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Cellular peptidyl-prolyl *cis/trans* isomerase Pin1 facilitates replication of feline coronavirus

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ABSTRACT

Although feline coronavirus (FCoV) causes feline infectious peritonitis (FIP), which is a fatal infectious disease, there are no effective therapeutic medicines or vaccines. Previously, *in vitro* studies have shown that cyclosporin (CsA) and FK506 inhibit virus replication in diverse coronaviruses. CsA and FK506 are targets of clinically relevant immunosuppressive drugs and bind to cellular cyclophilins (Cyps) or FK506 binding proteins (FKBPs), respectively. Both Cyp and FKBP have peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. However, protein interacting with NIMA (Pin1), a member of the parvulin subfamily of PPIases that differs from Cyps and FKBPs, is essential for various signaling pathways. Here we demonstrated that genetic silencing or knockout of Pin1 resulted in decreased FCoV replication *in vitro*. Dipentamethylene thiuram monosulfide, a specific inhibitor of Pin1, inhibited FCoV replication. These data indicate that Pin1 modulates FCoV propagation.

1. Introduction

Coronaviruses (CoVs) cause severe diseases of the respiratory system, gastrointestinal tract, and the central nervous system in animals (Perlman and Netland, 2009). Feline CoVs (FCoVs) have been classified into two biotypes comprising the ubiquitous feline enteric CoV (FECV) and feline infectious peritonitis virus (FIPV) (Pedersen, 2009). Feline infectious peritonitis (FIP) is one of the most frequent causes of death in young cats, and classical symptoms of effusive/wet FIP, non-effusive/dry form of FIP, or a combination of the two can develop (Berg et al., 2005). Mortality is extremely high once clinical signs appear, although some cats can live with the disease for weeks, months, or even years (Pedersen, 2014). Nevertheless, FIP is currently incurable by drug treatment, and there are no effective prophylactic vaccines. Virus replication depends on a variety of host factors (de Haan and Rottier, 2006; Vogels et al., 2011; Zhang et al., 2010), which consequently represent potential antiviral targets. We have reported that cyclosporin A (CsA), a cellular cyclophilin (Cyp) inhibitor, can inhibit FCoV replication in cell culture (Tanaka et al., 2012). Although FK506 suppresses calcineurin and the nuclear factor pathway of activated T-cells (NFAT) at the same stage as CsA, FK506 did not affect FCoV replication (Tanaka et al., 2012). These data indicate that CsA does not exert inhibitory effects via the NFAT pathway. We have reported that CsA treatment caused a sustained reduction in pleural fluid volume and viral copy number in a cat diagnosed with effusive FIP (Tanaka et al., 2015). CsA is well known as a potent replication inhibitor of various human and animal CoVs (de Wilde et al., 2013; Pfefferle et al., 2011). Regarding requirements of Cyps in CoV replication, using small interfering RNA (siRNA) experiments, de Wilde et al. (2011) reported that both CypA and CypB did not affect severe acute respiratory syndrome CoV (SARS-CoV) replication (de Wilde et al., 2011). In contrast, human CoV NL63 replication depends on CypA but not CypB (Carbajo-Lozoya et al., 2014). FK506 inhibits human CoVs, SARS-CoV, NL63, and 229E (Carbajo-Lozoya et al., 2012), but each CoV requires different immunophilins as described above.

Cyps and FKBPs, two major families of peptidyl-prolyl *cis-trans* isomerase (PPIase) that catalyze the *cis-trans* isomerization of the prolyl peptide bond preceding proline residues, are targets of clinically relevant immunosuppressive drugs, CsA and FK506, respectively (Siekierka et al., 1989a, 1989b). The immunosuppressive activity of these drugs is unrelated to inhibition of PPIase

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activity, and neither Cyp nor FKBP genes are essential (Yaffe et al., 1997). In contrast, Protein Interacting with NIMA (Pin1), a member of the parvulin subfamily of PPIases that differs from Cyps and FKBPs, is essential for cell growth and requires a catalytically competent PPIase domain (Lu et al., 1996; Lu and Hunter, 1995). Its catalytic site is unique among other PPIase enzymes because it recognizes an unusual phosphorylated Ser/Thr–Pro motif in its substrates (Lu and Zhou, 2007; Lu et al., 2002; Wulf et al., 2005). Pin1 plays important roles in many cellular events, including cell cycle progression, cell proliferation, transcriptional regulation, and neoplasmic transformation. The protein has been linked to several diseases, such as cancer, Alzheimer's disease, and asthma (Lu and Zhou, 2007). Regarding infectious diseases, Pin1 directly interacts with the hepatitis C virus (HCV) NS5A and NS5B proteins and plays unique roles in HCV replication (Lim et al., 2011). Pin1 modulates human immunodeficiency virus type1 (HIV-1) infection by interaction with the capsid protein by uncoating and regulating APOBEC3G (Misumi et al., 2010; Watashi et al., 2008). However, there are no reports exploring the role of Pin1 in CoV replication.

In the present study, we examined the roles of Pin1 in FCoV replication. In conclusion, we report that Pin1 facilitates replication of FCoV, and a specific inhibitor of Pin1 inhibits both virus replication and protein expression *in vitro*. Therefore, Pin1 may be a potential target for FIP treatment as well as Cyps.

2. Materials and methods

2.1. Cell culture of virus

Felis catus whole fetus-4 (fcwf-4; American Type Culture Collection, VA, USA) cells were maintained in Dulbecco's modified Eagle's medium (D-MEM, Sigma–Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Tokyo, Japan). We propagated FIP virus FCoV (79-1146 strain was a gift from Dr. Tsutomu Hodatsu, Kitasato University, Japan) in fcwf-4 cells and then purified the virions by linear sucrose gradient ultracentrifugation.

2.2. Plasmid constructs

We isolated the feline peptidyl-prolyl *cis/trans* isomerase Pin1 gene from fcwf-4 cells, using the polymerase chain reaction (PCR) with primers 5′-TACCGAGCTCGGATCCACCATGGCGGACGAAGAGAAGCTG-3′ and 5′-GATATCTGCAGAATTCTCACTCCGTGCGCAGGATGATG-3′ for amplification. Total RNA of fcwf-4 cells was isolated using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. The RNA was reverse transcribed using a PrimeScript reverse transcriptase (RT)-PCR kit (Takara-bio, Shiga, Japan) before complementary DNA (cDNA) of Pin 1 was amplified with PrimeSTAR Max DNA Polymerase (Takara-bio). Pin1 specific primers are described above. The Pin1 gene was cloned into pEF6/Myc-His A vector (Invitrogen, Tokyo, Japan) which was digested with EcoRI and BamHI restriction enzymes using In-fusion HD Cloning Kit (Takara-bio) according to the manufacturer's protocol. The Pin1 sequence cloned into the vector was confirmed by Big-Dye sequencing analysis (Applied Biosystems Japan, Tokyo, Japan). For genetic knockout (KO) experiments with fcwf-4 cells, we constructed the plasmids using CRISPR/Cas9 systems. Briefly, we synthesized oligonucleotides to guide RNA to target Pin1 DNA (Table 1), and these were sub-cloned into the vector, pSpCas9 (BB)-2A-Puro (pX459: Addgene, Cambridge, MA, USA). The sequences of all constructed plasmids were confirmed using a Big-Dye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, CA, USA).

Table 1
Oligonucleotide sequences for single guide RNA targetting Pin1 gene.

Oligonucleotide name	Oligonucleotide sequence
Feline Pin1 Cas9-5 FW	5′-caccgaagagaagctgccgcccggc-3′
Feline Pin1 Cas9-5 RV	5′-aaacgccgggcggcagcttctcttc-3′

2.3. Transient expression of the Pin1 gene and infection with FCoV

After the cells were seeded 1 day prior to transfection at 2.5×10^5/well in 12-well plates, the plasmid vector containing the c-Myc-tagged Pin1 gene was transfected into fcwf-4 cells using Xtreme HD transfection reagent (Roche diagnosis, Tokyo, Japan) according to the manufacturer's instructions. The empty vector pEF6/Myc-His A was transfected into the cells to normalize the total amount of DNA per transfection. The cells transfected with the plasmids were infected with FCoV at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu) per cell, in order to study their effects on FCoV infection 24 h after transfection. The infected cells were collected after 20-h incubation and used for analysis.

2.4. Cell viability assay

We assayed WST-8 to evaluate the cytotoxicity of dipentamethylene thiuram monosulfide [DTM, (MP Biomedicals, LLC; Solon, OH, USA)] for fcwf-4 cells using the Cell Counting Kit-8 (Dojin Chemical Inc., Wako, Japan) according to the manufacturer's instructions.

2.5. Cells treated with dipentamethylene thiuram monosulfide or interferon-ω

fcwf-4 cells were incubated with or without various concentrations of DTM or interferon (IFN)-ω (Intercat, TORAY, Tokyo, Japan) for 30 min at 37 °C before we inoculated fcwf-4 cells with FCoV at a MOI of 1 pfu per cell to study their effects on FCoV infection. After adsorption for 1 h at 37 °C, medium containing the virus was removed, and the cells were rinsed three times with phosphate-buffered saline (PBS) and incubated with or without various concentrations of DTM or IFN-ω for 20 h before analysis by Western blotting and a quantitative (qRT-PCR) assay.

2.6. Real-time, quantitative reverse transcriptase-polymerase chain reaction

The fcwf-4 cells were infected at a MOI of 1 pfu per cell and then incubated with or without DTM. The medium was removed 20 h post-infection, and RNAiso-plus (Takara Bio) was added to the cells for RNA preparation according to the manufacturer's protocol. Total RNA was quantified using the One Step PrimeScript RT-PCR kit (Perfect Real Time; Takara-Bio). Viral cDNAs were quantified by real-time PCR using the forward and reverse primers for the FCoV-N gene (5′-TGGCCACACAGGGACAAC-3′) and (5′-AGAACGACCACGTCTTTTGGAA-3′) and the TaqMan probe (FAM-TTCATCTCCCCAGTTGACG-BHQ-1). Reaction mixtures were prepared according to the manufacturer's protocol, and sequences were amplified using a 7500 Sequence Detection System (Applied Biosystems, Tokyo, Japan). cDNA to the FCoV-N gene was cloned into the pcDNA3.1 vector (Invitrogen), which was then serially diluted to provide standards for FCoV gene quantification. The viral RNA copy number was normalized using the feline β-2-microglobulin (β2M) gene (GenBank accession no. **NM_001009876**). The β2M gene derived from fcwf-4 cells was cloned by PCR amplification using the following primers: fβ2M-F 5′-GGCGCGTTTTGTGGTCTTGGTC-3′ and fβ2M-R 5′-CACTTAACGACCTTGGGCTC-3′. The amplified PCR products were sub-cloned into pTAC-1 plasmids (BioDynamics Laboratory Inc. Tokyo, Japan) to provide standards for the β2M gene. We then quantified the feline β2M gene by real-time PCR using the forward (5′-CGCGTTTTGTGGTCTTGGTCTTGGT-3′) and reverse (5′-AAACCTGAACCTTTGGAGAATGC-3′) primers for the β2M gene and detected the gene using the TaqMan probe, TAMRA-CGGACTGCTCTATCTGTCCCACCTGGA-BHQ-2.

2.7. RNA interference

siRNA duplexes (Sigma–Aldrich, Tokyo, Japan) against feline Pin1 were used to silence Pin1 expression in fcwf-4 cells. These sequences of siRNA are shown in Table 2. A non-targeting siRNA (Bioneer corporation, Daejeon, Republic of Korea; SN-1023) was used to monitor transfection and knockdown efficiency. For transfection into the cells, 2×10^5 fcwf-4 cells per well in 12-well clusters were transfected with Opti-MEM reduced-serum medium (Life Technologies), containing 50 nM siRNA using Xtreme-siRNA transfection reagent (Roche diagnosis, Tokyo, Japan). The cells were infected with FCoV at 24 h post transfection (p.t.) at a MOI of 1 pfu per cell. The infected cells were collected at 48 h p.t. to be analyzed by Western blotting, RT-qPCR, and titration.

2.8. Knockout of Pin1 gene in the fcwf-4 cells by CRISPR/Cas9 systems and virus infection

Constructed plasmids were transfected into fcwf-4 cells with Xtreme HD transfection reagent. More than 12 cell lines were cloned after selection with puromycin (6 μg/mL) over a period of 2 weeks. Mutations of each cell line were confirmed by Western blot analysis and genomic DNA sequence analysis. KO cells were infected with FCoV 79-1146 strain at a MOI of 1 pfu per cell to study their effects on FCoV infection before the cells were collected for Western blot and RT-qPCR analysis, 20 h post infection.

2.9. Luciferase assay

Luciferase activities were quantified using pGL4.30 [luc2P/NFAT-RE/Hygro] (Promega, Tokyo, Japan), pRL-SV40 vectors, and the pGL3 promoter (Promega) for the NFAT response assay. The reporter assays proceeded using the Dual-Luciferase Reporter Assay System (Promega). The two reporter plasmids were co-transfected into fcwf-4 cells with or without 0.05 μg/mL phorbol 12-myristate-13-acetate (PMA; Sigma–Aldrich, Tokyo, Japan) and 0.142 μg/mL ionomycin (Sigma–Aldrich) to stimulate calcium signaling for each assay. Total cell lysates were prepared with reporter lysis buffer provided with the Dual-Luciferase Reporter Assay System at 48 h p.t. before the assay. Luciferase activities were quantified in triplicate assays using a Lumat LB9507 system (Berthold Technologies, Tokyo, Japan).

Table 2
siRNA sequences targeting to Pin1 used in this study.

siRNA name	Sequence
156–178	Forward: 5′-GCAGGGGUACUACUUUAAU-3′ Reverse: 5′-UUAAAGUAGUACACCCUGC-3′
157–179	Forward: 5′-CAGGGUGUACUACUUUAAUC-3′ Reverse: 5′-AUUAAAGUAGUACACCCUG-3′
159–181	Forward: 5′-GGGUGUACUACUUUAAUCAC-3′ Reverse: 5′-UGAUUAAGUAGUACACCC-3′
247–269	Forward: 5′-CGAGCCCACCAGGGUCC-3′ Reverse: 5′-UCGGACCCUGGUGGGCUC-3′
253–275	Forward: 5′-CACCAGGGUCCGAUGCUC-3′ Reverse: 5′-UGAGCAUCGGACCCUGGU-3′

2.10. Western blot analysis

The cell membranes were disrupted with cell lysis buffer [10 mM Tris–HCl, pH 7.8, 1 mM ethylenediamine tetraacetic acid (EDTA), 1% NP-40, and 0.15 M NaCl], including Complete Mini (Roche Diagnostics, Tokyo, Japan) at 20 h after infection. The cell lysates were resolved by electrophoresis on 12.5% SuperSep gels (WAKO, Tokyo, Japan) and Western blotted on to Immobilon-P membranes (Millipore, Tokyo, Japan). Non-specific protein binding was blocked with 5% non-fat dry milk, and then the membranes were incubated with the primary antibodies [anti-FCoV nucleocapsid (N) antibody (FIPV3-70; MyBioSource, CA, USA), anti-c-Myc antibody (Santa Cruz Biotechnology, CA, USA), anti-Pin1 antibody (Cell Signaling Technology, Tokyo, Japan), anti-Cyp B (Thermo Fisher Scientific, Yokohama, Japan), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Calbiochem, CA, USA)] for 1 h. Antigen signals were visualized by reacting proteins on the membranes with horseradish peroxidase-conjugated anti-mouse IgG antibody (Promega) and/or anti-rabbit IgG antibody (Promega) followed by an enhanced chemiluminescence substrate (Super-Signal West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific) according to the manufacturer's protocol.

2.11. Statistical analysis

The 50% inhibitory concentration (IC_{50}) was calculated with the R CRAN software drc package. Statistical significance was determined using the student's t test. For all data analyzed, a significance threshold of $p < 0.05$ was assumed. Values are expressed in some figures as means ± standard deviation (SD).

3. Results

3.1. Pin1 enhances FCoV replication and protein expression

To analyze the effects of Pin1 on FCoV replication, we cloned *F. catus* Pin1 gene from fcwf-4 cells. The predicted sequence of the Pin1 gene has been reported on the National Center for Biotechnology Information (NCBI) nucleotide database (GenBank accession no. **XM_003981844**). The sequence of the cloned Pin1 gene was exactly the same as the predicted nucleotide sequence (data not shown) by sequence analysis. First, the cMyc-tagged Pin1 gene was transfected into fcwf-4 cells to examine the effects of Pin1 on FCoV replication in cells infected with FCoV. The relative viral N protein expression was normalized with endogenous CypB protein (Fig. 1a and b). Western blotting and RT-qPCR analysis showed that both the virus protein expression and RNA replication in the fcwf-4 cells transfected with Pin1 gene were enhanced at a 1.5-fold higher level than the mock transfected cells (Fig. 1b and c).

3.2. Pin1 does not affect NFAT signaling in fcwf-4 cells

It has been reported that Pin1 interacts with the phosphorylated form of NFAT and inhibits calcium dependent activation of NFAT (Liu et al., 2001). To evaluate the effects of Pin1 on the calcineurin-NFAT pathway in fcwf-4 cells, the NFAT luciferase reporter plasmid pGL4.30 (luc/NFAT-RE/Hygro) and a normalized control plasmid pRL-SV40 were transfected into fcwf-4 cells that had been incubated with/without PMA and ionomycin. The NFAT signal is controlled by calcium stimulation; therefore, NFAT signal was not activated in the absence of PMA and ionomycin (Iampietro et al., 2014). As shown in Fig. 2, NFAT activities in the presence or absence of Pin1 expression were not significantly different. These results suggest that enhancement of FCoV replication by Pin1 expression does not correlate with the calcineurin-NFAT pathway.

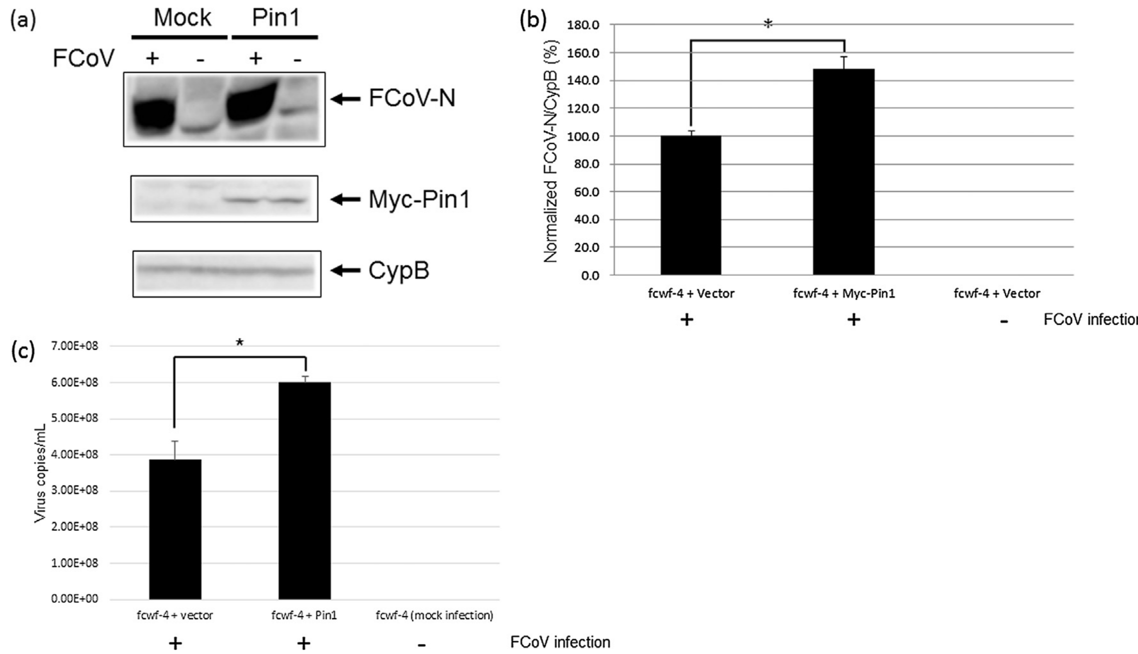

Fig. 1. Overexpression of Pin1 enhances FCoV replication. (a) fcwf-4 cells were transfected with the Myc-tagged Pin1 gene and incubated for 24 h. The cells were then infected with FCoV 79-1146 at MOI of 1 pfu per cell. After 20 h post infection, the cells were disrupted with lysis buffer and used for Western blotting analysis. FCoV-N protein normalized with endogenous CypB protein. (b) Total cell lysates infected with or without FCoV were harvested from cells transfected with plasmids and used for Western blotting analysis. Relative expression of N protein was normalized with CypB protein. The band intensity was quantified using Multi Gauge imaging software. Experiments were carried out in triplicates. Error bars indicate standard deviations. The asterisk indicates a significant difference (*, $p < 0.05$) from the value for the vector control. (c) fcwf-4 cells were transfected with an empty vector or Myc-tagged Pin1 vector and incubated for 24 h. The cells were then infected with FCoV after transfection. After 20 h post infection, extracellular RNAs isolated from culture supernatant were quantified by RT-qPCR analysis. Experiments were carried out in triplicates. Error bars indicate standard deviations. The asterisk indicates a significant difference (*, $p < 0.05$) from the value for the vector control.

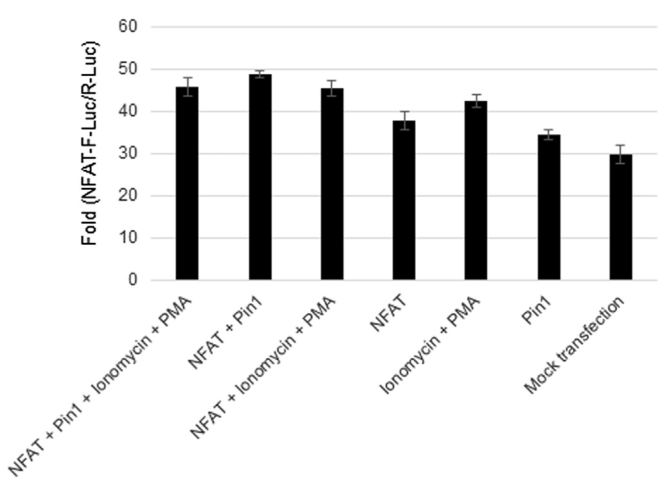

Fig. 2. Pin1 does not affect NFAT signaling in fcwf-4 cells. NFAT signaling in fcwf-4 cells and non-response to ionomycin and PMA stimulation. The plasmid vector [pGL4.30 (luc2P/NFAT-RE/Hygro)] containing the NFAT response element and fire fly luciferase was co-transfected with pRL-SV40 as a transfection control. After 48 h incubation with or without ionomycin and PMA, total cell lysates were prepared with reporter lysis buffer provided as part of the Dual-Luciferase Reporter Assay System at 48 h p.t. Both luciferase activities were then measured. Experiments were performed in duplicate. Error bars indicate standard deviations.

3.3. DTM, a specific inhibitor to Pin1, inhibits FCoV replication in a dose-dependent manner

To verify the effects of Pin1 on FCoV replication, we used a specific inhibitor (DTM) against Pin1 activity. DTM is known as a specific inhibitor against Pin1-PPIase activities with an EC50 value of 4.1 μM (Tatara et al., 2009). To analyze cell viability, we carried out a cytotoxicity assay using different concentrations of DTM. Treatment at concentration levels less than 50 μM did not show cellular toxicity (Fig. 3a). When fcwf-4 cells infected with FCoV were treated with DTM, the expression of the viral N protein by Western blotting analysis was suppressed by DTM treatment compared with that by DMSO treatment (Fig. 3b). RT-qPCR analysis showed that DTM inhibited viral replication in a dose-dependent manner with an IC_{50} of 1.2 μM (Fig. 3c). Using a dose of 10 μM DTM, inhibition equated to an approximately 2-log reduction compared with that of DMSO-treated fcwf-4 cells. These data show that Pin1 plays an important role in virus replication and protein expression. Additionally, we examined the effects of IFN because IFN often affects virus replication in infection. The effects of IFN-ω (50 or 5 IU) on FCoV were not significantly different from those of DMSO treated cells (Fig. 3d). These data indicate that IFN treatment of fcwf-4 cells does not affect virus replication.

3.4. Viral protein expression of FCoV is inhibited in fcwf-4 cells expressing siRNAs

We next confirmed the role of Pin1 in virus replication using genetic knockdown experiments. Five kinds of siRNA against feline Pin1 gene were transfected into fcwf-4 cells before cells were infected with FCoV. The greatest reduction of the FCoV N protein was found using siRNA 247–269 (approximately 50% reduction), and the second most influential siRNA was siRNA 159–181 (approximately 40% reduction), which was evident by Western blotting analysis after viral protein expression was normalized with GAPDH expression (Fig. 4a and b).

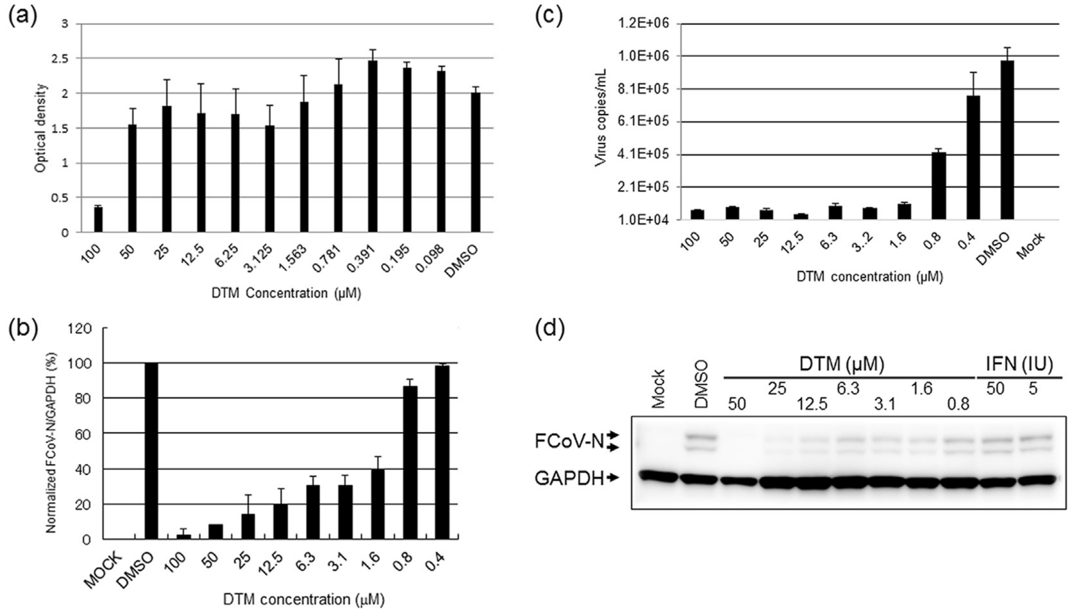

Fig. 3. DTM, a specific Pin1 inhibitor, inhibits FCoV replication. (a) fcwf-4 cells were incubated with DTM to examine cell toxicity using various concentrations of DTM. After 24-h treatment with DTM, cell viability was assessed by the WST-8 assay. Error bars indicate standard deviations. (b) fcwf-4 cells treated with various concentrations of DTM were infected with FCoV. At 20 h after infection, the cells were collected with cell lysis buffer and analyzed by Western blotting methods. DMSO not containing DTM was used as a positive control lane. (c) Total RNAs from the supernatants of infected cells were treated as described in the panel legend (b) and were extracted and analyzed by RT-qPCR. Each sample was assessed through triplicate measurements. Error bars indicate standard deviations. (d) fcwf-4 cells were incubated with or without DTM and IFN-ω after infection with FCoV. At 20 h after infection, the cells were lysed with cell lysis buffer and assessed by Western blotting analysis.

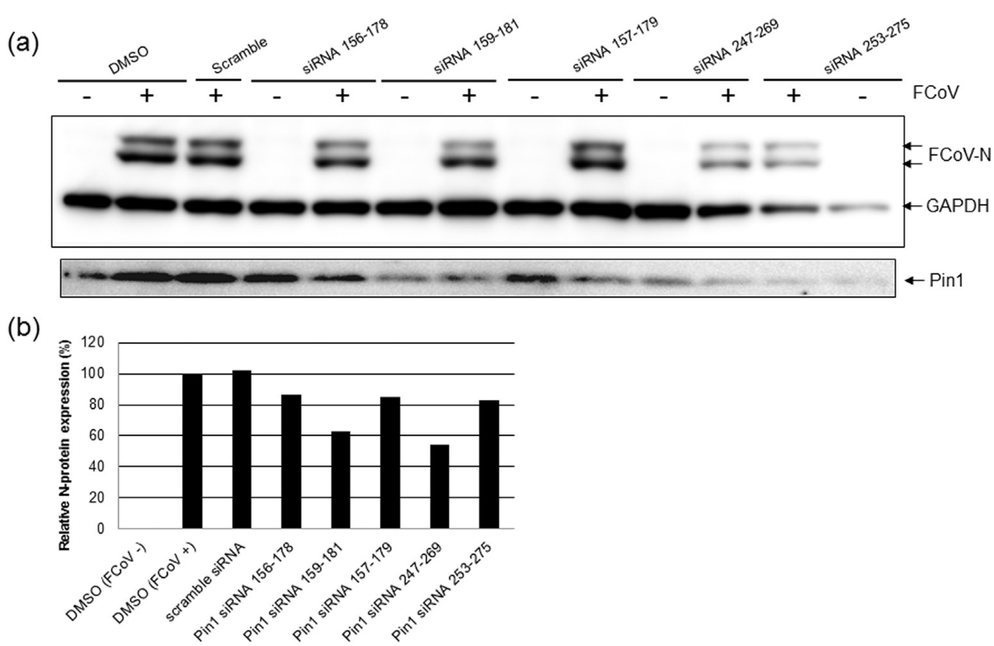

Fig. 4. fcwf-4 cells were transfected with various kinds of siRNA against Pin1 gene. (a) At 24 h after transfection, the cells were infected with FCoV and incubated for 20 h. Total cell lysates after incubation were assessed by Western blotting. Scramble siRNA was used for a negative control experiment. (b) Relative FCoV-N protein expression was normalized with GAPDH protein from the results of panel (a), and Western blotting experiments were indicated.

3.5. *Replication of FCoV is inhibited in Pin 1-knockout cell lines*

When infected fcwf-4 cells were treated with DTM, the reduction ratio was much greater than that in cells treated with siRNA. Therefore, to examine the role of Pin1 gene by other methods, we carried out genetic KO experiments in fcwf-4 cells using CRISPR/Cas9 systems. The cell viability and doubling times on the KO cells were not significantly different from the parent fcwf-4 cells (data not shown). We confirmed the protein expression levels of Pin1 by Western blot analysis. The results showed that the protein expression of Pin1 was almost undetectable. The results of Western blot and RT-qPCR analysis showed that virus replication in the Pin1-knockout cells infected with FCoV was suppressed by approximately one-log reduction compared with that in the infected parent fcwf-4 cells (Fig. 5a and b). These data indicate that Pin1 protein plays important roles in FCoV replication.

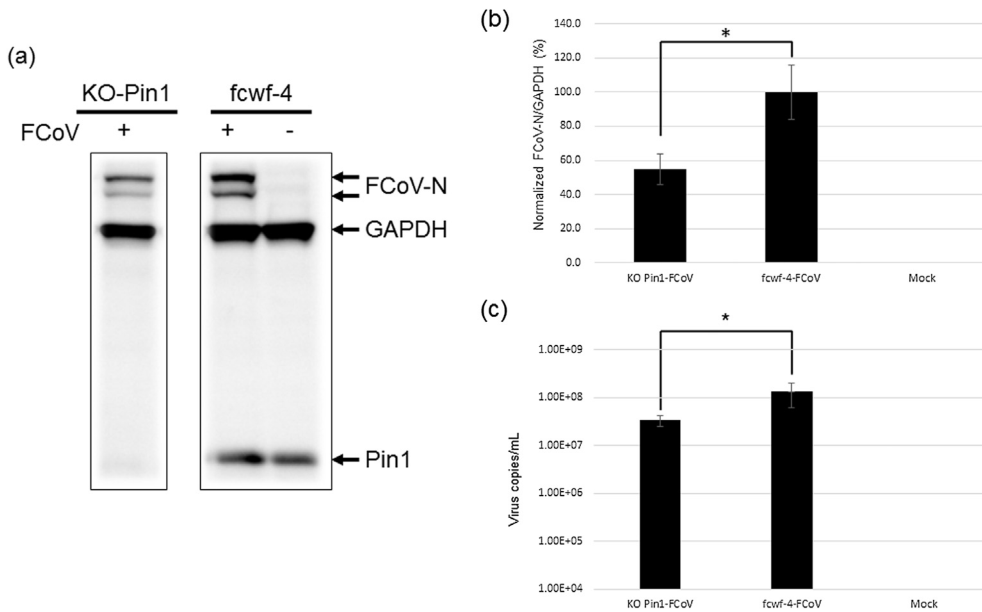

Fig. 5. KO cells for the Pin1 inhibit FCoV replication. (a) Stable KO cells for the Pin1 gene were infected with FCoV. At 20 h after infection, total cell lysates were prepared and used for Western blotting analysis. (b) Relative FCoV-N protein expression was normalized with GAPDH protein from the results of panel (a), Western blotting experiments were indicated. Each sample was assessed by triplicate measurements. Error bars indicate standard deviations. The asterisk indicates a significant difference (*, $p < 0.05$) from the value for the parent fcwf-4 cells. (c) Total RNAs from the supernatants of infected cells treated as described in the legend to panel (a) were extracted and analyzed by RT-qPCR. Each sample was assessed by triplicate measurements. Error bars indicate standard deviations. The asterisk indicates a significant difference (*, $p < 0.05$) from the value for the parent fcwf-4 cells.

4. Discussion

Pin1 is a chaperone protein which regulates protein folding as well as Cyps. However, its catalytic site is unique among other PPIase enzymes because it recognizes an unusual phosphorylated Ser/Thr–Pro motif in its substrates (Lu and Zhou, 2007; Lu et al., 2002; Wulf et al., 2005). We tried to use DTM in this study which specifically inhibits Pin1 activities *in vitro*. DTM inhibits FCoV replication in a dose-dependent manner. These results are compatible with the results of Pin1-siRNA experiments (Fig. 3b). NFAT signals in fcwf-4 cells did not respond to ionomycin-PMA stimulation or overexpression of Pin1. These results show that the fcwf-4 cell does not respond to NFAT signals. On the contrary, overexpression of Pin1 enhanced FCoV replication. Additionally, knockout of Pin1 expression suppressed viral replication and protein expression. However, N protein expression of FCoV was not completely inhibited by knockout of Pin1 (Fig. 5a–c) in this study. These data indicate that FCoV replication is affected not only by Pin1 activities but also by other host factors, such as cellular PPIases. To examine this hypothesis, further studies are needed to use multi-PPIase knockout cells by CRISPR/Cas9 system. Nevertheless, our studies indicate that Pin1 modulates FCoV replication. To date, no direct role has been reported for Pin1 which affects CoV replication.

The N protein of CoV forms a helical ribonucleoprotein structure through the wrapping of genomic RNA (gRNA) by the RNA chaperone domain (Spencer and Hiscox, 2006; Zuniga et al., 2007). The N protein may participate in the discontinuous transcription of subgenomic mRNAs (sgmRNAs) because depletion of N from the replicon reduces the synthesis of sgmRNA, but not gRNA (Zuniga et al., 2010). Glycogen synthase kinase-3 (GSK-3) is the kinase responsible for the phosphorylation of the serine–arginine (SR)-rich motif which is conserved in the N-protein of mouse hepatitis virus, SARS-CoV, and FCoV. Treatment with a GSK-3 inhibitor reduces the phosphorylation of N protein and reduces the viral titer and cytopathic effects (Wu et al., 2009). In this study, we could not show which viral or cellular protein interacted with Pin1 and how the complexes regulated FCoV replication. However, Pin1 did not directly interact with the N protein or the Nsp12 protein of FCoV (CoV RNA dependent RNA polymerase) in our experiments (data not shown). We speculate that Pin1 may directly or indirectly regulate the function of GSK-3. DDX1 is a member of the DEAD-box protein family, and knockdown of DDX1 reduced the quantities of longer viral RNAs of infectious bronchitis virus (IBV) (Wu et al., 2014). DDX1 has been identified as a member of the cellular interactomes for the IBV-N protein (Emmott et al., 2013), and an interaction between DDX1 and the non-structural protein 14 (nsp14) of IBV has been identified (Xu et al., 2010). N phosphorylation allows recruitment of DDX1 to the phosphorylated-N-containing complex, which facilitates template readthrough and enables longer sgmRNA synthesis (Wu et al., 2014). However, we do not have any evidence that Pin1 regulates GSK3 or DDX1 functions.

Pin1 interacts directly with HCV NS5A and NS5B proteins and plays unique roles in HCV replication (Lim et al., 2011). Additionally, Pin1 modulates HIV-1 infection by interaction with the capsid protein in uncoating and regulating APOBEC3G (Misumi et al., 2010; Watashi et al., 2008). CsA was shown to exert inhibitory effects on herpes simplex virus (Walev et al., 1991), vaccinia virus (Damaso and Keller, 1994), BK polyoma virus (Acott et al., 2008), HIV-1 (Billich et al., 1995; Streblow et al., 1998), and HCV (Watashi et al., 2005). These reports show that PPIase has important roles in replication of various viruses, as well as influencing uncoating and viral internalization into the cell. Considering these findings, for the first time, our study has revealed the essential roles of Pin1 in FCoV replication. Therefore, our study may contribute to the development of anti-FCoV or other CoVs inhibitors.

Acknowledgments

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