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Pilot Study on Degradation of Classical Swine Fever Virus Nonstructural 2 Protein in Cells

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Abstract: The Nonstructural 2 (NS2) protein plays an important role in the infection process of Classical Swine Fever Virus (CSFV), it is an autoprotease cleaving the NS2-3 polyprotein for the NS3 protein release. The NS2 protein is an easily biodegradable protein in cells and the biogradation pathways are not still very clear at present. The Swine Umbilical Vein Endothelial Cell (SUVEC) constucted by the lab is a good cell line model for studying the pathogenic mechanism of CSFV. In this study, the degradation of CSFV NS2 were observed by detecting the NS2 protein expression in SUVEC and PK-15 cells. The recombinant plasmid pEGFP-NS2 with complete *NS2* gene of CSFV was transfected into SUVEC and PK-15 cells, respectively. The NS2 RNA was determined by RT-PCR and expression of NS2 protein in cells were analyzed by fluorescence microscopy and western blot assay after transfected. The degradation of NS2 protein also were observed by fluorescence microscopy and Western-blot assay. The results shown that the NS2 protein is short-lived in cells, the biodegradation process could be inhibited by a proteasomal inhibitor (MG132), hinting the NS2 protein is degraded via proteasome pathway in cells and this degradation process was related to the phosphorylation of NS2 protein. This is a primary study on the degradation of CSFV NS2 protein. The future experiments will address the degradation mechanism of CSFV NS2 and find the phosphorylation amino acids of NS2 protein which maybe relate to protein degadation in cells.

Key words: Classical swine fever virus, NS2 protein, protein degradation, phosphorylation, China

INTRODUCTION

Classical Swine Fever (CSF) is classified as a notifiable disease according to the World Organization for Animal Health classification of diseases caused by Classical Swine Fever Virus (CSFV) (Dreier *et al*., 2007). Pestiviruses, together with flaviviruses and hepatitis C virus form the family Flaviviridae. Within the Flaviviridae family, the genus *Pestivirus* comprises CSFV, Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV) (Gallei *et al*., 2005). CSFV is a small envoloped virus with a positive-sense RNA genome of approximately 12.3 kb which encodes a single hypothetical polyprotein of 3,899 amino acids and is processed into four mature structural proteins (C, Erns, E1 and E2) and eight mature nonstructural proteins (namely Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) using viral protease and host cellular proteases (Elbers *et al*., 1996; Harada *et al*., 2000; Agapov *et al*., 2004; Gil *et al*., 2006). Except the leader protein Npro, all the mature nonstructural proteins are essential for the virus life cycle while the proteins NS3 to NS5B are sufficient for RNA replication (Behrens *et al*., 1998; Moser *et al*., 1999; Lai *et al*., 2000; Agapov *et al*., 2004; Gil *et al*., 2006). The biosynthesis of nostructural of CSFV were clarified in recently, the half-lives of NS2, NS3, NS4A, NS5A and NS5B aslo were revealed (Lamp *et al*., 2011). The NS3 protein was essential for high productivity of CSFV *in vivo* (Moulin *et al*., 2007). The NS3 serine protease cleaves at the downstream cleavage sites and possesses helicase and NTPase acitivities (Tamura *et al*., 1993; Warrener and Collett, 1995; Xu *et al*., 1997). As known that the main role of CSFV NS2 is as an autoprotease for cleaving the NS2-3 polyprotein in infected cells, NS2-mediated cleavage at the NS2-3 site is essential for the pestivirus life cycle and is temporally modulated by a 1:1 stoichiometric association of NS2 with the cellular cofactor Jiv (Lackner *et al*., 2005, 2006). Whether NS2, either alone or associated with NS3 has additional essential functions in the virus life cycle is unknown except for the role of uncleaved NS2-3 in BVDV morphogenesis identified (Agapov *et al*., 2004). The main function of NS2 protein was to cleave the precursor

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NS2-3, it's would be degraded when it has executed this founction. However, researchers do not find the revelant report in the NS2 protein degradation in CSFV infected cells.

It is well known that two protein degradation pathways exist in host cells, namely the lysosomal pathway and the proteasome pathway. The lysosomal pathway is used mainly as a degradation pathway for exogenous protein while the proteasome pathway plays a critical role in degradation of endogenous protein, especially in short-lived proteins and cellular signal transduction regulatory proteins (Ciechanover, 1998). Most of the proteins degraded by the proteasome pathway depend on ubiquitinylation. However, other proteins were not dependent on ubiquitinylation but were degraded directly through the proteasome pathway. The choice of pathway depended on the phosphorylation and dephosphorylation of the proteins. Some viruses could degrade self-protein by taking advantage of the host cell proteasome system to regulate the viral replication level for achieving a balance between virus and host cells thus providing a good intracellular environment for persistent infection (Gao *et al*., 2003).

Swine endothelial cells are commonly used as an *in vitro* model for studying features of the blood-brain barrier and some hemorrhagic diseases. The research group established a immortalized Swine Umbilical Vein Endothelial Cells (SUVECs) for studying the pathogenic mechanism of CSFV *in vitro* (Hong *et al*., 2007). Until now, there has been very little known about the degradation pathway of CSFV NS2 protein. A study shown that the -NH2 terminal of NS2 CSFV protein was highly hydrophobic and NS2 protein localized in Endoplasmic Reticulum (ER), there are at least four transmembrance region in NS2 protein and two internal signal peptide sequences were critical for NS2 protein to translocalize to ER (Guo *et al*., 2011). A study shown the CSFV NS2 protein interacted at least with 6 proteins of SUVEC (Kang *et al*., 2012). The SUVEC and PK-15 cell were as cell models for studying the CSFV NS2 protein degradation in this experiment. The degradation of CSFV NS2 were observed by detecting the NS2 protein expression in host cells. This is a primary study on the degradation of CSFV NS2 protein. The future experiments will address the degradation mechanism of CSFV NS2 and find the phosphorylation amino acids of NS2 protein which maybe relate to protein degadation in host cells.

MATERIALS AND METHODS

Cell and virus: The Swine Umbilical Vein Endothelial Cell line (SUVEC) was established and cultured as previously reported (Hong *et al*., 2007). Classical swine fever virus Shimen strain was acquried from China Institute of Veterinary Drug Cotrol.

Cloning and sequencing of *CSFV NS2* gene: Specific primers for amplifying the *CSFV NS2* gene were designed according to the nucleotide sequence of the CSFV Shimen strain (Accession Number: AF092448), the 1371 bp predicted fragment covered the whole coding region of NS2. Primer sequences are as follows: RT-primer 5'-CCC ATAGTGTCACATACCAG-3', Sense-primer (P1) 5'-GGAA AGATAGATGGCGGTTGG CAGC-3', Anti-sense-primer (R1) 5'-TCTAAGCACCCAGCCAAGGTGTTCCA-3.

Total RNA was extracted from SUVEC 72 h after inoculation with the CSFV Shimen strain. The first cDNA strands were synthesized with the RT primer according to the procedure of the first-strand cDNA Synthesis kit (Takara, China). The *NS2* gene was amplified by Polymerase Chain Reaction (PCR) with the first cDNA strands as templates. The conventional PCR reaction mixture for each of assay was 2x MasterMix 10 μL, 10 μM P1 primer 1 μL, 10 μM R1 primer 1 μL, 2 μL cDNA templates and made up to 20 μL with deionized distilled water. PCR was carried out according to the following procedures: denaturation at 95°C for 5 min followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, 90 sec at 72°C and a final extension of 10 min at 72°C. PCR amplicons were visualized on an ethidium bromide-stained 1.5% agarose gel and the fragment purified using the Agarose gel DNA purification kit (Takara, China). PCR products were cloned into vector pMD19-T (Takara, China) and the recombinant plasmid was transfected into competent *E. coli* DH5α (Tiangen Biotech, China). The positive recombinant (pMD-NS2) was verified by enzyme digestion (Bam H I/Hind III) and sequencing. Protein phosphorylation of NS2 was analyzed using online software (http://www.cbs.dtu.dk/services/NetPhos/).

Eukaryotic expression plasmid construction and transfection: The specific expression primers were designed according to the *CSFV NS2* gene sequences. Sense-primer (P2) 5'-GAAGTCGACGGAAAGATAGATG GCGGTT GGCAGC-3', containing a Sal I site; Anti-sense-primer (R2) 5'-GAAGGATCCTCT AAGCACCCAGCCAAG GTGTTCCA-3', containing a Bam H I site.

The *NS2* gene was cloned from the pMD-NS2 by PCR using P2/R2 primers, digested with Sal I/Bam H I and inserted into the pEGFP-C1 (Takara, China) digested with the same enzymes (Sal I/BamH I) to construct the recombinant eukaryotic expression plasmid. The positive plasmid was identified by sequencing and enzyme digiestion (Sal I/BamH I) and named pEGFP-NS2.

SUVECs and PK-15 cell were seeded onto a 15 mm^2 dish (Costar, USA) and transfected 24 h later when they were 50-60% confluent. Cells were transfected with the pEGFP-NS2 and the pEGFP-C1 control vector using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, USA). The untransfected negative control and transfected cells were passaged 48 h after transfection and G418 was added to the cell medium at a finial concentration of 1500 μg mL^{-1}. The medium containing G418 was replaced every 48 h. The positive cells with G418 resistance were cultured in G418 containing medium for 15 days after the negative control cells had died. The expression of green fluorescent protein in cells was screened by fluorescent microscopy to select the positive transfected cells for expansion.

RT-PCR and Western blot analysis: The *NS2* gene was investigated in pEGFP-NS2 transfected positive cells, pEGFP-C1 transfected positive cells and negative control cells by RT-PCR. The expression of NS2 protein was analyzed by Western-blot. Briefly, 60 h after transfection whole cell extracts were prepared by washing cells with Phosphate Buffer Solution (PBS), harvested by scraping and suspended in 1 mL PBS. Following centrifugation, cells were resuspended in cell lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 100 μg mL^{-1} PMSF and protease inhibitors) and centrifuged at 15,000x g for 30 min at 4°C. Cell extracts were collected in supernatant and boiled for 5 min in 2x loading buffer. The 25 μg of protein of each sample was resolved by 120 g L^{-1} Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to a Polyvinylidene Fluoride (PVDF) membrane (Millipore, USA) for 3 h. The membrane was blocked for 1 h with 20 g L^{-1} skim milk in TNT buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl and 0.05% Tween 20) then incubated with mouse anti-GFP mAb (Millipore, USA) overnight at 4°C and washed three times with PBS. The presence of primary antibodies was detected using horseradish peroxidase-conjugated goat anti-mouse antibody (Millipore, USA) incubated for 1 h at 37°C. The protein bands were visualized by enhanced chemiluminescence carried out according to the manufacturer's instructions (Millipore, USA). At the same time, detection of GAPDH was also used as an internal reference to test for discrepancy of protein expression in different cells.

Examination of CSFV NS2 protein degradation: Cells with stable expression of NS2 were seeded onto a 15 mm^2 dish (Costar, USA) and cultured for 24 h then the medium discarded and fresh medium containing MG132 (5 μM) (Merck, China) added for a further 24 h. Cells were washed twice with PBS and incubated with Hoechst33342 (Invitrogen, USA) at 37°C for 15 min and again washed twice with PBS. The fluorescene of cells were viewed under florescence microscopy (Nikon, Japan) to evaluate the foreign genes expression in tested cells. The expression of NS2 protein also were determined with RT-PCR on transcriptional level and Western-blot on protein level, respectively.

The SUVECs and PK-15 cells with stable expression of NS2 were seeded onto a 15 mm^2 dish and cultured for 24 h then the medium discarded and replaced with fresh medium containing Curcumin (100 μM) (Merck, China) and cultured for a further 24 h. Cells were washed twice with PBS and incubated with Hoechst33342 at 37°C for 15 min and then washed twice more with PBS. The cells were treated to determined the NS2 protein expression on transcription and protein levels as described.

RESULTS AND DISCUSSION

NS2 protein phosphorylation analysis and expression plasmid construction: The obtained *CSFV NS2* gene was 99.9% sequences similarity with the CSFV Shimen strain (AF092448). The high probability of phosphorylation sites in serine at amino acids 223 and 224 in threonine at 37 and 47 and in tyrosine at 204, 332 and 392 of NS2 protein (Fig. 1). NS2 protein would be phosphorylated with the protein kinases PKC and casein kinase CKII. These results suggested that the CSFV NS2 protein could easily be phosphorylated and subject to degradation. The eukaryotic expression plasmid containing the *NS2* gene was identified by PCR and Bam H I/Sal enzyme digestion. The sequencing analysis shown that the *NS2* gene was inserted correctly into pEGFP-C1 and the recombinant plasmid was named pEGFP-NS2.

Examination of NS2 protein degradation: The pEGFP-NS2 fusion protein were expressed in SUVECs and PK-15 cells analyzed by RT-PCR on transcription level and Western-blot on protein level, hinting both of transfected cells could be as the research models of protein degradation.

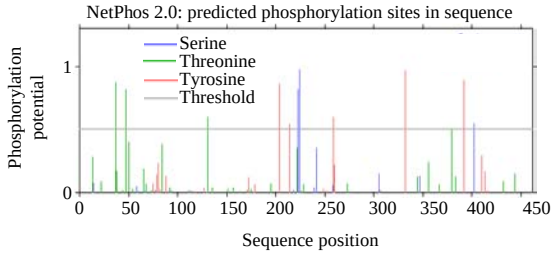

Fig. 1: Prediction of phosphorylation sites of CSFV NS2 protein. Score >0.5 shows strong possibility of phosphorylation

The NS2 protein were determined by RT-PCR, fluorscence examination and western-blot in cells treated with MG132 and control cells. The *NS2* genes have been detected by RT-PCR both in the cells (SUVECs and PK-15 cells transfected with pEGFP-NS2) treated with MG132 and those that untreated. No green fluorescence was obseved in MG132 treated cells transfected with pEGFP-NS2 but strong green fluorescence was observed in untreated cells (Fig. 2 and 3). Cells transfected with pEGFP-C1 vector also shown strong green fluorescence both in the cells treated with MG132 and those that untreated (Fig. 4). These results also were confirmed by Western blot (Fig. 5 and 6). The experiments hinted that the *NS2* gene could be transcribed well in transfected cells but was very easily degraded via the proteasome pathway and this degradation process could be inhibited with specific proteasome inhibitors.

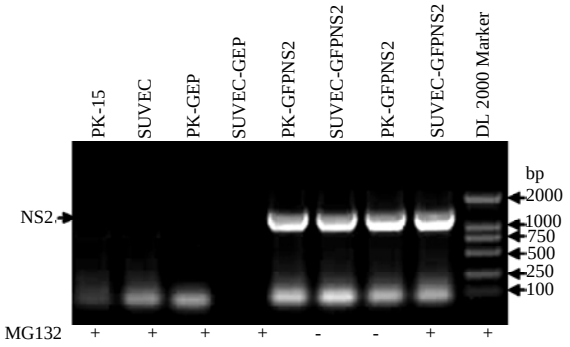

Fig. 2: Detection of CSFV NS2 mRNA in different cells. '+' means the cells treated with MG132; '-' means the cells untreated with MG132 (control)

The cells expressing NS2 protein treated with 100 μM curcumin shown significantly higher levels of green fluorescence compared with those not treated with curcumin (Fig. 7). These results demonstrated that the proteasome degradation pathway of CSFV NS2 protein could be inhibited by the protein kinase CK inhibitor-curcumin, hinting the NS2 protein degradation process was relative to protein phosphorylation by protein kinase CK.

The functions of CSFV-encoding proteins such as the structural C, Erns, E1, E2 and nonstructural N^{pro}, NS3, NS5B proteins have been studied extensively. Investigations on the nonstructural protein NS2 have focused on its autoprotease activity for high productivity of CSFV in host cells. In this study, the mRNAs of NS2 were transcribed well in the SUVEC and PK cells but no

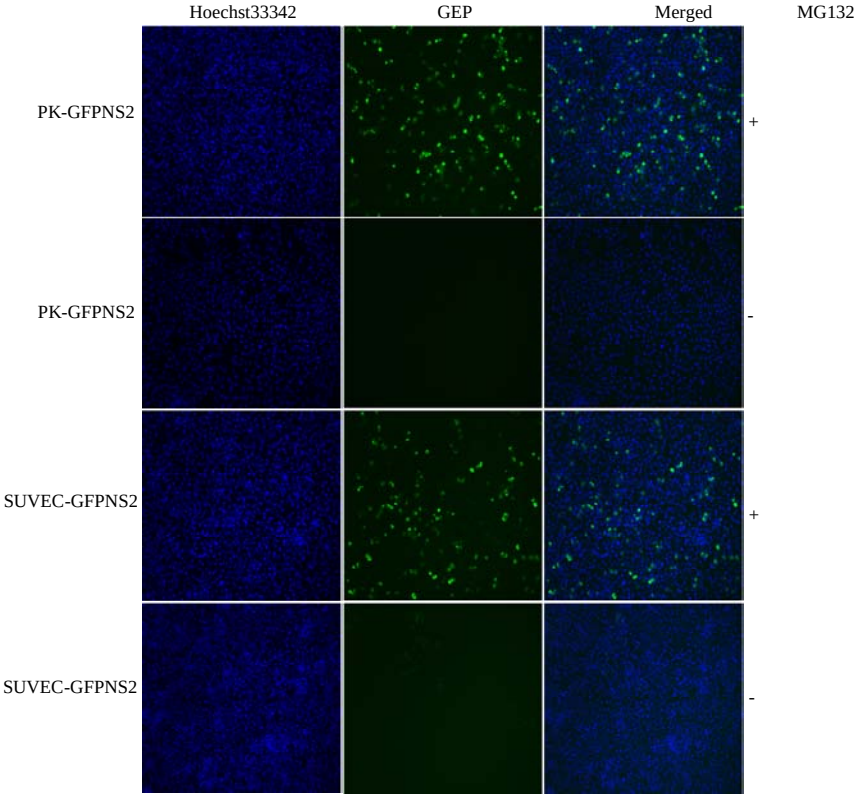

Fig. 3: Fluorescence observation of GFPNS2 fusion protein expressed in SUVEC-GFPNS2 and PK-GFPNS2 cell lines (100x). '+' means the cells treated with MG132. '-' means the cells untreated with MG132 (control)

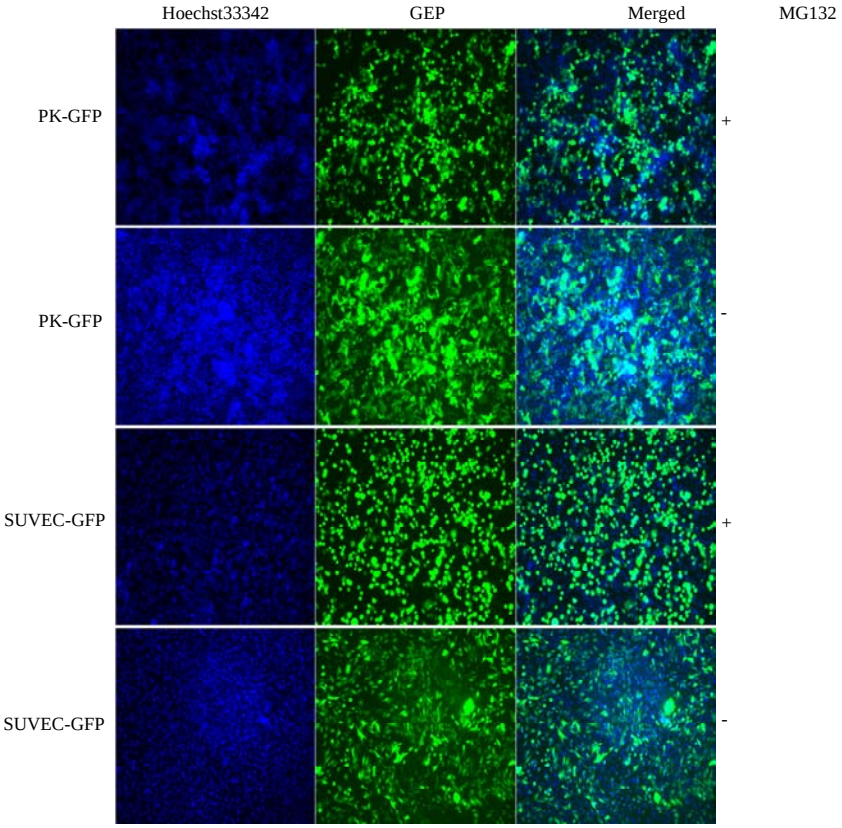

Fig. 4: Fluorescence detection of GFP protein expression in SUVEC-GFP and PK-GFP cell lines (100x). '+' means the cells treated with MG132. '-' means the cells untreated with MG132 (control)

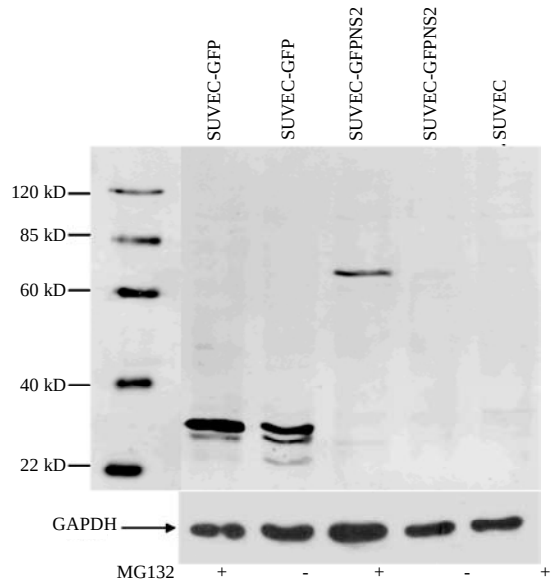

Fig. 5: Detection of GFPNS2 fusion protein expressed in SUVEC-GFPNS2 cell by western blot. '+' means the cells treated with MG132. '-' means the cells untreated with MG132 (control)

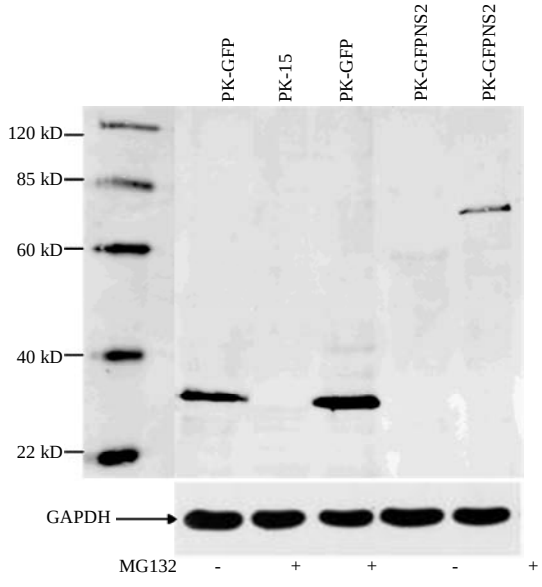

Fig. 6: Detection of GFPNS2 fusion protein expressed in PK-GFPNS2 cell by western blot. '+' means the cells treated with MG132. '-' means the cells untreated with MG132 (control)

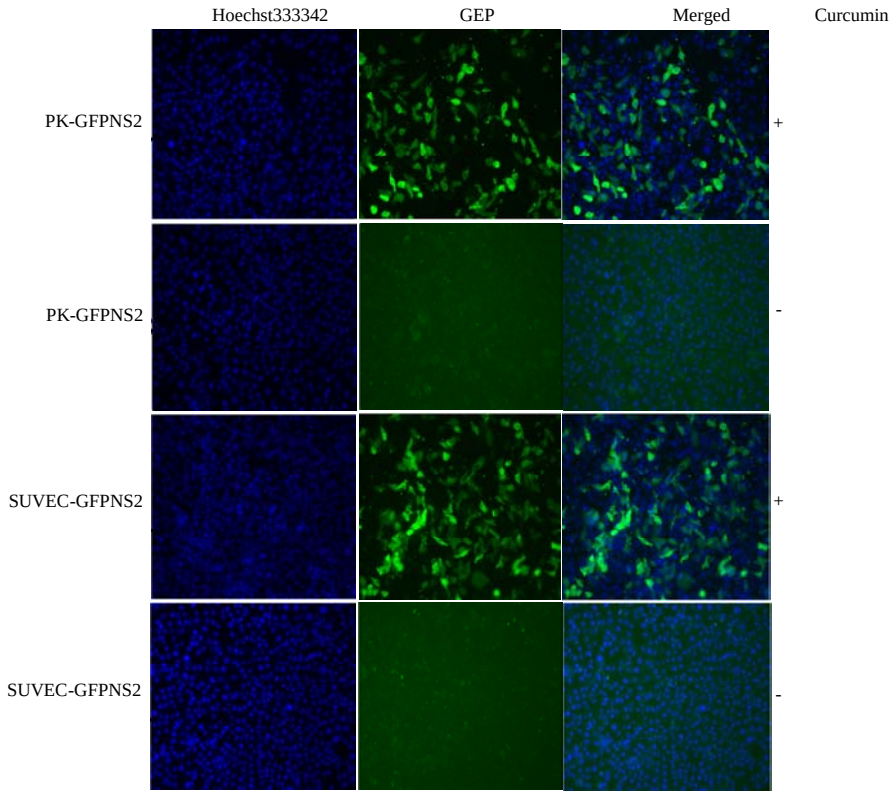

Fig. 7: Fluorescence detection of the inhibitory action of curcumin on the degradation of GFPNS2 protein (200x). '+' means the cells treated with curcumin. '-' means the cells untreated with curcumin (control)

specific NS2 fusion protein could be detected by Western blot and fluorescence examination. However, the NS2 protein expression was detected in both types of cells when treated with the proteasome inhibitor MG132. This indicated that NS2 protein was being expressed in both cells but was being degraded quickly via the proteasome pathway. The results also suggested that the NS2 protein was extremely easily degraded and this feature did not change in different cell lines. Earlier studies shown that major intracellular proteins were degraded by the lysosomal pathway and proteasome pathway and that most endogenous proteins were degraded by the proteasome pathway (Pickart, 2001). The proteasome system is a major mechanism for intracellular protein regulation and plays a critical role in regulating protein degradation mechanisms involving in the short-lived proteins and also some regulatory cell processing proteins such as cell cycle and signal transduction proteins. The rapid and effective degradation of these proteins is very important for maintaining cell physiological functions (Ciechanover, 1998). The proteasome pathway involves the processes of protein ubiquitination and phosphorylation. Phosphorylation occurs more frequently at serine, threonine and tyrosine residues (Dantuma and Masucci, 2002; Kalejta and Shenk, 2003). In viruses, the majority of proteins are degraded via the proteasome pathway, either by an ubiquitin-dependent or non-ubiquitin-dependent process (Jejcic *et al*., 2009; Yuksek *et al*., 2009). A similar study shown that the NS2 protein of the Hepatitis C Virus (HCV) was easily degraded in host cells. They detected the protein expression in host cells by Western blot and fluorescence examination and confirmed that the protein was degraded via the proteasome pathway, the serine in amino acid 168 being directly related to this process which was regulated by protease phosphorylation (Franck *et al*., 2005). The protein kinase CKII belongs to the multifunctional serine and threonine kinase. Virus-encoded proteins can be phosphorylated with CKII and this processing can accelerate the degradation processes. Bioinformatic analysis in this study shown a high probability of phosphorylation of serines at amino acids 223 and 224 of threonine at amino acids 37 and 47. The degradation of CSFV NS2 protein was significantly inhibited in the cells treated with curcumin, the specific inhibitor of CKII. These data provided an indirect

theoretical basis to NS2 protein degradation. In the future, the relationship of phosphorylation degradation and specific amino acid sites can be studied to clarify further the degradation mechanism of the CSFV NS2 protein.

Specific degradation characteristics of proteins are closely related to their specific functions; for example, the HCV NS2 is a membrane protein localized in the Endoplasmic Reticulum (ER) of the host cells (Yamaga and Ou, 2002; Lorenz *et al*., 2006) and it regulates the physiological and pathological responses of cells by causing ER cell stress (Moradpour *et al*., 2007). The short-lived characteristic of HCV NS2 protein plays a critical role in the persistence of the viral infection. In addition, the HCV NS5 protein with RNA-dependent RNA polymerase activity is essential for virus replication. The protein is degraded rapidly by the proteasome of the host cells to negatively regulate viral replication thus constituting a significant mechanism for the virus to evade the host cell's defenses and establish a persistent infection (Gao *et al*., 2003). Preliminary research has revealed that the CSFV NS2 protein has a similar characteristics to the HCV NS2 protein in that they both have self-protease activity (Pieroni *et al*., 1997; Lackner *et al*., 2006; Welbourn and Pause, 2007; Schregel *et al*., 2009), located in the ER of host cells and they play an indispensable role *in virus* particle assembly (Jones *et al*., 2007; Moulin *et al*., 2007; Murray *et al*., 2008; Yi *et al*., 2009).

CONCLUSION

The rapid degradation of CSFV NS2 protein in cells could negatively regulate the formation of CSFV virus particles and influence the course of pathogenesis. The degradation of the NS2 protein would therefore have important biological significance in enabling the establishment of persistent infection of CSFV. This study provides a primary data for further research on the role of the NS2 protein in CSFV persistent infection in animals.

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REFERENCES

Agapov, E.V., C.L. Murray, I. Frolov, L. Qu, T.M. Myers and C.M. Rice, 2004. Uncleaved NS2-3 is required for production of infectious bovine viral diarrhea virus. J. Virol., 78: 2414-2425.

Behrens, S.E., C.W. Grassmann, H.J. Thiel, G. Meyers and N. Tautz, 1998. Characterization of an autonomous subgenomic pestivirus rna replicon. J. Virol., 72: 2364-2372.

Ciechanover, A., 1998. The ubiquitin-proteasome pathway: On protein death and cell life. EMBO J., 17: 7151-7160.

Dantuma, N.P. and M.G. Masucci, 2002. Stabilization signals: A novel regulatory mechanism in the ubiquitin/proteasome system. FEBS Lett., 529: 22-26.

Dreier, S., B. Zimmermann, V. Moennig and I. Greiser-Wilke, 2007. A sequence database allowing automated genotyping of classical swine fever virus isolates. J. Virol. Methods, 140: 95-99.

Elbers, K., N. Tautz, P. Becher, D. Stoll, T. Rumenapf and H.J. Thiel, 1996. Processing in the pestivirus E2-NS2 region: Identification of proteins p7 and E2p7. J. Virol., 70: 4131-4135.

Franck, N., J. le Seyec, C. Guguen-Guillouzo and L. Erdtmann, 2005. Hepatitis C virus NS2 protein is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome. J. Virol., 79: 2700-2708.

Gallei, A., T. Rumenapf, H.J. Thiel and P. Becher, 2005. Characterization of helper virus-independent cytopathogenic classical swine fever virus generated by an *in vivo* RNA recombination system. J. Virol., 79: 2440-2448.

Gao, L., H. Tu, S.T. Shi, K.J. Lee, M. Asanaka, S.B. Hwang and M.M.C. Lai, 2003. Interaction with a ubiquitin-like protein enhances the ubiquitination and degradation of hepatitis C virus RNA-dependent RNA polymerase. J. Virol., 77: 4149-4159.

Gil, L.H.V.G., I.H. Ansari, V. Vassilev, D. Liang and V.C.H. Lai *et al*., 2006. The amino-terminal domain of bovine viral diarrhea virus npro protein is necessary for alpha/beta interferon antagonism. J. Virol., 80: 900-911.

Guo, K.K., Q.H. Tang, Y.M. Zhang, K. Kang and L. He, 2011. Identification of two internal signal peptide sequences: Critical for classical swine fever virus non-structural protein 2 to trans-localize to the endoplasmic reticulum. Virol. J., Vol. 8. 10.1186/1743-422X-8-236.

Harada, T., N. Tautz and H.J. Thiel, 2000. E2-p7 region of the bovine viral diarrhea virus polyprotein: Processing and functional studies. J. Virol., 74: 9498-9506.

Hong, H.X., Y.M. Zhang, H. Xu, Z.Y. Su and P. Sun, 2007. Immortalization of swine umbilical wein endothelial cells with human telomerase reverse transcriptase. Mol. Cells, 3: 358-363.

Jejcic, A., R. Daniels, L. Goobar-Larsson, D.N. Hebert and A. Vahlne, 2009. Small molecule targets env for endoplasmic reticulum-associated protein degradation and inhibits human immunodeficiency virus type 1 propagation. J. Virol., 83: 10075-10084.

Jones, C.T., C.L. Murray, D.K. Eastman, J. Tassello and C.M. Rice, 2007. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J. Virol., 81: 8374-8383.

Kalejta, R.F. and T. Shenk, 2003. Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein. Proc. Natl. Acad. Sci. USA., 100: 3263-3268.

Kang, K., K. Guo, Q. Tang, Y. Zhang, J. Wu, W. Li and Z. Lin, 2012. Interactive cellular proteins related to classical swine fever virus non-structure protein 2 by yeast two-hybrid analysis. Mol. Biol. Rep., 39: 10515-10524.

Lackner, T., A. Muller, M. Konig, H.J. Thiel and N. Tautz, 2005. Persistence of bovine viral diarrhea virus is determined by a cellular cofactor of a viral autoprotease. J. Virol., 79: 9746-9755.

Lackner, T., H.J. Thiel and N. Tautz, 2006. Dissection of a viral autoprotease elucidates a function of a cellular chaperone in proteolysis. Proc. Natl. Acad. Sci. USA., 103: 1510-1515.

Lai, V.C.H., W. Zhong, A. Skelton, P. Ingravallo and V. Vassilev*et al*., 2000. Generation and characterization of a hepatitis C virus NS3 protease-dependent bovine viral diarrhea virus. J. Virol., 74: 6339-6347.

Lamp, B., C. Riedel, G. Roman-Sosa, M. Heimann and S. Jacobi *et al*., 2011. Biosynthesis of classical swine fever virus nonstructural proteins. J. Virol., 85: 3607-3620.

Lorenz, I.C., J. Marcotrigiano, T.G. Dentzer and C.M. Rice, 2006. Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. Nature, 442: 831-835.

Moradpour, D., F. Penin and C.M. Rice, 2007. Replication of hepatitis C virus. Nat. Rev. Microbiol., 5: 453-463.

Moser, C., P. Stettler, J.D. Tratschin and M.A. Hofmann, 1999. Cytopathogenic and noncytopathogenic RNA replicons of classical swine fever virus. J. Virol., 73: 7787-7794.

Moulin, H.R., T. Seuberlich, O. Bauhofer, L.C. Bennett, J.D. Tratschin, M.A. Hofmann and N. Ruggli, 2007. Nonstructural proteins NS2-3 and NS4A of classical swine fever virus: Essential features for infectious particle formation. Virology, 365: 376-389.

Murray, C.L., C.T. Jones and C.M. Rice, 2008. Architects of assembly: Roles of *Flaviviridae* non-structural proteins in virion morphogenesis. Nat. Rev. Microbiol., 6: 699-708.

Pickart, C.M., 2001. Ubiquitin enters the new millennium. Mol. Cell, 8: 499-504.

Pieroni, L., E. Santolini, C. Fipaldini, L. Pacini, G. Migliaccio and N. La Monica, 1997. *In vitro* study of the NS2-3 protease of hepatitis C virus. J. Virol., 71: 6373-6380.

Schregel, V., S. Jacobi, F. Penin and N. Tautz, 2009. Hepatitis C virus NS2 is a protease stimulated by cofactor domains in NS3. Proc. Natl. Acad. Sci USA., 106: 5342-5347.

Tamura, J.K., P. Warrener and M.S. Collett, 1993. RNA-stimulated ntpase activity associated with the p80 protein of the pestivirus bovine viral diarrhea virus. Virology, 193: 1-10.

Warrener, P. and M.S. Collett, 1995. Pestivirus NS3 (p80) protein possesses RNA helicase activity. J. Virol., 69: 1720-1726.

Welbourn, S. and A. Pause, 2007. The hepatitis C virus NS2/3 protease. Curr. Issues Mol. Biol., 9: 63-69.

Xu, J., E. Mendez, P.R. Caron, C. Lin, M.A. Murcko, M.S. Collett and C.M. Rice, 1997. Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements and molecular model of an enzyme essential for pestivirus replication. J. Virol., 71: 5312-5322.

Yamaga, A.K. and J.H. Ou, 2002. Membrane topology of the hepatitis C virus NS2 protein. J. Biol. Chem., 277: 33228-33234.

Yi, M., Y. Ma, J. Yates and S.M. Lemon, 2009. Trans-complementation of an NS2 defect in a late step in hepatitis C virus (HCV) particle assembly and maturation. PLoS Pathogens, Vol. 5, No. 5. 10.1371/journal.ppat.1000403.

Yuksek, K., W.L. Chen, D. Chien and J.H.J. Ou, 2009. Ubiquitin-independent degradation of hepatitis C virus F protein. J. Virol., 83: 612-621.