Modulation of alpha interferon anti-hepatitis C virus activity by ISG15

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ISG15 has recently been reported to possess antiviral properties against viruses, both *in vivo* and *in vitro*. Knock-down of ISG15 gene expression by small interfering RNA followed by alpha interferon (IFN-α) treatment in Huh-7 cells resulted in an increased phenotypic sensitivity to IFN-α, as determined by measuring hepatitis C virus (HCV) RNA replication inhibition in stably transfected HCV replicon cells and in cells infected with genotype 1a HCVcc (infectious HCV). This IFN-α-specific effect, which was not observed with IFN-γ, correlated with an increase in expression of the IFN-α-inducible genes IFI6, IFITM3, OAS1 and MX1, whereas the expression of the non-IFN-α-inducible genes PTBP-1 and JAK1 remained unchanged. It has previously been reported that, unlike ISG15 knock-down, increased sensitivity to IFN-α after knock-down of USP18 occurs through the prolonged phosphorylation of STAT-1. Combination knock-down of ISG15 and USP18 resulted in a moderate increase in IFN-α-inducible gene expression compared with single ISG15 or USP18 knock-down. Furthermore, the phenotype of increased gene expression after ISG15 knock-down and IFN-α treatment was also observed in non-hepatic cell lines A549 and HeLa. Taken together, these results reveal a novel function for ISG15 in the regulation of the IFN-α pathway and its antiviral effect.

INTRODUCTION

Hepatitis C virus (HCV), a positive-sense single-stranded RNA virus with a genome of 9.6 kb, belongs to the family *Flaviviridae*. There are approximately 170 million HCV-infected individuals worldwide. The current standard of care for chronic hepatitis C patients includes the use of pegylated alpha interferon (Peg-IFN-α) and ribavirin. However, the sustained virological response (SVR) for genotype (GT)-1-infected patients treated with IFN-α and ribavirin is approximately 50 % (Manns *et al.*, 2007) and the mechanism by which IFN-α inhibits HCV replication is still unclear.

Type I IFN, including IFN-α, plays an important role in innate immunity against virus infections (Randall & Goodbourn, 2008). The canonical pathway for IFN-α signalling is through the phosphorylation of STAT-1 and STAT-2 by Jak1 and Tyk2 kinases, heterodimerization of phosphorylated STAT-1 and STAT-2, and association with IFN-regulatory factor (IRF)-9 to form IFN-stimulated gene

factor (ISGF)-3. This complex binds to the IFN-stimulated response element (ISRE) and results in transcriptional upregulation of many genes, including genes with antiviral properties, such as protein kinase R (PKR), 2′,5′-oligoadenylate synthetase (OAS1) and MX protein. Also upregulated are a subset of genes whose biological functions are still not clearly defined, such as IFIT-5, IFI6 (Itsui *et al.*, 2006; Zhu *et al.*, 2003), IFITM3 (Zhu & Liu, 2003) and ISG15 (see below).

ISG15, an IFN-α-inducible protein of 15 kDa, is a ubiquitin-like modifier that is upregulated rapidly after lipopolysaccharide or IFN induction (Haas *et al.*, 1987; Manthey *et al.*, 1998). This protein can be secreted in human monocytes, lymphocytes, A549 and NIH OVCAR-3 cells (D'Cunha *et al.*, 1996b). Secreted ISG15 has been shown to result in the release of IFN-γ from lymphocytes (Recht *et al.*, 1991), as well as to stimulate the proliferation and activation of natural killer (NK) cell-derived lympho-kine-activated killer cells (D'Cunha *et al.*, 1996a). *In vivo*, however, the lack of ISG15 does not affect NK-cell proliferation or activity (Osiak *et al.*, 2005).

ISG15 contains two ubiquitin-like domains. The C-terminal ubiquitin-like domain contains the conjugating domain sequence LRLRGG, with the two glycines being

required for conjugation. Conjugation of ISG15 to cellular proteins occurs with the help of ubiquitin-like enzymes UBE1L, UbcH8 and Herc5 (Wong *et al.*, 2006; Yuan & Krug, 2001; Zhao *et al.*, 2004), whilst USP18/UBP43 is the ubiquitin-specific protease that unlinks ISG15 from its interacting protein (Malakhov *et al.*, 2002). *In vitro* experiments have demonstrated that ISG15 can be conjugated to a wide variety of cellular proteins, including STAT-1, RIG-I and transcriptional factors (Zhao *et al.*, 2005). Although the functional significance of this conjugation is still unclear, it has been postulated that the conjugation prevents proteosomal degradation of cellular proteins (Liu *et al.*, 2003).

More recently, ISG15 has been demonstrated to possess antiviral activity against selected DNA and RNA viruses. $ISG15^{-/-}$ mice are more susceptible to influenza A and B viruses, herpes simplex virus 1, Sindbis virus and gammaherpesvirus 68 infection (Lenschow *et al.*, 2007). In addition, $ISG15^{-/-}$ mice are more susceptible to vaccinia viruses lacking the viral E3 protein (Guerra *et al.*, 2008). Also, overexpression of ISG15 in IFN-$\alpha/\beta R^{-/-}$ mice attenuates Sindbis virus infection, and this occurs through the ISG15 LRLRGG motif (Lenschow *et al.*, 2005). Another group, however, reported that ISG15 has modest antiviral activity against Sindbis virus *in vitro*, but has no antiviral activity against Sindbis virus *in vivo* (Zhang *et al.*, 2007). The conflicting results were attributed to the use of different strains of Sindbis virus and different routes of virus inoculation into mice (Zhang *et al.*, 2007). Interestingly, $ISG15^{-/-}$ mice are not susceptible to vesicular stomatitis virus or lymphocytic choriomeningitis virus infection (Osiak *et al.*, 2005), which could indicate a selectivity of the antiviral property of ISG15.

In vitro, ISG15 has been reported to possess antiviral activity against human immunodeficiency virus (HIV)-1, Ebola virus and influenza A and B viruses. In the case of HIV-1, ISG15 blocks ubiquitination of HIV-1 Gag to Tsg101, which subsequently blocks HIV-1 virion release (Okumura *et al.*, 2006). Also, ISG15 conjugates to Nedd4 ubiquitin ligase and prevents Ebola virus-like particle release from cells (Malakhova & Zhang, 2008; Okumura *et al.*, 2008). Similarly, influenza B virus NS1 protein binds to ISG15 and prevents ISGylation of cellular proteins after virus infection (Yuan & Krug, 2001), and IFN-induced ISG15 conjugation is required for inhibiting influenza A virus replication (Hsiang *et al.*, 2009).

We set out to investigate whether ISG15 could have direct anti-HCV activity or whether it could have an effect through modulation of the antiviral properties of IFN-α. Knock-down of ISG15 gene expression by small interfering RNA (siRNA) followed by IFN-α treatment results in an increased phenotypic sensitivity of Huh-7 cells to IFN-α, as determined by measuring HCV RNA inhibition in stably transfected HCV replicon cells and in cells infected with GT-1a HCVcc (infectious HCV). This IFN-α effect correlated with an increase in expression of the IFN-α-

inducible genes IFI6, IFITM3, OAS1 and MX1, and was also observed in non-Huh-7 cell lines. Taken together, these results suggest a novel function for ISG15 in the regulation of the IFN-α pathway and its antiviral effect.

METHODS

Cell culture. 2209-23 is a Huh-7 cell line stably transfected with a GT-1b HCV replicon (Klumpp *et al.*, 2006) and was used in siRNA gene knock-down and IFN-α EC$_{50}$ determination experiments. Rof-0c is a Huh-7 cell line cured of the GT-1b replicon by using an HCV polymerase inhibitor. HeLa (a human immortal cell line derived from cervical cancer cells) and A549 (carcinomic human alveolar basal epithelial cells) cell lines were obtained from the ATCC. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with Glutamax and 100 mg sodium pyruvate ml^{-1}, 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin/streptomycin. A549 cells were cultured in F12 medium supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin. All reagents were from Invitrogen.

siRNA gene knock-down and quantitative (q)RT-PCR analysis. On-TARGET select plus siRNAs targeted against ISG15, STAT-2 and PTBP-1 and a control siRNA (siGenome RISC-free siRNA; siCtrl) were purchased from Dharmacon, whilst siRNAs targeted against USP18 were purchased from Ambion, Inc. Cells were transfected with 10 nM (for single siRNA transfection) or 20 nM (for combination transfections) siRNA for 4 or 18 h using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, followed by treatment with 5 IU IFN-α ml^{-1} (IFN-α2a or Roferon; Hoffmann-LaRoche, Inc.) or 100 IU IFN-α ml^{-1} for Rof-0c cells. Three days post-treatment, total cellular RNA was isolated by using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was carried out with 2 µl total cellular RNA in a 10 µl reaction by using Taqman RT reagents (Applied Biosystems).

HCV replicon RNA levels were quantified as described previously (Klumpp *et al.*, 2006).

To quantify the levels of IFN-α-induced genes, primers and probes (FAM-labelled) were designed against IFITM3, IFI6, ISG15, OAS1, MX1, PTBP-1 and JAK1 (Supplementary Table S1, available in JGV Online). Primers and probe for USP18 were purchased from Applied Biosystems. Ribosomal protein S9 (RPS9) was used as the endogenous control to normalize gene-expression levels.

Changes in gene expression in cells transfected with siRNA and treated with IFN-α were expressed as a ratio to the levels observed in siRNA control-transfected and untreated cells, set arbitrarily as 1. Graphs were plotted by using GraphPad Prism 4 software.

Compounds. Recombinant IFN-α (IFN-α2a; Roferon) was obtained from F. Hoffmann-La Roche; recombinant human IFN-γ was purchased from R&D Systems.

Compound EC$_{50}$ determinations after ISG15 knock-down in HCV replicon cells. IFN-α or IFN-γ EC$_{50}$ values were determined after siRNA gene knock-down in replicon cells as follows: 2209-23 cells were seeded in six-well plates at a density of 200 000 cells per well and transfected with 10 nM control or relevant siRNA by using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. For STAT-2 knock-down, 2209-23 cells seeded in six-well plates at a density of 200 000 cells per well were subjected to two siRNA transfections, 3 days apart. Prior to the second siRNA transfection, cells were split and reseeded at a density of 200 000 cells in a new six-well plate. Eighteen hours post-siRNA transfection,

cells were trypsinized and seeded into 96-well plates, and EC_{50} values were determined as described previously (Klumpp *et al.*, 2006).

Compound EC_{50} determinations after ISG15 knock-down in HCVcc-infected cells. IFN-α EC_{50} values were determined after siRNA gene knock-down in Rof-0c cells infected with GT-1a H77-S infectious HCV (HCVcc). The virus stocks were generated by transfecting the full-length H77-S RNA genome into Rof-0c cells as described by Yi *et al.* (2006). Rof-0c cells were transfected with siRNA as described above and then, 18 h post-transfection, plated at 10 000 cells per well into a 96-well plate. Four hours post-plating, the cells were treated with 3-fold serial dilutions of IFN-α. Twenty-four hours post-plating, the medium was removed and 90 μl H77-S virus stock was added per well, as well as IFN-α (in 3-fold dilutions) to maintain the IFN-α treatment. Three days post-infection, total cellular RNA was isolated by using an RNeasy kit (Qiagen) according to the manufacturer's instructions. The EC_{50} values for IFN-α were determined as described previously (Klumpp *et al.*, 2006).

Immunoblot. Three days post-siRNA transfection and IFN-α treatment, cells were lysed in SDS-lysis buffer (Invitrogen). Samples were passed through a fine-gauge needle followed by heating at 90 °C to denature proteins. A 20 μl aliquot of each sample was resolved on a denaturing SDS/10 % (v/v) polyacrylamide gel and probed with anti-ISG15, anti-STAT-1 and phosphorylated STAT-1 antibodies (Cell Signaling Technology), anti-IFITM3 antibody (Brem *et al.*, 2003), anti-HCV NS5A antibody (a gift of Dr Ralf Bartenschlager, Abteilung Molekulare Virologie, Universität Heidelberg, Germany) or anti-actin antibody (Amersham Biosciences) as an endogenous control. Protein signal-intensity levels were measured by using enhanced chemiluminescence on a Typhoon scanner (Amersham Biosciences).

Statistical methods. The $\log_{10}$ values of EC_{50} or $\log_2$ values of qRT-PCR data for all groups were compared by using a two-way ANOVA model including terms of group and experiment, followed by post-hoc comparisons using a Tukey test.

RESULTS

Effective knock-down of ISG15 mRNA and protein level in Huh-7 cells

ISG15 gene knock-down in Huh-7 cells was performed by using four ISG15 siRNA oligos (#1, #2, #3 and #4). The most efficient knock-down was observed with siRNA #2, with a knock-down of 87 % compared with the siRNA control (siCtrl) (Fig. 1a), whereas siRNAs #1 and #4 achieved knock-down of approximately 42 and 70 %, respectively. Knock-down using siRNA #3 (20 %) was not effective; it therefore served as an inactive ISG15 siRNA control.

Also, transfection of the siRNA control (RISC-free/siCtrl) or PTBP-1 siRNA did not affect the gene-expression levels of ISG15 compared with untransfected cells, indicating that siRNAs do not affect ISG15 gene-expression levels. PTBP-1 siRNA was functional in reducing the levels of PTBP-1 gene expression by 61 % (data not shown).

The effect of ISG15 siRNA after IFN-α treatment was evaluated. Cells were first transfected with ISG15 siRNA #2 for 4 h prior to treatment with 5 IU IFN-α ml^{-1}, as this would allow suppression of ISG15 gene expression that is
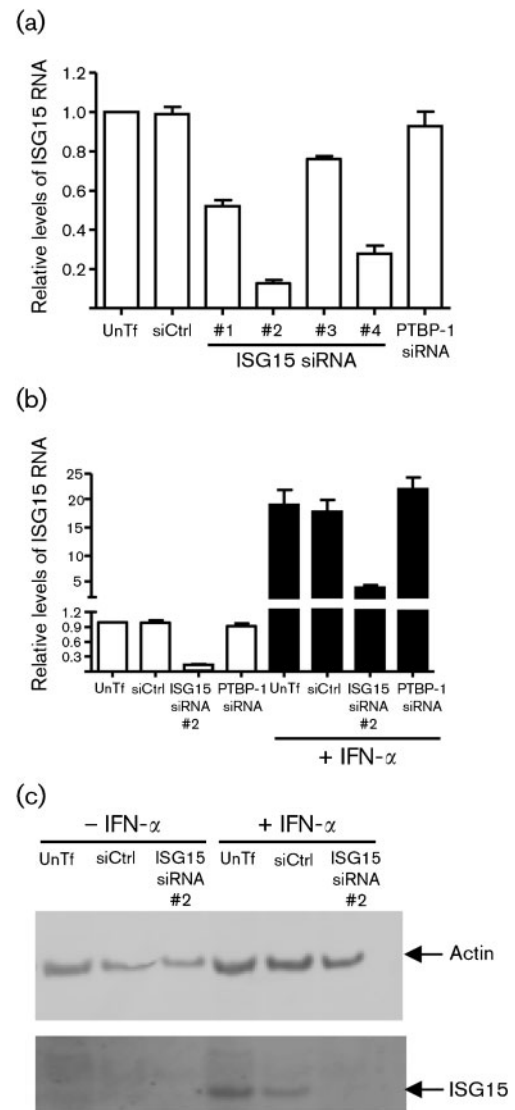

Fig. 1. Efficient knock-down of ISG15 by siRNA. Cells were transfected with siRNAs for 4 h, followed by replacement of medium with or without 5 IU IFN-α ml^{-1}. Three days post-transfection, cells were harvested for (a, b) qRT-PCR of ISG15 gene-expression levels and (c) Western blot analysis of ISG15 protein levels. Graphs represent means + SD from at least three independent experiments. UnTf, Untransfected cells.

induced by IFN-α. As shown in Fig. 1(b), low-dose IFN-α treatment resulted in an approximately 20-fold increase in the levels of ISG15 mRNA compared with untreated cells, whereas transfection with ISG15 siRNA #2 achieved an 80 % knock-down of ISG15 mRNA levels (compared with siRNA control-transfected and IFN-α-treated cells) (Fig. 1b). As a control, PTBP-1 siRNA transfection did not have an effect on ISG15 gene-expression levels after IFN-α treatment (Fig. 1b). As siRNA control transfection had no effect on gene-expression levels, all subsequent graphs were plotted with the siRNA control set arbitrarily at 1.

Correlating with the mRNA data, ISG15 protein levels were increased similarly upon treatment with low-dose IFN-α and subsequently knocked down completely upon treatment with ISG15 siRNA (Fig. 1c). Using 5 IU IFN-α ml^{-1}, we were not able to detect ISGylated proteins, which were, however, observed when cells were treated with 4000 IU IFN-α ml^{-1} (data not shown).

Knock-down of ISG15 gene expression results in an IFN-α-sensitive phenotype for the HCV replicon

ISG15 has been reported to conjugate to cellular proteins involved in the type I IFN-response pathway, including STAT-1 (Malakhov et al., 2003). To study whether ISG15 is involved directly in the mechanism by which IFN-α inhibits HCV replicon replication, we examined the sensitivity of the HCV replicon to IFN-α after knocking down ISG15. As controls, USP18 and STAT-2 were also knocked down (data not shown). As shown in Fig. 2, knock-down of USP18 resulted in an increase in sensitivity to IFN-α (EC$_{50}$=0.06±0.01 IU ml^{-1}) as reported previously (Randall et al., 2006), whereas knock-down of STAT-2 resulted in a decrease in sensitivity to IFN-α (EC$_{50}$=1.08±0.16 IU ml^{-1}).

Knock-down of ISG15 resulted in a 4-fold increase in sensitivity of the HCV replicon to IFN-α (EC$_{50}$=0.06± 0.01 IU ml^{-1}) compared with siRNA control-transfected cells (EC$_{50}$=0.24±0.05 IU ml^{-1}), a result similar to the effect observed after USP18 knock-down (Fig. 2). This increased sensitivity, as determined by EC$_{50}$ values, after ISG15 knock-down was found to be statistically significant ($P<0.01$).
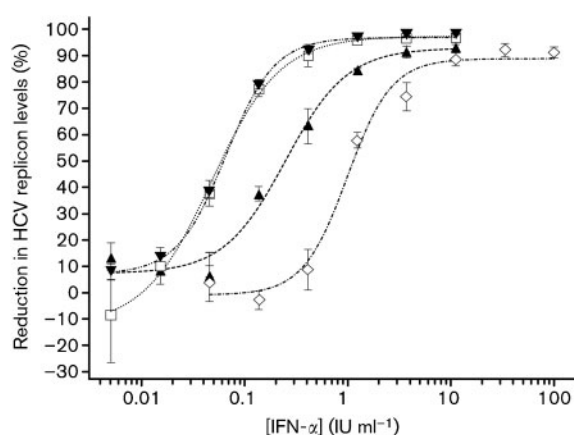
IFN-γ utilizes a different signalling pathway from IFN-α and has previously been reported also to have anti-HCV activity (Frese et al., 2002). Interestingly, knock-down of ISG15 (EC$_{50}$=0.17±0.03 ng ml^{-1}) did not result in a change in the HCV replicon sensitivity to IFN-γ compared with the siRNA control (EC$_{50}$=0.16±0.02 ng ml^{-1}).

Knock-down of ISG15 gene expression results in an IFN-α-sensitive phenotype for HCVcc

To determine whether the increased sensitivity to IFN-α after ISG15 knock-down is restricted to the HCV subgenomic replicon, we investigated whether the sensitivity to IFN-α of the GT-1a infectious virus (HCVcc) was affected similarly by ISG15 knock-down. Knock-down of ISG15 followed by infection of cells with HCV resulted in a 4-fold increase in sensitivity to IFN-α (EC$_{50}$=0.97±0.39 IU ml^{-1}) compared with siRNA control-transfected cells (EC$_{50}$=3.85±1.42 IU ml^{-1}), a result consistent with the HCV replicon data, which was statistically significant ($P<0.05$). Knock-down of USP18 followed by infecting cells with GT-1a HCVcc resulted in a 7-fold increase in sensitivity to IFN-α (EC$_{50}$=0.55±0.27 IU ml^{-1}) (Fig. 3), a result consistent with a published report using GT-2a HCVcc (Randall et al., 2006).

Knock-down of ISG15 gene expression results in reduced HCV RNA levels in both the HCV replicon and HCVcc

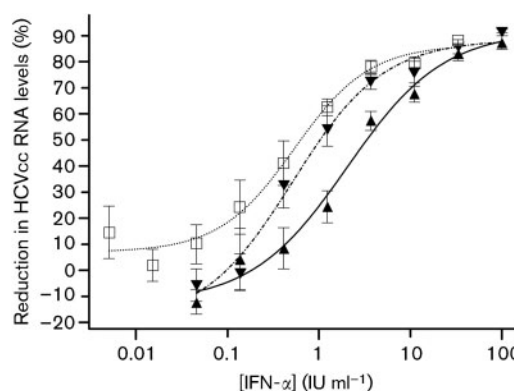Evaluation by qRT-PCR of the levels of HCV RNA after ISG15 knock-down and IFN-α treatment showed that

Fig. 2. Knock-down of ISG15 results in increased sensitivity of HCV replicon levels to IFN-α. Cells were transfected with siRNAs for 18 h, replated and treated with 3-fold dilutions of IFN-α for 3 days. Cells were then lysed and read using *Renilla* luciferase reagent. Data represent means±SD from three independent experiments. ▲, siRNA control; □, USP18 siRNA; ◇, STAT-2 siRNA; ▼, ISG15 siRNA #2.

Fig. 3. Knock-down of ISG15 results in increased sensitivity of infectious HCVcc to IFN-α. Cells were transfected with siRNAs for 18 h, replated and treated with 3-fold serial dilutions of IFN-α. Twenty-four hours post-plating, cells were infected with H77-S virus and 3-fold dilutions of IFN-α. Three days post-IFN-α treatment, cells were harvested for qRT-PCR analysis to determine HCV 5′ untranslated region (UTR) RNA levels. Data represent means±SD from four independent experiments. ▲, siRNA control; □, USP18 siRNA; ▼, ISG15 siRNA #2.

transfection of replicon cells with ISG15 siRNA did not affect the levels of HCV RNA (Fig. 4a). Upon treatment of siRNA control-transfected cells with 5 IU IFN-α ml^{-1} ($20 \times EC_{50}$), HCV RNA levels were reduced by 95 % compared with untreated cells. Knock-down of ISG15 followed by treatment with 5 IU IFN-α ml^{-1} resulted in a 99 % reduction of HCV replicon levels compared with siRNA control-transfected and untreated cells, or a further 72 % reduction of HCV RNA levels compared with siRNA control-transfected and IFN-α-treated cells ($P<0.01$) (Fig. 4a).

Similarly, in the GT-1a HCVcc system, there was an 83 % reduction in HCV RNA levels upon treatment of siRNA control-transfected cells with 100 IU IFN-α ml^{-1}, compared with siRNA control-transfected and untreated cells (Fig. 4b). With ISG15 knock-down and IFN-α treatment, we observed a further reduction of HCV RNA levels by 62 % compared with siRNA control-transfected and IFN-α-treated cells (Fig. 4b), which was statistically significant ($P<0.01$). Both of these results are consistent with the observation of increased sensitivity of the HCV replicon and GT-1a HCVcc to IFN-α after ISG15 knock-down.

Increased expression of IFN-α-inducible genes in HCV replicon- and HCVcc-infected cells after ISG15 knock-down and IFN-α treatment

IFN-α treatment of cells induces the transcriptional upregulation of a set of genes that are distinct from IFN-γ (Cheney et al., 2002). As an increase in the phenotypic sensitivity of HCV replicon- and HCVcc-infected cells to IFN-α (but not to IFN-γ) was observed, we investigated the expression levels of a number of IFN-α-inducible genes (IFITM3, IFI6, OAS1 and MX1), as well as non-IFN-α-inducible genes (PTBP-1 and JAK1), by qRT-PCR after ISG15 knock-down and IFN-α treatment, to get an insight into the mechanism of this sensitization. Knock-down of

ISG15 mRNA followed by IFN-α treatment resulted in approximately 2.8- and 3.3-fold increases in the expression of the IFN-stimulated genes (ISGs) IFITM3 and IFI6, respectively (Fig. 5a, b), and 8-fold and 3.8-fold increases in OAS1 and MX1 gene expression, respectively (Fig. 5c, d), compared with cells transfected with siRNA control and treated with IFN-α. This increase was only observed when cells were transfected with ISG15 siRNA and treated with IFN-α; in cells that were transfected with ISG15 siRNA but not treated with IFN-α, there were no significant changes in expression levels of IFITM3, IFI6, OAS1 or MX1 (Fig. 5a–d), suggesting a role for ISG15 in the modulation of IFN-inducible gene expression and IFN antiviral activity. As shown in Fig. 5(g), a reduction in ISG15 protein levels was accompanied by an increase in the levels of IFITM3, a result consistent with the qRT-PCR data (Fig. 5g).

It has been reported previously that the mechanism by which knock-down of USP18 results in an increase in HCV sensitivity to IFN-α is through the prolongation of STAT-1 phosphorylation (Randall et al., 2006). Thus, we investigated whether ISG15 knock-down similarly results in prolonged STAT-1 phosphorylation. As shown in Fig. 5(h), we were able to detect phosphorylated STAT-1 18 h after USP18 knock-down and IFN-α treatment. However, phosphorylated STAT-1 was barely detectable 4 h after ISG15 knock-down and IFN-α treatment and was no longer detectable at 7 h, clearly suggesting a different mechanism for ISG15.

The expression of IFN-α-inducible genes was also evaluated upon knock-down of ISG15 in HCVcc-transfected cells (75 % in untreated and 55 % in IFN-α-treated cells) (Fig. 6a). Similar increases in IFITM3 (3.4-fold), IFI6 (2.6-fold), OAS1 (3.3-fold) and MX1 (2.4-fold) gene-expression levels were observed in HCVcc-infected cells subjected to ISG15 knock-down followed by IFN-α treatment (Fig. 6b–e). All observed increases in IFITM3, IFI6, OAS1 and MX1 gene-expression levels after ISG15
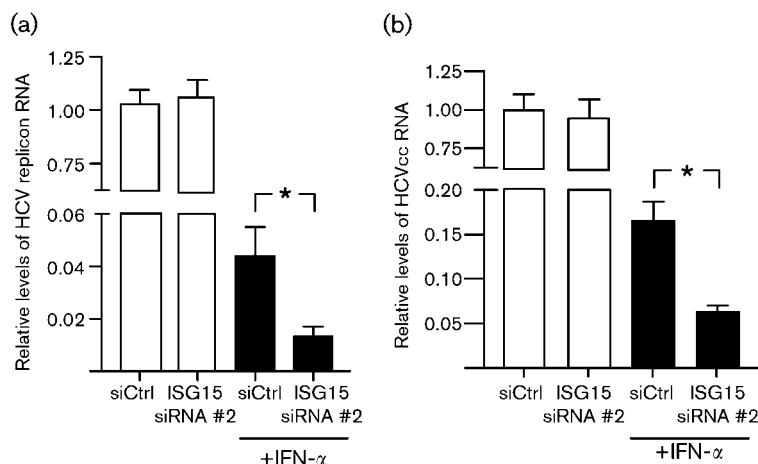

(a)

(b)

Fig. 4. Knock-down of ISG15 followed by IFN-α treatment further reduces HCV replicon and HCVcc RNA levels. (a) siRNAs were transfected into cells for 4 h, followed by replacement of medium with or without 5 IU IFN-α ml^{-1}. Cells were harvested 3 days post-transfection for qRT-PCR analysis of HCV neomycin phosphotransferase II gene levels. (b) Cells were transfected with siRNAs for 18 h, replated and treated with or without 100 IU IFN-α ml^{-1}. Twenty-four hours post-plating, the cells were infected with H77-S virus and 100 IU IFN-α ml^{-1}. Three days post-IFN-α treatment, cells were harvested for qRT-PCR analysis of HCV 5′ UTR RNA levels. Graphs represent means+SD from at least three independent experiments. *$P<0.01$.
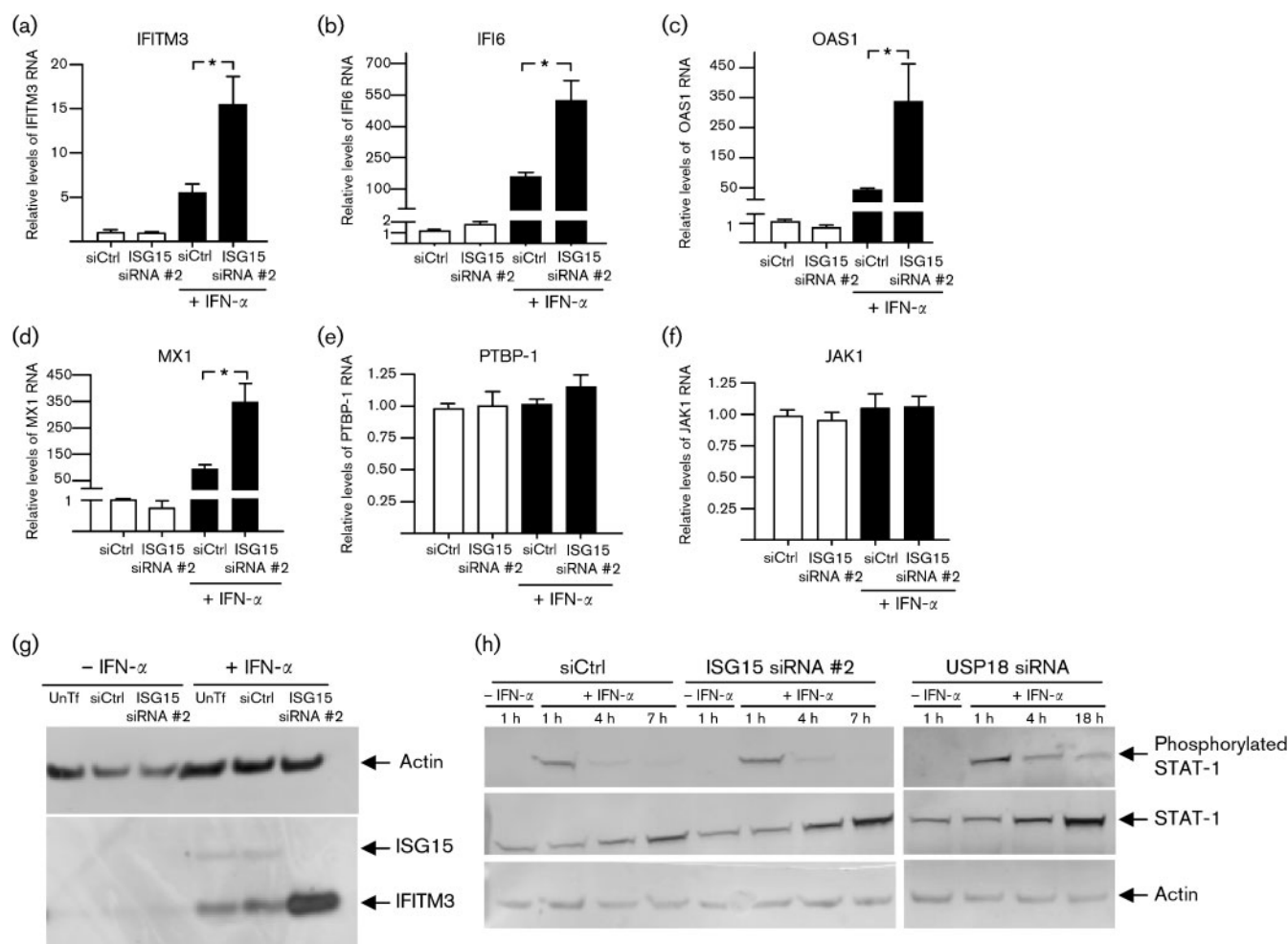
Fig. 5. Knock-down of ISG15 by siRNA followed by IFN-α treatment results in increased expression levels of IFN-α-inducible genes and proteins in HCV replicon cells, whereas non-IFN-α-inducible genes were not affected, and the mechanism of the increased gene expression is not through prolonged STAT-1 phosphorylation. Cells were transfected with siRNAs for 4 h, followed by replacement of fresh medium with or without 5 IU IFN-α ml^{-1}. Cells were harvested 3 days post-transfection for qRT-PCR analysis of (a) IFITM3, (b) IFI6, (c) OAS1, (d) MX1, (e) PTBP-1 and (f) JAK1 gene-expression levels, or (g) Western blot analysis of ISG15 and IFITM3 protein levels. (h) Cells were transfected with siRNAs for 4 h, followed by replacement of fresh medium with or without 5 IU IFN-α ml^{-1}. Cells were harvested at the indicated time points for Western blot analysis of phosphorylated and non-phosphorylated STAT-1 protein. Graphs represent means + SD from three independent experiments. UnTf, Untransfected cells. *$P < 0.01$.

knock-down and IFN-α treatment in HCV replicon- or HCVcc-infected cells were determined to be statistically significant compared with siRNA control-transfected and IFN-α-treated cells ($P < 0.01$).

To determine whether the increased gene expression after ISG15 knock-down and IFN-α treatment was specific to IFN-α-inducible genes, we measured expression levels of two non-IFN-α-inducible genes, PTBP-1 and JAK1. As shown in Figs 5(e–f) and 6(f–g), PTBP-1 or JAK1 gene-expression levels were not changed significantly (<1.2-fold) after ISG15 knock-down and IFN-α stimulation in HCV replicon-infected (Fig. 5e, f) or HCVcc-infected (Fig. 6f, g) cells.

To confirm that the observed increase in gene-expression levels after efficient ISG15 mRNA knock-down and IFN-α treatment was not due to an off-target effect, we evaluated the gene-expression levels of IFITM3, IFI6, OAS1 and MX1 after transfection of four individual ISG15 siRNAs and IFN-α treatment. As shown in Fig. 1(a), siRNA #3 did not effectively knock down ISG15 mRNA in the absence of IFN-α. After treatment with IFN-α, siRNA #3 did not reduce ISG15 mRNA levels significantly (12 %), whereas siRNAs #1, #2 and #4 reduced ISG15 mRNA levels by 50, 81 and 67 %, respectively (Fig. 7a). Similarly, gene-expression levels of IFITM3, IFI6, OAS1 and MX1 were not increased significantly after transfection with siRNA #3 compared with siRNA control-transfected and IFN-α-
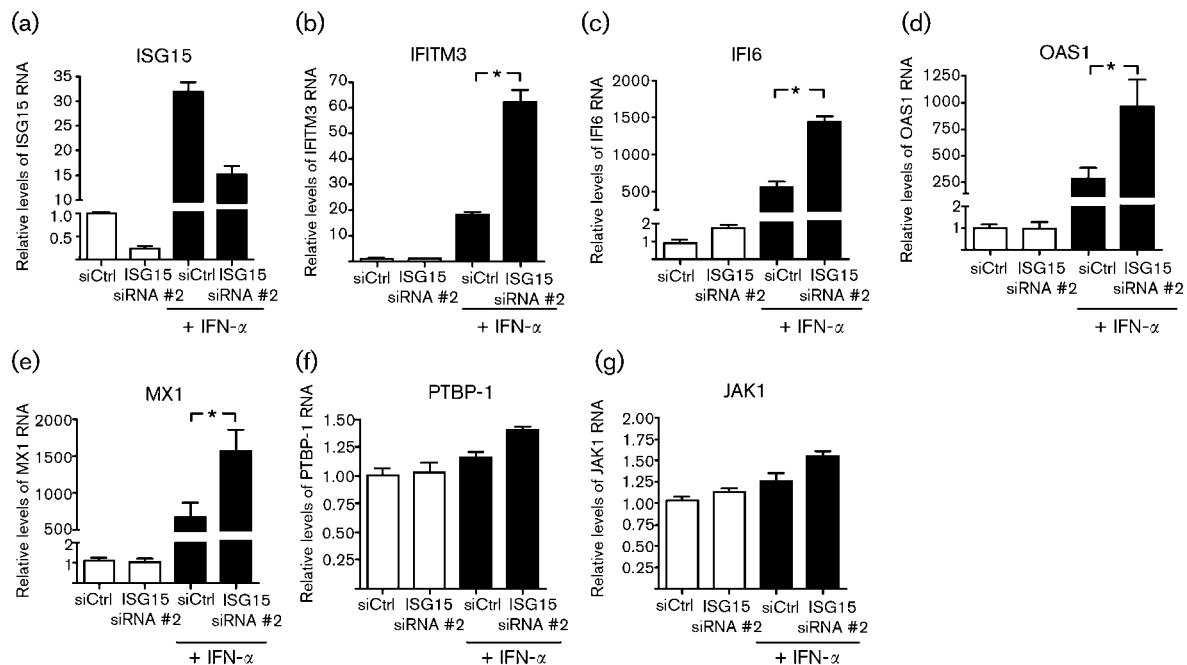
Fig. 6. Knock-down of ISG15 by siRNA followed by IFN-α treatment results in increased gene-expression levels of IFN-α-inducible genes in HCVcc-infected cells, whereas non-IFN-α-inducible genes were not affected. Cells were transfected with siRNAs for 18 h, replated and treated with or without 100 IU IFN-α ml^{-1}. Cells were harvested 4 days post-IFN-α treatment for qRT-PCR analysis of (a) ISG15, (b) IFITM3, (c) IFI6, (d) OAS1, (e) MX1, (f) PTBP-1 and (g) JAK1 gene-expression levels. Graphs represent means + SD from four independent experiments. *$P < 0.01$.

treated cells (Fig. 7b–e). On the other hand, gene-expression levels of IFITM3, IFI6, OAS1 and MX1 were increased by approximately 2-fold (for IFITM3) to 5-fold (for OAS1) after transfection of cells with siRNAs #1, #2 and #4 compared with siRNA control-transfected and IFN-α-treated cells. These increases were determined to be statistically significant ($P < 0.01$). Thus, the fact that siRNA #3 did not effectively knock down ISG15 and did not result in increased ISG expression indicated that the phenotype is specific for ISG15 and not due to an off-target effect. Similarly, PTBP-1 and JAK1 gene-expression levels were not affected significantly by siRNA transfection and IFN-α treatment (<1.2-fold change) (Fig. 7f, g).

Combination knock-down of ISG15 and USP18 results in a moderate increase in IFN-α-inducible gene expression

As knock-down of USP18 or ISG15 results in increased sensitivity to IFN-α, we determined whether the combined knock-down of ISG15 and USP18 could further sensitize cells to IFN-α. As shown in Fig. 8(a, b), knock-down of ISG15 resulted in increased USP18 gene expression (1.6-fold) after IFN-α treatment and, similarly, knock-down of USP18 resulted in increased ISG15 gene expression (2.5-fold) after IFN-α treatment. Furthermore, combination knock-down of ISG15 and USP18 resulted in effective knock-down of ISG15 gene expression after IFN-α

treatment compared with siRNA control and IFN-α treatment. USP18 gene expression was also downregulated in the double knock-down compared with the ISG15 knock-down, but was not suppressed compared with the siRNA control. This could be due to the fact that ISG15 knock-down upregulates the expression of USP18 after IFN-α treatment.

The combined knock-down of ISG15 and USP18 after IFN-α treatment resulted in an increase in expression of IFN-α-inducible genes compared with the individual knock-down of ISG15 or USP18 followed by IFN-α treatment (<2-fold for IFITM3, IFI6 and MX1, and an approx. 4-fold increase for OAS1), whereas PTBP-1 and JAK1 gene-expression levels remained unchanged (Fig. 8c–h).

Knock-down of USP18 resulted in increased ISG15 protein levels, whereas combination knock-down resulted in no detectable ISG15 proteins (Fig. 8i). HCV NS5A protein levels were detectable in the absence of IFN-α treatment, with or without siRNA transfection, whereas in the presence of IFN-α treatment, HCV NS5A protein levels were reduced to very low levels and became undetectable when both ISG15 and USP18 genes were knocked down simultaneously (Fig. 8i). These data suggest that the sensitization of cells to IFN-α by simultaneous knock-down of the ISG15 and USP18 genes is greater than when each gene is knocked down individually, suggesting an interaction between these two genes (Fig. 8c–f), but also
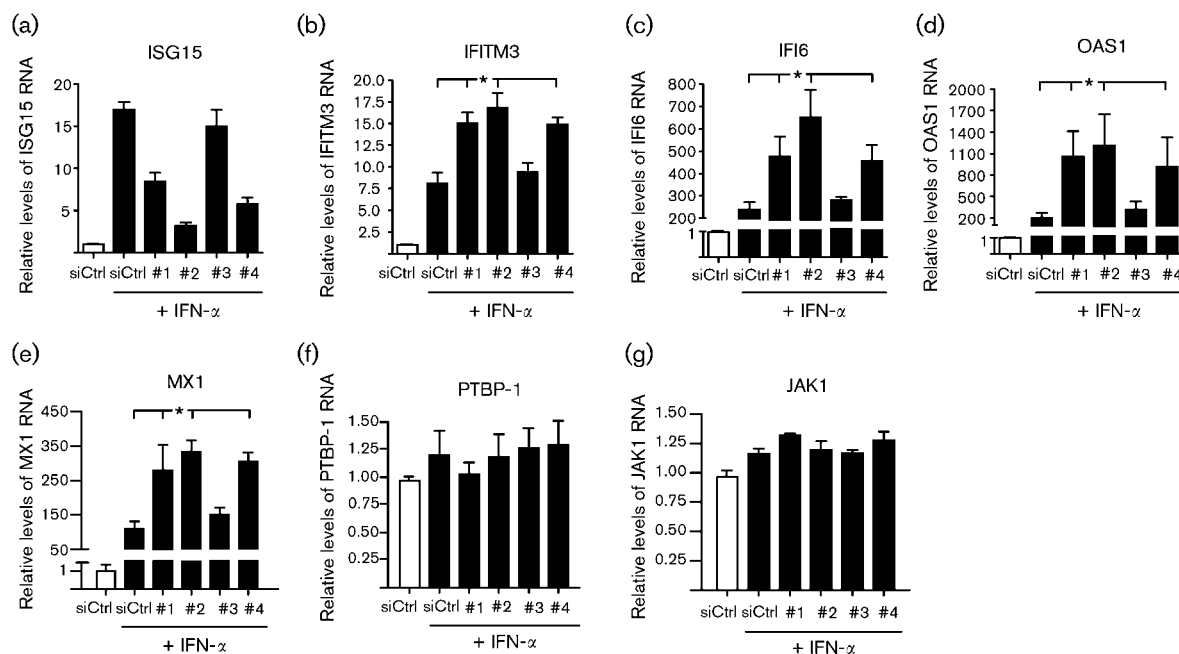
Fig. 7. Induction of IFN-α-induced gene expression after ISG15 knock-down and IFN-α treatment is specific. siRNAs were transfected into cells for 4 h, followed by replacement of fresh medium with or without 5 IU IFN-α ml^{-1}. Cells were harvested 3 days post-treatment for qRT-PCR analysis of (a) ISG15, (b) IFITM3, (c) IFI6, (d) OAS1, (e) MX1, (f) PTBP-1 and (g) JAK1 gene-expression levels. Graphs represent means + SD from at least three independent experiments. *$P < 0.01$.

DISCUSSION

A number of studies have shown that ISG15 has antiviral properties against selected viruses (Guerra *et al.*, 2008; Hsiang *et al.*, 2009; Lenschow *et al.*, 2005, 2007; Malakhova & Zhang, 2008; Okumura *et al.*, 2006, 2008; Osiak *et al.*, 2005; Yuan & Krug, 2001; Zhang *et al.*, 2007). We present herein results demonstrating that knock-down of ISG15 alone does not result in any changes in HCV RNA levels in the HCV replicon or HCVcc, indicating that ISG15 does not possess direct antiviral activity against HCV *in vitro*. In addition, we present data demonstrating that ISG15 can negatively regulate the IFN-α signalling pathway.

It was reported previously that overexpressing ISG15 alone has no effect on HCV replication (Itsui *et al.*, 2006; Jiang *et al.*, 2008; Randall *et al.*, 2007). It is possible that, without the concomitant overexpression of ISG15-conjugating enzymes, conjugation to cellular proteins that can regulate HCV replication may not occur, as basal levels of these enzymes may be very low. Another possibility is that the cellular proteins that are conjugated to ISG15 exist at very low levels, and may need to be induced by IFN-α. As such, the functional activity of ISG15 may not be observable only by overexpressing ISG15.

Surprisingly, knock-down of Ube2L6 did not result in any change in sensitivity of the HCV replicon to IFN-α (EC$_{50}$=0.22 ± 0.02 IU ml^{-1}). This indicates that ISG15 conjugation is not required for modulation of the IFN-α signalling pathway, a result consistent with those of Kim *et al.* (2006). Conversely, there could exist other, unidentified conjugation enzymes that may compensate for the loss of Ube2L6.

Knock-down of ISG15 resulted in an increased sensitivity of the HCV replicon and HCVcc to IFN-α, and also resulted in increased ISG expression. This suggests that the increased ISG expression amplifies the antiviral state within IFN-α-treated cells, resulting in the increased sensitivity of the HCV replicon and HCVcc to IFN-α. This phenotype was not observed for IFN-γ, thus suggesting that ISG15 has a specific effect on the modulation of IFN-α-inducible genes.

The increase in ISG expression after ISG15 knock-down and IFN-α treatment was not restricted to Huh-7-derived cells. We similarly observed this phenotype in the non-hepatic cell lines A549 and HeLa (Supplementary Fig. S1, available in JGV Online). This indicates strongly that ISG15 may possess a universal role in the modulation of the IFN-α pathway.

In our studies, we were unable to detect prolonged phosphorylation of STAT-1 after ISG15 knock-down and IFN-α treatment (Fig. 5h), compared with siRNA control or USP18-knocked-down and IFN-α-treated cells, a result consistent with those of Osiak *et al.* (2005). Together, these
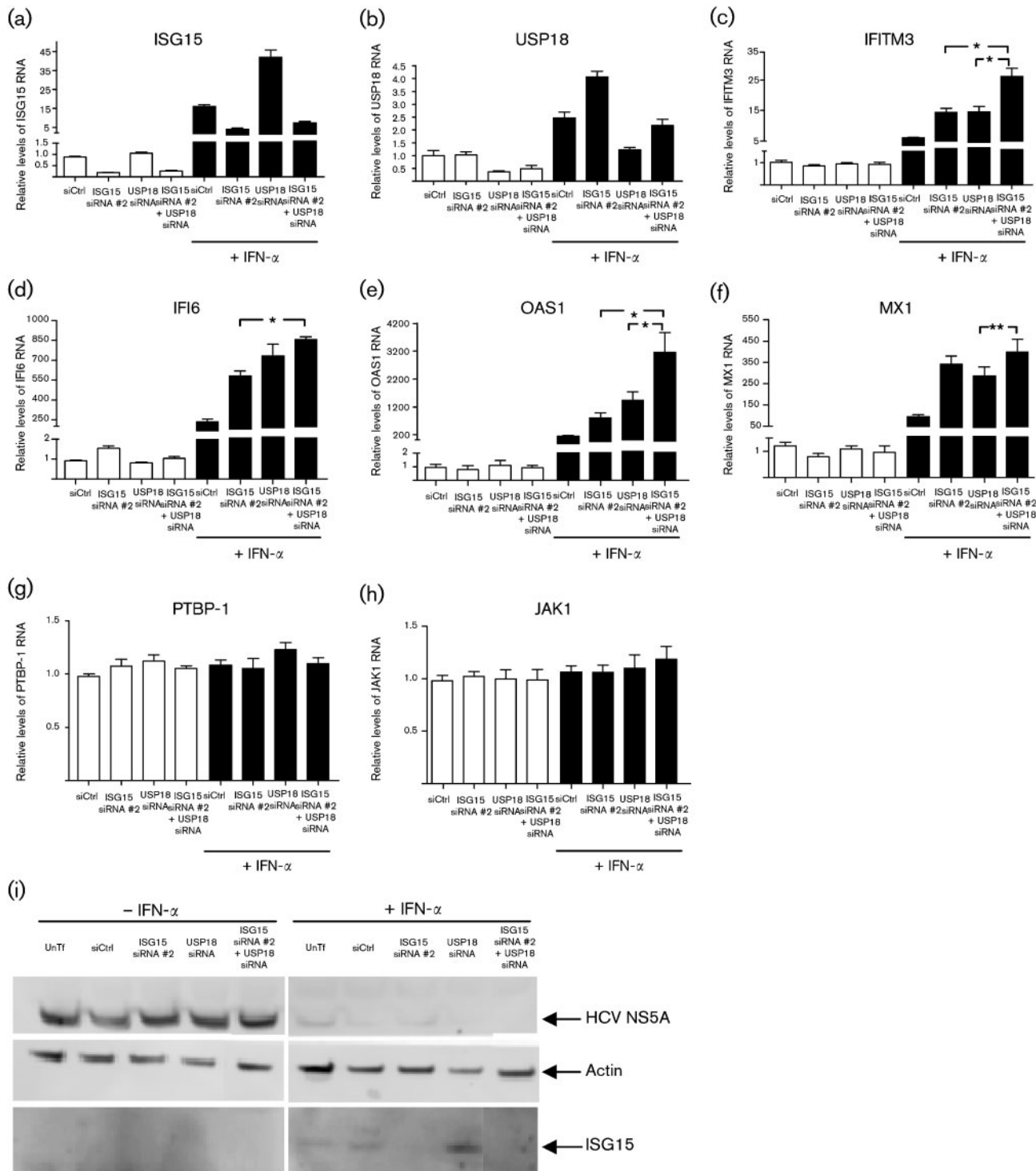
Fig. 8. Combination knock-down of ISG15 and USP18 results in a slight increase in IFN-α-inducible gene expression compared with single ISG15 or USP18 knock-down. siRNAs were transfected into cells for 4 h, followed by replacement of fresh medium with or without 5 IU IFN-α ml^{-1}. Cells were harvested 3 days post-treatment for qRT-PCR analysis of (a) ISG15, (b) USP18, (c) IFITM3, (d) IFI6, (e) OAS1, (f) MX1, (g) PTBP-1 and (h) JAK1 gene-expression levels, or (i) Western blot analysis of HCV NS5A and ISG15 protein levels. Graphs represent means + SD from at least four independent experiments. UnTf, Untransfected cells. *$P<0.01$; **$P<0.05$.

results indicated that the increased sensitivity of the HCV replicon and HCVcc to IFN-α after ISG15 knock-down occurs through a different signalling pathway from USP18. Furthermore, the modest increase in IFN-α-induced gene expression after combination knock-down of ISG15 and USP18 (Fig. 8) suggests that ISG15 and USP18 are modulating the IFN-α signalling pathway at different levels.

Moreover, the sensitivity of the HCV replicon to IFN-γ, which also uses phosphorylated STAT-1 for signal transduction, was not affected by ISG15 knock-down. This again suggests that ISG15 regulation of the IFN-α pathway does not include STAT-1, but may affect STAT-2, IRF-9 or the p38 MAPK pathway, or could occur downstream of the JAK–STAT signalling pathway.

HCV proteins have been reported to be capable of blocking the type I IFN signalling pathway (Gale & Foy, 2005) and ISG15 has also been demonstrated to be capable of conjugating to influenza NS1 viral protein (Yuan & Krug, 2001). However, as ISG15 has never been demonstrated to conjugate to HCV proteins, coupled with the fact that the increase in IFN-α sensitivity after ISG15 knock-down and IFN-α treatment was also observed in the parental Huh-7, A549 and HeLa cell lines, all of which do not carry HCV replicons, we believe that the effect of ISG15 on the IFN-α signalling pathway is independent of ISG15 conjugation to HCV proteins or HCV replication.

To date, ISG15 has been reported to being capable of regulating two different signalling pathways (Kim *et al.*, 2008; Takeuchi *et al.*, 2006). Takeuchi *et al.* (2006) demonstrated that ISG15 regulates the nuclear factor (NF)-κB pathway by conjugating to protein phosphatase 2Cβ, thereby enhancing NF-κB activity by suppressing dephosphorylation of I-κB (Takeuchi *et al.*, 2006), and Kim *et al.* (2008) reported that ISG15 negatively regulates the innate immune response through ISGylation and modulating RIG-I protein levels (Kim *et al.*, 2008; Takeuchi *et al.*, 2006). However, after ISG15 knock-down and IFN-α treatment in a Huh-7.5-derived cell line (Gee *et al.*, 2008) that is RIG-I defective, an increase in IFN-α-induced gene expression was still observed (P. K. Chua & I. Nájera, unpublished results). Thus, it is unlikely that ISGylated RIG-I is the negative regulator that is controlling IFN-α-induced gene expression in our experimental setting.

Furthermore, we did not detect IFN-β gene expression in the 2209-23 cell line after ISG15 knock-down and IFN-α treatment (data not shown). Stimulation of 2209-23 cells with poly I : C, a strong inducer of IFN-β gene expression in parental Huh-7 cells, also did not induce IFN-β gene expression. These data indicate strongly that (i) 2209-23 cells are defective in IFN-β gene expression, and (ii) increased ISG expression after ISG15 knock-down and IFN-α treatment in 2209-23 cells is not due to IFN-β production.

It has been postulated that a high baseline hepatic ISG expression may play an important role in the lack of response to IFN-α-based therapies (Chen *et al.*, 2005).

Interestingly, microarray analysis of liver biopsies from IFN responders and non-responders showed that non-responders had higher levels of ISG15 gene expression than responders (Asahina *et al.*, 2008; Asselah *et al.*, 2008; Chen *et al.*, 2005; Sarasin-Filipowicz *et al.*, 2008). Thus, it can be rationalized from our experimental data, together with the reported clinical data, that ISG15 gene knock-down in non-responders together with IFN-α treatment could possibly enhance treatment efficacy. In addition, ISG15 could potentially serve as a useful biomarker for predicting SVR in patients on IFN-α therapy.

In conclusion, we report here a novel role for ISG15 in regulating HCV sensitivity to IFN-α, as well as the regulation of ISG expression and thus modulation of IFN-α anti-HCV activity. Furthermore, we hypothesize that ISG15 may possess pleiotropic properties, one of which could be involved in the direct antiviral response against specific viruses, whilst another is to dampen the IFN-α response.

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