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RNA interference screening of interferon-stimulated genes with antiviral activities against classical swine fever virus using a reporter virus

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ABSTRACT

Classical swine fever (CSF) caused by classical swine fever virus (CSFV) is a highly contagious and often fatal disease of pigs, which leads to significant economic losses in many countries. Viral infection can induce the production of interferons (IFNs), giving rise to the transcription of hundreds of IFN-stimulated genes (ISGs) to exert antiviral effects. Although numerous ISGs have been identified to possess antiviral activities against different viruses, rare anti-CSFV ISGs have been reported to date. In this study, to screen anti-CSFV ISGs, twenty-one ISGs reported previously were individually knocked down using small interfering RNAs (siRNAs) followed by infection with a reporter CSFV expressing *Renilla* luciferase (Rluc). As a result, four novel anti-CSFV ISGs were identified, including natural-resistance-associated macrophage protein 1 (NRAMP1), cytosolic 5′-nucleotidase III A (NT5C3A), chemokine C-X-C motif ligand 10 (CXCL10), and 2′-5′-oligoadenylate synthetase 1 (OAS1), which were further verified to exhibit antiviral activities against wild-type CSFV. We conclude that the reporter virus is a useful tool for efficient screening anti-CSFV ISGs.

1. Introduction

Classical swine fever virus (CSFV) is the etiologic agent of classical swine fever (CSF), a highly contagious and fatal disease of pigs. The disease results in significant economic losses to the pork industry in many countries. CSFV belongs to the genus *Pestivirus* within the family *Flaviviridae* (Vilcek and Nettleton, 2006) and it is a small, enveloped, and positive-sense RNA virus, whose genome contains a 5′-untranslated region (5′-UTR), 3′-UTR, and a single large open reading frame (ORF) encoding a polyprotein of 3898 amino acids that is co- and posttranslationally processed by cellular and viral proteases to yield four structural and eight nonstructural (NS) proteins in the following order: N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Bintintan and Meyers, 2010; Heimann et al., 2006; Moulin et al., 2007; Rümenapf et al., 1993; Ruggli et al., 1996).

Like other viruses, CSFV induces host defense responses upon infection, including the innate immune response, which leads to the production of interferons (IFNs). Subsequently, IFNs activate the JAK-STAT signaling pathway by binding their cognate receptors, resulting in the transcription of numerous IFN-stimulated genes (ISGs) to exert antiviral activities (Borden et al., 2007; Medzhitov, 2009; O'Neill and Bowie, 2010; Takeuchi and Akira, 2010). Importantly, most ISGs can disrupt distinct steps of the viral replication cycle, including entry, uncoating, genome replication, particle assembly, and egress. Currently, insight into the effector functions of ISGs-encoded proteins has been limited primarily to a handful of molecules. For example, protein kinase R (PKR), interferon-inducible transmembrane proteins (IFITM), viperin, 2′-5′-oligoadenylate synthetase/ribonuclease L (OAS/RNase L), and ISG15 have been uncovered to instigate an antiviral state against hepatitis C virus (HCV) (Bigger et al., 2001; Helbig et al., 2005; Metz et al., 2013; Schoggins et al., 2011). Furthermore, few anti-CSFV ISGs have been reported, with only myxovirus resistance protein 1 (Mx1) identified to have an antagonistic effect on CSFV (Yan et al., 2014).

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To date, wild-type (wt) viruses combining with gene microarray, proteomic, or the transcriptome sequencing are widely used to screen antiviral ISGs (de Veer et al., 2001; Hou et al., 2014; Pichlmair et al., 2011). In contrast with wt viruses, reporter viruses expressing enhanced green fluorescent protein (EGFP), firefly luciferase (Fluc), or *Renilla* luciferase (Rluc) can be used to sensitively and conveniently quantify viral replication and gene expression (Fang et al., 2006, 2008; Kim et al., 2007; Liu et al., 2011; Li et al., 2013b; Shen et al., 2014).

In this study, a reporter virus expressing Rluc in combination with a series of small interfering RNAs (siRNAs) targeting twenty-one previously reported ISGs was used to screen anti-CSFV ISGs, resulting in the identification of four novel anti-CSFV ISGs, natural resistance-associated macrophage protein 1 (NRAMP1), cytosolic 5′-nucleotidase III A (NT5C3A), chemokine C-X-C motif ligand 10 (CXCL10), and 2′-5′-oligoadenylate synthetase 1 (OAS1).

2. Materials and methods

2.1. Cells and viruses

CSFV-permissive porcine kidney cell line (PK-15) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Shanghai, China) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and incubated at 37 °C with 5% CO_2. The CSFV Shimen strain (GenBank accession no. AF092448.2), vSM-Fluc, or rescued reporter virus was propagated in PK-15 cells in DMEM supplemented with 2% FBS.

2.2. Construction of the infectious cDNA clone pSM-Rluc

The infectious cDNA clone pSM-Rluc (Fig. 1) was constructed based on the CSFV Shimen strain full-length infectious cDNA pBRCISM (Li et al., 2013a). Briefly, the *Rluc* gene amplified by PCR from the pRL-null vector (Promega, Madison, WI, USA) was introduced between amino acids 13 and 14 of N^{pro} by standard overlapping PCR with the following primer pairs: N^{pro}Rluc-1-F (5′-CCC TCG AGA TGC TAT GTG GAC GAG GGC ATG-3′, the *Xho*I site underlined)/N^{pro}Rluc-1-R (5′-CTG GAT CAT AAA CTT TCG AAG TCA TGT TTG TTT TGT ATA AAA GTT CAA A-3′), N^{pro}Rluc-2-F (5′-TTG AAC TTT TAT ACA AAA CAA ACA TGA CTT CGA AAG TTT ATG ATC CAG-3′)/N^{pro}Rluc-2-R (5′-CCA CTC CCA TTG GTT TTT GTT TTT GTT CAT TTT TGA GAA CTC GCT C-3′) and N^{pro}Rluc-3-F (5′-GCG AGT TCT CAA AAA TGA ACA AAA ACA AAA ACC AAT GGG AGT GGA G-3′)/N^{pro}Rluc-3-R (5′-CTC TAG AGG GGC CCT ATG GTA GAC CG-3′, the *Xba*I site underlined). Then, the fusion fragment was digested with *Xho*I and *Xba*I and subsequently ligated into pBRCISM cut with the same enzymes. Finally, the infectious clone named pSM-Rluc was verified by multiple restriction endonuclease digestion and sequencing.

2.3. Generation of the reporter CSFV vSM-Rluc

The reporter CSFV was rescued as described previously (Li et al., 2013a). Briefly, PK-15 cells of 80% confluence were transfected with 4 μg of pSM-Rluc using the X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany) and incubated for 6 h at 37 °C in a humidified 5% CO_2 incubator and subsequently washed three times with DMEM and maintained in DMEM supplemented with 2% FBS for 2 d. Then, the transfected cells were passaged blindly four times and the virus was harvested by three cycles of freeze-thawing followed by centrifugation at 1000 × *g* for 10 min. The supernatant was preliminarily tested by a commercial CSFV antigen-capture ELISA (IDEXX, Liebefeld-Bern, Switzerland).

2.4. Indirect immunofluorescence assay (IFA)

The CSFV Shimen strain- or vSM-Rluc-infected PK-15 cells were washed with PBS and fixed with cold ethanol for 30 min at −20 °C. The fixed cells were then incubated for 2 h at 37 °C in the presence of CSFV-specific anti-E2 monoclonal antibody (MAb) at a dilution of 1:400 and washed five times with PBS. Then, the cells were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG at a dilution of 1:100 (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. Finally, the cells were washed again with PBS and analyzed under fluorescence microscope (Nikon, TE2000U, Melville, NY, USA).

2.5. Virus titration

At different time points postinfection, cells were lysed by three cycles of freeze-thawing and the supernatant was clarified by centrifugation at 1000 × *g* for 10 min. Tenfold dilutions of the clarified supernatant were distributed to each of 4 wells of a 96-well plate seeded with PK-15 cells. The titers were determined by IFA at 48 h postinfection (hpi) and calculated according to the Reed and Münch (1938) method.

2.6. One-step growth curve of vSM-Rluc

PK-15 cells grown in a 24-well plate were infected with vSM-Rluc or the CSFV Shimen strain at a multiplicity of infection (MOI) of 0.1. After 2 h at 37 °C, the infected cells were washed and incubated in fresh medium at 37 °C and 5% CO_2. At different time points postinfection, the viruses were harvested by three cycles of freeze-thawing and titrated on PK-15 cells according to the Reed and Münch (1938) method.

2.7. Renilla luciferase (Rluc) activity assay

PK-15 cells grown in 48-well plates were infected with vSM-Rluc at a 100 median tissue culture infective dose ($TCID_{50}$), and a *Renilla* luciferase assay system (Promega, Madison, WI, USA) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) was used to detect the Rluc activities in relative light units (RLU) according to the manufacturer's instructions.

2.8. RT-PCR and real-time RT-PCR

Total RNA of virus-infected PK-15 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with

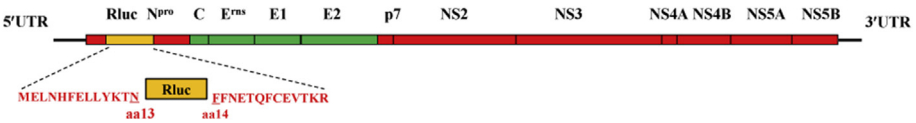

Fig. 1. Schematic diagram of the infectious cDNA clone pSM-Rluc. The Rluc gene was inserted to pBRCISM, creating pSM-Rluc. The structural proteins (green) and nonstructural proteins (red) of CSFV genome are depicted. In addition, the N^{pro}Rluc fusion gene is enlarged, in which the Rluc gene (yellow) is introduced between the amino acids 13 and 14 of N^{pro} (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)