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Heat shock protein 70 is associated with CSFV NS5A protein and enhances viral RNA replication

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ABSTRACT

The non-structural 5A (NS5A) protein of classical swine fever virus (CSFV) is proven to be involved in viral replication and can also modulate cellular signaling via to its ability to interact with various cellular proteins. Here, HSP70/NS5A complex formation is confirmed by coimmunoprecipitation and GST-pulldown studies. Additionally, the N-terminal amino acids (29–240) of NS5A were identified as the interaction region through in vivo deletion analyses, and confocal microscopy showed that NS5A and HSP70 colocalized in the cytoplasm. Overexpression of HSP70 via the eukaryotic expression plasmid pDsRED N1 or lentivirus significantly promoted viral RNA synthesis. Whereas the knockdown of HSP70 by lentivirus-mediated shRNA or inhibition by quercetin markedly decreased the viral load. These data suggest that HSP70 plays a critical role in the viral life cycle, particularly during the virus RNA replication period. The investigation of HSP70 protein functions may be beneficial for developing new strategies to treat CSFV infection.

Introduction

Classical swine fever virus (CSFV) is a member of Pestivirus within the Flaviviridae family (Cuthbert, 1994), which possesses a single-stranded, positive-sense RNA genome, coding for one polyprotein which is subsequently processed into four mature structural proteins (C, E^{rns}, E1 and E2) and eight non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) by cellular and viral proteases (Tang et al., 2010, 2011). Classical swine fever (CSF) is caused by CSFV, previously List A disease of pigs by the World Organization for Animal Health (OIE) with high mortality rates, characterized by high fever, multiple hemorrhages, thrombocytopenia and respiratory and gastrointestinal symptoms (Kleiboeker, 2002; Ning et al., 2014). CSFV is reported can establish a persistent infection through prevent host cell apoptosis and induce immune depression (Bensaude et al., 2004; Johns et al., 2010), moreover, CSFV is able to cross the placenta and establish an infection in the developing fetus (Charleston et al., 2001), leading to huge economic losses in the pig industry. At present, the vaccines used against CSFV are usually implemented to prevent CSF while the effective therapies are limited (Moormann et al., 2000). Thus, to identify novel host targets or pathways for effective treatment during CSFV infection is very important, which is also essential to clarify the relationship between virus and the host cells (Pei et al., 2014).

The viral non-structural protein 5A (NS5A), consisting of 497 amino acids, is a phosphorylation protein that can bind to multiple host proteins and also is a component of the viral replicase complex (Sheng et al., 2014). It has been reported that NS5A in the endoplasmic reticulum can suppress the internal ribosome entry site (IRES) located in the 5′UTR, interact with the 3′UTR, induce oxidative stress, and regulate viral replication (Chen et al., 2012; He et al., 2012; Isken et al., 2014; Sheng et al., 2010; Xiao et al., 2009). In Hepatitis C virus (HCV), also a member of the Flaviviridae family, the NS5A protein is an essential component of the viral RNA replication complex and also to modulate the host cell environment (Frank et al., 2006; Tellinghuisen et al., 2004). In addition, HCV NS5A plays an essential role in the modulation of both viral and cellular RNA translation (He et al., 2003; Kalliampakou et al., 2005; Wu et al., 2008). However, the mechanism by which NS5A participates in viral particle production or regulates host cellular functions remains unknown.

As we all known, virus, especially positive sense genome RNA viruses, largely depend on the host cells to complete their life cycles. Heat shock protein 70 (HSP70) is highly induced by fever and highly conserved family of genes in eukaryotes, the phylogenetic conservation suggests that play a host protective role (James et al., 1997; Mackowiak, 2000; Rassow et al., 1997). HSP70s are involved in a remarkable variety of cellular processes, including the folding of newly synthesized proteins, refolding of misfolded

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or aggregated proteins, translocation of organellar and secretory proteins across membranes, protein complex assembly and disassembly, receptor signaling, and to interfere with host antiviral responses (Hartl and Hayer-Hartl, 2002; Kim et al., 2012; Mayer and Bukau, 2005; Nagy et al., 2011; Wang et al., 2004).

In this study, we first confirmed that HSP70 can interact with the CSFV NS5A protein through co-immunoprecipitation and GST-pulldown assays and found that HSP70 colocalize with NS5A in SUVEC cells using confocal microscopy. We also found that HSP70 can positive affect viral proliferation in ST cells. These findings indicate that HSP70 may play an important role in the viral life cycle.

Results

NS5A binds to HSP70 in vivo

In our previous report, we found that CSFV NS5A can interact with HSP70(Zhang et al., 2014). To further investigate interactions between CSFV NS5A and HSP70, we examined their ability to form a co-immunoprecipitatable complex. Lysates from SUVECs transfected to express NS5A-Flag were immunoprecipitated with ANTI-FLAG M2 Affinity Gel, and the proteins in the complexes were analyzed by western blotting with an anti-Flag or anti-HSP70 antibody. HSP70 was detected in the immunoprecipitates obtained with the anti-Flag antibody (Fig. 1A). Reciprocal co-IP experiments also showed that the anti-Myc antibody precipitated NS5A-Flag (Fig. 1C). Consistent with these results, endogenous HSP70 was co-immunoprecipitated with NS5A-Flag from SUVECs. Next, we co-expressed exogenous NS5A-Flag and HSP70-Myc in SUVECs and expressed HSP70-Myc as a control to detect interactions between NS5A and HSP70. The results showed that exogenous NS5A and HSP70 were both detected in the immunoprecipitate (Fig. 1B). As we know, there is a huge difference in compared to expression in the context of the viral polyprotein and individually expressed NS5A is dysfunctional in many aspects (Shi et al., 2002). SUVECs were infected with CSFV firstly and then cotransfected with plasmid of pFlag-NS5A and pMyc-HSP70. Similar results were comparable with the former one (Fig. 1b) that is obtained by co-precipitation by use anti-Flag antibody (data not shown). These experiments indicated the specificity of the interaction between NS5A and HSP70 in vivo whether SUVECs were infected or noninfected with CSFV.

NS5A directly binds to HSP70 in vitro

After demonstrating a direct interaction between recombinant NS5A and HSP70 in vivo, we then determine whether this interaction occurs in vitro. Recombinant full-length HSP70-GST fusion protein was expressed in and purified from bacteria, and pNS5A-Flag was transfected into HEK293T cells. The interaction between HSP70 and NS5A were tested by GST-pulldown analyses. Full-length HSP70-GST was immobilized on glutathione agarose, and NS5A-Flag was added to assay binding. As a negative control for HSP70-GST binding, a bacterial GST-containing lysate was added. The proteins in the complexes were analyzed by western blotting with an anti-GST or anti-Flag antibody. As shown in Fig. 1D, NS5A-Flag was detected in an HSP70-GST and NS5A-Flag complex. These findings indicated the interaction of HSP70 and NS5A in vitro.

The N-terminal region of NS5A is required for interaction with HSP70

The regions of NS5A required for interaction with HSP70 were further investigated. Three deletion mutants of NS5A were constructed with a Flag tag at the C terminus (Fig. 2A); each was transfected into SUVECs, and the interaction of HSP70 with NS5A-Flag was determined using a co-IP assay. Based on the detection of endogenous HSP70, the N-terminal region of NS5A, from amino acids 29 to 240, is required for the interaction with HSP70 (Fig. 2B).

CSFV NS5A co-localizes with HSP70

To determine whether the CSFV NS5A protein colocalizes with HSP70, laser-scanning confocal microscopy was performed on SUVECs co-expressing pEGFP-NS5A and pERED-HSP70 (Fig. 3). HSP70 was more diffusely distributed in the cytoplasm but did colocalize with NS5A in the endoplasmic reticulum. Compare with the only cotransfection NS5A-GFP and HSP70-RED, they distribute punctate in the endoplasmic reticulum. When cells infected with CSFV, NS5A-GFP and HSP70-RED distribute more widely in the cytoplasm maybe due to in the context of the polyprotein of CSFV, but they still colocalized. This result along with a previous yeast two-hybrid screen and the NS5A-Flag co-immunoprecipitation with HSP70 suggest an interaction between NS5A and the HSP70 protein.

Overexpression of HSP70 promotes CSFV propagation

Previous studies have shown that the NS5A protein is involved in virus replication. To understand whether HSP70 is involved in CSFV replication, the impact of HSP70 overexpression on CSFV replication was studied in the ST cell line by measuring the level of CSFV RNA. When HSP70 was overexpressed either by lentivirus (Fig. 4A and B) or a eukaryotic expression plasmid (Fig. 4D and F), the levels of CSFV RNA were significantly increased compared with the levels observed in control cells (Fig. 4C and F).

Lentivirus-mediated HSP70 knockdown and quercetin treatment reduce CSFV replication

We then tested the effects of HSP70 inhibition by quercetin or knockdown by shRNA on CSFV replication. Fist, the cell viability upon silencing and inhibition of HSP70 is tested by MTT methods. The dose of the quercetin is selected at 100 μM (Fig. 5A) and shRNA mediated knockdown HSP70 have no lethality to cells (Fig. 5B). ST cells were infected with CSFV and tested by RT-qPCR to determine CSFV RNA replication. As expected, knockdown of HSP70 (Fig. 6A, C and D) resulted in a significant decrease of intracellular viral RNA levels (Fig. 6B and E). These results indicated potentially effects of HSP70 knockdown and suggested that HSP70 has important functions in the CSFV life cycle.

Modulation of cellular Hsp70 and effect on CSFV replication

CSFV NS5A has previously been reported to participate in CSFV gene replication. Here, we report that NS5A can interact with HSP70 and that HSP70 overexpression promotes and HSP70 knockdown reduces CSFV replication.

We next investigated HSP70 protein expression during CSFV propagation. To determine whether CSFV infection affects HSP70, mock-infected and CSFV-infected cellular lysates were collected at the indicated time and then analyzed. Total cellular RNA was extracted, and the expression of CSFV and HSP70 mRNA was quantified by real-time RT-PCR (Fig. 7A and C), and cellular lysates were analyzed to detect HSP70 protein expression by western blotting (Fig. 7B). The results showed higher levels of HSP70 protein at 24 h post-exposure to CSFV, whereas its expression level was decreased at 48 h. So we speculated that HSP70 may play a role at the gene replication phase of CSFV, and further study was needed.

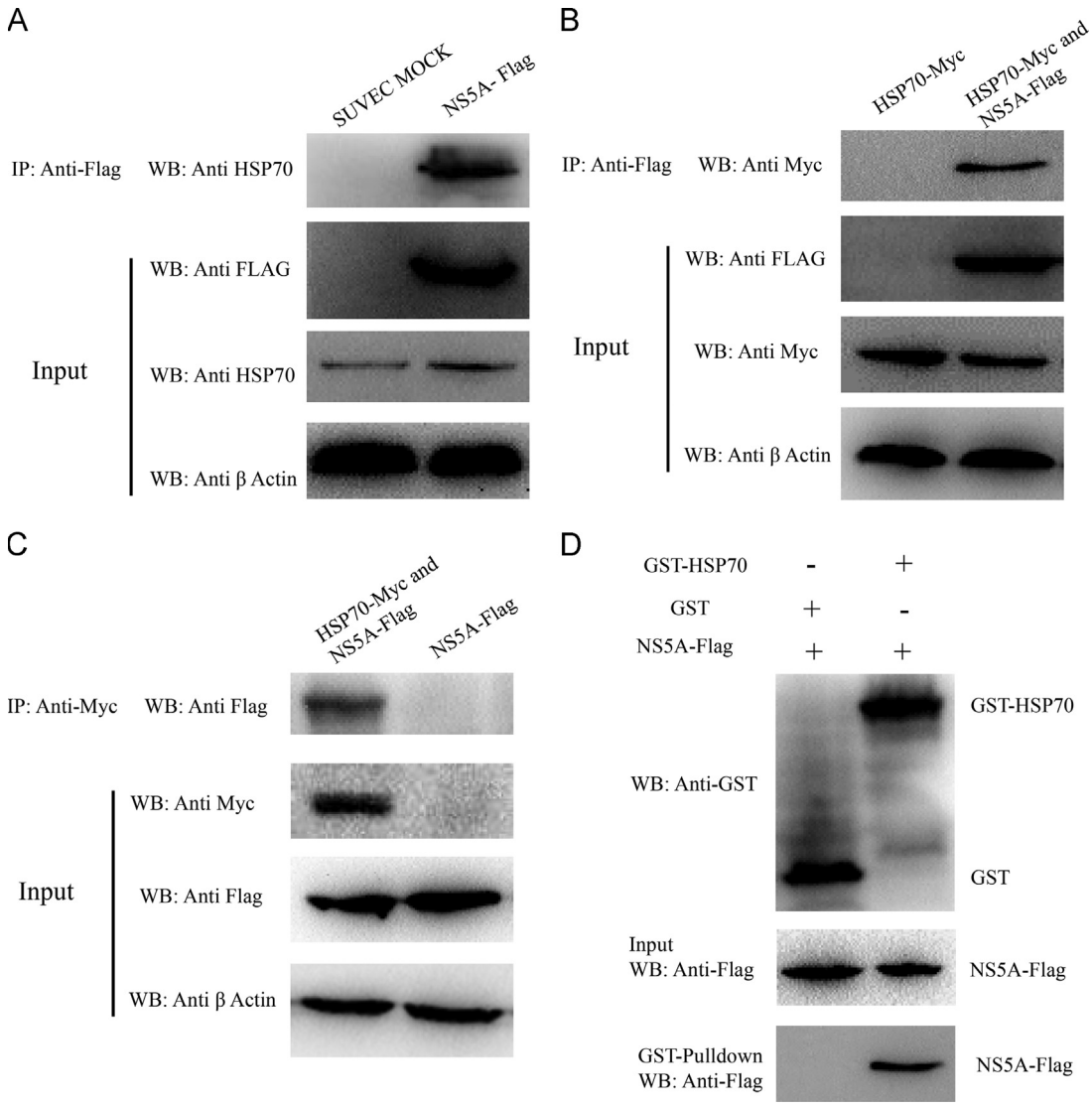

Fig. 1. HSP70 interacts with NS5A in SUVEC. (A) Coimmunoprecipitation assay demonstrates that endogenous HSP70 binds NS5A-Flag in transfected cells. SUVEC cells were transfected pNS5A-Flag plasmid for 48 h and harvested. Cell lysates from transfected and untransfected control cells were immunoprecipitated with antibody against Flag followed by Western blot analysis. (B) Exogenous HSP70-Myc and NS5A-Flag coexpression in SUVEC cells. Cell lysates from cotrasfected HSP70-Myc and NS5A-Flag or transfected HSP70-Myc cells were immunoprecipitated with antibody against Flag followed by Western blot analysis. (C) Reciprocal co-IP experiments showed that the anti-Myc antibody precipitated NS5A-Flag. (D) GST pulldown assay. Glutathione beads conjugated to GST or the GST-HSP70 protein fusion protein were incubated with recombinant NS5A-Flag protein. After washing, proteins were eluted from the beads and SDS-PAGE was performed. The expression of NS5A was detected by immunoblotting with anti-Flag mAb. GST and GST-HSP70 protein expression was confirmed by immunoblotting with rabbit anti-GST pAb.

Discussion

Viruses depend on host factors to carry out important functions such as gene duplication and protein synthesis because of their limited genome coding capacity. During evolution, viruses have acquired unique multifunctional proteins. Recently, through binding to the 3′-UTR of the viral RNA genome and modulating the RdRp activity of NS5B, CSFV NS5A has been shown to regulate CSFV viral RNA replication(Sheng et al., 2012a, 2012b). NS5A also can modulate cellular signaling and host cellular responses through its ability to interact with various cellular proteins (Zhang et al., 2014).

In classical swine fever virus, we found in our previous studies that NS5A could interact with HSP70 by a yeast two-hybrid screen (Zhang et al., 2014). There have been many studies showing NS5A/HSP70 interactions in HCV, however, there have no study about NS5A/HSP70 interactions in CSFV. Gonzalez et al. (2009) found HCV NS5A can interact with heat shock proteins 40 and 70. HSP72 plays a positive regulatory role in HCV RNA replication by interact with NS5A protein (Chen et al., 2010). More recently it is found that HSC70 and HSP70 both binding with HCV NS5A and play distinct roles in the virus life cycle (Khachatoorian et al., 2014). In the present study, we confirmed a direct interaction between CSFV NS5A and HSP70 both in vivo and vitro. We transfected an NS5A-Flag plasmid alone or along with an HSP70-Myc plasmid into SUVECs and were able to successfully pull down a NS5A-Flag/HSP70 or NS5A-Flag/HSP70-Myc complex by coimmunoprecipitation assays (Fig. 1A–C). We further proved that HSP70 binds directly to CSFV NS5A in vitro through GST-pulldown analyses utilizing purified recombinant GST-HSP70 proteins (Fig. 1D). The results might indicate that no other viral or host protein is required for the interaction between NS5A and HSP70. Confocal microscopy also showed that NS5A and HSP70 colocalize in SUVECs (Fig. 4). These studies fully illustrated that the CSFV NS5A protein can interact with HSP70 protein.

Through co-immunoprecipitation analyses, we showed that the N-terminal domain (N720 and N804) of NS5A directly binds to HSP70; in contrast, the C terminus (C687) of NS5A cannot interact with NS5A (Fig. 2), suggesting NS5A interact with HSP70 through its N terminal. We speculated that this difference is caused by the

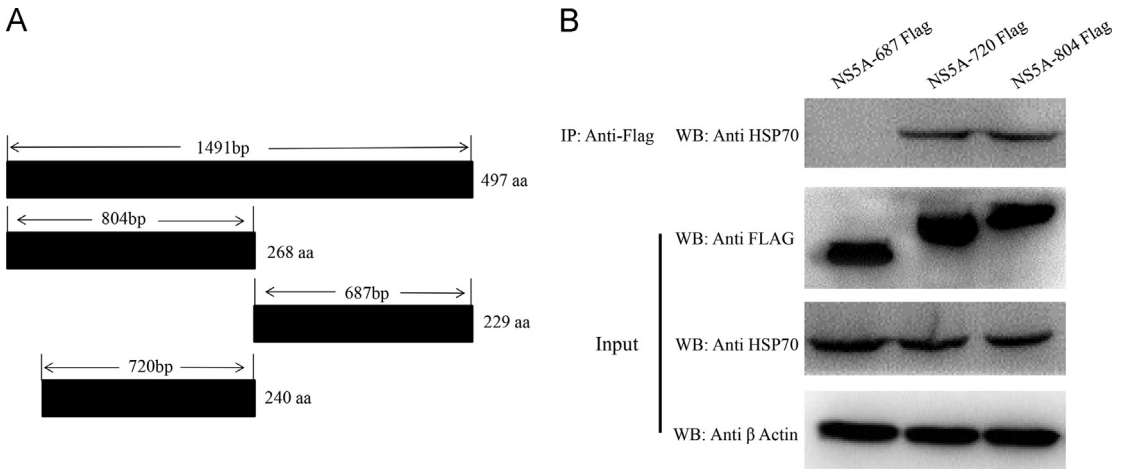

Fig. 2. The N-terminal region of NS5A is required for association with HSP70 protein. (A) Schematic representation of the protein domains of CSFV NS5A protein and the individual deletion mutant form of NS5A tested in this study. (B) Co-IP analysis of the association of endogenous HSP70 with Flag-tagged NS5A or mutant forms of NS5A in SUVEC cells transfected with the indicated expression plasmids.

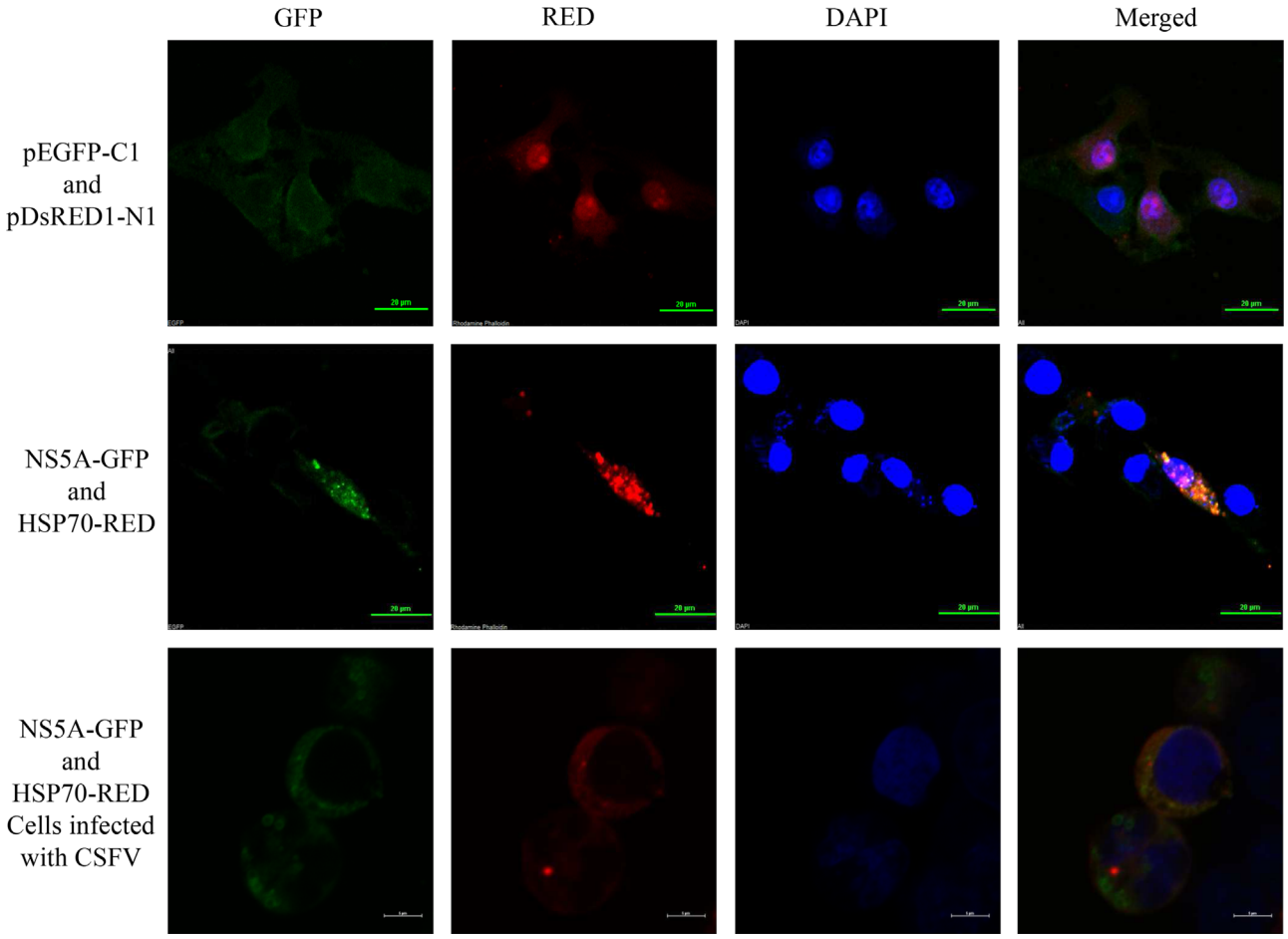

Fig. 3. NS5A colocalizes with HSP70. SUVEC cells were co-transfected with pEGFP-NS5A and pERED-HSP70, pEGFP-N1 and pERED-N1 as control, and analyzed by laser confocal microscopy after 48 h. All cells were stained with DAPI. The pERED-HSP70 fusion protein showed was colocated with pEGFP-NS5A in the endoplasmic reticulum whether infected or uninfected with CSFV.

fact that although NS5A is a phosphorylated protein that localizes to the endoplasmic reticulum, the N terminus could be responsible for its interactions with cellular protein to modulate its function. It was recently reported that the C-terminal end of NS5A is necessary for the assembly and production of CSFV infectious particles (Sheng et al., 2014). The N terminus of BVDV NS5A has been demonstrated to form an in-plane amphipathic-helix that anchors the protein to intracellular membranes, a feature that is believed to be essential for the formation of the functional replication complex (Tellinghuisen et al., 2006). However, which amino acids are involved and the mechanism by which N-terminal domain of NS5A modulates viral RNA replication require further studies.

Furthermore, we verified that HSP70 positively regulates CSFV replication. The overexpression of HSP70 via lentivirus or a eukaryotic

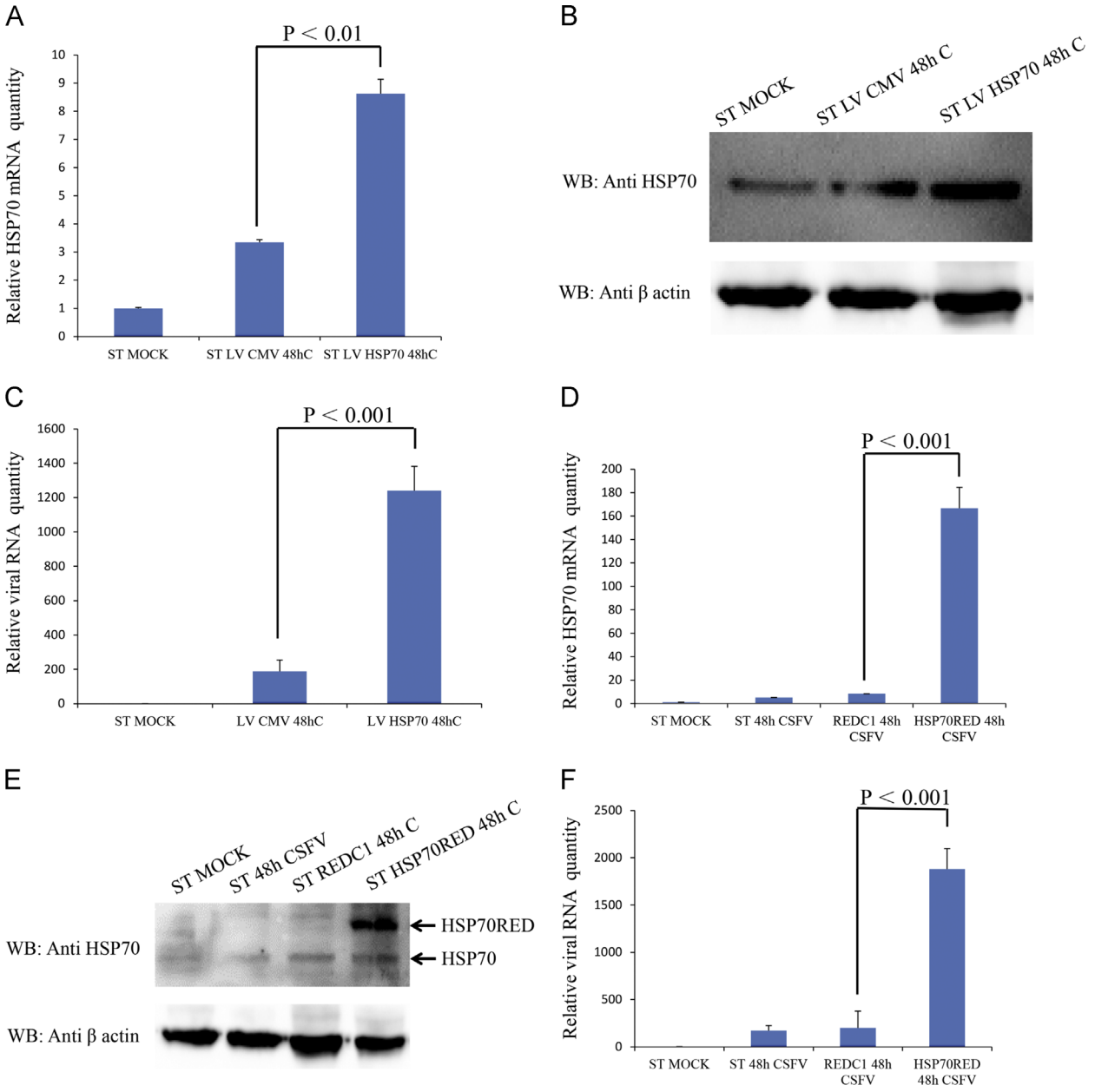

Fig. 4. Overexpression of HSP70 increases CSFV propagation. (A and B) overexpression of HSP70 mediated by lentivirus, showed the changes of HSP70 both in gene and protein leverl. (C) The change of CSFV RNA expression level when overexpression of HSP70 mediated by lentivirus. (D and E) Overexpression of HSP70 mediated by eukaryotic expression vector pDsREDN1, showed the changes of HSP70 both in gene and protein leverl. (F) The change of CSFV RNA expression level when overexpression of HSP70 mediated by eukaryotic expression vector.

expression plasmid significantly increased the virus gene level (Fig. 4). The results showed the transient overexpression HSP70 mediated by pDsREN N1 increases HSP70 approximately 16 fold and stimulates replication 9 fold (Fig. 4D and F), stable lentiviral transfer increases HSP70 mRNA by 2.5 fold and RNA replication increase 6 fold (Fig. 4A and C). We speculated that the overexpression of HSP70 protein was mediated by pDsRED N1, of which expression increased more than lentiviral contributing to the higher transfection efficiency of pDsRED N1. Conversely, the knockdown of HSP70 mediated by lentivirus or quercetin resulted in a significant decrease in virus production (Fig. 6), which was comparable to the reduction of virus observed by HSP70 knockdown in previous study (Gonzalez et al., 2009). HSP70 is involved in many viral life cycle activities, such as transcription, cellular transformation, viral genome replication, and increased virion assembly (Sullivan and Pipas, 2001). Hsp70 is a chaperone interacting with many proteins and might also interact with additional viral proteins. It has been reported that HSP70 positively regulates porcine circovirus type 2 and hepatitis C virus replication (Chen et al., 2010; Liu et al., 2013; Parent et al., 2009).

Generation of large amount of viral proteins during infections frequently leads to cellular stress and the up-regulation of HSP's expression. For instance, during West Nile virus, a flavivirus, infection, the authors found that the various isoforms of HSP70s were up-regulated, while HSP90s were down-regulated (Pastorino et al., 2009). But HSP70 chaperone family is always involved in replication of many RNA virus, such as HSP72 is associated with the hepatitis C virus replicase complex and enhances viral RNA replication (Chen et al., 2010). In the current study, we demonstrated that HSP70 can interact with CSFV NS5A and promote CSFV gene replication. Thus, our results are consistent with the notion that HSP70 plays a role in viral replication. We speculated that HSP70/NS5A interaction may play a role in viral RNA replication, and a further study is needed to verify this hypothesis.

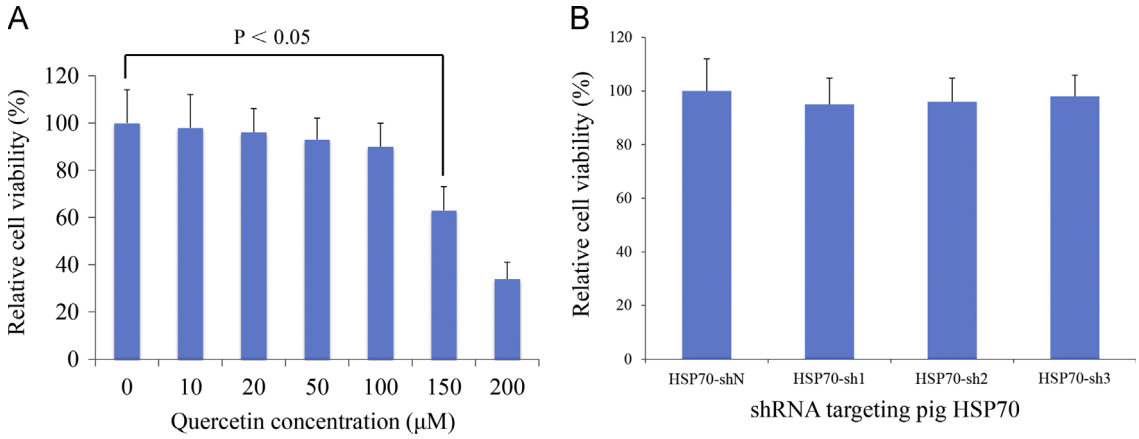

Fig. 5. The cell viability upon silencing and inhibition of HSP70 is tested by MTT methods. (A) ST cells were treated with different concentrations of quercetin, MTT (2 μg/mL) was added to each well and incubated at 37 °C for 4 h, light absorbance of each well was measured at 490 nm in microplate reader. (B) ST cells were treated with lentivirus-based shRNA to knockdown HSP70, MTT (2 μg/mL) was added to each well and incubated at 37 °C for 4 h, light absorbance was measured at 490 nm in microplate reader.

Recently, the HCV protein NS5A has been implicated in the regulation of HCV genome replication, IRES-mediated translation of the viral polypeptide, and viral assembly (Appel et al., 2008; He, 2003). Accordingly, we speculated that the NS5A/HSP70 complex may impact one or more of these stages of the viral life cycle. Viral replication was modestly reduced by HSP70 knockdown and more strongly by quercetin treatment. Further studies to determine the role of HSPs alone and in complex with NS5A with regard to assembly and secretion are beyond the scope of this study but are currently being investigated.

In summary, we demonstrated that the HSP70 chaperone can interact with CSFV NS5A protein and promote CSFV RNA replication. The importance of HSP chaperones to CSFV replication represents a target for the development of anti-CSFV therapies. Indeed, several HSP inhibitors have been shown to effectively attenuate virus production (Gonzalez et al., 2009; Okamoto et al., 2006).

Materials and methods

Plasmid constructs and virus

CSFV (Shimen strain) was purchased from the Control Institute of Veterinary Bio-products and Pharmaceuticals (China).

The NS5A-GFP plasmid encoding the CSFV NS5A protein with a GFP fusion protein at its C terminus was constructed by cloning the NS5A gene into the pEGFP-C1 vector (Clontech) using the EcoRI and BamHI restriction enzymes. The cDNA for the swine HSP70 protein (GenBank accession no. NM_001123127.1) was cloned into the pDsRED1-N1, pCDNA3.1(+) (Clontech), and lentivector CD513B-1 vectors using EcoRI and BamHI to generate the HSP70-RED, HSP70-Myc, and LV-HSP70 plasmids, respectively. For the bacterial expression of the glutathione S-transferase (GST)-tagged HSP70 protein, the HSP70 encoding region was cloned into the pGEX-6P-1 vector (28–9546-48; GE Healthcare), using EcoRI and BamHI to create pGST-HSP70. NS5A-Flag, NS5A-687-Flag, NS5A-720-Flag, and NS5A-804-Flag were cloned into the pCDNA3.1(+) vector using BamHI and XhoI. All primers are listed in Table 1, and all plasmids were confirmed by restriction digestion and sequencing.

Cell culture

HEK293T cells were cultured in high glucose Dulbecco's-modified Eagle's medium (DMEM; GIBCO, UK) containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids and 10% fetal bovine serum (FBS) (GIBICO, UK). Swine umbilical vascular endothelial cells (SUVECs) and swine testicular (ST) cells were cultured in high-glucose DMEM (GIBCO, UK) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, USA) and 50 μg/ml heparin (Sigma-Aldrich, USA). In the experiments, quercetin known as inhibitor of HSP synthesis (Liu et al., 2013) was diluted in dimethyl sulfoxide (DMSO), and used at a final concentration of 100 μM. For different experiments of CSFV infection, cells were grown to approximately 70% confluence in cell culture plates and were infected with CSFV at an multiplicity of infection (MOI) of 1, as described previously(Lin et al., 2014), The control was infected with the same volume of cell culture solution.

Antibodies

ANTI-FLAG M2 Affinity Gel utilizes a purified murine IgG_1 monoclonal antibody (SIGMA-ALDRICH®, A2220). Anti-c-Myc Agarose Affinity Gel antibody produced in rabbit (SIGMA-ALDRICH®, A7470). Anti-HSP70 produced in rabbit (SIGMA-ALDRICH™, QC21915), an anti-β-actin mouse monoclonal antibody (Tianjin Sungene Biotech, KM9001, China), an anti-GST-Tag mouse polyclonal antibody, and anti-Flag-Tag mouse monoclonal antibody and an anti-c-Myc mouse monoclonal antibody (CWBIO, China) were used. The secondary antibody was HRP-labeled Goat Anti-Mouse IgG or Goat Anti-Rabbit IgG (Beyotime, China).

Lentivectors construction and lentivirus production

The primers used to over-express HSP70 to generate pCDH-CMV-HSP70 (Table 1) contained EcoRI and BamHI restriction sites. The HSP70 gene was cloned into the over-expression lentivector pCDH-CMV-MCS-EF1-GreenPuro (CD513B-1) (SBI, Mountain View, CA, USA) to generate pCDH-CMV-HSP70. The three pairs of shRNA targeting pig HSP70 and a negative control shRNA listed in Table 2 were cloned into pCDH-U6-MCS-EF1-GreenPuro (SBI, Mountain View, CA, USA) to generate the pCDH-U6-HSP70-sh1, pCDH-U6-HSP70-sh2, pCDH-U6-HSP70-sh3 and pCDH-U6-HSP70-shN lentivectors, respectively. HEK-293T cells were transfected with the constructs along with three other plasmids (pGag/Pol, pRev, pVSV-G) into HEK293T cells using TurboFect (Thermo scientific) according to the manufacturer's protocol. The knockdown or overexpression experiments were performed as follows. ST cells (4×10^6/well) were seeded in a 6-well plate. After 24 h, 50% of the medium in each well was removed, and polybrene was added to a final concentration of 8 g/ml. The shRNA or HSP70 overexpressing lentiviruses (MOI=1) were added to the cells. After an overnight

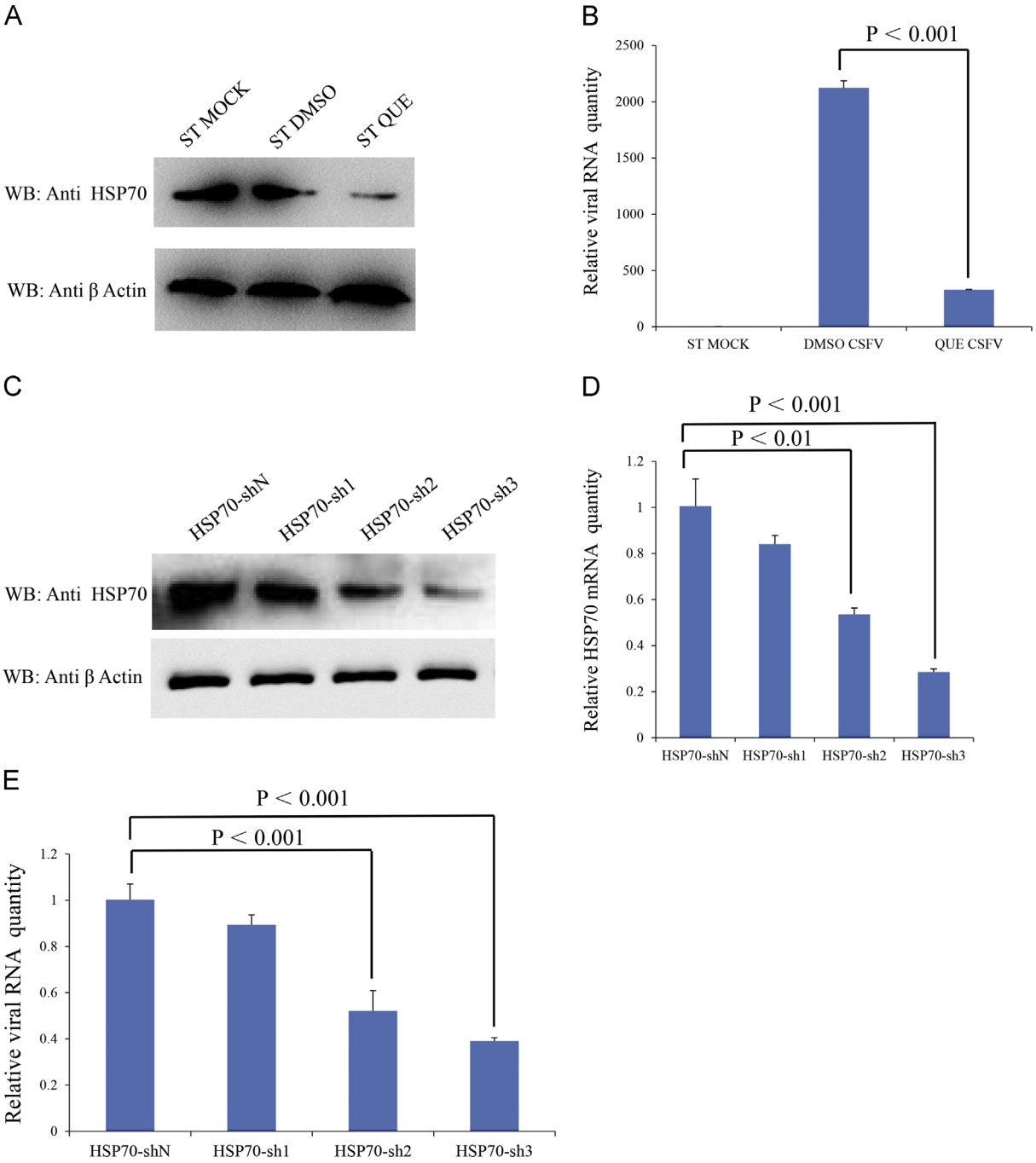

Fig. 6. CSFV replication is inhibited by quercetin or reduced by shRNA-mediated knockdown of HSP70. (A) Western blot analysis of cells treated with the above noted. (B) Intracellular viral RNA levels assay by RT-qPCR demonstrates that treated by quercetin significant decrease in intracellular virus levels. (C) Western blot analysis of cells treated with the abobe noted. (D) Intracellular HSP70 production assay by RT-qPCR. (E) Intracellular viral RNA levels assay by RT-qPCR.

incubation at 37 °C, the medium was replaced with fresh medium and incubated for another 72 h. The cells were then used to evaluate the expression of HSP70 or for further experiments.

MTT assay

The cell viability upon silencing and inhibition of HSP70 was tested by MTT methods according to the manufacturer's instructions. Shortly, ST cells were seeded in 96-well culture dishes. Different concentrations of inhibitors were added in when the cell confluence reached to about 70–80%. After cultivation at 37 °C in incubator with 5% CO_2 for 24 h, the cell culture medium was removed from the wells. Then 50 μL MTT (2 μg/mL) was added to each well and incubated at 37 °C for 4 h. Subsequently, MTT was removed and 200 μL DMSO was added. To dissolve fomazan crystal, the 96-well culture plates were put in electronic oscillator for 10 min. Cell viability was quantified using a Multiskan FC Microplate Photometer (Thermo Scientific, USA). Three independent experiments were performed in triplicates.

Real-time RT-PCR

The target gene HSP70 and CSFV were quantified by real-time RT-PCR using the primers listed in Table 1. Total cellular RNA was isolated using TRIzol (Invitrogen), and cDNA was reverse transcribed from 1 μg of total RNA using a reverse transcription kit

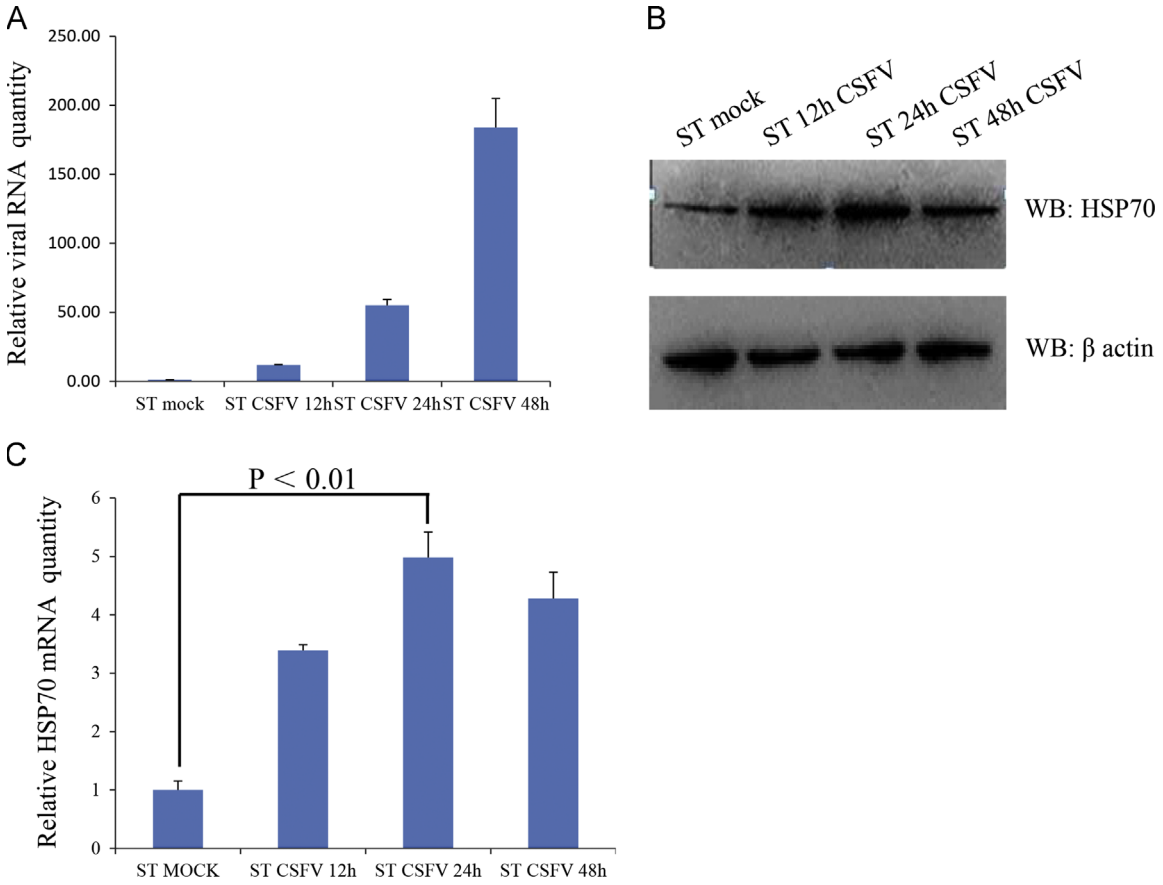

Fig. 7. The amount of HSP70 protein expression in the ST cells infected with CSFV virus. (A) Intracellular viral RNA levels assay by RT-qPCR. (B) Western blot analysis of cells treated with the above noted. (C) RT-qPCR analysis of intracellular HSP70 production treated with the above noted.

Table 1
Primers used in this study.

Primer	Sequence(5′–3′)	Use
NS5A-GFP-F	CCGAATTCTTCAAGTAATTACATACTAGAGC	Amplification of NS5A
NS5A-GFP-R	ACGGATCCCAGTTTCATAGAATACACTTTTGC	
HSP70-RED-F	CGGAATTCTGATGTCCGCTGCAAGAGAAG	Amplification of HSP70
HSP70-RED-R	CGGGATCCTCATCAACCTCCTCAATGACAG	
GST-HSP70-F	CGCGGATCCATGTCCGCTGCAAGAGAAG	Amplification of HSP70
GST-HSP70-R	CGCGAATTCTTAATCAACCTCCTCAATGACAG	
HSP70-Myc-F	ATTGGATCCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGTCCGCTGCAAGAGAAG	Amplification of HSP70
HSP70-Myc-R	ATTGAATTCTTAATCAACCTCCTCAATG	
NS5A-Flag-F	ATTGGATCCATGTCAAGTAATTACATACTAGAGC	Amplification of NS5A
NS5A-Flag-R	ATTCTCGAGTCACTTATCGTCGTCATCCTTGTAATC CAGTTTCATAGAATACAC	
NS5A-687-Flag-F	CGCGGATCCATGGTAGTGGTGGATACAACTGAC	Amplification of NS5A-687
NS5A-687-Flag-R	ATTCTCGAGTCACTTATCGTCGTCATCCTTGTAATCCAGTTTCATAGAATACAC	
NS5A-720-Flag-F	CGCGGATCCATGCCTGCCCCTTTCAGCTGTG	Amplification of NS5A-720
NS5A-720-Flag-R	ATTCTCGAGTCACTTATCGTCGTCATCCTTGTAATCAGCAGGCTGCAAGGTTATTTC	
NS5A-804-Flag-F	ATTGGATCCATGTCAAGTAATTACATACTAGAGC	Amplification of NS5A-804
NS5A-804-Flag-R	ATTCTCGAGTCACTTATCGTCGTCATCCTTGTAATCAGCAGGCTGCAAGGTTATTTC	
pCDH-CMV-HSP70-F	CGGAATTCATGTCCGCTGCAAGAGAAG	Amplification of HSP70
pCDH-CMV-HSP70-R	CGGGATCCTTAATCAACCTCCTCAATG	
β actin -F	**CAAGGACCTCTACGCCAACAC**	Quantitative real-time PCR for detection of β actin
β actin-R	**TGGAGGCGCGATGATCTT**	
CSFV-F	**GATCCTCATACTGCCCACTTAC**	Quantitative real-time PCR for detection of CSFV
CSFV-R	**GTATACCCCTTCACCAGCTTG**	
HSP70-F	**GCAGGCAGCAGTGTTGATGG**	Quantitative real-time PCR for detection of HSP70
HSP70-R	**TGGTGGCATTCCTCTGGATTAG**	

(Takara Bio, Dalian, China). RNA expression was normalized by the housekeeping gene β-actin. Quantitative real-time RT-PCR was carried out using SYBR ExScriptTM RT-PCR Kit (Takara Bio, Dalian, China). Reactions were performed under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 30 s. the relative transcript levels were analyzed using the ΔΔCt method as specified by the manufacturer (Schmittgen and Livak, 2008).

Table 2
Short hairpin RNA (shRNA) inserts.

shRNA	Sequence (loop in bold letters) (5′–3′)
pCDH-U6-HSP70-sh1S	GATCCGCTAATTGGGCGCAAGTTTGCCAAGAGGCAAACTTGCGCCCAATTAGCTTTTTG
pCDH-U6-HSP70-sh1A	AATTCAAAAAGCTAATTGGGCGCAAGTTTGCCTCTTGGCAAACTTGCGCCCAATTAGCG
pCDH-U6-HSP70-sh2S	GATCCGGCTCAGATCCATGATATTGTCAAGAGACAATATCATGGATCTGAGCCTTTTTG
pCDH-U6-HSP70-sh2A	AATTCAAAAAGGCTCAGATCCATGATATTGTCTCTTGACAATATCATGGATCTGAGCCG
pCDH-U6-HSP70-sh3S	GATCCGGGAACTGAACAAGAGCATCACAAGAGTGATGCTCTTGTTCAGTTCCCTTTTTG
pCDH-U6-HSP70-sh3A	AATTCAAAAAGGGAACTGAACAAGAGCATCACTCTTGTGATGCTCTTGTTCAGTTCCCG
pCDH-U6-HSP70-shNS	GATCCGATGAAATGGATAGAAGTACACAAGAGTGTACTTCTATCCATTTCATCTTTTTG
pCDH-U6-HSP70-shNA	AATTCAAAAAGATGAAATGGATAGAAGTACACTCTTGTGTACTTCTATCCATTTCATCG

Western blots

Cells grown in 6-well plates were rinsed once with PBS, detached by scraping into cell lysis buffer for western and IP (Beyotime, China) containing the proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF) (Beyotime, China). The cells were lysed for 30 min on ice and the insoluble material was removed by centrifugation at 13,000*g* for 10 min at 4 °C. The protein concentration was determined with BCA Protein Assay Kit (Beyotime Biotech, China, P0012S). SDS-PAGE and western blotting were carried out using standard methods. Briefly, equivalent amounts of cell lysate protein were separated on polyacrylamide-Tricine gels (12% polyacrylamide). After SDS-PAGE, the proteins were transferred onto 0.45-μm polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The membranes were blocked with 5% non-fat milk powder (Yili Industrial Group Co., Ltd., Hollyhock, China) in TBST (50 mM Tris HCl, with 150 mM NaCl, PH 7.4, containing 0.5% Tween 20) for 2 h at RT. After washing, the membranes were incubated with an anti-β-actin antibody or primary antibodies (diluted in 2% non-fat milk powder according to the manufacturer's instructions) with shaking overnight at 4 °C. Thereafter, a secondary HRP-labeled goat anti-mouse or anti-rabbit IgG (H+L) antibody (Bioss, Bei Jing, China) diluted at 1:5000 in blocking solution was added to the membranes and incubated for 2 h at RT. After washing 3 times with TBST for 10 min each, detection was performed using chemiluminescence luminol reagents (SuperSignal West PicoTrial Kit, Pierce).

Co-immunoprecipitation assays

SUVECs transfected with NS5A-Flag or co-transfected to express NS5A-Flag and HSP70-Myc for 48 h were harvested in cell lysis buffer for western blot and IP (Beyotime, China) containing PMSF (Beyotime, China). The lysate was collected and utilized for coimmunoprecipitation assays using ANTI-FLAG M2 Affinity Gel (SIGMA-ALDRICH®, A2220) or Anti-c-Myc Agarose Affinity Gel antibody according to the manufacturer's instructions. Briefly, 50 μl of the resin stored in 50% glycerol was centrifuged at 8000*g* for 30 s and rinsed twice with 500 μl of TBS (50 mM Tris HCl, with 150 mM NaCl, PH 7.4). A 1000 μl sample of cell lysate was added to the washed resin and the samples were agitated overnight at 4 °C. The resin was washed three times with 500 μl of TBS and the sepharose pellet was resuspended in 2 × SDS sample buffer. After boiling, the supernatant was subjected to SDS-PAGE analysis.

GST-pulldown assays

For GST-pulldown assays, GST or GST-HSP70 protein were generated by expression in *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) using the pGEX6p-1 plasmid. The proteins were bound to glutathione agarose (Thermo Scientific, 21516) according to the manufacturer's instruction and the beads were washed four times with 1:1 wash solution (TBS (25 mM Tris–HCl, 0.15 M NaCl, pH 7.2): Pull-Down Lysis Buffer and incubated for 2 h at 4 °C with recombinant Flag-tagged NS5A harvested from transfected HEK293T cells. The eluted proteins were detected by SDS-PAGE and immunoblotting.

Confocal microscopy

SUVECs were cultured for 24 h on glass coverslips in six-well plates at a density of $\sim 2 \times 10^6$ cells/well and transfected with 3 μg of pEGFP-NS5A and 3 μg of pERED-HSP70 or the same amount of empty vector using TurboFect (Thermo scientific, #R0531) according to the manufacturer's instructions. After a 48- h incubation, the cells were washed with cold 1 × PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and incubated with DAPI at 37 °C for 20 min. Images were viewed by laser-scanning confocal microscopy (LSM510 META; Zeiss, Germany).

Statistical analysis

Data analyses were performed as mean ± SD. Differences in each groups were examined for statistical significance using Student's *T* test and *P* values less than 0.05 was considered statistically significant.

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