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BST2/Tetherin inhibits hepatitis C virus production in human hepatoma cells

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ABSTRACT

Hepatitis C virus (HCV) infection is a common cause of chronic hepatitis and is currently treated with alpha interferon (IFN-α)-based therapies. IFN-induced cell membrane protein BST2 (also known as CD317, HM1.24 or tetherin) has been reported to tether a broad range of lipid-enveloped viruses on cell surfaces. However, whether HCV is sensitive to BST2 remains controversial. Here we established a Huh7.5-BST2-TO cell line, in which BST2 expression is regulated by tetracycline. Our results showed that the effect of BST2 on inhibiting HCV production was dependent on its expression level. Highly expressed BST2 reduced the yield of cell-free HCV virions but did not affect the efficiency of HCV infection and genome replication. Co-localization of HCV core protein and BST2 was detected by immunofluorescence in certain cells with high expression, but not in cells with low BST2 expression. Furthermore, inhibition of IFN-α induced BST2 expression in Huh7.5 cells by siRNA technology slightly reduced the antiviral response of the cytokine against HCV, but only at low IFN-α concentration. While overexpression of BST2 inhibited HCV replication in this system, BST2 is therefore not likely to be a major contributor to the antiviral effect of IFN-α.

1. Introduction

Hepatitis C virus (HCV) is an enveloped positive strand RNA virus and is a member of the genus Hepacivirus in the family of *Flaviviridae* (Lindenbach and Rice, 2005). The interferon (IFN) family of cytokines is as key component of the innate immune response against HCV infection. It has been demonstrated previously by our group and others that the expression of IFN-inducible proteins, such as viperin, ISG20, ISG56, ADAR 1 and GBP1, can inhibit HCV replicon replication and thus might modulate an IFN response against HCV (Hayashi et al., 2005; Taylor et al., 2005; Chang et al., 2006; Helbig et al., 2005; Marcello et al., 2006; Itsui et al., 2009; Jiang et al., 2008; Zhou et al., 2011). However, the role of ISG15 in regulating HCV infection remains controversial. Kim et al. demonstrated that HCV replication is inhibited by overexpressing ISG15 in the HCV subgenomic replicon cells (Kim et al., 2008). However, several other studies reported that ISG15 decreased phenotypic sensitivity to IFN-alpha treatment as a regulator of the IFN response (Chua et al., 2009; Broering et al., 2010). More recently, using a RNA interference-based "gain of function" screen, several new ISGs with proven antiviral activity have been indentified in sub-genome HCV replicon system (Metz et al., 2012). However, the HCV replicon does not permit analysis of ISG against the complete viral life cycle, such as viral entry, assemble and release.

BST2, also known as CD317, HM1.24 or tetherin, is a type I IFN-induced cell membrane protein that potently inhibits the release of many enveloped viruses. These include all retroviruses tested as well as members from five other families, such as *Filoviridae* (Ebola and Marburg viruses), *Arenaviridae* (Lassa fever virus), *Herpesviridase* (Kaposi's sarcoma-associated herpesvirus), *Paramyxoviridae* (Sendai virus and Nipah virus), and *Rabdoviridae* (vesicular stomatitis virus) (Jouvenet et al., 2009; Neil et al., 2008; Sakuma et al., 2009; Weidner et al., 2010; Kong et al., 2012). BST2 tethers budding virions on the cell surface, which are subsequently endocytosed and degraded in the lysosomes (Neil et al., 2008; Yang et al., 2010). Our previous observation demonstrated that expression of BST2 did not inhibit HCV replicon replication in HEK293 cells (Jiang et al., 2008). In this study, we now report that expression of BST2 in Huh7.5 cells is able to reduce the yield of virion production in HCV cell culture system.

2. Materials and methods

2.1. Cells and virus production

Huh7.5 and HEK293T cells were maintained in Dulbecco's modified minimal essential medium (DMEM, Invitrogen, Carlsbad, CA)

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supplemented with 10% fetal bovine serum (Blight et al., 2002). Plasmid containing the full-length HCV Jc1 cDNA was cloned from chemically synthesized DNA oligomers (GenScript, Piscataway, NJ) (Pietschmann et al., 2006). HCV RNA was transcribed *in vitro* with the MEGAscript kit (Ambion, Austin, TX) and electroporated into Huh7.5 cells to produce infectious HCV (Qu et al., 2011). The virus yield ($TCID_{50}$/ml) was determined as previously described (Guo et al., 2001). For production of high titer virus stock, the infected Huh7.5 cells were cultured in DMEM-containing 2% FBS between 7–9 days postinfection. The pool of supernatant was cleared of cells by passage through a 0.45-mm-pore-size filter, concentrated 20-fold by using Amicon Ultra-15 centrifugal filter Units (10,000 NMWL, Millipore, Billerica, MA). The infectivity of HCV ($TCID_{50}$/ml) was determined and adjusted to 5×10^5 TCID50 per milliliter. HCV pseudoparticles (HCVpp) were produced by using Viral Safe Lentiviral Packaging System (Cell Biolabs, San Diego, CA) as previous reported (Bartosch et al., 2003). The cDNA sequence encoding the HCV genotype 2a strain JFH1 aa 132–746 representing the C terminus of core, E1 and E2 were cloned into the expression vector pCAGEN (Addgene, Cambridge, MA) A packageable construct pLenti-Luc (Cell Biolabs) containing a luciferase gene under control of the cytomegalovirus (CMV) promoter was used as reporter. HEK293T cells were transfected with the plasmids of Lentiviral Packaging System to produce the HCVpp as manual. Cell lines were infected with HCVpp (MO = 10) absence of any entry enhancer (e.g. polybrene). Seventy-two hours postinfection, cells were lysed in 1× passive lysis buffer (Promega, Fitchburg, WI) and luciferase activity was monitored by the Renilla luciferase assay system (Promega).

2.2. Plasmid construction and establishment of cell lines

In a Tet-Off system, expression of genes is controlled by tetracycline (Tet) response element (TRE) and can be repressed by Tet and its derivatives in a dose–response manner. Here we used the Tet-Off system to regulate the differential expression of BST2 in Huh7.5 cells. To construct the BST2 expression vector, BST2 cDNA was released from plasmid pcDNA5/RFT/BST2 (Jiang et al., 2010) and inserted into pTRE2 vector (Clontech, Heidelberg, Germany) digested with the same restriction enzymes BamH I and Not I. To construct plasmid pcDNA5/RFT/BST2CV5, BST2 cDNA was amplified from pcDNA5/RFT/BST2 with a pair of primers (Forward: 5′-GAGCTTAAGATGGCATCTACTTCGTATGACTA-3′. Reverse: 5′-CACGCGGCCGCCCTG CAGCAGAGCGCTGAGGCCC-3′). The PCR product was digested by Afl II and Not I and ligated with vector fragment recovered from Afl II and Not I restricted pcDNA5/RFT/Viperin-CV5 (Jiang et al., 2010). To establish stable Tet-Off expressing Huh7.5 cell lines (Huh7.5-BST2-TO) and carboxyl-terminally V5 tagged BST2 cells (Huh7.5-BST5cv5), Huh7.5 cells were co-transfected with plasmids pTRE-BST2 and pTet-off (Clontech) or pCDNA5/FRT/BST2CV5 and pcDNA3 at a molar ratio of 9 to 1 and selected with 250 μg/ml G418. G418-resistant cell clones that express BST2 or BST2V5 were detected the respective proteins in cell lysates using Western blot.

2.3. Indirect immunofluorescence

Cells were fixed with PBS containing 2% paraformaldehyde and penetrated by incubating with PBS containing 0.1% Triton X-100. Cells were then blocked and incubated with mouse monoclonal antibody against HCV NS3 (Clone H23, Abcam, Cambridge, MA), core (C7-50, Abcam), or rabbit polyclonal anti-BST2 antibody (Proteintech, Chicago, IL). Bound primary antibody was visualized by Alexa Fluor 488-conjugated goat anti-mouse IgG or Alesa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen). Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, Invitrogen). HCV infected cell foci were detected with an In-Cell Western assay.

2.4. Western blot assay

Cell monolayers were washed once with phosphate-buffered saline buffer and lysed with 1× Laemmli buffer. A fraction of the cell lysate was separated on sodium dodecyl sulfate 12% polyacrylamide gels and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with blocking buffer (LI-COR Biotechnology, Lincoln, Nebraska) and probed with primary antibodies against BST2 (Proteintech), HCV NS3 (Abcam), and β-actin (Millipore). Secondary antibodies were IRDye 800CW goat anti-Mouse IgG and IRDye 680CW goat anti-Rabbit IgG. Membrane was scanned by Odyssey Infrared Imaging System (LI-COR Biotechnology).

2.5. RNA extraction and Northern blot hybridization

Total cellular RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA was fractionated on a 1.5% agarose gel containing 2.2 M formaldehyde and transferred onto nylon membranes. Membranes were hybridized with riboprobes specific for plus-stranded HCV RNA under the conditions described previously (Guo et al., 2001).

2.6. RNA quantification by qRT-PCR

Supernatant HCV RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed using SuperScript III (Invitrogen). Quantitative PCR (qPCR) reaction was performed on Applied Biosystems 7500 thermocycler using the following probe (6-carboxyfluorescein [FAM]-5′-CCTTGTGG TACTG CCTGA-3′-MGBNFQ [molecular-groove binding nonfluorescence quencher]; Applied Biosystems), forward and reverse primers (5′-AGCGTTGGGTTGCGAAAG-3′ and 5′-CACTCGCAAGCGCCCT-3′, respectively). The standard curve was generated using serial 10-fold dilutions of *in vitro* transcribed full-length HCV RNA (Qu et al., 2011).

2.7. siRNA knockdown

Huh7.5 cells were transfected with SMART*pool* siRNA (100nM final concentration) targeting BST2 or non-targeting siRNA (control) by following manufacturer protocol (Thermo Dharmacon, Lafayette, CO). Six hours post transfection, cells were left untreated or treated with the indicated concentrations of IFN-α for 24 h and followed by infection with HCV at a MOI of 0.05. One set of cells was harvested without infection at 24 h post IFN treatment and the levels of BST2 in the cell lysates were determined by Western blot. Intracellular viral protein expression, RNA replication, and virus yield were determined at two days postinfection.

3. Results

3.1. Establishment of Huh7.5-BST2-TO cell line

Huh7.5 is a human hepatoma (Huh7)-derived cell line that is highly permissive to HCV infection (Blight et al., 2002 and Lindenbach et al., 2005). To test whether BST2 could inhibit the release of HCV virions from infected hepatocytes, we established the Huh7.5-derived stable cell line by which BST2 protein is regulated by Tet (Huh7.5-BST2-TO). As shown in Fig. 1B, BST2 expression was tightly regulated by the tetracycline, and its expression correlated

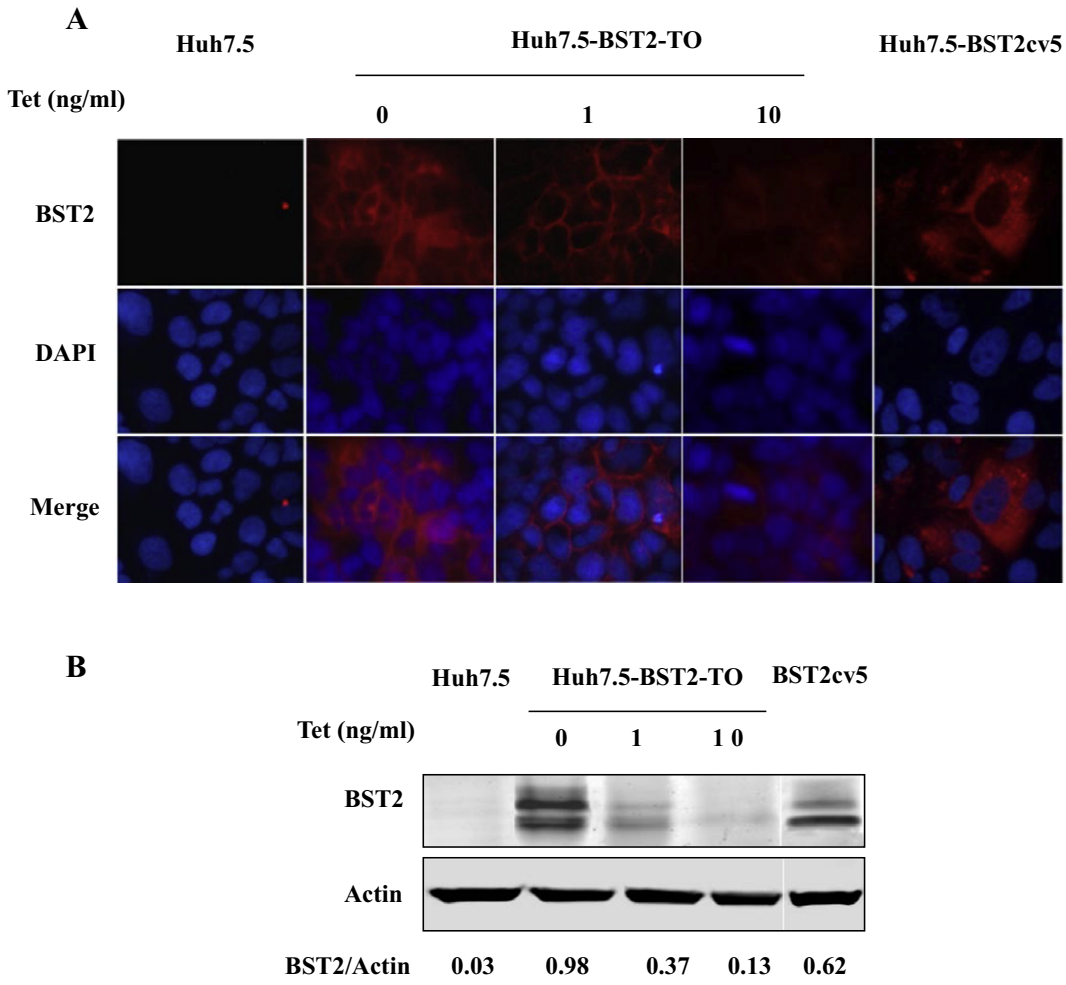

Fig. 1. Characterization of BST2-expressing cell lines. (A) Expression and subcellular localization of BST2 and its variant in cell lines were visualized by indirect immunofluorescence. Huh7.5-BST2-TO cells were cultured in complete DMEM supplemented 0, 1 or 10 ng/ml of Tet. Parental Huh7.5 cells left were used as negative controls. Cell nuclei were stained with DAPI. (B) BST2 expression in the lysates of to the cell lines was detected by a Western blot with a rabbit anti-BST2 antibody. β-Actin served as a loading control.

with Tet dosage. Immunofluorescence showed that BST2 is localized predominantly to the peripheral zone of cells, most likely on the plasma membrane in Huh7.5 cells. However, high expression of BST2 led to a cytoplasmic distribution in the Huh7.5-BST2 cell without Tet. In marked contrast, BST2cv5 is exclusively distributed in the cytoplasm (Fig. 1A).

3.2. Expression of BST2 inhibits HCV infection

To test the effects of BST2 on HCV infection, parental Huh7.5 and Huh7.5-BST2-TO were infected with HCV at a MOI of 0.015. While HCV infection had spread into approximately 100% of parental Huh7.5 cells at day 4 postinfection, only partial Huh7.5-BST2-TO cells were infected with HCV (Fig. 2A). Furthermore, the proportion of infected cells negatively correlated with the BST2 expression levels, suggesting that BST2 inhibited at least one stage of the HCV replication cycle. The intracellular levels of HCV nonstructural proteins (Fig. 2B), the yield of infectious virions (Fig. 2C), and extracellular viral RNA (Fig. 2D) were also negatively correlated with the levels of BST2 expression in Huh7.5-BST2-TO cells, which is consistent with the immunostaining. Compared with wild-type BST2, the expression of BST2cv5 had a neglectable antiviral effect against HCV.

3.3. BST2 inhibits the later stages of HCV life cycle

To determine at which stage BST2 effects HCV replication, a synchronized infection assay (or one step growth curve) was performed. Briefly, parental Huh7.5, Huh7.5-BST2-TO cells were infected with HCV at a MOI of 3, the highest MOI we could achieve with our concentrated virus stock. Based on the Poisson distribution, such a multiplicity of infection will ensure that over 90% of cells are initially infected with at least one infectious HCV virion. Two days after infection, viral yields and viral RNA/proteins were determine in culture media and harvested cells, respectively. Compared to parental Huh7.5 cells, expression of BST2 did not affect the frequency of NS3-positive cells (Fig. 3A), intracellular levels of NS3 protein and viral RNA (Fig. 3C). HCV replication did not significantly differ between these cells. However, exogenous BST2 decreased HCV virions and RNA titer by 6- and 5.6-fold compared to parental cells. Moreover, decreasing BST2 levels increased HCV virions and RNA titer. These observations demonstrated that the BST2 expression correlated with the HCV inhibition in cells (Fig. 3D and E). Subcellular distribution of BST2 and HCV structural core proteins was revealed in the Huh7.5-BST2-TO infected with HCV (Fig. 3B). Consistent with a previous report (Ye et al., 2012), most of cells with low BST2 expression localized at plasma membrane (yellow arrow), and most of the HCV core protein was

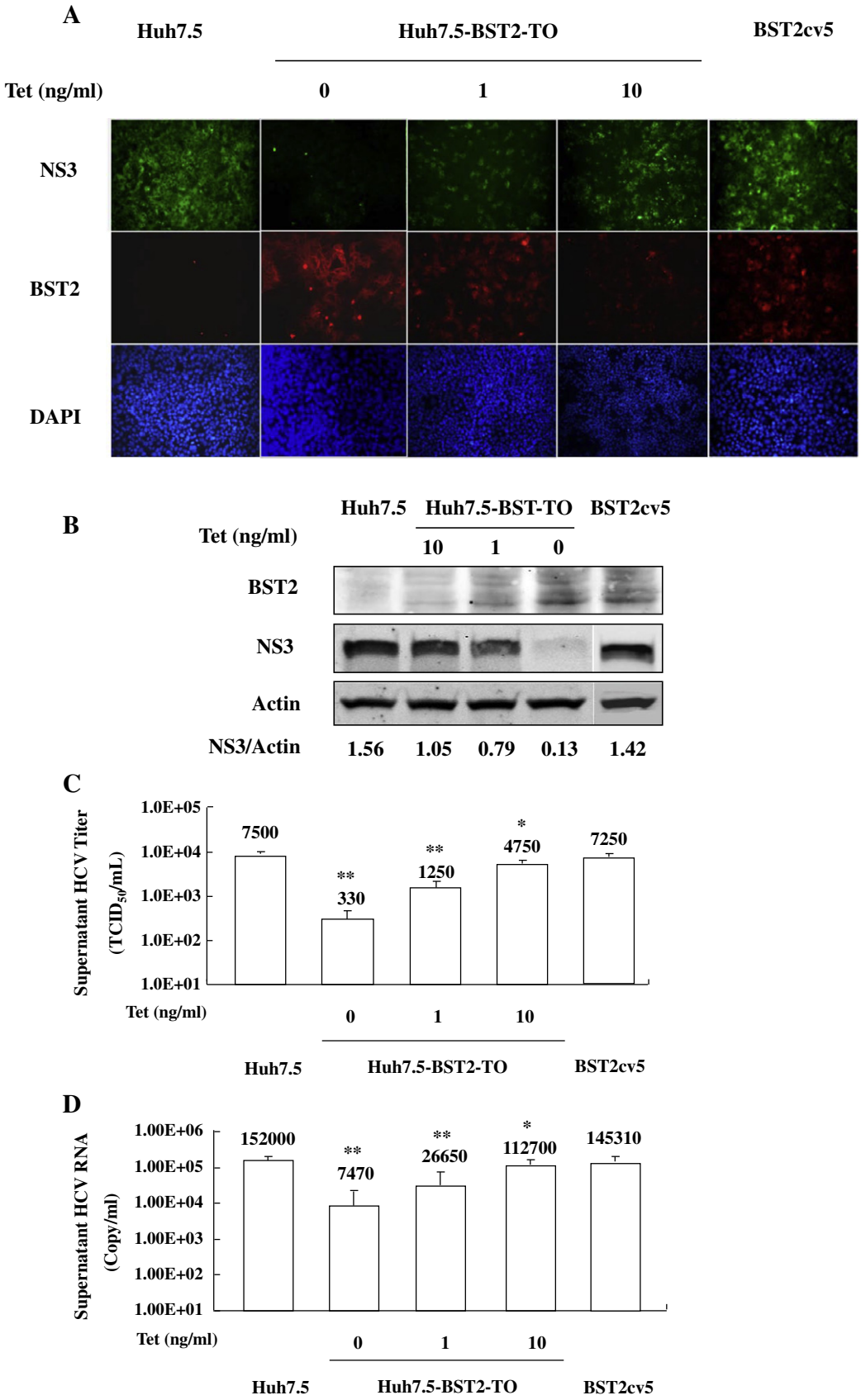

Fig. 2. BST2 expression inhibits HCV infection of Huh7.5 cells. Huh7.5-BST2-TO were cultured in 0, 1, 10 ng/ml of tetracycline for 1 day and then infected with HCV at a MOI of 0.015 and harvested at day 4 postinfection. (A) Cells were double-stained for HCV NS3 (top panel, green) and BST2 (middle panel, red). Cell nuclei were stained with DAPI (bottom panel, blue). The levels of intracellular HCV NS3 protein (B) were determined by Western blot assays. β-Actin served as loading controls. Virus yields ($TCID_{50}$/ml) (C) and cell-free viral RNA (copies/ml) (D) in culture media were determined by $TCID_{50}$ and qRT-PCR assays and expressed as mean ± standard error ($n = 3$). The values from the Huh7.5-BST2-TO and Huh7.5-BST2cv5 cells were compared with that from parent Huh7.5 cells. p values were calculated using Student's t test. $^{**}p < 0.01$; $^{*}p < 0.05$.

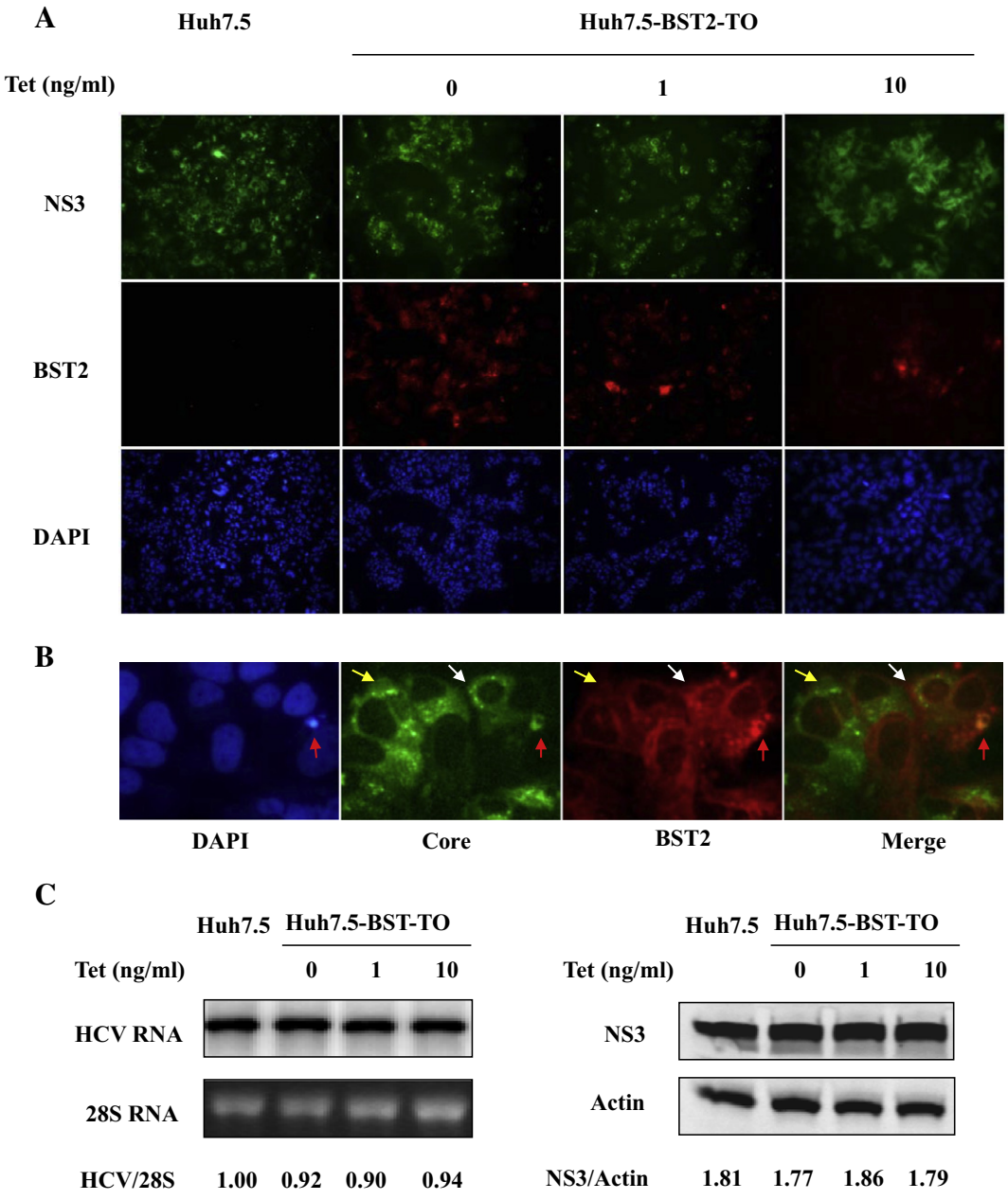

Fig. 3. BST2 inhibits HCV infection of Huh7.5 cells. Huh7.5-BST2-TO were cultured in 0, 1, 10 ng/ml of tetracycline for 1 day and then infected with HCV at a MOI of 3 and harvested at 2 days postinfection. Expressions of BST2, HCV NS3 and core proteins are showed by indirect immunofluorescence staining. Cell nuclei were stained with DAPI. The levels of intracellular HCV RNA (right panel of 2C) and HCV NS3 protein (left panel of 2C) were determined by Northern blot and Western blot assays, respectively. 28s rRNA and β-actin served as loading controls. Supernatant infectious HCV titers were determined and expressed as $TCID_{50}$/ml as mean ± standard error (D, $n = 3$). Extracellular HCV RNA was determined by qRT-PCR assay (E, $n = 3$). Cells were infected with HCVpp (MOI = 10) and the luciferase activity was determined for evaluation of efficiency of viral entry ($n = 3$). The values from the Huh7-BST2-TO cells were compared with that from parent Huh7 cells (F). p values were calculated using Student's t test. $^{**}p < 0.01$; $^{*}p < 0.05$.

localized to the cytoplasm, only a few of them were co-localized with BST2. However, in the cells with high BST2 expression (white arrow), BST2 mostly exhibited a punctate-like cytoplasmic distribution. This suggested that highly expressed BST2 could also localize to the cytoplasmic bodies. Furthermore, coexpression of viral core and BST2 was clearly observed in a body in the high BST2 expressing cells (red arrow). A DAPI stained agglomerate was also observed in this body, suggesting that the BST2 might tether the core protein with the viral genome. This might provide direct evidence that BST2 interacts with HCV *in vitro*. However, high BST2 expression may exceed most situations of endogenous BST2 expression levels. It is therefore unclear if the observed co-localization is physiologically relevant. Expression of BST2 did not affect the HCVpp luciferase activity (Fig. 3F), suggesting that BST2 does not inhibit HCV entry. Conclusively, these results imply that BST2 inhibits the later stages of HCV life cycle, but does not affect viral entry, viral genome replication and viral protein expression.

3.4. BST2 is a functional mediator of IFN-α-induced antiviral response against HCV

To further investigate the physiological role of BST2 in IFN-α-induced antiviral response against HCV infection, we determine whether depleting IFN-α-induced BST2 expression in Huh7.5 cells compromises the antiviral effect of this cytokine. Transfection of SMART*pool* siRNA targeting BST2, but not nontargeting siRNA

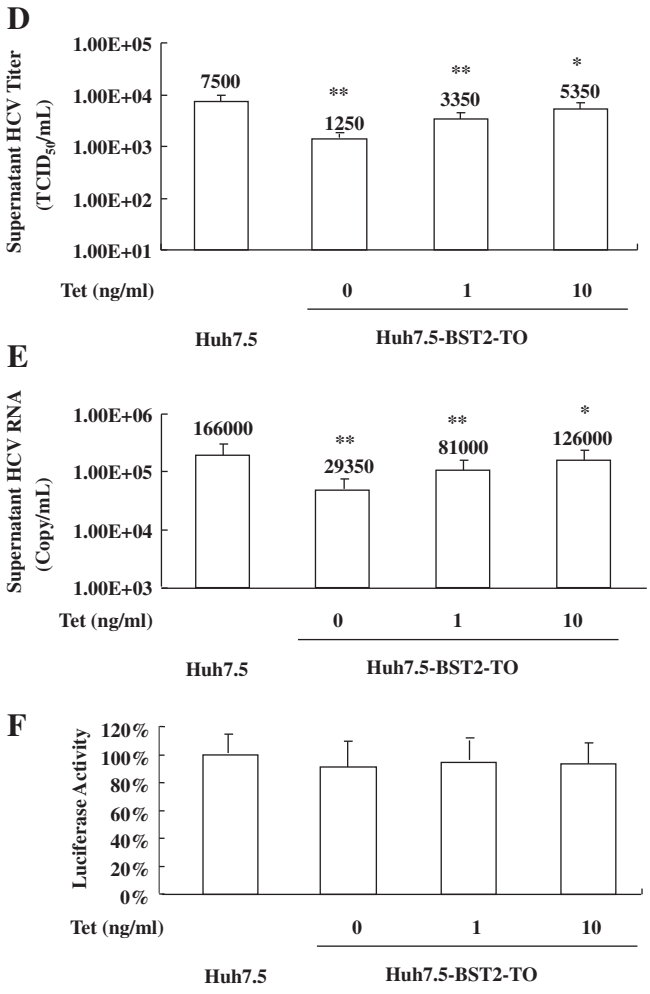

Fig. 3. (*continued*)

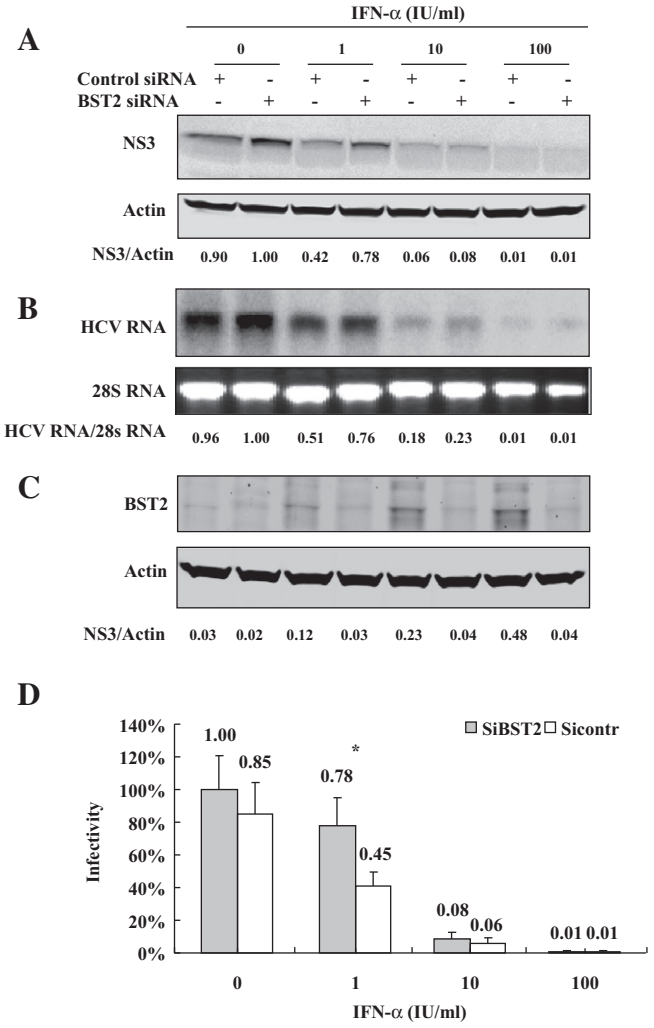

Fig. 4. BST2 is a functional mediator of IFN-α-induced antiviral response against HCV. Huh7.5 cells were transfected with either SMART*pool* siRNA targeting BST2 or non-targeting siRNA (control). Six hours post-transfection, cells were left untreated or treated with indicated concentrations of IFN-α for 24 h and followed by infection with HCV at a MOI of 0.05. Two days after infection, cells were harvested and the levels of intracellular HCV NS3 (A) and HCV RNA (B) were determined by Western blot and Northern blot, respectively. β-Actin and 28s rRNA served as loading controls. To determine the siRNA knockdown efficiency, one set of cells was harvested without infection at 24 h post IFN treatment and the levels of BST2 in the cell lysates were determined by Western blot assay. β-Actin served as loading controls (C). Virus yield at day two after infection were determined and normalized as the percentage of control siRNA transfected cells without IFN-α treatment ($n = 3$) (D). The values from the BST2 siRNA knockdown were compared with that from control siRNA knockdown cells. p values were calculated using Student's t test. $^{*}p < 0.05$.

(control), efficiently reduced IFN-α-induced BST2 expression in Huh7.5 cells (Fig. 4). Interestingly, reduction of IFN-α-induced BST2 expression only moderately attenuated the ability of IFN-α to inhibit HCV infection at lower concentrations (1 IU/ml), but not at higher concentrations. This might be attributed to the compensation of other antiviral pathways that are activated at the higher concentrations of IFN-α treatment.

4. Discussion

HCV replication occurs on a lipid raft membrane structures. It has been proposed that infectious HCV particles are assembled while core particles bud through the endoplasmic reticulum membrane. These particles as then transported to the secretory pathway via the trans-Golgi network (Jones and McLauchlan, 2010). HCV is unable to bud via the membrane of infected cells, in which BST2 functions to tether budding virions. However, BST2 is expressed at HCV replication and assembly sites including detergent-insoluble subcellular domains, endoplasmic reticulum, lipid rafts membrane web structures, and the trans-Golgi network (Dube et al., 2009; Gastaminza et al., 2008; Kupzig et al., 2003; Miyanari et al., 2007). Recently, it was shown that tetherin has negligible activity in restricting HCV from hepatocytes (Ye et al., 2012). Another group demonstrated that BST2 moderately restricts HCV release from Huh7.5 hepatocytes, while the virus lacks a mechanism to counteract this restriction (Dafa-Berger et al., 2012). In this study, we used a BST2 Tet-inducible system in Huh7.5-BST2-TO cells to show that HCV production was inhibited by BST2 overexpression in a concentration-dependent manner.

BST2 is a transmembrane protein that contains a short N-terminal cytoplasmic domain, a membrane-spanning alpha-helix; a coiled-coil ectodomain and a C-terminal GPI anchor (Kupzig et al., 2003; Schubert et al., 2010; Swiecki et al., 2011). The protein is localized at the plasma membrane as well as the membranes of multiple intracellular vesicles (Dube et al., 2009; Kupzig et al., 2003). Consistent with these observations, our study showed that BST2 preferably localized at plasma membrane but exhibits a marked cytoplasmic distribution in high BST2 expressing Huh7.5 cells (Figs. 1A and 3B). At the plasma membrane, BST2 is found within cholesterol-enriched lipid rafts, presumably due to its C-terminal GPI modification (Kupzig et al., 2003). The variant BST2cv5 exhibited predominantly cytoplasmic distribution and

an altered protein sizes by western blot. This suggested that the addition of 14 amino acid residues of V5 epitope tag at the C-terminus of BST2 inhibited the C-terminal GPI modification. BSTcv5 did not effect the virion yield suggests that the C-terminal GPI anchor might be required for BST2 to inhibit HCV production (Fig. 2).

BST2 does not inhibit HCV entry and genome replication, but reduced the virion yield (Figs. 2 and 3). Particularly, the highly expressed BST2 Huh7.5 cells showed a marked inhibition of cell-free virion yield, in which an abundant cytosolic expression of BST2 was detected. Furthermore, a co-localization of HCV structural core protein, viral genome, and BST2 was detected in the intracellular bodies, but only in high BST2 expressing cells (Fig. 3B). It is unknown whether this colocalization represents a direct tether of HCV virion in the intracellular membrane or an endocytosis and degradation in the lysosomes. However, BST2 might directly interact with the HCV particles bearing genome.

Knockdown of BST2 expression in INF-α-Huh7.5 cells demonstrates a moderate physiological role of BST2 against HCV infection at low level of interferon treatment (Fig. 4). This also implies that interferon-induced BST2 seems more efficient than the BST2 in the Huh7.5-BST2-TO cells. Additional studies are needed to assess if different modifications between the IFN-α-induced BST2 and exogenous BST2 produce the different effects on virion yields. However, the effect of BST2 on HCV inhibition is discerned only at low-level interferon treatment. This indicated that BST2 might be not a major contributor to the antiviral effect of IFN-α. In summary, although the molecular mechanism by which BST2 inhibits HCV release remains to be determined, our results demonstrate that BST2 is not likely to be a major contributor to the antiviral effect of IFN-α.

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