ISGs in iPS-HLCs after treatment of IFNα.
iPS-HLCs and Huh7.5.1 cells were treated with 100 pg/ml recombinant IFNα for 6 or 24 h. mRNA levels of ISGs (ISG15, ISG56, and MxA) following IFNα treatment were determined by real-time RT-PCR analysis. The data before IFNα treatment on each cells was normalized to 1. The data are expressed as the mean ± S.D. (n = 4).

immune responses in iPS-HLCs, the HCV genomic RNA levels in iPS-HLCs were determined after inoculation with HCVcc. The HCV genome was detected on day 1, and increased by approximately 5-fold on day 2 (Fig. 4A). HCV genome levels significantly declined on day 3, then remained low but constant up to day 6. iPS-HLCs and Huh7.5.1 cells exhibited similar levels of HCV genome on days 1 and 2; however, the level of HCV genome in Huh7.5.1 cells gradually increased up to day 6. Approximately 100-fold increases in the HCV genome copy numbers were found on day 6 in Huh7.5.1 cells, compared with those on day 1. Expression of NS5A protein was also found in iPS-HLCs on day 2, followed by a reduction in the NS5A protein levels on day 4 (Fig. 4B). NS5A-positive iPS-HLCs were almost entirely absent on day 6. On the other hand, NS5A-positive Huh7.5.1 cells gradually increased after inoculation. We also examined the HCVcc production in the culture supernatants of iPS-HLCs following inoculation. Virus titers in the culture supernatants on day 4 were below the level of detection (data not shown). In order to examine whether the inhibitory effects of anti-HCV agents on HCV infection can be evaluated in iPS-HLCs, cyclosporine-A (CsA) or recombinant IFN-α was added to iPS-HLCs after inoculation with HCVcc. The HCV genome copy numbers were reduced more than 10-fold by treatment with CsA and recombinant IFN-α (Fig. 4C), indicating that iPS-HLCs are available for the evaluation of anti-HCV agents.

While the mRNA levels of type I IFNs were not significantly up-regulated in iPS-HLCs after inoculation with HCVcc, the mRNA levels of the type III IFNs and the ISGs were significantly increased on day 2, and declined to the background levels after day 4 (Fig. 5). In particular, the IFNλ1 and IFNλ2 mRNA levels were elevated by more than 100-fold on day 2. The mRNA expression profiles of the type III IFNs and the ISGs were similar. Huh7.5.1 cells expressed undetectable or background levels of type III IFN and ISG mRNAs following inoculation with HCVcc. MxA protein levels were also elevated on day 3 following inoculation with HCVcc (Supplemental Fig. 2). These data suggest that after HCVcc was inoculated into iPS-HLCs, the HCVcc genome significantly replicated in iPS-HLCs. Subsequently, the HCVcc genome should be recognized by PRRs, including RIG-I and TLR3, resulting in up-regulation of ISGs and elimination of the HCVcc genome.

4. Discussion

The aim of this study was to evaluate the relationship between HCV infection profiles and HCV-induced innate immune responses in iPS-HLCs. The relationship is highly important, since the innate immune responses are involved in spontaneous HCV clearance following acute infection. In this study, type III IFNs were more highly up-regulated in iPS-HLCs, compared with type I IFNs, following inoculation with HCVcc. Efficient induction of type III IFN expression in PHHs and non-human primate livers after HCVcc inoculation has been reported in several studies (Okamoto et al., 2014; Park et al., 2012; Thomas et al., 2012; Yang et al., 2011). The RIG-I-IPS-1 signal and TLR3-TRIF play important roles in the induction of type III IFNs in hepatocytes (Okamoto et al., 2014; Yoshio et al., 2013). Both type I and III IFNs activate the JAK/STAT pathway, inducing activation of ISGs. Although recombinant type I IFNs possess efficient anti-HCV activities and have been used as an anti-HCV drug, type III IFNs also exhibit efficient anti-HCV activities (Nakagawa et al., 2013; Park et al., 2012). In this study, the mRNA levels of type III IFNs were more than 30-fold elevated in iPS-HLCs on day 2 following inoculation with HCVcc, while type I IFNs mRNA levels did not appear to be increased. These data suggest that type III IFNs mainly mediated anti-HCV activities in iPS-HLCs following inoculation.

Although previous studies have reported widely disparate levels of induction of type I IFNs after inoculation with HCVcc in PHHs and human embryonic stem (ES) cell-derived and iPS cell-derived hepatocyte-like cells (Carpentier et al., 2014; Helle et al., 2013; Yan et al., 2017; Yang et al., 2011), they generally reported much lower type I IFN expression levels than type III IFN expression levels after HCVcc infection. In the present study, type I IFNs were hardly induced in iPS-HLCs after inoculation with HCVcc; however, transfection with total RNA recovered from HCV replicon subgenomic RNA-expressing cells resulted in significant elevation of type I IFN mRNA levels. These data indicate that iPS-HLCs are able to produce type I IFNs *via* innate immune responses.

Following inoculation with HCVcc, efficient expression of ISGs, including MxA and ISG56, was found in iPS-HLCs. Previous studies demonstrated that MxA, ISG15, and ISG56 are involved in the suppression of HCV infection (Itsui et al., 2011; Janes et al., 2010; Raychoudhuri et al., 2011). Induction of ISGs is considered to contribute to a

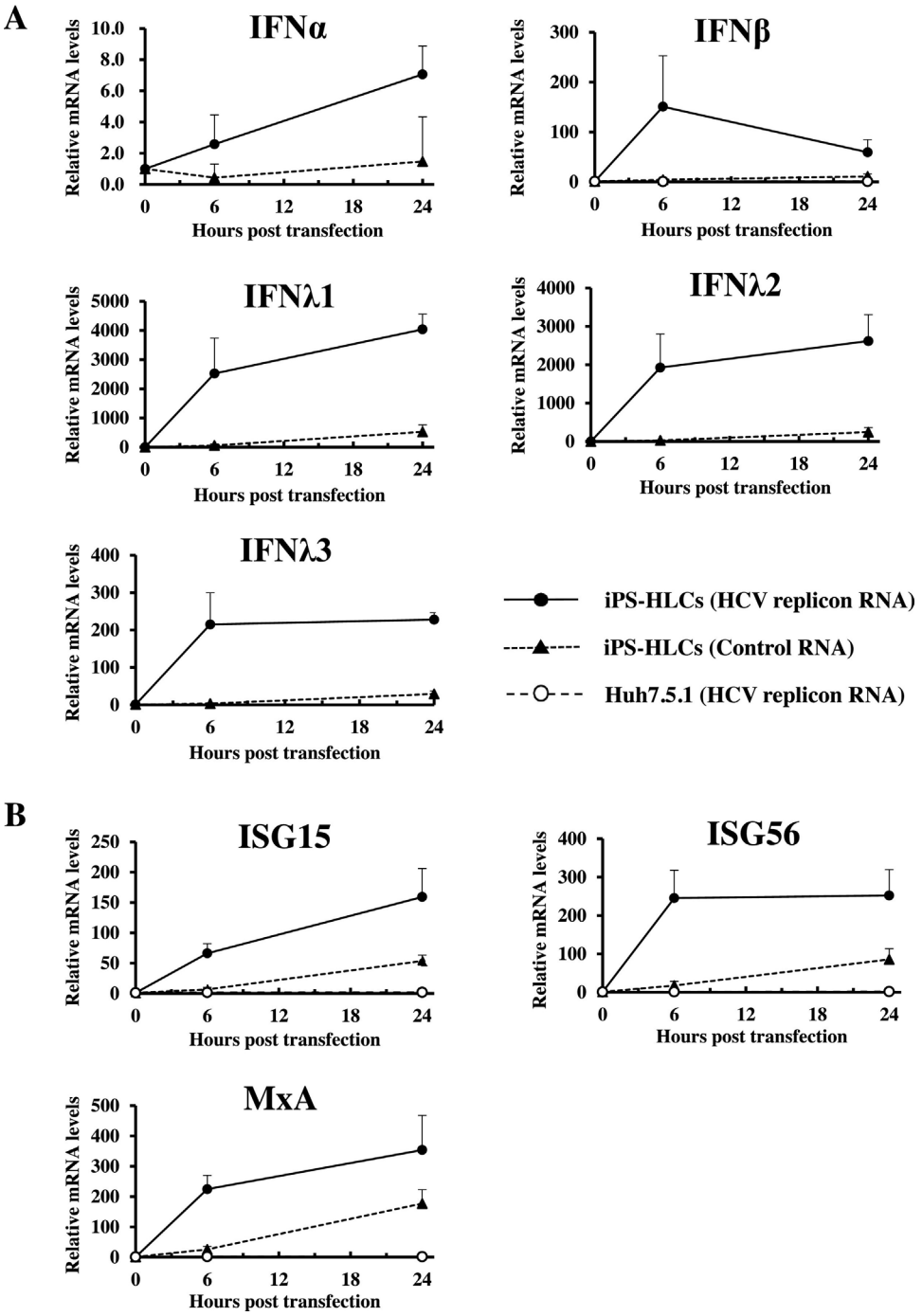

Fig. 3. Expression levels of type I and III IFNs and ISGs in iPS-HLCs after transfection with HCV subgenomic replicon-expressing cell-derived RNA.
iPS-HLCs and Huh7.5.1 cells were transfected with 10 μg/ml of total RNA extracted from Huh7.5.1 1b Feo cells (HCV subgenomic replicon RNA) or from Huh7.5.1 cells (Control RNA) for 6 h. mRNA levels of (A) type I and III IFNs and (B) ISGs were determined by real-time RT-PCR analysis. The data before transfection on each cells was normalized to 1. The data are expressed as the mean ± S.D. (n = 3–4). mRNA levels of IFNα and IFNλ1 − 3 in Huh7.5.1 cells were undetectable.

reduction in the HCV genome levels after day 3 following HCVcc inoculation in iPS-HLCs. Both type I and III IFNs induce ISG expression *via* the JAK/STAT signaling pathway (Dickensheets et al., 2013). In order to examine the involvement of the JAK/STAT signaling pathway in the reduction in the HCV genome levels after day 3, iPS-HLCs were treated with a JAK inhibitor. Treatment with a JAK inhibitor failed to significantly restore the HCV genome levels (data not shown). Previous studies reported that IRFs, which are activated by RIG-I ligands, directly induce ISG expression in an IFN-independent manner (Grandvaux et al., 2002; Lazear et al., 2013). The HCV genome would induce ISG expression in a JAK/STAT-independent pathway in the presence of a JAK inhibitor, leading to a reduction in the HCV genome copy numbers.

Following acute infection with HCV in patients, HCV persistently infects the hepatocytes, leading to chronic hepatitis. In this study, HCV genome levels were examined for 6 days, although most of the previous studies exhibited the HCV infection profiles in PHHs and stem cell-derived hepatocytes only up to 72 h after inoculation (Helle et al., 2013; Wu et al., 2012; Zhou et al., 2014). Copy numbers of HCV genome in iPS-HLCs significantly increased on day 2, and then reduced on day3 following inoculation; however, the HCV genome levels remained low but constant up to day 6. Although iPS-HLCs were cultured for 6 days following infection in this study, we confirmed that iPS-HLCs can be cultured for more than 7 days while maintaining the hepatocyte-specific gene expression profile (data not shown). Our results demonstrated that the effects of relatively long-term infection with HCV on hepatocyte function can be evaluated in iPS-HLCs.

Another advantage of iPS-HLCs is that they can be used to evaluated drug metabolism, including induction of drug metabolizing enzymes.

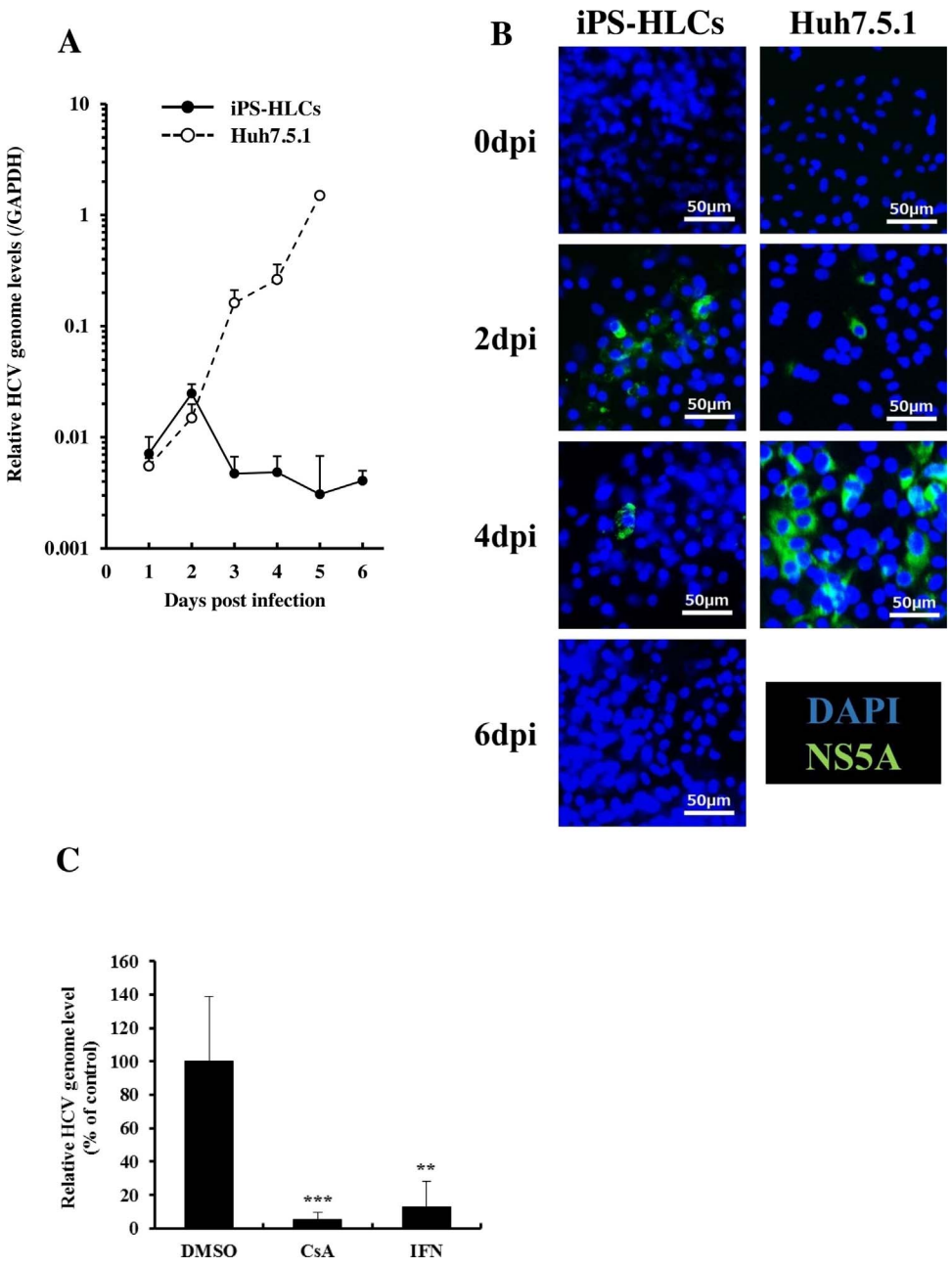

Fig. 4. Infection profiles of HCVcc in iPS-HLCs. (A-C) iPS-HLCs and Huh7.5.1 cells were inoculated with HCVcc at an MOI of approximately 1. (A) Total RNA was daily extracted from the cells following inoculation with HCVcc. HCV genomic RNA levels were determined by real-time RT-PCR analysis. (B) Cells were immunostained with anti-HCV NS5A antibody (green) every other day postinfection. Nuclei were counterstained with DAPI (blue). (C) iPS-HLCs were infected with HCVcc for 6 h at an MOI of approximately 0.1. At 24 h postinfection, cells were treated with DMSO, CsA (1 μg/ml), or IFNα (100 pg/ml) for 48 h. After treatment, intracellular HCV genomic RNA levels were determined by real-time RT-PCR analysis. HCV genomic RNA levels in mock-infected cells were below the level of detection. The data are expressed as the mean ± S.D. (n = 3–5).

Several anti-HCV agents affect the expression and/or activities of cytochrome P450 (CYP), which is the most important enzyme in drug metabolism, and are metabolized by CYP (Burger et al., 2013; Khalilieh et al., 2015). CYP activities largely affect the anti-HCV activities of anti-HCV agents. The activity levels of CYP in Huh7 cells are much lower than those in PHHs (Choi et al., 2009). iPS-HLCs thus make it possible to evaluate the hepatotoxicities and anti-HCV activities of candidate compounds and their metabolites.

In summary, we demonstrated that the HCV genome can replicate in iPS-HLCs, leading to induction of innate immunity and clearance of the HCV genome. These data suggest that iPS-HLCs are a promising *in vitro* HCV infection model for evaluation of the interaction between HCV infection and cellular responses, including innate immunity. Recently, genome editing technology in various types of stem cells has been dramatically improved (Hockemeyer and Jaenisch, 2016; Waddington et al., 2016). Genome editing of human iPS cells would further improve the availability and usefulness of iPS-HLCs as an *in vitro* HCV infection model.

Conflict of interest

The authors declare no conflict of interest.

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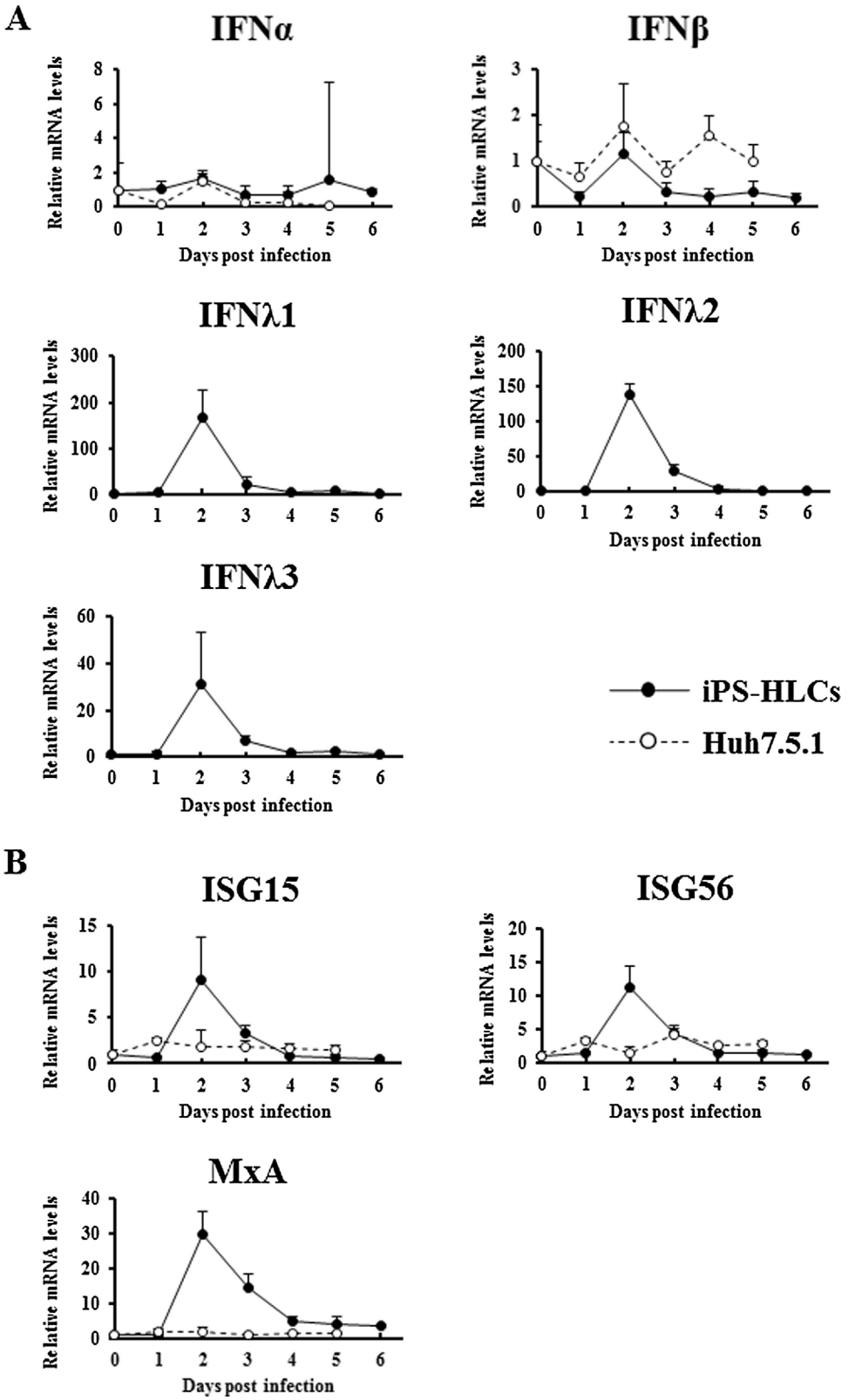

Fig. 5. HCV-induced innate immune responses in iPS-HLCs.
iPS-HLCs were inoculated with HCVcc as described above. mRNA levels of (A) type I and III IFNs and (B) ISGs were determined by real-time RT-PCR analysis. The data before inoculation with HCVcc was normalized to 1. The data are expressed as the mean ± S.D. (n = 3). mRNA levels of IFNλ1-3 in Huh7.5.1 cells were undetectable.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2017.09.004.

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