Screening of cellular proteins that interact with the classical swine fever virus non-structural protein 5A by yeast two-hybrid analysis

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Classical swine fever virus (CSFV), the pathogen of classical swine fever (CSF), causes severe hemorrhagic fever and vascular necrosis in domestic pigs and wild boar. A large number of evidence has proven that non-structural 5A (NS5A) is not only a very important part of viral replication complex, but also can regulate host cell's function; however, the underlying mechanisms remain poorly understood. In the current study, aiming to find more clues in understanding the molecular mechanisms of CSFV NS5A's function, the yeast two-hybrid (Y2H) system was adopted to screen for CSFV NS5A interactive proteins in the cDNA library of the swine umbilical vein endothelial cell (SUVEC). Alignment with the NCBI database revealed 16 interactive proteins: DDX5, PSMC3, NAV1, PHF5A, GNB2L1, CSDE1, HSPA8, BRMS1, PPP2R3C, AIP, TMED10, POLR1C, TMEM70, METAP2, CHORDC1 and COPS6. These proteins are mostly related to gene transcription, protein folding, protein degradation and metabolism. The interactions detected by the Y2H system should be considered as preliminary results. Since identifying novel pathways and host targets, which play essential roles during infection, may provide potential targets for therapeutic development. The finding of proteins obtained from the SUVEC cDNA library that interact with the CSFV NS5A protein provide valuable information for better understanding the interactions between this viral protein and the host target proteins.

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1. Introduction

Classical swine fever (CSF) is a highly contagious and fatal viral disease of domestic pigs and wild boar, classified as a notifiable (previously List A) disease by the World Organization for Animal Health (OIE) because of its considerable socio-economic impact on the pig industry. Classical swine fever virus (CSFV) is the pathogen of CSF, and together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), it is classified as a member of the genus *Pestivirus* within the Flaviviridae family (Becher and Thiel 2002). Hepatitis C virus (HCV), the major cause of post-transfusion hepatitis, also belongs to this family (Cuthbert 1994). Although attenuated CSFV C strain vaccine obtained by passaged CSFV in rabbit has been wildly used to control CSFV infection in the clinic, so far, there are no effective therapies to counter act CSFV infection. Therefore, identifying novel pathways and host targets that play essential roles during infection will provide potential targets for therapeutic development.

CSFV is a small, enveloped virus with a 12.3 kb single positive-sense RNA genome. Its genome consists of a single large open reading frame (ORF), which is flanked by a 5′untranslated region (5′ UTR) and a 3′untranslated region (3′ UTR). A polyprotein is first synthesized, and subsequently processed into four structural proteins (C, E^{rns}, E1 and E2) and

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eight non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)(Tang *et al.* 2010, 2011).

The function of the CSFV NS5A has been extensively explored. Encoding by 497 amino acids NS5A can repress the internal ribosome entry site (IRES) located in the 5′ UTR, interact with the 3′ UTR, and regulate viral replication (Xiao *et al.* 2009; Sheng *et al.* 2010; Chen *et al.* 2012). Recently a study showed that the CSFV NS5A protein can induce oxidative stress in the host endothelial cells (He *et al.* 2012); however, the exact function of the protein remains unknown. In particular, the functions of NS5A in the modulation of the host cell environment have not been reported so far. In HCV, NS5A protein is reported as an essential component of the viral RNA replication complex and modulates the host cell environment (He *et al.* 2001; Tellinghuisen *et al.* 2004). In addition, the HCV NS5A plays an essential role in the modulation of viral and cellular RNA translation (He *et al.* 2003; Kalliampakou *et al.* 2005; Wu *et al.* 2008).

To better understand the regulatory functions of the CSFV NS5A in the host cell, we tried to identify and analyse the cellular proteins that interact with NS5A via a yeast two-hybrid screening, and to analyse its intricate functions. Identification of cellular cofactors and their mechanisms of action is a fundamental aspect of virus–host interaction research. Screening of genome-wide cDNA libraries has become an efficient way of systematically discovering cellular cofactors which are essential for various aspects of viral life cycle. The yeast two-hybrid (Y2H) system is an effective and accurate method for researching protein interactions, and which is frequently used to search unknown cellular interactive proteins by using a certain protein (Huang *et al.* 2002; Sun *et al.* 2005; Johns *et al.* 2010; Kang *et al.* 2012).

Previous studies have shown that NS5A protein is localized in the endoplasmic reticulum (He *et al.* 2012). In this study, the bait vector, pGBKT7-NS5A-721, was constructed to express a 240-amino-acid domain of the CSFV NS5A protein. The pGBKT7-NS5A-721 vector expressed peptides localized both in the nucleus and cytoplasm. And the SUVEC cDNA library was screened with the bait vector in a Y2H system. We report here that screened protein genes provide useful information for studying the role of NS5A in the interaction between CSFV and the host cell.

2. Materials and methods

2.1 *Construction of CSFV NS5A-GFP fusion vector and bait vector*

The CSFV NS5A protein itself cannot enter the nucleus of eukaryotic cells; however, when using the Y2H system, the bait protein must be able to enter the nucleus. Thus, we computer-analysed the NS5A gene of CSFV and found that in the 804 bp sequence there is a low complexity region. Furthermore, it has been reported that there is a membrane positioning sequence in the first 84 bp of the N-terminus (Brass *et al.* 2007). Based on these findings, we divided NS5A into five fragments (figure 1). To amplify the 721 bp fragment, which expression product would locate to both in the nucleus and cytoplasm of the NS5A gene, the primers used are listed in table 1 (underlined is the recognition sequence of the *Eco*RI and *Bam*HI restriction enzyme). pEGFP-NS5A (conserved in our lab) was used as the template, PCR was carried out to amplify the each fragment as standard procedures. PCR products were detected by 1.5% agarose gel electrophoresis, purified from the gel with Gel extraction kit (Biotech, China) and digested with restriction enzymes (*Eco*RI/*Bam*HI; Takara, China). Thereafter, the fragment were cloned into the pEGFP-C1 expression vector or the yeast expression vector pGBKT7 (Clontech, USA), and then the authenticity and right orientation of the cloned sequence were confirmed by restriction digestion and sequencing.

2.2 *Confocal microscopy*

The SUVEC cell line was established in our laboratory and was cultured at 37°C and 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO, UK) containing 10% heat-inactivated fetal serum (FCS) (Hyclone, China), 50 μg/mL heparin (Sigma-Aldrich, USA), and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) as previously described (Hong *et al.* 2007). The subcellular localization of the five CSFV NS5A constructs was detected with confocal microscopy, as previously described. Briefly, SUVECs were grown on glass coverslips in 6-well tissue culture plates, washed with Hank's balanced salt solution (HBSS), incubated with Hoechst33342 (10 ng/mL; Beyotime, China) at 37°C for 15 min, and then washed twice with HBSS. Cells were then incubated with ER-tracker Red probe (10 ng/mL; Invitrogen) at 37°C for 15 min and washed with DMEM without serum. Images were viewed by laser confocal scanning microscopy (LSM510 META; Zeiss, Germany).

2.3 *RT-PCR and Western blotting*

The purified plasmid containing pGBKT7-NS5A-721 and pGBKT7 were transformed into strain AH109. About 1 mm transformant colonies were cultured in 6 mL SD/-Trp liquid medium at 30°C with shaking at 250 rpm until the OD600 reached 0.4–0.6. Total RNA was extracted from 5 mL cultured yeast using RNA pure Yeast Kit (Tarunion, China). The isolated RNA was then reverse-transcribed to cDNA with Oliga dT according to the manufacturer's

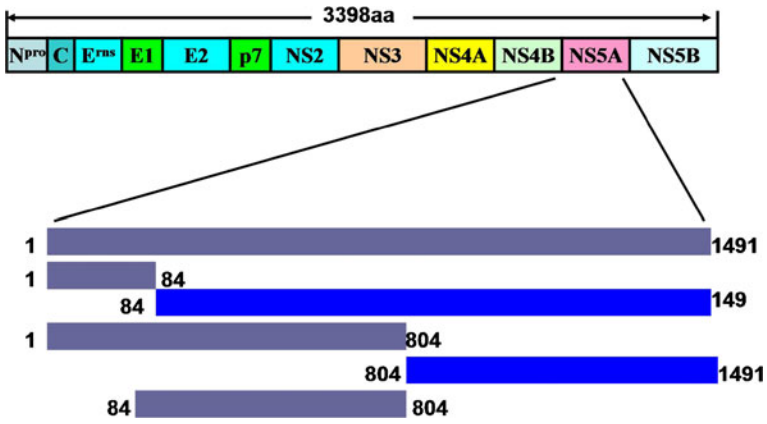


Figure 1. Schematic representation of the CSFV NS5A deletion-mapping results. Based on the fact that the CSFV NS5A protein contains a low complexity region and a membrane positioning sequence, NS5A gene was divided into five sections as shown in the figure.

instructions. RT-PCR was performed using 2 μL cDNA as a template with the primer pairs: NS5A-721-F and NS5A-721-R (table 1). PCR was carried out as standard procedure. Another 5 mL cultures were harvested at 4°C. Then, 100 μL acid-washed glass beads, 300 μL of 6 M urea, 40 μL 100 mM dithiothreitol, and 260 μL loading buffer (50 mM Tris pH 6.8, 2% SDS, 0.05% bromophenol blue, 10% glycerol) were added to the pellet, followed by vortexing for 5 min and boiling for 5 min. After being centrifuged at 12,000 rpm for 5 min at 4°C, the supernatant was collected. SDS-PAGE and Western blotting were carried out by standard methods using anti-c-Myc (the plamid of pGBKT7 contain a c-Myc label) and antiβ-actin monoclonal antibodies (Abcam, California, USA) at a dilution of 1:500. The secondary antibody used was HRP-labelled Goat Anti-Mouse IgG (Beyotime, China).

Table 1. Primers used for the construction of fivesubclones of the CSFV NS5A and bait vector

Primers	Sequence (5′ → 3′)
NS5A/1-84-F	5′-CC***GAATTC***TTCAAGTAATTACATTC TAGAGCTCCTG-3'
NS5A/1-84-R	5′-AA***GGATCC***GGCCCAGCTGATTGC-3'
NS5A/84-1491-F	5′-AA***GAATTC***TCCTGCCCCTTTCAGT TGTG-3'
NS5A/84-19491-R	5′-AC***GGATCC***CAGTTTCATAGAATAC ACTTTTGC-3'
NS5A/1-804-F	5′-CC***GAATTC***TTCAAGTAATTACATT CTAGAGCTC-3'
NS5A/1-804-R	5′-TA***GGATCC***AGCAGGCTGCAAGGTT ATC-3'
NS5A/84-804-F	5′-TC***GAATTC***TGTAGTGGTGGATACA ACTG-3'
NS5A/84-804-R	5′-TA***GGATCC***AGCAGGCTGCAAGGTT ATC-3'
NS5A/804-1491-F	5′-TC***GAATTC***TGTAGTGGTGGATACA ACTGACG-3'
NS5A/804-1491-R	5′-AC***GGATCC***CAGTTTCATAGAATAC ACTTTTGC-3'
NS5A-721-F	5′-AA***GAATTC***CCTGCCCCTTTCAGTTG TG-3'
NS5A-721-R	5′-TA***GGATCC***AAGCAGGCTGCAAGGT TATC-3'

2.4 *Screening of the SUVEC library and selection of the CSFV NS5A interactive clones*

Matchmaker TM Library Construction & Screening Kits, Advantage TM 2 PCR Kit, Yeast strain AH109, library construction pGADT-Rec vector, SD/-Trp/-Leu/-His/-Ade, SD/-Trp/-Leu medium and X-α-Gal were purchased from Clontech (USA). Yeast nitrogen base without amino acids (YNB) was purchased from Wolsen (China). Acid glass beads were purchased from Sigma (St. Louis, MO, USA). Fresh yeast competent cells were prepared by the TE/LiAc method. The LiAc cotransformation method was used to transform the bait vector pGBKT7-NS5A-721, dscDNA and the linearized DNA-AD vector pGADT-Rec simultaneously into yeast competent cells which were then grown on SD/-His/-Leu/-Trp medium for 3–6 days at 30°C. Colonies of 2–3 mm in diameter were transferred to SD/-His/-Leu/-Trp/-Ade medium and grown for 3–6 days at 30°C. Next, all the colonies on the SD/-His/-Leu/-Trp/-Ade medium were then inoculated on to SD/-His/-Leu/-Trp/-Ade-X-α-Gal medium and cultivated 3 times on the same medium. Blue colonies were considered as positive clones, and were inoculated into 5 mL SD/-Trp/-Leu liquid medium and incubated for 1–3 days in a shaker at 30°C. Yeast plasmids were extracted from the cells using the TIANprep Yeast plasmid DNA kit (Tiangen China). Initially, the NS5A-721

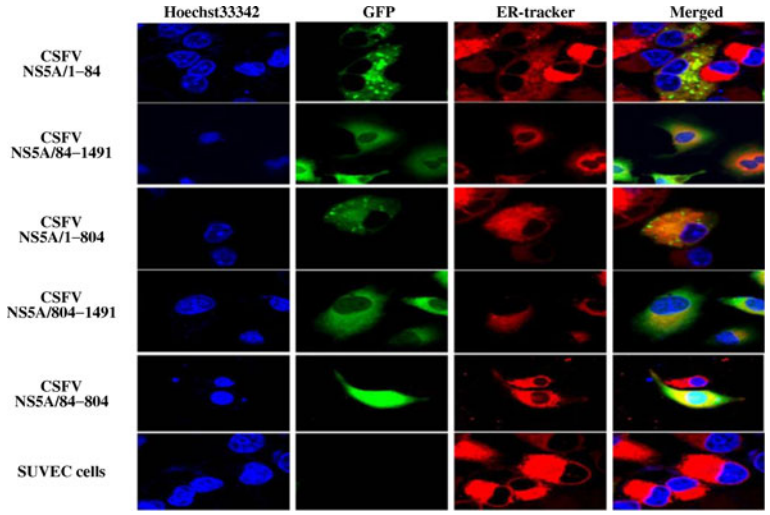


Figure 2. Detection of the five CSFV NS5A GFP fusion proteins and their subcellular localization in SUVECs by confocal microscopy. All cells were stained with Hoechst33342 and ER-tracker. The GFP fusion protein showed that NS5A/84-804 was located both in the nucleus and the cytoplasm.

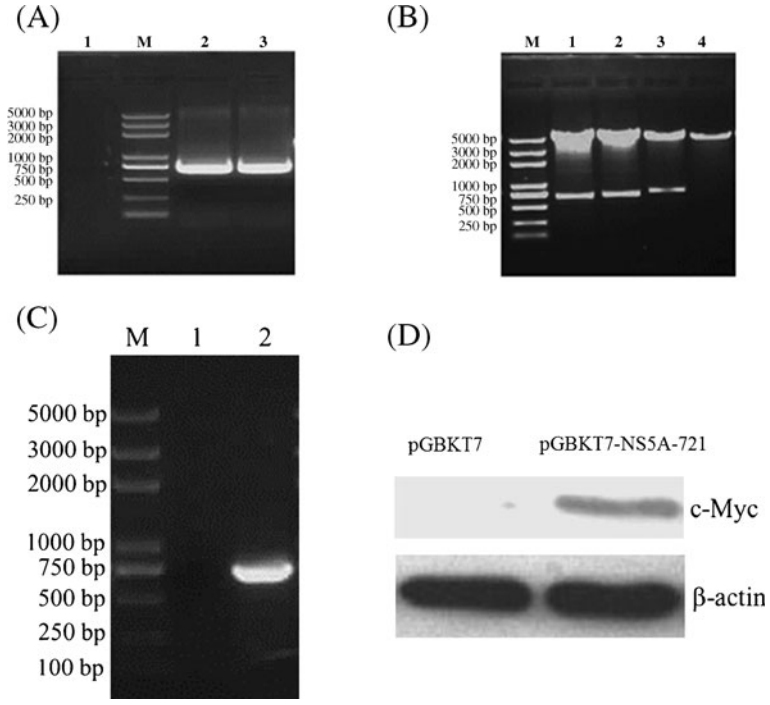


Figure 3. Construction and identification the bait vector. (**A**) Gel electrophoresis image of the target gene. Lane M is Tans 2000plus DNA Marker; lanes 2 and 3 are the 721bpfragment of the NS5A the bait protein that can transport into the nucleus; lane 1 is a negative control. (**B**) Gel electrophoresis image of the identification of the recombinant plasmid by enzyme digestion. Lane M is Tans 2000plus DNA marker; lanes 1–3 are pGBKT7-NS5A-721 digested by*Eco*RI and *Bam*HI; lane 4 is pGBKT7-Rec digested by *Eco*RI and *Bam*HI. (**C**) Gel electrophoresis image of theRT-PCR to the target gene. Lane M is Tans 2000plus DNA marker; the template of lane 1 is cDNA from pGBKT7; the template of lane 2 is cDNA from pGBKT7-NS5A-721. (**D**) Expression of bait fusion protein in AH109 yeast. AH109 yeast transformed with pGBKT7-NS5A-721 or empty vector pGBKT7 as a control were lysed and resolved on SDS-PAGE for Western blotting using anti-c-MYC and anti-β actin.

fragment of the bait plasmid was amplified using theSNS5A-721 and ANS5A-721 primers to exclude false-positive clones resulting from library plasmids activation of library plasmids. The extracted plasmid, which contains the NS5A-721 fragment as a second template, was amplified from the cDNA fragment library using LD-PCR primers. The plasmids of both PCR results were identified as positive candidate genes, in line with expectations. The procedure above was processed according to the Yeast Protocols Handbook (Clontech).

Murine p53 and SV40 large T-antigen, which are known to interact in Y2H assays, were used as positive controls. Lamin C, which does not interact with most other proteins, was expressed from pGBKT7-Lam as a negative control for any fortuitous interaction between an unrelated protein and either the pGADT7-T control or DNA-AD plasmids.

2.5 *Confirmation and sequencing analysis of rescuing library plasmid positive clones*

Because the plasmids from each yeast colony might be a mixture of bait plasmid and at least one type of AD/library plasmid, we needed to separate the plasmids in *E. coli.* The isolated plasmids were transformed into DH5α competent cells (Tiangen Biotech, China), which were then grown on LB medium containing 50 μg/mL ampicillin to select for AD/library plasmids only. Each isolated cDNA fusion plasmid was transformed again with the bait plasmid into the yeast competent cells AH109. Clones that grew on SD/-Trp/-Leu/-His/-Ade plates were confirmed as positive clones. The cDNA insertion was amplified by PCR using the Advantage 2 PCR kit (Clontech). The plasmids and PCR products were sequenced and blasted in GenBank.

2.6 *Functional classification and pathway analysis*

Functional classification analysis was based on Gene Ontology and the UniProt database. Identified proteins were organized into functional classifications based on the biological process. Pathway analysis was mainly based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa *et al.* 2002). The data that were chosen had to have GO categories with a *P*-value, and pathways that involved identified proteins.

2.7 *Construction of the NS5A–cellular protein interaction network*

An NS5A–cellular protein interaction network was constructed based on the data of identified proteins. According to the experimental results, the interaction score of NS5A

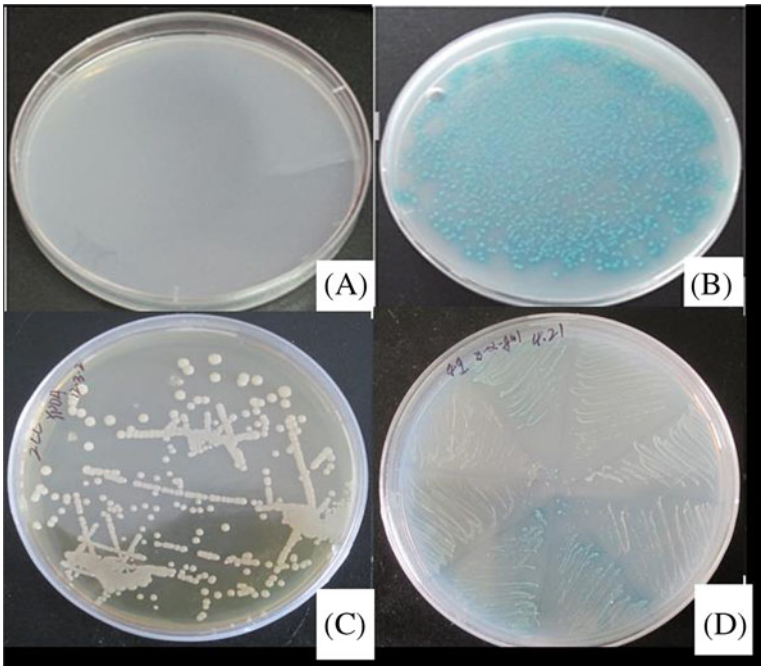


Figure 4. Screening of the SUVEC cDNA library by cotransformation. (**A**) and (**B**) are the negative and positive controls of transformed competent cells grown on the SD/-His/-Leu/-Trp/-Ade/X-α-Gal plat. (**C**) is one of the SD/-His/-Leu/-Trp plates with transformed colonies. (**D**) is the SD/-His/-Leu/-Trp/-Ade/X-α-Gal plate on which the blue colonies were potential positive clones.

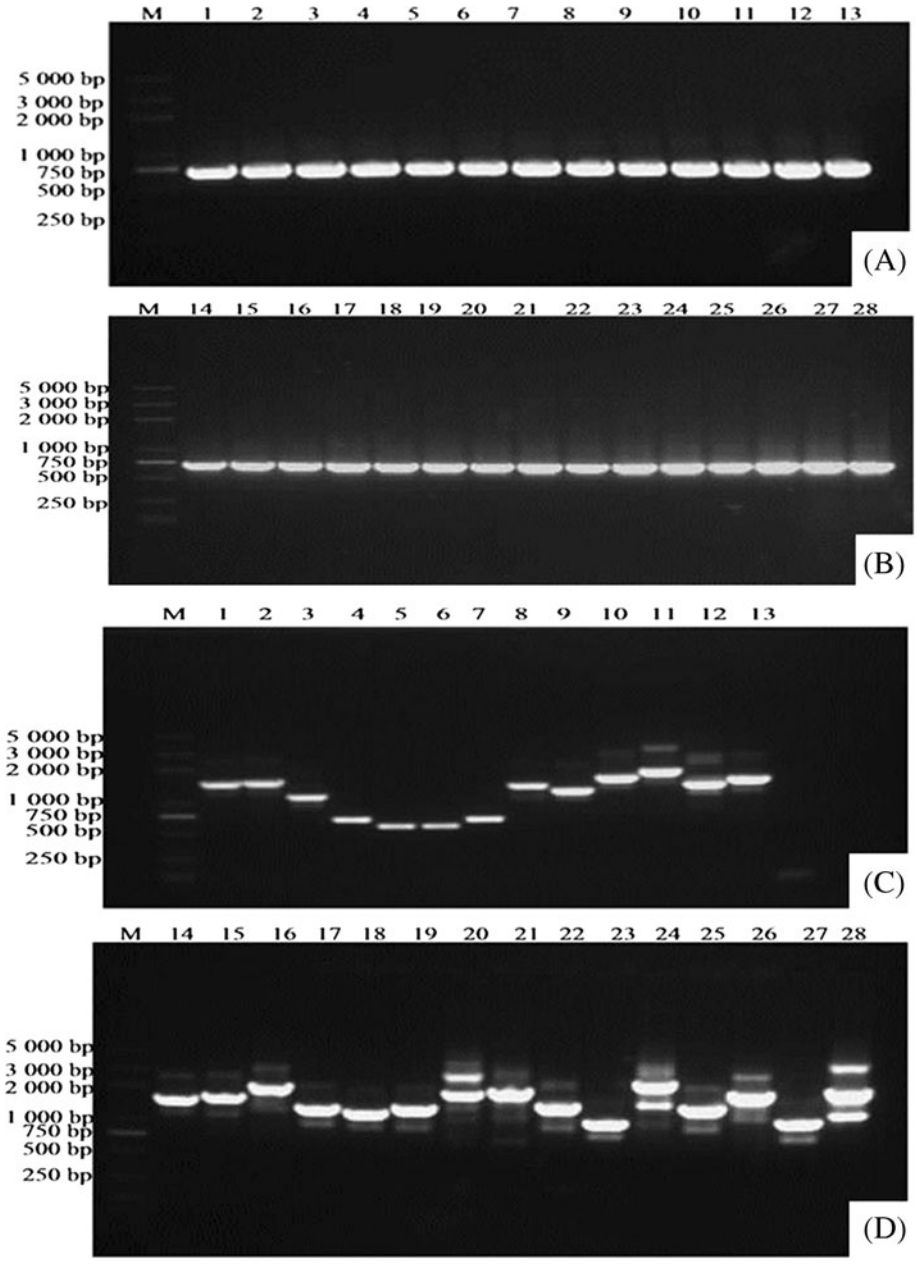


Figure 5. Identification of positive clones. Plasmids from the blue colonies, which were potential positive clones that grew on SD/-His/-Leu/-Trp/-Ade/X-α-Gal plates, were amplified to ensure that both the bait gene and cDNA were in the same colony. (**A**) and (**B**) show the PCR results of the bait gene from the plasmid extract. (**C**) and (**D**) show the PCR results of the dscDNA genes.

with 16 host cell proteins (DDX5, PSMC3, NAV1, PHF5A, GNB2L1, CSDE1, HSPA8, BRMS1, PPP2R3C, AIP, TMED10, POLR1C, TMEM70, METAP2, CHORDC1 and COPS6) was set to 0.999. Based on the correlation between the proteins in the STRING 9.0 database (Szklarczyk *et al.* 2011), the NS5A–cellular protein interaction network was constructed using the Cytoscape v2.8.1 software. NS5A protein is a core protein. In the current report, nodes were applied to represent the proteins, and the interactions between proteins were expressed by straight lines between the nodes. The most central protein tended to be more essential than non-central proteins in the modular organization of the NS5A–cellular protein interaction network. A sub-network was used to identify a group of same-coloured

Table 2. The results of the positive clonesBLAST to theGenBank

Protein no.	Protein name	Gene	NCBI protein accession no.		Max identity (%)	No. of clones
			Pig	Human		
1	ATP-dependent RNA helicase DDX5	DDX5	AK350880.1	AB451257.1	96	6
2	Proteasome (prosome, macropain) 26Ssubunit, ATPase, 3	PSMC3	AK232134.1	BC106920.2	91	4
3	neuron navigator 1	NAV1	XM_003130607.3	BC007523.1	89	1
4	PHD finger protein 5A	PHF5A	AK351244.1	BCO75808.1	87	1
5	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	GNB2L1	AK398315.1	BC000366.2	91	1
6	highly similar to Actin, cytoplasmic 2	CSDE1	AY550069.1	AK316361.1	93	2
7	heat shock 70 kDa protien 8	HSPA8	AK399549.1	BC042163.1	91	1
8	breat cancer metastasis-suppressor 1	BRMS1	AK392644.1	AF159141.1	87	2
9	Phosphatase 2 (formerly 2A), regulatory subunit B", gamma	PPP2R3C	AK343977.1	BC012563.1	93	2
10	aryl hydrocarbon receptor interacting protein	AIP	XM_003122456.1	BC104827.1	91	3
11	Transmembraneemp224-like trafficking protein 10	TMED10	AK235607.1	BC001496.2	88	1
12	Polymerase (RNA) I polypeptide C, 30 kDa	POLRIC	AK234320.1	BC008863.2	87	2
13	transmembrane protein 70	TMEM70	AK392799.1	BC002748.2	91	2
14	Methionylaminopeptidase 2	METAP2	AK239778.1	AK315559.1	95	1
15	cycteine and histidine-rich domain(CHORD)-containing, zinc binding protein 1	CHORDC1	None	AK315559.1	95	1
16	COP9 constitutive photomorphogenic homolog subunit 6	COPS6	AK394846.1	BC002520.2	91	1

proteins. These proteins, which have a relationship with the same cellular protein, interacted with NS5A in the network, thus implying that they shared similar biological functions.

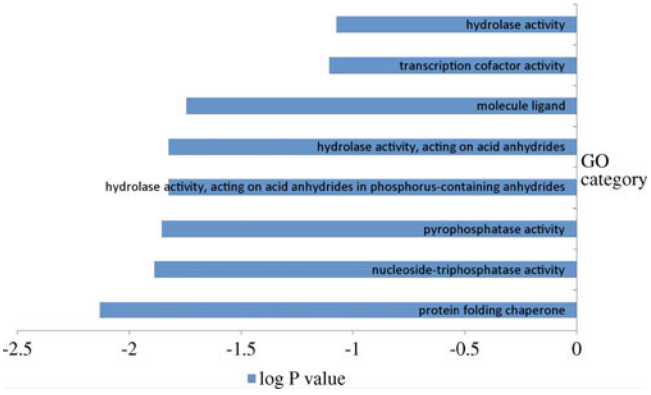


Figure 6. Functional classification of the identified proteins. Functional classification of the identified proteins was performed according to GO biological processes. All GO categories with a *P*-value were chosen. These identified proteins were sorted by the enrichment of GO categories. The vertical axis is the GO category and the horizontal axis is the enrichment of GO.

3. Results

3.1 *Expression and subcellular localization of five fragments of CSFV NS5A*

The gene encoding the NS5A protein of CSFV was divided into five fragments (figure 1). Each part of NS5A was amplified by PCR from a pEGFP-NS5A plasmid. The products were identified by nucleotide electrophoresis, then purified and verified by sequence analysis, and finally inserted into the pEGFP-C1 plasmid (data not shown). The expression and subcellular localization of each construct of the CSFVNS5A protein was investigated by confocal fluorescence microscopy in SUVEC stable cell lines (figure 2). The results showed that only NS5A/84-804, with a 721 bp fragment, was located both in the nucleus and cytoplasm. This indicated that NS5A-721 could be used to construct the bait vector for the Y2H system.

3.2 *Construction and identification of the bait vector*

The 721 bp fragment of the CSFV NS5A gene was amplified by PCR from NS5A-GFP. The product was

identified by nucleotide electrophoresis, then purified and verified by sequencing, and finally inserted into the pGBKT7 plasmid. The recombinant plasmid pGBKT7-NS5A-721 was confirmed by PCR (figure 3A) and double restriction enzyme digestion by *Eco*RI/*Bam*HI (figure 3B). Moreover, sequencing analysis revealed that the gene was correctly inserted into pGBKT7 in the right reading frame. Finally, the bait vector pGBKT7-NS5A-721 was transformed into the yeast competent AH109, which is correct express confirmed both by RT-PCR (figure 3C) and Western blotting (figure 3D).

3.3 *Screening for cellular proteins that interact with the CSFV NS5A protein*

The pGBKT7-NS5A-721 plasmid, dscDNA and a linearized pGADT-Rec plasmid were transformed into the yeast competent cells, AH109, which were then sprayed onto19 SD/-His/-Leu/-Trp plates and cultured at 30°C for 3–6 days until colonies appeared. A representative plate is shown in figure 4C. The AH109 cells containing pGBKT7-Lam plasmids negative control and the pGBKT7-53 plasmid positive control indicated that the cotransformation was successful (figure 4A and B). As for the screened transformants, 168 clones grew on SD/-His/-Leu/-Trp plates and their diameter was larger than 2 mm, and 48 clones grew on SD/-His/-Leu/-Trp/Ade plates. After 3 inoculations on SD/-His/-Leu/-Trp/Ade/X-α-Gal plates, 34 blue colonies were initially identified as positive candidate colonies (figure 4D).

3.4 *Identification of positive clones*

These 34 colonies were inoculated in SD/-Leu/-Trp liquid medium to cultivate for extraction of the plasmids. The bait NS5Agene was detected by PCR in 28 colonies (figure 5A and B), while the other six were negative (data is not shown).The positive samples were sequentially tested by PCR for the library plasmids, and all of them were positive. As shown in figure 5C and D, the inserted fragments of the library plasmids were mostly between 1000 and 2000 bp.

3.5 *Positive plasmid confirmation and sequence analysis*

According to figure 4D (just shows a part of the positive colonies), 28 isolated colonies, of which 32 target plasmids were confirmed positive after a second cotransformation of bait plasmid with cDNA plasmid into the Y2H competent cells. The sequences of the inserted fragments of the positive plasmids were aligned with Basic Local Alignment Search Tool (BLAST) on the NCBI website against the human non-refSeq and the pig non-refSeq databases (table 2). While most genes identified by the BLAST against the pig database were unknown, the results of the BLAST against the human gene database indicated that there were 16 interactive proteins: DDX5, PSMC3, NAV1, PHF5A, GNB2L1, CSDE1, HSPA8, BRMS1, PPP2R3C, AIP, TMED10, POLR1C, TMEM70, METAP2, CHORDC1 and COPS6.

3.6 *Functional classification and pathway analysis*

The identified proteins were sorted by the enrichment of GO categories, and based on the UniProt database. The enriched GOs targeted by the 16 identified proteins contained protein folding chaperone, nucleoside-triphosphatase activity, pyrophosphatase activity, hydrolase activity that acts on acid anhydrides in phosphorus-containing anhydrides, hydrolase activity that acts on acid anhydrides, molecule ligand, transcription cofactor activity, hydrolase activity, etc. (figure 6). Pathway analysis was mainly based on the KEGG bioinformatics database.

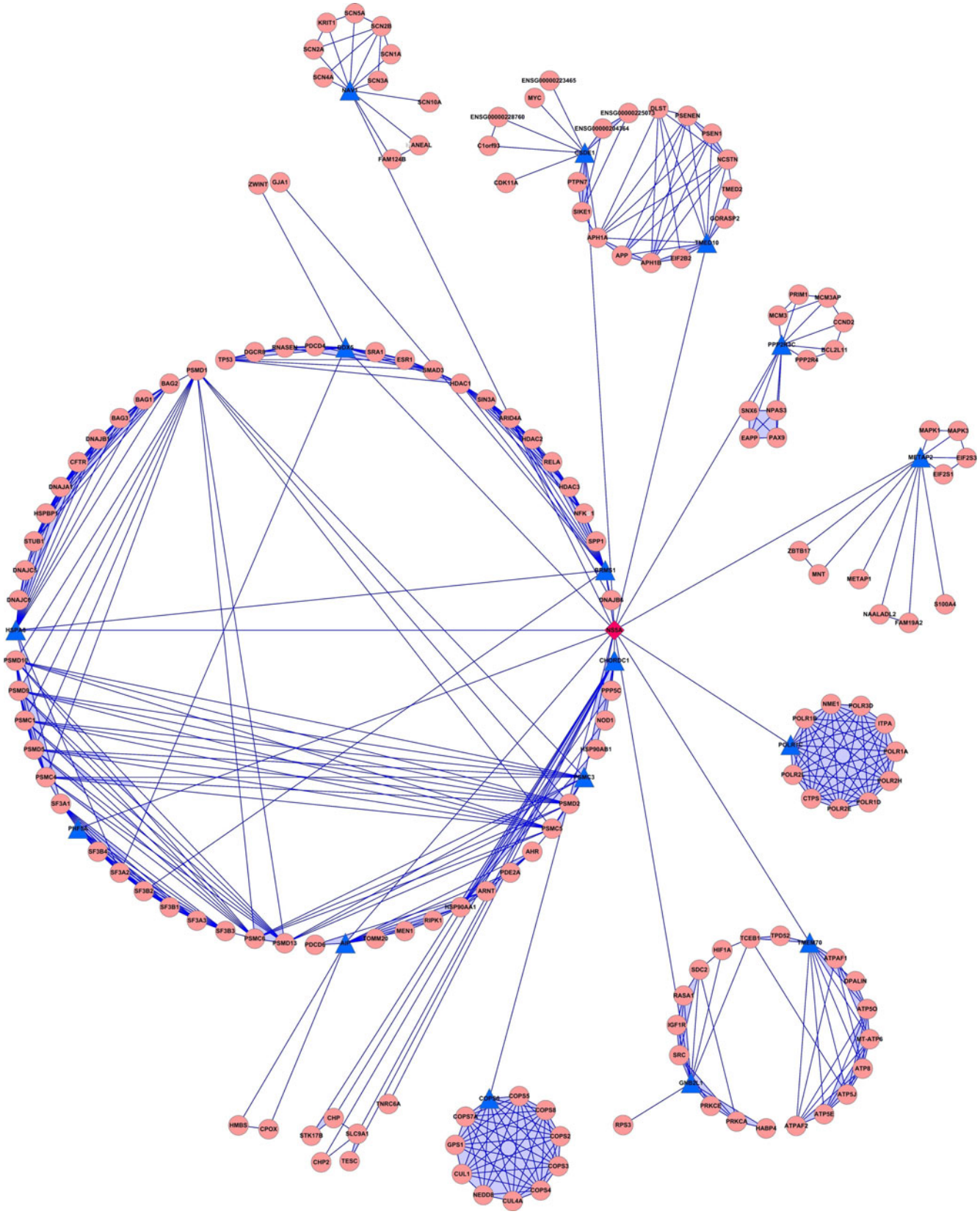
3.7 *Construction of the CSFV NS5A–cellular protein interaction network*

In the NS5A–cellular protein interaction network, BRMS1, DDX5, HSPA8, PHF5A, AIP, PSMC3 and CHORDC1 are the most remarkable node proteins, whereas CSDE1, TMED10, TMEM70 and GNB2L1 are sub-remarkable node proteins. NAV1, PPP2R3C, METAP2, POLR1C and COPS6 interact with NS5A while also involved with some other cellular proteins (figure 7). These notable node proteins tend to be more essential than non-central proteins in the modulation and organization of the NS5A–cellular protein interaction network.

4. Discussion

In the current study, the Y2H system was used to identify host cell proteins that interact with the CSFV NS5A protein. Sixteen host cell proteins were ascertained by sequence alignment and database retrieval. The Y2H system is usually used to study protein–protein interactions. Its feasibility and

Figure 7. The network of the CSFV NS5A and cellular proteins. The NS5A-cellular protein interaction network was constructed based on ▶ the screened proteins in this study and the STRING 9.0 database. The core protein NS5A is marked as a diamond. The identified proteins related to NS5A are marked as triangles, and the host cell proteins related to the identified proteins are marked as circles. HSPA8, TMEM70, CHORDC1, PSMC3 and COPS6 belong to the most central protein interaction network of cellular protein; therefore, they might be of great significance to the NS5A–cellular protein interaction network.


SCN5A
KRIT1
SCN2B
SCN2A
SCN1A
SCN4A
SCN3A
NAV1
SCN10A
ANEAL
FAM124B
ENSG00000223465
MYC
ENSG00000228760
ENSG00000225073
DLST
PSENEN
ENSG00000204364
PSEN1
C1orf93
CDSE1
NCSTN
CDK11A
PTPN7
TMED2
SIKE1
GORASP2
APH1A
EMC10
APP
APH1B
EIF2B2
ZWINT
GJA1
PRIM1
MCM3AP
MCM3
CCND2
PPP2R5C
BCL2L11
PPP2R4
SNX6
NPAS3
EAPP
PAX9
MAPK1
MAPK3
METAP2
EIF2S3
EIF2S1
ZBTB17
MNT
METAP1
S100A4
NAALADL2
FAM19A2
TP53
DGCR8
RNASEN
PDCD4
NCOA5
SRA1
ESR1
SMAD3
HDAC1
SIN3A
ARID4A
HDAC2
RELA
HDAC3
NFK_1
SPP1
PSMD1
BAG2
BAG1
BAG3
DNAJB1
CFTR
DNAJA1
HSPBP1
STUB1
DNAJC1
DNAJC8
HSPA4
PSMD10
PSMD6
PSMC1
PSMD5
PSMC4
SF3A1
PRPF3E
SF3B4
SF3A2
SF3B2
SF3B1
SF3A3
SF3B3
PSMC6
PSMD13
PDCD6
AIP
TOMM20
MEN1
RIPK1
HSP90AA1
ARNT
PDE2A
AHR
PSMC5
PSMD2
PSMC3
HSP90AB1
NOD1
PPP5C
CHORDC1
HSPA8
DNAJB6
NME1
POLR3D
POLR1B
ITPA
POLR1E
POLR1A
POLR2J
POLR2H
CTPS
POLR1D
POLR2E
TCEB1
TPD52
TMED10
HIF1A
ATPAF1
SDC2
OPALIN
RASA1
ATP5O
IGF1R
MT-ATP6
SRC
ATP8
GNB2L1
ATP5J
PRKCB
ATP5E
PRKCA
HABP4
ATPAF2
RPS3
COPS6
COPS5
COPS8
COPS7A
COPS2
GPS1
COPS3
CUL1
NEDD8
COPS4
CUL4A
HMBS
CPOX
CHP
TNRC6A
STK17B
SLC9A1
CHP2
TESC

effectiveness in verifying known protein interactions and screening candidate proteins, which specifically interact with target proteins have been confirmed and extended to cell cycle regulation, signal transduction, drug discovery, and other virus–host interaction fields.

The identified protein functions in diverse biological processes and signalling pathways were formed through GO and pathway analysis. A protein–protein interaction network was constructed based on the correlation relationships among individual proteins. The potential roles of some of these identified proteins in relation to pathogenesis and host antiviral response are discussed below.

The proteins we screened by the Y2H method from the SUVEC cDNA library, such as ATP-dependent RNA helicase (DDX5), PHD finger protein 5A, RNA polymerase 1, and aryl hydrocarbon receptor interacting protein, all of these proteins have relationships with RNA transcription, which is involved in every step of the host cell RNA transcription. Generally, viruses cannot replicate without the help of the host cell, as its life cycle needs host protein participation. Previous studies have shown that HCV need the help of the host cell proteins that are related to transcription to complete its RNA transcription process (Tai *et al.* 2009). The results showed that the RNA transcription process of CSFV may need the help of the host cell proteins which are related to transcription. Interestingly, in the research of HCV transcription, Owsianka and Patel (Owsianka and Patel 1999) found that the C protein of HCV can interact with DDX3. After the HCV C protein interacts with the host cell protein, DDX3, the inhibition of DDX3's role in the transcription process can reduce the host cell protein expression, suggesting that the interaction may play an important role in the HCV pathogenic mechanism (Mamiya and Worman 1999).

Cardiovascular system anomaly caused by CSFV infection plays a pivotal role in the pathogenesis of CSFV. Our Y2H results confirmed that nerve navigator 1 (NAV1) and methionine aminopeptidase 2 (MetAP2) interact with the CSFV NS5A protein. Previous research found that they have a close relationship with vascular function. These studies reported that the vascular system and nervous system were associated at the birth phase, and that they have the same migration route, and hence they could be influenced by the same growth signal (Chang *et al.* 2002; Chi *et al.* 2003). Furthermore, it has been shown that NAV1is also expressed in vascular endothelial cells, in tissues and organs during the process blood vessels formation, and NAV1 can regulate vascular and, especially, microvascular generation (Mukouyama *et al.* 2002). MetAP2 also plays an important role in the process of angiogenesis (Griffith *et al.* 1998). Nevertheless, elucidating which role is played by the interaction between NAV1and the CSFV NS5A protein in the process needs further research.

Molecular partners are not related in terms of composition of the cells but have common characteristics, which can assist other proteins to be composed and correctly assembled while they do not belong to the composition of the protein. Through the Y2H assay we found that the CSFVNS5A protein can interact with heat shock protein 70 (HSP70), the intracellular protein transmembrane protein 70 (TMEM70), and CHORDC1 containing protein 1 (CHORD-). HSP70 is an important molecular partner in cells, as it participates in the regulation of the cell cycle, oxidative-stress-caused DNA damage repair and embryo development (Gu *et al.* 2012). Another study found that HSP70 can participate in HCV virus replication enzyme formation and improve the replication of the virus particles, playing an important role in the viral replication process (Serva and Nagy 2006). It is also known that Hsp70 participates in the regulation of myocardial apoptosis following ischaemia reperfusion, neurodegenerative and other chronic diseases such as diabetes, neuropathy and epilepsy (Bironaite *et al.* 2013). Our study shows for the first time that the NS5A protein of CSFV interact with HSP70; however, the function of HSP70 in CSFV virus replication needs to be elucidated. Although there is no direct interaction between p23 and CHORD-I, it has been reported that a complex was formed by p23, CHORD-I and heat shock protein 90, which can regulate the function of HSP90 (Karagöz *et al.* 2011). HSP90 is a molecular protein involved in the regulation of a variety of intracellular signalling pathways in animal cells. Interestingly, it has been shown that there is an interaction between HSP70 and HSP90 (Pratt and Toft 2003). Therefore, in the current study, the interactions observed between the CSFV NS5A and HSP70, and p23 and CHORD I indicate that the CSFV NS5A protein may affect the SUVEC cell function by regulating HSP70 and HSP90.

In addition, we found that the CSFV NS5A protein can interact with the proteasome 26S subunit 3 and COP9 constitutive photomorphogenic subunit 6 (COPS6), which belong to the proteasome biodegradation pathway (Nickell *et al.* 2009; Gallastegui and Groll 2010; Nezames and Deng 2012). An important way of degrading cell proteins involves mediation by proteasomes. This result indicates that the CSFV NS5A protein may be degraded by the proteasome pathway, and the CSFV NS5A protein may be degraded associated with other intracellular proteins, which can affect the normal physiological function of the host cell. Further studies are needed to verify this effect. In an endoplasmic reticulum stress reaction, host cells promote the endoplasmic reticulum transport of proteins into the cytoplasm, faster protein ubiquitination, and degeneration by the proteasome (Friedlander *et al.* 2000; Rao and Bredesen 2004; Schüller *et al.* 2005). In the current study, among the screened cellular proteins, we observed that proteasome 26S subunit 3 and

COPS6 are involved in protein degradation. The proteasomal degradation pathway is essential for many cellular processes, including cell cycle, regulation of gene expression, and responses to oxidative stress.

In conclusion, a wide range of interactions exists between the CSFV NS5A protein and intracellular proteins of SUVECs. Our results indirectly illustrate that the CSFV NS5A protein may play a vital role in the regulation of the host cell physiological function after infection of CSFV. Additionally, this study provides new directions for further research on how the CSFV NS5A protein affects SUVEC physiological functions. Namely, how do the CSFV NS5A protein and these intracellular proteins work, and are the intracellular protein expression levels increased or reduced when interacting with the CSFV NS5A protein? These questions need to be further explored.

Currently, the pathogenesis of CSFV is not very clear, and we poorly understand the interactions between virus-encoded proteins and host cell molecules. In this study, the Y2H system was adopted to screen for host cell proteins that interact with the CSFV NS5A. The screened proteins have laid the experimental foundations for further research on the mechanism by which NS5A affects the host cell life cycle and the pathogenesis of classical swine fever virus.

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