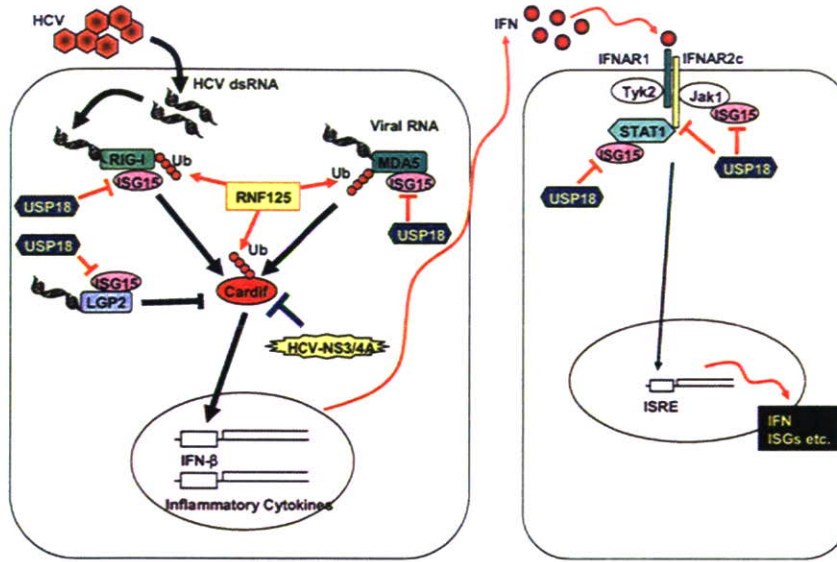
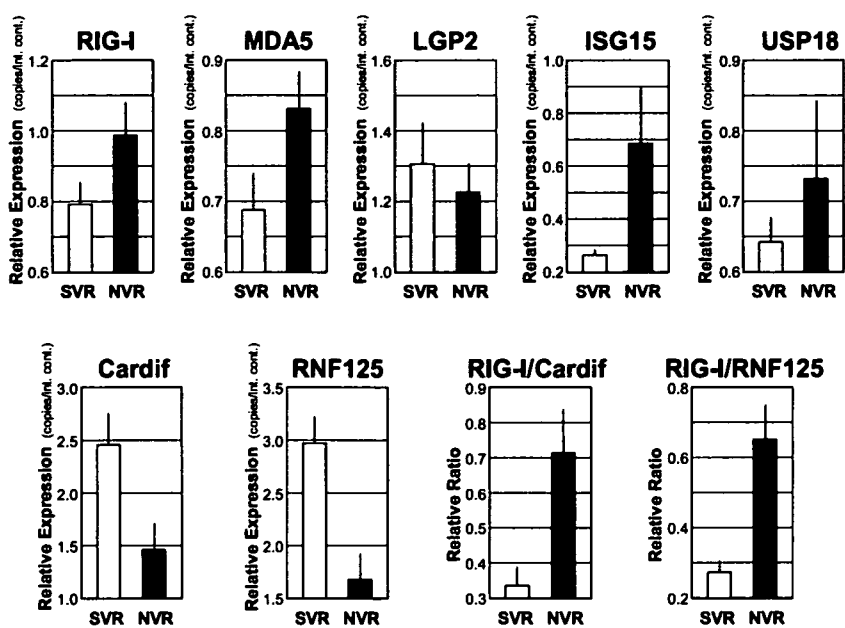


Figure 5

Supplementary Figure 1 for Online Publish and Review





Supplementary Figure 2 for Online Publish and Review

Smad4 is Essential for Down-regulation of E-cadherin Induced by TGF- β in Pancreatic Cancer Cell Line PANC-1

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Smad4 is a tumour suppressor gene frequently deleted in pancreatic cancer. To investigate the roles of Smad4 deficiency in invasive and metastatic capabilities of pancreatic cancer, we examined the effects of Smad4 deficiency on regulation of the invasion suppressor E-cadherin in pancreatic cancer cell line PANC-1. TGF- β decreased expression of E-cadherin and β -catenin proteins at the plasma membrane, increased Snail and Slug mRNA expression, and induced fibroblastoid morphology in PANC-1 cells. These effects of TGF- β were abrogated in Smad4-knocked-down PANC-1 cells. We also found that TGF- β -induced down-regulation of E-cadherin expression was partially inhibited in Snail- and Slug-knocked-down PANC-1 cells. Thus, Smad4 mediates down-regulation of E-cadherin induced by TGF- β in PANC-1 cells, at least in part, through Snail and Slug induction. These results suggest that Smad4 deficiency observed in invasive and metastatic pancreatic cancer might not be linked to the loss of E-cadherin.

Key words: pancreatic cancer, E-cadherin, Smad4, TGF- β .

Abbreviations: TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor; siRNA, small-interfering RNA; EMT, epithelial mesenchymal transition.

Smad4 was originally identified as a tumour suppressor gene (DPC4: deleted in pancreatic cancer, locus 4) functionally inactivated in one-half of pancreatic adenocarcinoma (1). Subsequent studies have revealed that Smad4 belongs to the Smad gene family, which encodes intracellular signalling mediator of the transforming growth factor- β (TGF- β) superfamily of cytokines (2, 3). TGF- β binds to two different types of serine/threonine kinase receptors, termed type I and type II. The activated heteromeric complex of TGF- β type I and type II receptors induces phosphorylation of Smad2 and Smad3, which form hetero-oligomeric complexes with Smad4. The complexes then translocate to the nucleus and regulate transcriptional responses together with DNA binding co-factors.

Since TGF- β is a potent inhibitor of the growth of epithelial cells (4), the tumour-suppressor function of Smad4 in pancreatic cancer has been attributed to its capacity to mediate TGF- β -induced growth inhibition. However, recent studies propose that loss of Smad4 may be associated with invasive and metastatic capabilities of the tumour through various mechanisms (5–8). For example, Schwarte-Waldhoff *et al.* (7) reported that Smad4-deficient pancreatic cancer cells showed increased vascular endothelial growth factor (VEGF) expression

associated with their capabilities of *in vivo* metastasis through enhanced angiogenesis.

E-cadherin is a 120 kDa transmembrane glycoprotein that connects cells via homotypic interactions (9). Its cytoplasmic domain associates with a family of submembranous cytoplasmic proteins termed the catenins: α -catenin, β -catenin, γ -catenin and p120 (10). They link E-cadherin to the cytoskeleton, thus acting as a scaffold for the maintenance of cell architecture. The E-cadherin–catenin complexes represent the main adhesion system responsible for the maintenance of cell–cell contacts in epithelial tissues (10).

It is now recognized that E-cadherin may act as a tumour suppressor (11). Numerous studies have shown that down-regulation of E-cadherin expression or functional perturbation of the E-cadherin–catenin complexes has been found to occur very frequently during the progression of carcinomas and is associated with an invasive and metastatic phenotype of the tumours. Regarding pancreatic cancer, loss of membranous E-cadherin expression was associated with high grade and advanced stage of the cancer (12–14). Furthermore, Perl *et al.* (15) elegantly showed that the loss of E-cadherin expression coincided with the transition from well differentiated adenoma to invasive carcinoma in a transgenic mouse model of pancreatic β -cell carcinogenesis, giving further support for a role of E-cadherin as an ‘invasive suppressor’ of pancreatic cancer.

In this study, we therefore hypothesized that loss of Smad4 might be linked to down-regulation of the

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invasive suppressor E-cadherin in pancreatic cancer cells, resulting in acquisition of highly metastatic capabilities of the tumour. Contrary to our expectations, the current results using Smad4-knockdown pancreatic cancer cell lines suggest that Smad4 is essential for down-regulation of E-cadherin induced by TGF- β in pancreatic cancer cell lines and Smad4 deficiency may not be associated with selective loss of E-cadherin observed in advanced pancreatic cancer.

MATERIALS AND METHODS

Cell Lines—The Grade III undifferentiated human pancreatic cancer cell line, PANC-1 (16), was purchased from the American Type Culture Collection (Rockville, MD, USA). Human pancreatic adenocarcinoma cell lines PK-8 and PK-9 were obtained from Dr M. Kobari (Sendai, Japan) (17). Human keratinocyte cell line HaCaT cells were previously obtained from Dr N.E. Fusenig (DKFZ, Heidelberg, Germany) (18). These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen, Carlsbad, CA) containing 10% FCS and 100 μ g/ml streptomycin.

Establishment of Smads-knocked-down Cell Lines—We have established Smad2-knocked-down, Smad3-knocked-down, Smad4-knocked-down PANC-1 cells and control Smad4-intact PANC-1 cells, which are designated PANC-1-S2KD, PANC-1-S3KD, PANC-1-S4KD and PANC-1-puro, by using the stable RNA interference (RNAi) method as previously described (19, 20). PK-8 and HaCaT cells stably transfected with the plasmid expressing siRNA that targeted Smad4 (S4KD) or with the empty plasmid (puro) were also established as previously described (19, 20).

Reagents—Human recombinant TGF- β 1 was purchased from R&D Inc. (Minneapolis, MN, USA). PD98059, SB203580 and LY294002 were purchased from CALBIOCHEM (San Diego, CA, USA) and SB202190, SP600125 were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Human Snail and Slug siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Transfection of Snail and Slug siRNAs into PANC-1 cells was performed according to the manufacturer's instruction.

Western Blot—Western blot analysis was performed by using antibodies for E-cadherin (Beckton Dickinson, San Jose, CA, USA), Smad2/3, Smad4 (Transduction Laboratories Inc.) and β -actin (Cell Signaling Technology Inc., Beverly, MA, USA) as previously described (19, 20).

Immunofluorescence—Cells were grown to confluency on 8-well chamber slides, rinsed with PBS and fixed with ice-cold 100% methanol for 5 min at -20°C . The fixed cells were incubated with primary antibody, anti human E-cadherin (dilution 1:50 in PBS+1% BSA) (Beckton Dickinson) for 1 h followed by FITC secondary antibodies (dilution 1:50 in PBS+1% BSA) (Dako Cytomation, Kyoto, Japan). Samples were mounted with mounting medium (Dako Cytomation) and examined under an ultraviolet light microscope.

Quantitative Real-time PCR—Quantitative real-time PCR with specific primers and probes (purchased from Applied Biosystems) was performed using the ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) as previously described (19, 20).

ELISA—Cells were cultured in serum-free DMEM for indicated times and the amounts of TGF- β 2 and VEGF in the culture supernatants were determined by using the human TGF- β 2 and VEGF ELISA kit (R&D Inc.).

Data Analysis—Values represent the mean \pm SD of triplicate samples per group. An unpaired Student's *t*-test was used for the statistical analysis of the results. $P < 0.05$ was considered to be significant.

RESULTS

Smad4 Deficiency Attenuates Down-regulation of E-cadherin Induced by TGF- β in Human Pancreatic Carcinoma Cell Line PANC-1—The human pancreatic carcinoma cell line, PANC-1, expressed functional TGF- β type I and II receptors and Smads (21–23). In addition, Nakajima *et al.* (24) recently reported that TGF- β decreased E-cadherin expression in PANC-1 cells with a change to a fibroblastoid morphology (24). Consistent with these findings, Western blot and immunofluorescence study showed that control PANC-1-puro cells expressed E-cadherin, which was down-regulated by addition of TGF- β (Fig. 1A and B). PANC-1-puro cells also showed a change from epithelial to spindle fibroblastic shaped morphology upon TGF- β stimulation (Fig. 1B). We also found significant β -catenin expression at the plasma membrane in PANC-1-puro cells, which was down-regulated by TGF- β (Fig. 1B). In contrast, treatment with TGF- β did not affect E-cadherin expression, epithelial morphology, and β -catenin expression at the plasma membrane in Smad4-knocked-down PANC-1-S4KD cells (Fig. 1A and B). These results indicated that Smad4 is essential for TGF- β -induced down-regulation of E-cadherin in PANC-1 cells.

Smad4 Deficiency Attenuates Down-regulation of E-cadherin Induced by TGF- β in PK-8, PK-9 and HaCaT Cell Lines—To determine whether Smad4 is essential for TGF- β -induced down-regulation of E-cadherin in other cell lines, we examined the effect of Smad4 deficiency on TGF- β -induced down-regulation of E-cadherin in human pancreatic cancer cell line PK-8 and human keratinocyte cell line HaCaT. Both cell lines express Smad4 and respond to TGF- β (25, 26). Similar to the results obtained in PANC-1 cells, immunofluorescence study showed that TGF- β down-regulated E-cadherin expression in control, but not in Smad4-knocked-down, PK-8 and HaCaT cells (Fig. 2A and B). We also found that PK-9 human pancreatic cancer cell line, which lacked Smad4 expression (25), expressed E-cadherin, which was not affected by stimulation with TGF- β (Fig. 2C). These results suggest that an essential role of Smad4 in TGF- β -induced down-regulation of E-cadherin is not specific to PANC-1 cells and may be a general phenomenon.

Smad4 Deficiency Attenuates TGF- β -induced Expression of Snail and Slug mRNAs in PANC-1 Cells—Transcriptional repression mechanisms have emerged

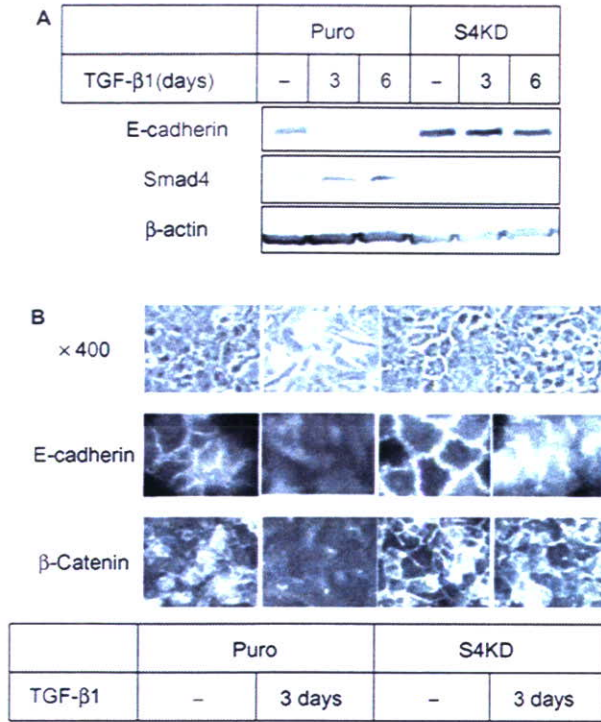


Fig. 1. Smad4 deficiency attenuates down-regulation of E-cadherin induced by TGF-β in human pancreatic carcinoma cell line PANC-1. Smad4-knockdown PANC-1 cells (PANC-1-S4KD) and control Smad4-intact PANC-1 cells (PANC-1-puro) were established as described in the section 'Materials and Methods'. (A) Cells were stimulated with 10 ng/ml TGF-β for 3 or 6 days and, then, cell lysates were subjected to Western blot analysis with specific antibodies for E-cadherin, Smad4 and β-actin. (B) (Upper panels) Phase contrast microphotographs showing morphology of a representative PANC-1-puro and PANC-1-S4KD cells in response to 72 h stimulation of 10 ng/ml TGF-β; (middle and bottom panels) cells were stimulated with 10 ng/ml TGF-β for 72h and, then, were subjected to immunofluorescence analysis with specific antibodies for E-cadherin and β-catenin. Similar results were obtained at least in three independent experiments.

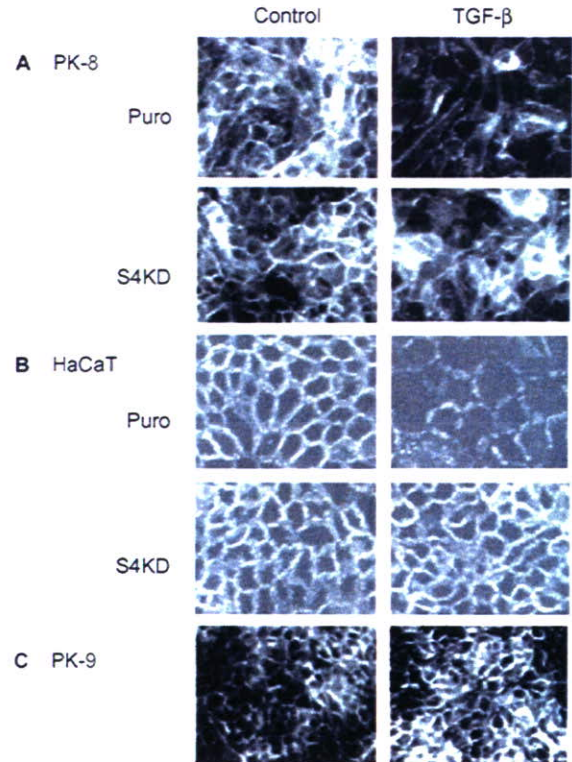


Fig. 2. Smad4 deficiency attenuates down-regulation of E-cadherin induced by TGF-β in human pancreatic carcinoma cell lines PK-8 and PK-9 and in human keratinocyte cell line HaCaT. Smad4-knockdown PK-8 cells and HaCaT cells (PK-8-S4KD, HaCaT-S4KD) and control Smad4-intact PK-8 and HaCaT cells (PK-8-puro, HaCaT-puro) were established as described in the section 'Materials and Methods'. PK-8-puro or PK-8-S4KD (A), HaCaT-puro or HaCaT-S4KD (B), or Smad4-deficient PK-9 cells (C) were stimulated with 10 ng/ml TGF-β for 3 days and, then, were subjected to immunofluorescence analysis with specific antibodies for E-cadherin and β-catenin. Similar results were obtained at least in three independent experiments.

as one of the crucial processes for the down-regulation of E-cadherin expression during development and tumour progression (27). Several E-cadherin transcriptional repressors have been characterized (Snail, E12/E47, ZEB-1, SIP-1 and Slug) and shown to act through an interaction with proximal E-boxes of the E-cadherin promoter (27-29). Recently, TGF-β was reported to be a regulator of Snail and Slug expression and involved in down-regulation of E-cadherin (30, 31). We thus examined the effects of Smad4 deficiency on Snail and Slug expression in PANC-1 cells. As shown in Fig. 3A, real-time PCR analysis showed that expression of Snail and Slug mRNA was induced by TGF-β in PANC-1-puro cells. Induction of Snail mRNA by TGF-β was observed in the presence of cyclohexamide (CHX), suggesting that *de novo* protein synthesis is not required for the Snail mRNA induction (Fig. 3A). On the other hand, Slug mRNA expression was increased in the presence of CHX alone and TGF-β did not up-regulate Slug mRNA

expression in the presence of CHX. Thus, *de novo* protein synthesis may be required for the Slug mRNA induction (Fig. 3A). In PANC-1-S4KD cells, TGF-β-induced Snail and Slug expression was abrogated (Fig. 3B). To determine whether TGF-β/Smad4-dependent Snail and Slug expression is required for TGF-β-induced down-regulation of E-cadherin, we examined the effects of Snail or Slug deficiency on TGF-β-induced down-regulation of E-cadherin in PANC-1 cells. As shown in Fig. 4A and B, TGF-β-induced down-regulation of E-cadherin was partially suppressed in Snail- and Slug-knockdown PANC-1 cells. These results suggest that TGF-β/Smad4-dependent Snail and Slug expressions are, at least in part, required for TGF-β-induced down-regulation of E-cadherin in PANC-1 cells.

Smad4 Deficiency Attenuates TGF-β-induced TGF-β and VEGF Production in PANC-1 Cells—Previous studies have suggested that Smad4 deficiency augments TGF-β and VEGF production of pancreatic cancer cells,

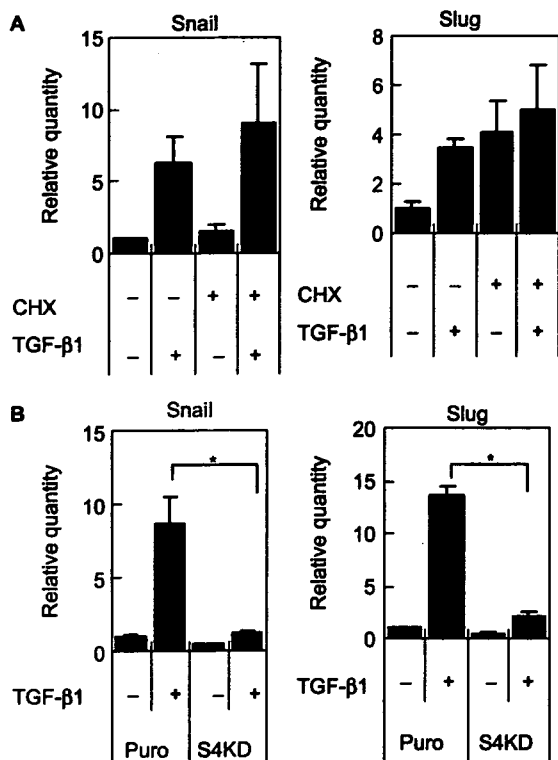


Fig. 3. Smad4 deficiency attenuates TGF-β-induced expression of Snail and Slug mRNAs in PANC-1 cells. (A) cDNAs were obtained from PANC-1-puro after 6 h stimulation with 10 ng/ml TGF-β in the presence or absence of 10 μg/ml cyclohexamide (CHX). Then, real-time PCR was performed using specific primers for Snail, Slug, and GAPDH. (B) cDNAs were obtained from PANC-1-puro and PANC-1-S4KD cells after 6 h stimulation with 10 ng/ml TGF-β and, then, real-time PCR was performed using specific primers for Snail, Slug, and GAPDH. The ratio of each gene to that of GAPDH was calculated, and the value of 1.0 was assigned to the PANC-1 wild-type cells that were incubated without TGF-β1. Values represent the mean ± SD of triplicate samples per group. *P < 0.05 compared with corresponding control. Similar results were obtained at least in three independent experiments.

which is associated with invasive and metastatic capabilities of pancreatic cancer cells (7, 8). Thus, we further examined the effects of Smad4 deficiency on TGF-β and VEGF production in PANC-1 cells (Fig. 5). Real-time PCR analysis showed that Smad7 mRNA induction, which was dependent of Smad4 (32), was abolished in PANC-1-S4KD cells, confirming the specific-knockdown of Smad4 in the cells. TGF-β1 significantly induced TGF-β1 and TGF-β2 mRNA expression in PANC-1-puro cells whereas it was abrogated in PANC-1-S4KD cells. In addition, ELISA assay showed that TGF-β-induced VEGF production was partially, but significantly, suppressed in PANC-1-S4KD cells (Fig. 5B). These results indicated that Smad4 is involved in TGF-β-induced TGF-β and VEGF expression in PANC-1 cells.

Smad3, but not Smad2, Deficiency Attenuates Down-regulation of E-cadherin Induced by TGF-β in PANC-1— Finally, we examined the effects of deficiency of other

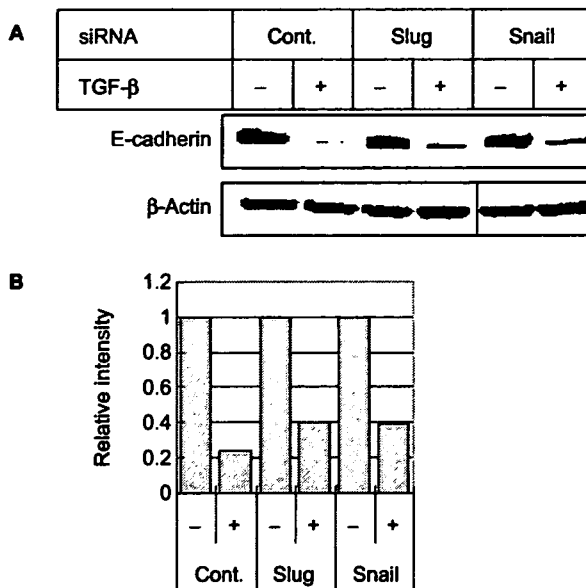


Fig. 4. Snail and Slug deficiency partially attenuates TGF-β-induced down-regulation of E-cadherin expression in PANC-1 cells. (A) PANC-1 cells were transfected with Snail, Slug, or control siRNAs according to the manufacturer's instruction. At 72 h after the transfection, cells were stimulated with 10 ng/ml TGF-β for 3 days and, then, cell lysates were subjected to western blot analysis with specific antibodies for E-cadherin and β-actin. (B) The density of the detected bands (E-cadherin and β-actin) was quantified in a Chemi Doc XRS-J, and the ratio (E-cadherin/β-actin) was calculated and presented below the western blot panel.

Smads or non-Smad pathways on down-regulation of E-cadherin induced by TGF-β in PANC-1 cells. As shown in Fig. 6A, Smad2-knockdown and Smad3-knockdown PANC-1 cells showed reduced expression of Smad2 and Smad3. Smad3, but not Smad2, deficiency attenuated down-regulation of E-cadherin induced by TGF-β in PANC-1 cells. In addition, an ERK inhibitor PD98059 and a p38 inhibitor SB203580 marginally suppressed down-regulation of E-cadherin induced by TGF-β in PANC-1 cells (Fig. 6B). In contrast, a JNK inhibitor SP600125 and a PI3K inhibitor LY294002 did not affect down-regulation of E-cadherin induced by TGF-β in PANC-1 cells (Fig. 6B). It was shown that SB203580 (a p38 inhibitor) was not completely specific to p38 and it also inhibited TGF-β signaling (33). Therefore, we examined the effects of SB202190, another selective p38 kinase inhibitor, on the down-regulation of E-cadherin induced by TGF-β in PANC-1 cells. SB202190 did not affect TGF-β-induced decrease of E-cadherin expression (Fig. 6B). These results suggested that Smad3 was also necessary for down-regulation of E-cadherin induced by TGF-β in PANC-1 cells and the ERK pathway was only partially involved in the regulation of E-cadherin.

DISCUSSION

Recent studies suggest that loss of Smad4 may enhance tumourgenicity by mechanisms independent of growth

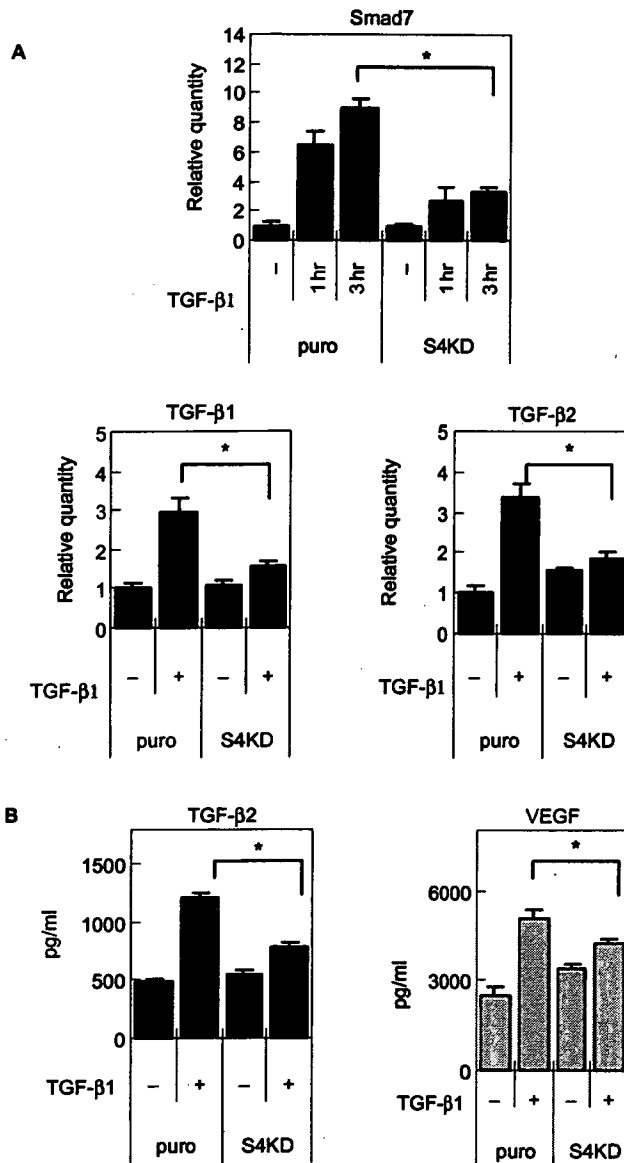


Fig. 5. Smad4 deficiency attenuates TGF-β-induced TGF-β and VEGF production in PANC-1 cells. (A) cDNAs were obtained from PANC-1-puro and PANC-1-S4KD cells after indicated hours or 12h stimulation with 10 ng/ml TGF-β1 and, then, real-time PCR was performed using specific primers for Smad7, TGF-β1, TGF-β2 and GAPDH. The ratio of each gene to that of GAPDH was calculated, and the value of 1.0 was assigned to the PANC-1 wild-type cells that were incubated without TGF-β1. (B) Culture supernatants were obtained from PANC-1-puro and PANC-1-S4KD cells after 48h stimulation with 10 ng/ml TGF-β1 and, then, concentrations of TGF-β2 and VEGF were measured by ELISA. Values represent the mean ± SD of triplicate samples per group. *P < 0.05 compared with corresponding control.

inhibition by TGF-β. Indeed, loss of Smad4 expression occurs late in the development of pancreatic cancer (34) and may be associated with invasive and metastatic capabilities of the tumour through various mechanisms (5-8). Our current results, however, suggest that Smad4

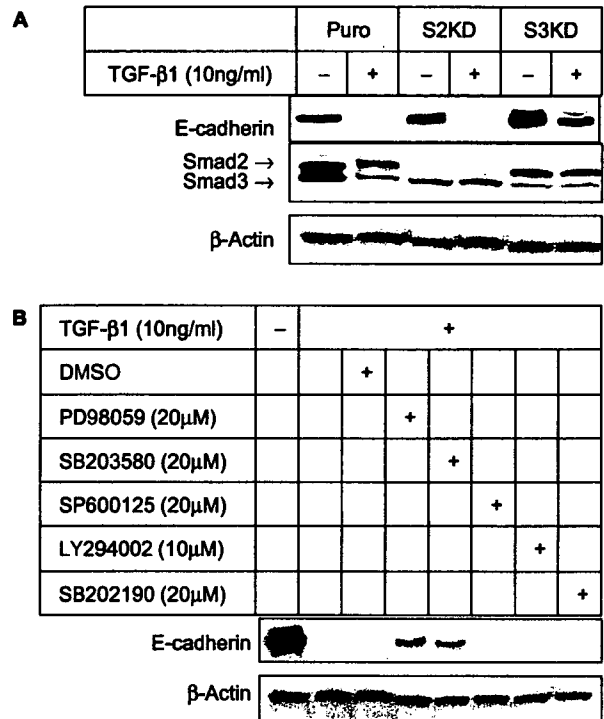


Fig. 6. Smad3, but not Smad2, deficiency attenuates down-regulation of E-cadherin induced by TGF-β in PANC-1 cells. (A) Smad2- and Smad3-knockdown PANC-1 cells (PANC-1-S2KD, PANC-1-S3KD) and control Smad4-intact PANC-1 cells (PANC-1-puro) were established as described in the section 'Materials and Methods'. Cells were stimulated with 10 ng/ml TGF-β for 6 days and, then, cell lysates were subjected to Western blot analysis with specific antibodies for E-cadherin, Smad2/3, and β-actin. (B) PANC-1-S4KD or PANC-1-puro cells were stimulated with 10 ng/ml TGF-β in the presence or absence of indicated concentrations of several inhibitors (PD98059, SB203580, SP600125, LY294002, SB212190) for 6 days and, then, cell lysates were subjected to western blot analysis with specific antibodies for E-cadherin and β-actin. Similar results were obtained at least three independent experiments.

is essential for TGF-β-induced down-regulation of E-cadherin in PANC-1 cells, at least in part, through up-regulation of E-cadherin repressors Snail and Slug (Figs 1, 3 and 4). In addition, Smad4 also appears to be required for TGF-β-induced TGF-β and, although partially, for TGF-β-induced VEGF production by PANC-1 cells (Fig. 5). Thus, loss of Smad4 might not be associated with loss of E-cadherin expression and also up-regulation of TGF-β and VEGF production frequently observed in advanced pancreatic cancer (12-14, 35).

We showed that Smad4 deficiency abrogated down-regulation of E-cadherin induced by TGF-β in PANC-1 cells (Fig. 1). However, Muller *et al.* (6) previously reported that Smad4 induced E-cadherin in colon cancer cells, which was in contrast to our results. They showed that re-expression of Smad4 at physiological levels in Smad4-deficient SW480 colon cancer cell line transcriptionally induced E-cadherin and re-established epithelial morphology of the cells although they did not examine expression of Snail family of E-cadherin repressors in the cells. Thus, it is possible that Smad4

deficiency might give different impacts on colon and pancreatic cancer cells.

We showed that Smad4 deficiency abrogated TGF- β -induced TGF- β and VEGF production in PANC-1 cells (Fig. 5). These results were also in contrast to the findings by Subramanian *et al.* (8) and Schwarte-Waldhoff *et al.* (7). They reported that Smad4 deficiency activated endogenous TGF- β signalling and expression, and VEGF production, respectively, in pancreatic cancer cell lines. Different pancreatic cancer cell lines used in their (BxPC-3 cells, Hs766T cells) and our (PANC-1 cells) studies might explain the different outcomes. This issue, however, remains to be determined in future analysis.

We showed that Smad3, but not Smad2, deficiency attenuated down-regulation of E-cadherin induced by TGF- β in PANC-1 cells (Fig. 6A). It is reported that Smad3 null mutant mice are viable and fertile in contrast to Smad2 null mice that show early embryonic lethality, suggesting distinct roles of Smad2 and Smad3 *in vivo* (36). In addition, *in vitro* studies suggest that Smad2 and Smad3 have unique, non-overlapping roles in control of target gene expression by TGF- β (37, 38). Our results also suggest the specific role for Smad2 and Smad3 for TGF- β signalling.

Ellenrieder *et al.* (39) reported TGF- β induced epithelial mesenchymal transition (EMT) through MAPK/ERK pathway in PANC-1 cells. They showed that TGF- β induced a fibroblastoid morphology, up-regulated mesenchymal markers, and down-regulated epithelial markers in PANC-1 cells although they did not examine E-cadherin expression as an epithelial marker. Our results showed that PD98059, an ERK inhibitor used in their study, only marginally affected TGF- β -induced decrease of E-cadherin in PANC-1 cells (Fig. 6B). Therefore, E-cadherin expression might undergo different regulation (i.e. Smad-dependent regulation) by TGF- β from other EMT-associated molecular events.

Recently, Han *et al.* (40) suggest that down-regulation of E-cadherin/catenin complex by TGF- β may not be associated with metastatic capabilities of skin cancers. They showed that chemically induced skin papilloma in double transgenic mouse expressing TGF- β and dominant negative TGF- β type II receptor (DNRII) progressed to metastasis without losing expression of the membrane-associated E-cadherin/catenin complex and at a rate higher than those observed in non-transgenic, TGF- β 1-transgenic, or DNRII-transgenic mice. Thus, mechanisms independent of E-cadherin loss might be also involved in invasive and metastatic capabilities of pancreatic cancer.

In summary, we demonstrated that Smad4 was essential for down-regulation of E-cadherin induced by TGF- β in pancreatic cancer cell line PANC-1 and also in other TGF- β -responsive cell lines. Our results suggest that mechanisms independent of loss of E-cadherin might be involved in invasive or metastatic capabilities of advanced pancreatic cancer cells that lack Smad4. However, the *in vitro* system may not be relevant to the *in vivo* situation of pancreatic cancer. Thus, the roles of Smad4 deletion in the invasive/metastatic phenotype of pancreatic cancer should be investigated using the *in vivo* experimental models in future studies.

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Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response

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Viral infections activate cellular expression of type I interferons (IFNs). These responses are partly triggered by RIG-I and mediated by Cardif, TBK1, IKK ϵ and IRF-3. This study analysed the mechanisms of dsRNA-induced IFN responses in various cell lines that supported subgenomic hepatitis C virus (HCV) replication. Transfection of dsRNA into Huh7, HeLa and HEK293 cells induced an IFN expression response as shown by IRF-3 dimerization, whilst these responses were abolished in corresponding cell lines that expressed HCV replicons. Similarly, RIG-I-dependent activation of the IFN-stimulated response element (ISRE) was significantly suppressed by cells expressing the HCV replicon and restored in replicon-eliminated cells. Overexpression analyses of individual HCV non-structural proteins revealed that NS4B, as well as NS34A, significantly inhibited RIG-I-triggered ISRE activation. Taken together, HCV replication and protein expression substantially blocked the dsRNA-triggered, RIG-I-mediated IFN expression response and this blockade was partly mediated by HCV NS4B, as well as NS34A. These mechanisms may contribute to the clinical persistence of HCV infection and could constitute a novel antiviral therapeutic target.

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INTRODUCTION

Type I interferon (IFN) plays a central role in eliminating virus, not only following clinical therapeutic application but also as a cellular immune response (Samuel, 2001; Taniguchi & Takaoka, 2002). Hepatitis C virus (HCV) infection is characterized by persistence and replication of the virus in the liver, despite an intact host immune system (Alter, 1997). Indeed, even after administration of the currently most potent IFN reagents, as many as half of the patients are refractory to the treatment and fail to eradicate the virus (Fried *et al.*, 2002). These features have led to speculation that HCV escapes from or attenuates the host antiviral response (Katze *et al.*, 2002).

Cellular antiviral responses are primarily mediated by IFN and IFN-stimulated genes (ISGs), including 2,5-oligoadenylate synthetase, dsRNA-dependent protein kinase R (PKR) and MxA proteins, as well as by as yet uncharacterized genes (Itsui *et al.*, 2006; Stark *et al.*, 1998). A study of experimental chimpanzee HCV infection has shown that various cytokines and chemokines are induced in the liver during the course of acute HCV infection and its clearance, and that a considerable proportion of the genes is induced by type I IFN (Bigger *et al.*, 2001).

Control of expression of ISGs is mediated by binding of type I IFNs to their receptors. Following receptor binding, STAT1 and STAT2 are phosphorylated to form ISGF-3, which translocates to the nucleus and binds the IFN-stimulated response element (ISRE), located in the promoter/enhancer region of ISGs, and activates transcription of ISGs (Samuel,

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2001; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002). ISRE-dependent gene expression is also mediated by binding of the ISRE by molecules such as IRF-1, IRF-3 and IRF-7 (Kanazawa *et al.*, 2004). IRF-3 is a transducer of virus-mediated signalling and plays a critical role in the induction of cellular antiviral responses (Lin *et al.*, 1998; Sato *et al.*, 2000; Taniguchi *et al.*, 2001; Yoneyama *et al.*, 1998). Transcriptional activation and suppression of IRF-3 are inversely correlated with the level of HCV replication *in vitro* (Yamashiro *et al.*, 2006). Following virus infection, IRF-3 is phosphorylated by two cytoplasmic kinases, TBK1 and IKK ϵ (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus and predominantly activates expression of the IFN- β gene and certain ISGs (Doyle *et al.*, 2002; Nakaya *et al.*, 2001; Taniguchi & Takaoka, 2002).

RIG-I is a recently identified cytoplasmic DExD/H box RNA helicase that participates in recognition of virus-related dsRNA as a pathogen-related molecular pattern (Yoneyama *et al.*, 2005). RIG-I contains two caspase-recruitment domains (CARDs) in the N terminus and a DExD/H box RNA helicase in the C terminus. MDA5 has been identified as another CARD-containing DExD/H box RNA helicase (Andrejeva *et al.*, 2004). More recently, an adaptor molecule of RIG-I and MDA5, Cardif (also known as IPS-I, MAVS and VISA), has been identified by four independent groups (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). On association with dsRNA, RIG-I or MDA5 causes conformational changes and homo-oligomerization, and binds the CARD of Cardif (Saito *et al.*, 2007). Cardif subsequently recruits the kinases TBK1 and IKK ϵ , which catalyse phosphorylation and activation of IRF-3 (Yoneyama *et al.*, 1998).

The IRF-3-mediated IFN- β induction pathway could be a target for viruses to counteract antiviral responses and promote their replication in host cells. Ebola virus, bovine viral diarrhoea virus (BVDV) and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins (Basler *et al.*, 2003; Schweizer & Peterhans, 2001; Talon *et al.*, 2000). There are several reports that HCV proteins interact with IFN-mediated antiviral systems. The NS5A and E2 proteins have been reported to interfere with the action of IFN by inhibiting the activity of PKR (He & Katze, 2002). It was reported recently that the HCV NS3A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif (Foy *et al.*, 2003; Meylan *et al.*, 2005).

The HCV subgenomic replicon is an *in vitro* model that simulates autonomous cellular replication of HCV genomic RNA (Lohmann *et al.*, 1999). Expression of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs (Blight *et al.*, 2000; Frese *et al.*, 2002; Guo *et al.*, 2001), suggesting intact IFN receptor-mediated cellular responses. In contrast, viral expression persists in the absence of the exogenous IFN. Baseline expression levels of ISG were substantially decreased in cells

expressing the HCV replicon compared with parental Huh7 cells (Kanazawa *et al.*, 2004). These findings led us to speculate that intracellular virus-induced antiviral responses are attenuated or caused to malfunction by the expression of viral proteins.

In this study, we investigated cell lines that support subgenomic HCV replication and HCV cell culture for the dsRNA-induced cellular IFN expression pathway. Here, we report that RIG-I- and Cardif-mediated IFN gene activation is uniformly attenuated in several replicon-expressing cell lines of different lineages and, more importantly, that the HCV NS4B protein is involved in the suppression of antiviral IFN responses.

METHODS

Plasmids. Plasmids pEF-flagRIG-I and Δ RIG-I expressed full-length and C-terminally truncated RIG-I protein, respectively (Yoneyama *et al.*, 2004). The plasmid pER-flagRIG-IKA (RIG-IKA) has a point mutation in the putative ATP-binding site of the RIG-I helicase domain and was used as a negative control for Δ RIG-I and RIG-I full transfection assays. Expression plasmids for full-length Cardif (Cardif), Cardif CARD (CARD) and CARD-truncated Cardif (Δ CARD) were provided by Dr J. Tschopp (University of Lausanne, Switzerland) (Meylan *et al.*, 2005). Expression plasmids for toll-like receptor 3 (TLR3) and TIR domain-containing adaptor inducing IFN- β (TRIF), the transmembrane receptor of dsRNA and the adaptor molecule of TLR3, respectively, were provided by Dr S. Akira (Osaka University, Japan). Plasmids expressing HCV NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B were amplified from HCV pCV-J4-L4S (Yanagi *et al.*, 1997) by PCR and subcloned. The DNA fragments were inserted into the vector pcDNA4/TO/*myc*-His (Invitrogen). Nucleotide sequences were confirmed by sequencing. Plasmids TOPO-NS34A (HCV N), TOPO-NS4B (HCV N) and pcDNA-NS4B (HCV JFH1) expressed Myc-tagged NS34A and NS4B proteins derived from the HCV N (Beard *et al.*, 1999) and HCV JFH1 (Wakita *et al.*, 2005) strains, as indicated. Plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. Plasmid pIFN β -Fluc was constructed by cloning the human IFN- β promoter region, spanning nt -110 to -36, upstream of the firefly luciferase gene of pGL3 Basic (Promega). Plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega), which expressed the *Renilla* luciferase protein, was used for correction of transfection efficiency.

Cell culture. HCV strain JFH1-infected Huh7.5.1, Huh7, Huh7.5.1 (kindly provided by Dr F. Chisari, The Scripps Institute, CA, USA; Zhong *et al.*, 2005), HeLa and HEK293 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C with 5% CO₂. Cells expressing the HCV replicon were cultured in medium containing 100 μ g G418 (Wako) ml⁻¹.

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVbneo-delS (designated pRep-N), was derived from an HCV clone of strain N, genotype 1b, and pSGR-JFH1 was derived from HCV JFH1, genotype 2a (Guo *et al.*, 2001; Wakita *et al.*, 2005). These replicons were reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising *Renilla* luciferase and neomycin phosphotransferase to construct pRep-Reo-1b and pRep-Reo-2a, respectively (Tanabe *et al.*, 2004; Yokota *et al.*, 2003). RNA was synthesized from the replicons using T7 polymerase (Promega) and transfected into Huh7,

HeLa and HEK293 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/1bReo, Huh7/2aReo, HeLa/2aReo and 293/2aReo). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A and NS5A protein expression levels and with the levels of replicon RNA (Yokota *et al.*, 2003).

Transient transfection. Transient DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ISRE reporter assays were carried out as previously described (Nakagawa *et al.*, 2004). To analyse IFN expression in HCV JFH1 cell cultures, a total of 1×10^5 Huh7.5.1, JFH-1 infected Huh7.5.1 and IFN-treated Huh7.5.1 cells were seeded into 24-well plates the day before transfection. Plasmids pISRE-TA-Luc and Δ RIG-I (200 ng each) were transfected using 1 μ l Lipofectamine 2000. RIG-IKA was used as a control. Luciferase assays were performed on day 3 post-transfection.

For further study, 400 ng of each non-structural protein was added to 1×10^4 Huh7 or HEK293 cells that had been seeded into 96-well plates the day before transfection. pISRE-TA-Luc and Δ RIG-I (40 ng each) were transfected using 0.5 μ l Lipofectamine 2000. RIG-IKA was used as a control.

Western blotting. Preparation of the cytoplasmic and nuclear fractions of cell lysates was carried out as described previously (Tanabe *et al.*, 2004). Protein (20 μ g) was separated using NuPAGE 4–12% Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

RT-PCR. Interleukin (IL)-8 mRNA was detected by RT-PCR as described previously (Itsui *et al.*, 2006). The primers used were IL8-S (5'-GCACAACTTTCAGAGACAGCAGAGCACAC-3') and IL8-AS (5'-CAGAGCTGCAGAAATCAGGAAGGCTGCCAA-3').

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-protein disulphide isomerase (PDI) or anti-Myc antibodies and subsequently with Alexa 488- or Alexa 568-labelled secondary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence).

Luciferase reporter assays. Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX; PerkinElmer) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were carried out in triplicate and the results expressed as means \pm SD.

MTS assay. To evaluate cell viabilities, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

IRF-3 dimer formation is attenuated in cells expressing the HCV replicon

In the HCV replicon-expressing cell lines Huh7/Rep-Reo-2a, HeLa/Rep-Reo-2a and 293/Rep-Reo-2a, replicon expression

levels corresponded well to internal *Renilla* luciferase activities. Expression of the HCV replicon was suppressed by IFN in a dose-dependent manner (data not shown).

Activation of RIG-I or MDA5 induces phosphorylation and homodimerization of IRF-3. Following transfection of poly(I:C) into Huh7, HeLa or HEK293 cells, IRF-3 dimers were detected (Fig. 1). However, in cells supporting HCV replicons, IRF-3 dimer formation was almost completely abolished. These findings showed that expression of HCV proteins blocked activation of dsRNA-mediated IFN expression and that these effects were consistently found in several cell lines of different origin.

The HCV replicon suppresses RIG-I/Cardif-induced IFN responses

ISRE reporter activities did not increase in naïve Huh7, HeLa or HEK293 cells following transfection of poly(I:C), whilst overexpression of full-length RIG-I increased poly(I:C)-mediated ISRE reporter activity in Huh7 and HEK293 cells (data not shown). In RIG-I-overexpressing Huh7 cells, transduction with an HCV replicon abolished the poly(I:C)-induced ISRE activation, and elimination of the replicon by IFN treatment restored these ISRE responses (Fig. 2a). Consistent results were obtained by overexpression of Δ RIG-I, a constitutively active form. Transfection of Δ RIG-I in Huh7 and HEK293 cells induced ISRE activation, whilst these responses were abolished or significantly suppressed in cell lines expressing HCV replicons and were recovered by eliminating the replicon by IFN treatment (data not shown). Similarly, ISRE activation by overexpression of Cardif, an adaptor molecule of RIG-I, was almost completely blocked in replicon-expressing cells and was recovered by eliminating the replicon from the cells (data not shown). The RIG-I-mediated IFN response was

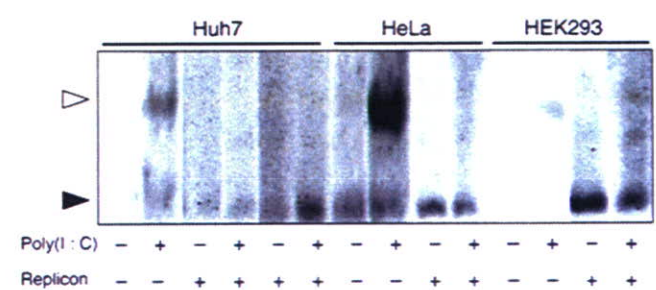


Fig. 1. Double-stranded RNA-induced IRF-3 dimer formation in cell lines that support HCV subgenomic replication. Poly(I:C) was transfected into naïve Huh7, HeLa and HEK293 cells, and into corresponding cell lines expressing the HCV replicon. Six hours after transfection, cell lysates were prepared, separated in polyacrylamide gels and blotted onto PVDF membrane. The membrane was immunoblotted with anti-IRF-3 and visualized by chemiluminescence (see Methods). The positions of the IRF-3 dimer (open arrowhead) and monomer (closed arrowhead) are indicated.

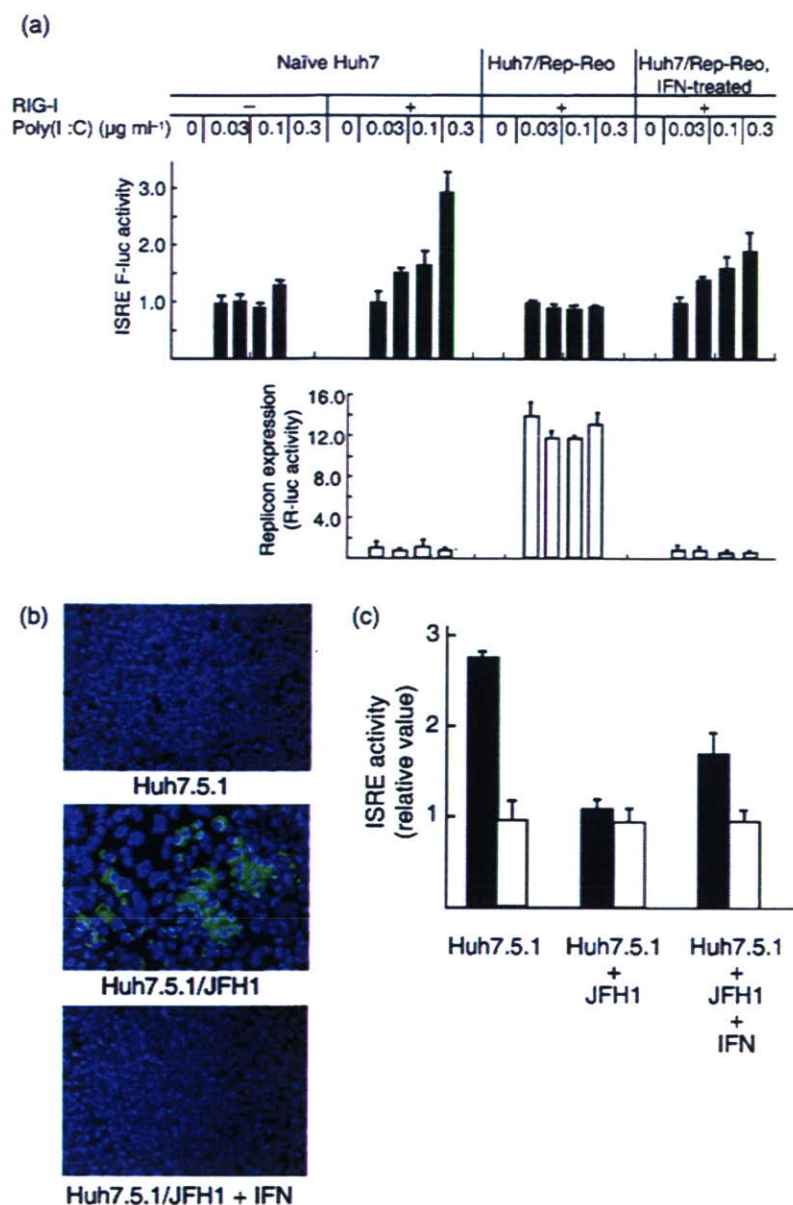


Fig. 2. Suppression of dsRNA-induced, RIG-I-mediated ISRE activation by HCV replication. (a) The HCV replicon suppresses transcriptional activation after poly(I:C) stimulation. The RIG-I expression plasmid and pISRE-TA-Luc were transiently transfected into the cell lines indicated. The following day, the amounts of poly(I:C) indicated were transfected into the corresponding cell lines and dual luciferase assays were carried out 8 h after transfection. Filled bars indicate ISRE-regulated firefly luciferase (F-luc) activities and open bars indicate *Renilla* luciferase (R-luc) activities representing replicon expression levels. In both graphs, scales for the y-axis are shown as relative values. Assays were carried out in triplicate and results are shown as means \pm SD. (b) Immunofluorescence microscopy results. Huh7.5.1 cells infected with HCV JFH1 (Huh7.5.1/JFH1) and JFH1-infected cells from which the virus had been eliminated by IFN treatment (Huh7.5.1/JFH1 + IFN) were incubated with anti-core primary antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). (c) ISRE activation by Δ RIG-I overexpression. The plasmid pISRE-TA-Luc was co-transfected with Δ RIG-I (filled bars) or RIG-I-KA (empty bars) into naive Huh7.5.1, Huh7.5.1/JFH1 or Huh7.5.1/JFH1 + IFN cells. Luciferase assays were carried out 8 h after transfection. The y-axis indicates ISRE-regulated luciferase activity shown as relative values. Assays were carried out in triplicate and results are shown as means \pm SD.

also suppressed in HCV JFH1 virus cell culture. In JFH1-infected Huh7.5.1 cells, Δ RIG-I-induced ISRE reporter activation was significantly suppressed, but was recovered in IFN-treated, virus-eliminated cells (Fig. 2b and c). These results demonstrated that RIG-I- and Cardif-mediated antiviral responses were substantially suppressed by both subgenomic and genomic viral replication in both hepatocyte- and non-hepatocyte-derived host cells.

NS34A and NS4B are responsible for suppressing RIG-I-mediated IFN responses

We next sought to define which HCV proteins were responsible for inhibition of the RIG-I- and IRF-3-mediated IFN induction pathway. We constructed expression plasmids that expressed the non-structural proteins

NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B (Fig. 3a). We transfected each expression plasmid with simultaneous activation of the RIG-I pathway by overexpression of Δ RIG-I, Cardif, TBK1 and IKK ϵ (Fig. 3b–e). Expression of full-length non-structural (NS345) and NS34A proteins inhibited ISRE activation mediated by expression of RIG-I and Cardif but not that mediated by TBK1 and IKK ϵ . Interestingly, it was found that NS4B also inhibited ISRE activation mediated by expression of RIG-I and Cardif, but not by TBK1 and IKK ϵ . Consistent with Fig. 3(b), overexpression of NS4B significantly suppressed Δ RIG-I-induced activation of the authentic IFN- β promoter (Fig. 3f).

Another group has studied IFN antagonism of flavivirus non-structural proteins and has reported that HCV NS4B did not affect IFN responses (Muñoz-Jordán *et al.*, 2005).

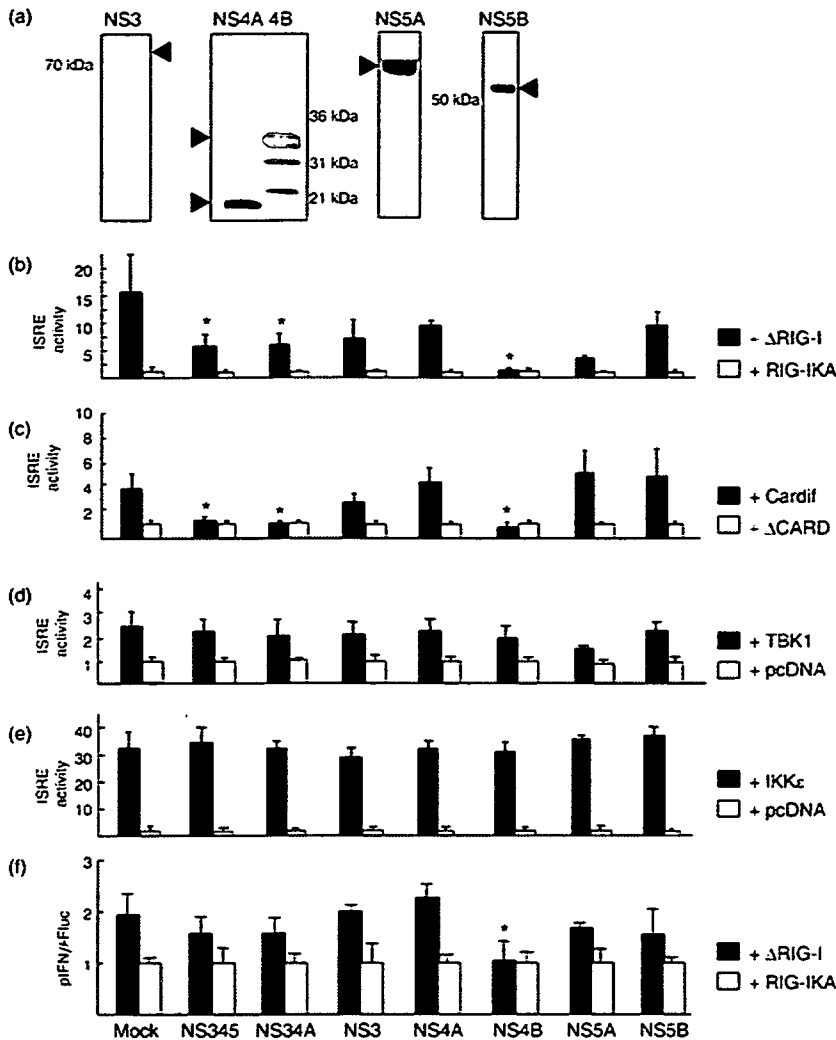


Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, the indicated plasmids expressing Δ RIG-I (b), Cardif (c), TBK1 (d) and IKK ϵ (e), and the indicated plasmids expressing individual HCV non-structural proteins. Plasmids RIG-IKA, Δ CARD or pcDNA were used as negative controls as indicated. Twenty-four hours after transfection, luciferase activities were measured. The y-axis shows relative values. Assays were carried out in triplicate and results are given as means \pm SD. *, $P < 0.05$. (f) pIFN- β and pRL-CMV were co-transfected into Huh7 cells, with plasmids expressing individual HCV non-structural proteins and plasmid expressing Δ RIG-I. Luciferase activities were measured 24 h after transfection. The y-axis shows relative values. Assays were carried out in triplicate and results are given as means \pm SD. *, $P < 0.05$. Plasmid RIG-IKA was used as a negative control.

To investigate strain-specific differences in the characteristics of NS4B proteins, we performed co-transfection assays using NS4B expression constructs from HCV N (Beard *et al.*, 1999) and JFH1 (Wakita *et al.*, 2005) strains, as well as HCV strain J4 (Fig. 4a and b). All NS4B constructs suppressed Δ RIG-I- or Cardif-mediated ISRE activation. These results suggested that the above-described effects of NS4B were independent of HCV strain.

NS4B has been reported to induce an unfolded protein response or endoplasmic reticulum (ER) stress through ATF6 or IRE1-X box protein (XBP1) pathways (Zheng *et al.*, 2005). The ER stress induces production of IL-8, which has been reported to interfere with the IFN system (Polyak *et al.*, 2001). Therefore, we detected expression of IL-8 using RT-PCR in cells with and without overexpression of NS4B. As shown in Fig. 4(c), no significant difference was observed in IL-8 mRNA levels among mock-, NS34A- and NS4B-transfected cells. These results showed that NS4B overexpression in the present study did not induce expression of IL-8 and that the IFN-antagonizing effects of NS4B were independent of IL-8.

It has been reported that NS34A suppresses the TLR3-mediated IFN response (Breiman *et al.*, 2005; Ferreon *et al.*, 2005). However, overexpression of HCV non-structural proteins did not suppress ISRE activation that was induced by overexpression of TLR-3 or TRIF (Fig. 5a and b), nor did NS34A from two different HCV strains, J4 and N, show significant suppression of TRIF-mediated ISRE activation (Fig. 5c). Although strain-specific differences might be involved, these data suggest that neither NS34A nor NS4B affect the TLR3-triggered, TRIF-mediated IFN expression signalling pathway.

The NS4B N terminus is involved in inhibition of the RIG-I-mediated pathway

Given the result that NS4B suppressed the RIG-I-mediated IFN expression pathway, we next investigated which domain of NS4B was responsible. We constructed plasmids that expressed truncated NS4B in which the protein-coding frame was truncated at four positions corresponding to the five transmembrane domains (Lundin *et al.*, 2003) (Fig. 6a).

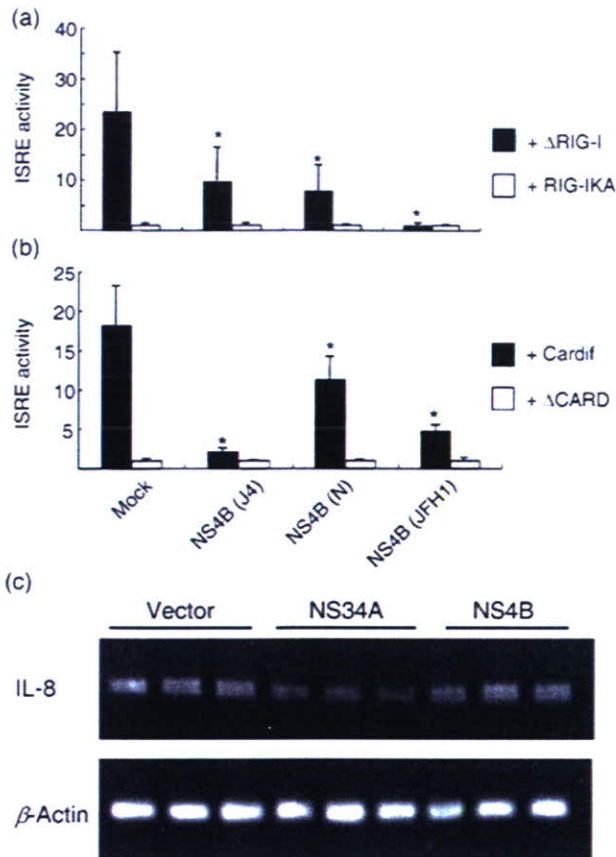


Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing Δ RIG-I (a) or Cardif (b). Luciferase activities were measured 24 h after transfection. The y-axis shows relative values. Assays were carried out in triplicate and results are given as means \pm SD. *, $P < 0.05$. (c) Semi-quantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from Huh7 cells transfected with empty vector or with NS34A or NS4B expression plasmid.

Expression and subcellular localization of NS4B truncated proteins were visualized by indirect immunofluorescence assays (Fig. 7). Each of the NS4B truncated proteins was localized predominantly to the perinuclear rim as dense spots. Some of the spots were similar to the staining of the ER-resident host protein PDI, consistent with previous reports (Lindström *et al.*, 2006; Lundin *et al.*, 2006). These truncated expression plasmids were co-transfected with Cardif expression plasmids into Huh7 cells. As shown in Fig. 6(b), Cardif-mediated ISRE activation was significantly suppressed by co-transfection of NS4Bt1–156 and NS4Bt1–186, as well as full-length NS4B, whilst transfection of NS4Bt90–260 and NS4Bt110–260 did not significantly suppress Cardif-mediated ISRE activation. The shortest construct, NS4Bt131–260, partially retained the ability to reduce ISRE activity. These results suggested that the

N-terminal domain of NS4B, which includes aa 1–110, might function directly to suppress RIG-I-mediated IFN expression responses.

DISCUSSION

The recent discovery of cytoplasmic dsRNA sensor molecules has resulted in rapid expansion of knowledge about the IFN-mediated virus defence pathway (Yoneyama *et al.*, 2004). Several reports suggest that viruses target the IFN system to establish replication in the host cells (Kato *et al.*, 2006). We have confirmed that the dsRNA-triggered, IRF-3-mediated IFN activation pathway was blocked in several replicon-supporting cell lines (Fig. 1). Similarly, the dsRNA responses were substantially suppressed in HCV JFH-1 cell culture compared with parental Huh7 cells (Fig. 2b and c). Overexpression analyses showed that RIG-I- and Cardif-mediated ISRE activation was significantly suppressed in HCV replicon-expressing cells, which recovered after elimination of the replicon by IFN treatment (Fig. 2a). In contrast, TBK1- or IKK ϵ -mediated ISRE activation was not suppressed in replicon-expressing cells. Overexpression of individual HCV non-structural proteins revealed that not only NS34A but also NS4B inhibited the ISRE activation signal (Figs 3, 4 and 5). These results suggested that HCV non-structural proteins suppress the IFN induction pathway and that the target host molecule could be Cardif or an unknown adaptor molecule acting between Cardif and TBK1/IKK ϵ .

NS4B protein is a 27 kDa hydrophobic integral membrane protein that is localized in the ER with other non-structural proteins. Studies on other flaviviruses such as Kunjin virus and BVDV support the notion that NS4B may indeed be an essential part of the replication mechanism (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000; Li & McNally, 2001; Qu *et al.*, 2001). These systems have demonstrated that intact NS4B is necessary in a *cis* configuration in the polyprotein for maintaining viral replication (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000). Furthermore, single mutations in NS4B alter the cytopathic effects of BVDV and even mediate changes in the cellular tropism of Dengue virus (Hanley *et al.*, 2003; Qu *et al.*, 2001). In HCV, the search for cell-culture-adaptive mutations in HCV subgenomic replicons has led to the generation of mutations in the NS4B region that confer higher replication levels and resistance to IFNs, as well as broadening the tropism for different cell lines (Lohmann *et al.*, 2003; Sumpter *et al.*, 2004; Zhu *et al.*, 2003). These pieces of evidence may imply that NS4B is not only part of the replication machinery but may also have other functions that enable establishment of viral replication.

NS4B truncation assays showed that RIG-I/Cardif-mediated ISRE activation was significantly suppressed by expression of N terminus-containing constructs (Fig. 6). These results imply that the N-terminal domain of NS4B, which is located between positions 1 and 110, may be essential for suppressing IFN expression responses in host

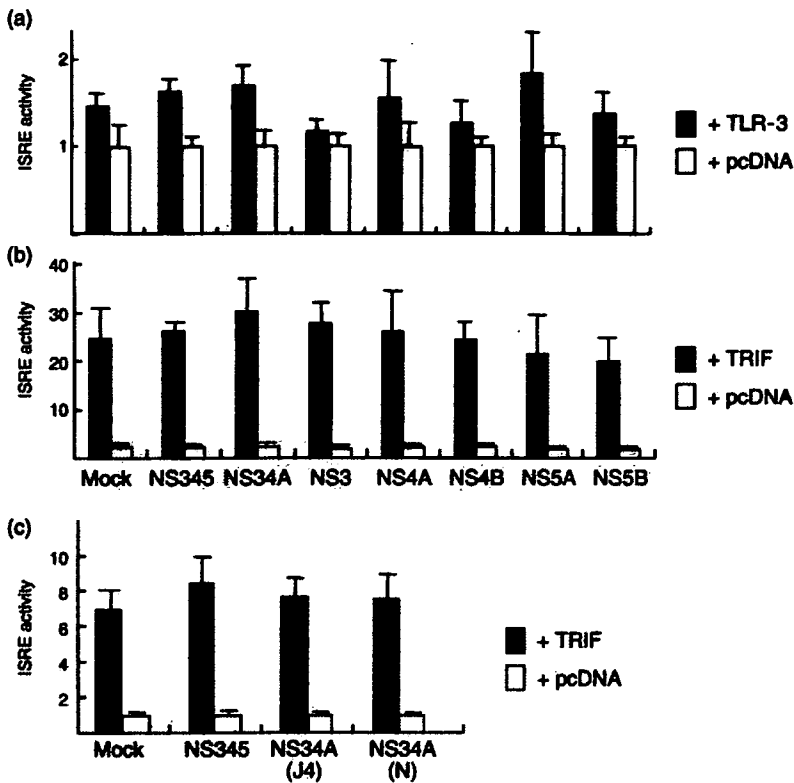


Fig. 5. Co-transfection analyses of HCV non-structural proteins and plasmid expressing TLR-3 or TRIF. (a, b). The following plasmids were co-transfected into Huh7 cells: pSRE-TA-Luc, pRL-CMV, plasmids expressing TLR-3 or TRIF and plasmids expressing individual HCV non-structural proteins, as indicated. Luciferase activities were measured 24 h after transfection. The y-axis shows relative values. Assays were carried out in triplicate and results are given as means \pm SD. (c) NS34A (J4) and NS34A (N) denote plasmids expressing HCV NS34A derived from HCV strains J4 and N, respectively. The NS4B plasmids indicated were co-transfected with pSRE-TA-Luc, pRL-CMV and plasmids expressing TRIF or pcDNA. Luciferase activities were measured 24 h after transfection. The y-axis shows relative values. Assays were carried out in triplicate and results are given as means \pm SD.

cells. Lindström *et al.*, (2006) investigated single point mutations in NS4B that negatively affected expression efficiency of the HCV replicon and reported that most of

the active mutations were located around the N-terminal domain. A distinctive feature of NS4B is that it requires membrane rearrangement to form its native structure

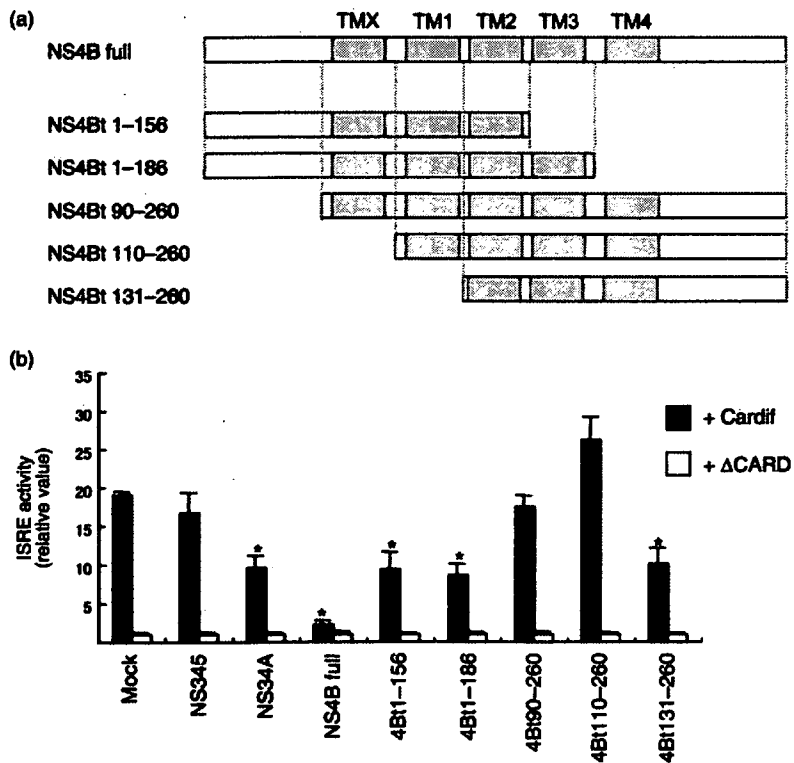


Fig. 6. Co-transfection analyses using truncated NS4B expression constructs. (a) Truncated constructs of NS4B. The protein-coding frame of NS4B was truncated in five constructs corresponding to the five transmembrane domains, as reported by Lundin *et al.* (2003). (b) The truncated NS4B plasmids, pSRE-TA-Luc and the Cardif- or ΔCARD-expressing plasmids indicated were co-transfected into Huh7 cells. Luciferase activities were measured 24 h after transfection. Results are given as means \pm SD.

(Lindström *et al.*, 2006; Lundin *et al.*, 2006). The HCV polyprotein is translated from a single reading frame and subjected to proteolytic cleavage by the host signal peptidase and two viral proteases (Grakoui *et al.*, 1993). The mature form of NS4B is localized in the ER and constitutes a subcellular structure called the membrane-associated focus (MAF) (Gretton *et al.*, 2005). Once the NS4B is cleaved, the N-terminal peptide of NS4B is translocated from the cytoplasmic to the luminal side, giving it a fifth transmembrane region (Lundin *et al.*, 2006). The N-terminal amphipathic helix (AH) 1 of NS4B

is necessary for this translocation and for MAF formation; NS4B molecules that were truncated at the AH1 lacked the ability to create the MAF, to translocate and to replicate (Elazar *et al.*, 2004; Lindström *et al.*, 2006).

In our assay, NS4Bt131–260 regained the ability to reduce ISRE activity. As we confirmed that all mutants co-localized with the ER, there may be some effect of the N-terminal localization of this mutant. The precise mechanism of NS4B suppression is still not clear and further experiments are needed.

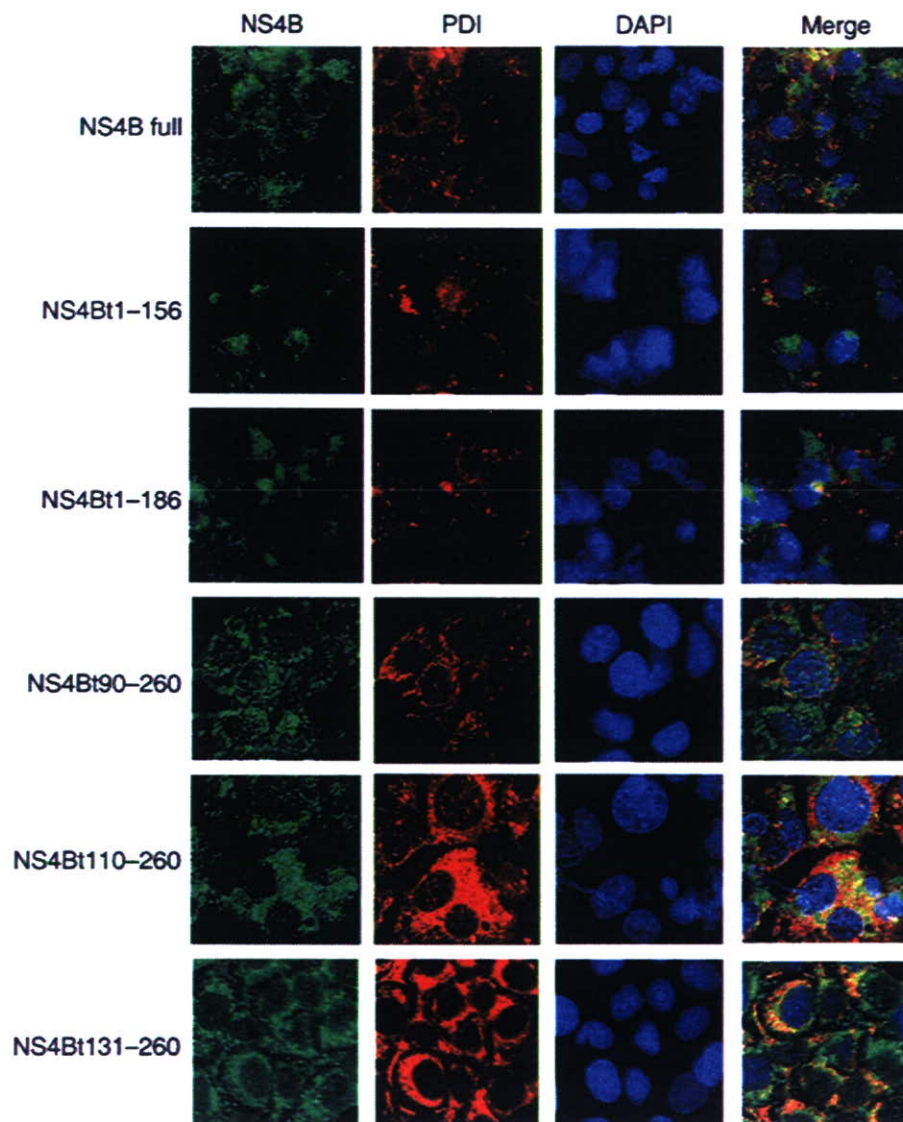


Fig. 7. Indirect immunofluorescence analysis of truncated NS4B proteins. The NS4B constructs indicated were transiently transfected into Huh7 cells. After 48 h, cells were labelled with anti-Myc or anti-PDI antibody. NS4B proteins were immunostained with Alexa Fluor 488-labelled goat anti-mouse IgG, whilst PDI was stained with Alexa Fluor 568-labelled goat anti-rabbit IgG. DAPI staining revealed the nuclear chromatin. Representative immunofluorescence images derived from a number of experiments are shown as four images of a single focal plane of Huh7 cells, showing NS4B proteins (green), PDI (red), DAPI staining (blue) and the superimposed images (merge).

NS4B has been reported to induce an unfolded protein response or ER stress (Zheng *et al.*, 2005). Accumulation of unfolded or misfolded proteins in the ER is detected by three ER sensor proteins, ATF6, IRE-1 and PERK-like ER kinase (PERK), and triggers the unfolded protein response as a stress response and induces expression of molecular chaperon proteins, global shut-off of protein translation and apoptotic cell death (Mori, 2000). Therefore, it may be possible that transgenic overexpression of NS4B induces ER stress and suppresses overall protein synthesis, including that of IFNs. In our experiments, however, NS4B suppressed the ISRE-mediated IFN gene activation but did not suppress non-specific protein expression, as demonstrated by *Renilla* luciferase activity in the control plasmid driven by the herpes simplex thymidine kinase promoter. In addition, the growth and viability of cells that overexpressed NS4B did not differ from untransfected cells or from those transfected with the other HCV proteins. IL-8 overproduction induced by ER stress was not observed in our NS4B-overexpressing cells (Fig. 4c). These findings indicated that the inhibitory effect of NS4B is specific to the IFN induction pathway and is not a non-specific effect through ER stress.

In conclusion, we have shown that dsRNA-induced IFN expression was suppressed by NS4B. These virus–host interactions probably contribute to HCV persistence and to the pathogenesis of HCV-associated liver disease.

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