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The ubiquitin-proteasome system is required for the early stages of porcine circovirus type 2 replication

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ABSTRACT

Porcine circovirus type 2 (PCV2) is the primary causative agent of porcine circovirus-associated diseases (PCVAD). It has been shown that the ubiquitin-proteasome system (UPS) is correlated with viral infection, but its role in PCV2 replication remains unknown. In the present study, we explored the interplay between PCV2 replication and the UPS in PK15 cells and found that treatment with a proteasome inhibitor (MG132 and lactacystin) significantly decreased the PCV2 titer at the early infection stage. We further revealed that inhibition of the UPS did not affect virus entry but decreased viral protein expression and RNA transcription potentially in a cell cycle-dependent manner. PCV2 infection has little effect on the chymotrypsin-like activity, and the gene-silencing of ubiquitin reduced the PCV2 titer, which indicates that the effective replication of PCV2 may be related to protein ubiquitination. Taken together, our data suggested that PCV2 replication requires the UPS machinery, which may represent a potential antiviral target against PCV2.

Introduction

Porcine circovirus type 2 (PCV2), which belongs to the genus *circovirus* of the family *Circoviridae*, is the most important pathogen among the porcine circovirus-associated diseases (PCVAD), including postweaning multisystemic wasting syndrome (PMWS) (Segales et al., 1997), porcine dermatitis and nephropathy syndrome (PDNS) (Ramos-Vara et al., 1997), reproductive failure, porcine respiratory disease complex (PRDC), granulomatous enteritis, necrotizing lymphadenitis (Chae, 2005), and acute pulmonary edema (APE) (Cino-Ozuna et al., 2011). The main target cells of PCV2 are considered to be macrophage/monocyte-lineage cells and other antigen-presenting cells (Rosell et al., 1999). PMWS is associated with swine immunosuppressive or immunodepressive diseases, which pave the way for secondary infection by *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004), porcine reproductive and respiratory syndrome virus (Harms et al., 2001), and porcine parvovirus (Allan et al., 1999).

The ubiquitin-proteasome system (UPS) coupled with the autophagy-lysosome system is the major protein degradation pathway in eukaryotic cells (Wong and Cuervo, 2010). The UPS, which regulates many protein targets within cells, plays an important role in a variety of biological processes. The cell cycle-regulated proteins, including cyclins (Glotzer et al., 1991), p27 (Pagano et al., 1995), and p35 (Patrick et al., 1998), are modulated by ubiquitination. The proteasome degrades the majority of the cellular proteins and generates most peptides presented on major histocompatibility complex (MHC) class I molecules (Rock et al., 1994). In addition, ubiquitin-activating enzyme E1 is required for MHC class I antigen presentation (Michalek et al., 1993). Additionally, nuclear factor-kappa B (NF-κB) is activated after IκBα is phosphorylated and consequently ubiquitinated (Chen et al., 1995), and the p105 NF-κB precursor for the p50 subunit also requires the proteasome and ubiquitin conjugation (Palombella et al., 1994). In cultured rat hippocampal neurons, the synaptic activity regulates the postsynaptic composition and signaling through the UPS (Ehlers, 2003). These above-mentioned studies demonstrate that the UPS not only plays a role in the complete degradation of polypeptides but also regulates the cell cycle, MHC I molecule-mediated antigen processing, and signaling pathways. Furthermore, the UPS also plays major roles in cell viability, DNA repair, protein synthesis, and stress response (Ciechanover and Iwai, 2004; Jentsch et al., 1987; Varshavsky, 2005).

Because the UPS has a vital role in many fundamental cellular processes, many viruses have reprogrammed the machinery according to their needs. Evidence shows that the UPS regulates the replication of rotavirus (Lopez et al., 2011), human immunodeficiency virus (Klinger and Schubert, 2005), Coxsackievirus B3 (Luo et al., 2003), flavivirus (Fernandez-Garcia et al., 2011), and vaccinia virus (Satheshkumar et al., 2009). In addition, Liu et al. demonstrated that porcine p53-induced RING-H2 (pPirh2), an E3 ubiquitin ligase, interacts with the ORF3 protein of PCV2 (Liu et al., 2007). We also found that the UPS-associated proteins are differently expressed in

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PCV2-infected porcine alveolar macrophages (Cheng et al., 2012). However, it is unknown whether the replication of PCV2 requires the UPS.

In the present study, we demonstrated that the inhibition of the UPS suppresses PCV2 replication at the early infection stage through the degradation of viral DNA copies, protein expression, and viral transcription potentially in a cell cycle-dependent manner. These results illustrate an important role of UPS in the regulation of the early stages of PCV2 replication.

Results

Effects of proteasome inhibitors on PCV2 infection in PK15 cells

To determine whether the UPS is involved in PCV2 replication, the specific proteasome inhibitors MG132 and lactacystin were used to explore the effect of UPS inhibition on PCV2 titer. We first evaluated the cell viability of MG132- and lactacystin-treated cells. The results showed that the cell survival was greater than 80% after treatment with MG132 or lactacystin at concentrations up to 10 μM (Fig. 1A).

We then assessed the effects of MG132 and lactacystin on the PCV2 titer. The cell supernatants were collected at 12 hours post infection (hpi), and the viral DNA copies were determined by absolute quantitative real-time PCR. Compared with DMSO-treated PK15 cells, MG132 (5 μM) inhibited the PCV2 titer in the cultured cells by 20.91%, and lactacystin (10 μM) inhibited the PCV2 titer by 28.09% (Fig. 1B). These data indicate that UPS regulates the early stages of PCV2 replication in PK15 cells.

Effects of proteasome inhibitors on different stages of PCV2 infection

To identify which step(s) in the virus lifecycle are targeted by proteasome inhibitors, we first quantified the viral DNA copies after applying the inhibitors at different time points. After the cells were incubated for 12 hpi, the supernatants were subjected to a viral copy assay. As shown in Fig. 2A, exposure to the inhibitors at 2 hpi significantly inhibited the number of viral DNA copies, and this inhibition was almost the same as that observed when the inhibitors were added at −2 hpi or 0 hpi. This finding suggests that the inhibition of viral replication by the proteasome inhibitors is likely not dependent on the blockade of viral entry into the host cells.

We further observed the effects of MG132 or lactacystin on viral protein expression and RNA transcription. A western blot analysis showed that both proteasome inhibitors (MG132 and lactacystin) decreased the expression of the PCV2 ORF2-encoded capsid protein in a dose-dependent manner (Fig. 2B). In addition, the transcriptional levels of ORF2 were reduced in MG132-treated cells compared to the levels observed in DMSO-treated cells (Fig. 2C). Taken together, these results indicate that the proteasome inhibitors decrease the number of viral DNA copies through suppression of viral protein expression and RNA transcription at the early infection stage.

Proteasome inhibitors induce G2/M phase arrest

As the smallest DNA virus infecting mammals, PCV2 replication depends on cellular enzymes expressed during the S phase of cells growth (Tischer et al., 1987). Experimental evidence shows that the UPS plays an important role in the regulation of the cell cycle, including the induction of S, G2/M arrest, and apoptosis (Han et al., 2009). To investigate whether the downregulation of the PCV2 titer is due to the effect of proteasome inhibitors on cell cycle progression, a flow cytometric analysis was performed based on the DNA content observed in nuclei stained with propidium iodide (PI, Sigma-Aldrich). As shown in Fig. 3, the percentages of cells in the sub-G1 phase and S phase were approximately 1% and 30%, respectively, and these percentages were not significantly influenced by treatment with MG132. However, a dose-dependent increase in the cell population at the G2/M phase was observed after treatment with MG132, and the increased cell populations after this treatment ranged from 44.69 ± 9.84% to 57.45 ± 5.93%. Additionally, the percentages of cells in the G0/G1 phase, which ranged from 22.83 ± 11.60% to 12.22 ± 7.90%, were significantly decreased in a dose-dependent manner with MG132 (Fig. 3B). A similar trend was also found in the PK15 cells treated with lactacystin (Fig. 3C). Taken together, the results reveal that proteasome inhibitors influence the cell cycle progression by prolonging the G2/M phase, which suggests that inhibition of the UPS may suppress the replication of PCV2 in a cell cycle-dependent manner.

Proteasome activity in PCV2-infected PK15 cells

There are two successive steps involved in protein degradation: the target protein substrates are ubiquitinated through their covalent attachment to ubiquitin, and ubiquitinated proteins are degraded in

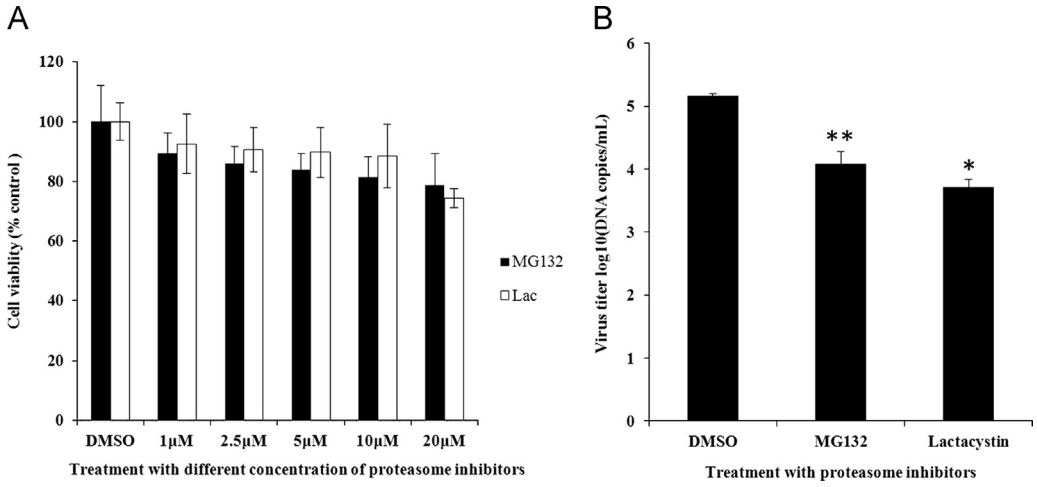

Fig. 1. Effects of proteasome inhibitors on PCV2 infection in PK15 cells. (A) Cell viability of inhibitor-treated PK15 cells. PK15 cells were treated with proteasome inhibitors (MG132 and lactacystin) at the indicated concentrations. The cell viability after treatment was assessed using the MTT assay. The MTT absorbance values of the DMSO-treated cells were defined as 100% survival. The values are the means ± SD ($n=5$). (B) PCV2 titers in the inhibitor-treated PK15 cells. After infection with PCV2, the PK15 cells were incubated with DMEM containing the inhibitors. The supernatant was collected at 12 hpi, and the virus titer was determined through absolute quantitative real-time PCR. The statistical analysis was performed using SPSS Statistics 17.0: significance was defined as $P<0.05$ (*), and high significance was defined as $P<0.01$(**).

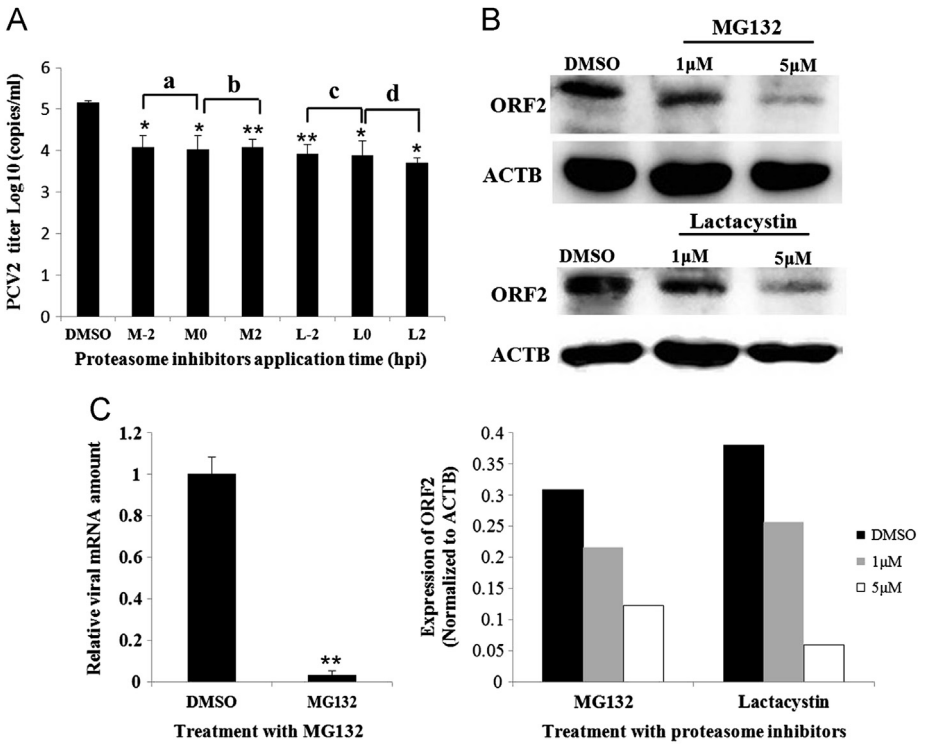

Fig. 2. Proteasome inhibition decreases ORF2 expression and viral RNA transcription in PCV2-infected PK15 cells. (A) Proteasome inhibitors were added to PK15 at −2 hpi, 0 hpi and 2 hpi. The supernatants were harvested at 12 hpi, and absolute quantitative real-time PCR was performed. *$P<0.05$ and **$P<0.01$ compared with DMSO-treated cells. M and L indicate treatment with MG132 and lactacystin, respectively; −2, 0, and 2 indicate the time at which the inhibitor was added. The following statistical differences were found between the groups: (a and b) M-2 and M2 compared with M0: $p_{M\text{-}2/M0}=0.854$, $p_{M2/M0}=0.837$, (c and d) L-2 and L2 compared with L0: $p_{L\text{-}2/L0}=0.88$, $p_{L2/L0}=0.564$. (B) After PCV2 infection, PK15 cells were incubated in the DMEM containing DMSO or inhibitors. The cell lysate was collected at 12 hpi, and a western blot assay for the detection of viral capsid protein ORF2 and ACTB was performed. The protein levels were quantified by densitometric analysis using Image-Pro Plus (version 4.5) and normalized with the ACTB levels. (C) The total cellular RNA of PCV2-infected PK15 cells treated with DMSO or MG132 was subjected to real-time RT-PCR analysis. The samples were normalized using the DMSO-treated PK15 cells as calibrators and GAPDH as the reference gene.

the 26S proteasome. To elucidate the role of the UPS in PCV2 infection, we investigated the proteasome activity in PK15 cells after PCV2 infection. The PK15 cells were first infected with PCV2 and then incubated with a fluorogenic proteasome peptide substrate, and PK15 cells incubated with MG132 or lactacystin were used as the positive control. The cell medium was then collected for measurement of the chymotrypsin-like (CT-like) activity (Luo et al., 2003). The results show that PCV2 infection did not affect the proteasomal CT-like activity, whereas treatments with MG132 or lactacystin inhibited ~40% of the proteasomal CT-like activity in the PK15 cells (Fig. 4). This observation suggests that protein ubiquitination may play a role in the PCV2 replication.

Knockdown of ubiquitin by siRNA reduces PCV2 infection

The ubiquitination of cellular proteins starts with their covalent conjugation to ubiquitin (Hershko and Ciechanover, 1992). To investigate the role of ubiquitination in PCV2 replication, we used an ubiquitin-specific siRNA (S117) to knockdown the expression of the *Sus Scrofa* ubiquitin B gene (GI: 156105184). As shown in Fig. 5, the viral DNA copies were reduced to 16.6% in the ubiquitin siRNA-transfected cells compared to the negative control (NC). These results suggest that protein ubiquitination may be a critical process during the PCV2 lifecycle.

Discussion

The UPS plays an important role in the degradation of intracellular proteins and regulates the cellular signaling pathway (Mukhopadhyay and Riezman, 2007). Many viruses have manipulated the UPS for their needs (Randow and Lehner, 2009; Shackelford and Pagano, 2005). For example, the UPS has been suggested to be required for viral immune evasion, for viral maturation and viral progeny release, for efficient viral replication, and for reactivation of virus from latency (Gao and Luo, 2006). In the present study, we show that treatment with proteasome inhibitors (MG132 and lactacystin) leads to a reduction in viral activity, as determined by decreases in the number of viral DNA copies, viral protein synthesis, and RNA transcription, likely in a cell cycle-dependent manner at the early viral infection stage. We further show that PCV2 infection has little effect on proteasomal activity and that the depletion of ubiquitin with siRNA reduces the genome copies of PCV2, which indicates that PCV2 infection may be correlated with protein ubiquitination.

A previous study showed that the inhibition of UPS impairs the viral infection cycle (Raaben et al., 2010). For instance, MG132 affects hepatitis E virus replication likely through the inhibition of viral transcription and/or translation without a significant effect on cellular translation (Karpe and Meng, 2012). Wang et al. (2011) demonstrated that cellular inhibitor of apoptosis protein 2 (cIAP2) can significantly reduce the levels of hepatitis B virus DNA replication via acceleration of the UPS-mediated decay of the polymerase. In the present study, we examined the effects of the proteasome inhibitors MG132 and lactacystin on PCV2 replication and found that the inhibition of the UPS reduces the number of viral DNA copies at 12 hpi (Fig. 1B). This finding suggests that the UPS is required for the early stage of PCV2 replication.

The UPS plays an important role in the different stages of the virus lifecycle, including viral entry, gene transcription, protein synthesis, assembly, and viral progeny release (Gao and Luo,

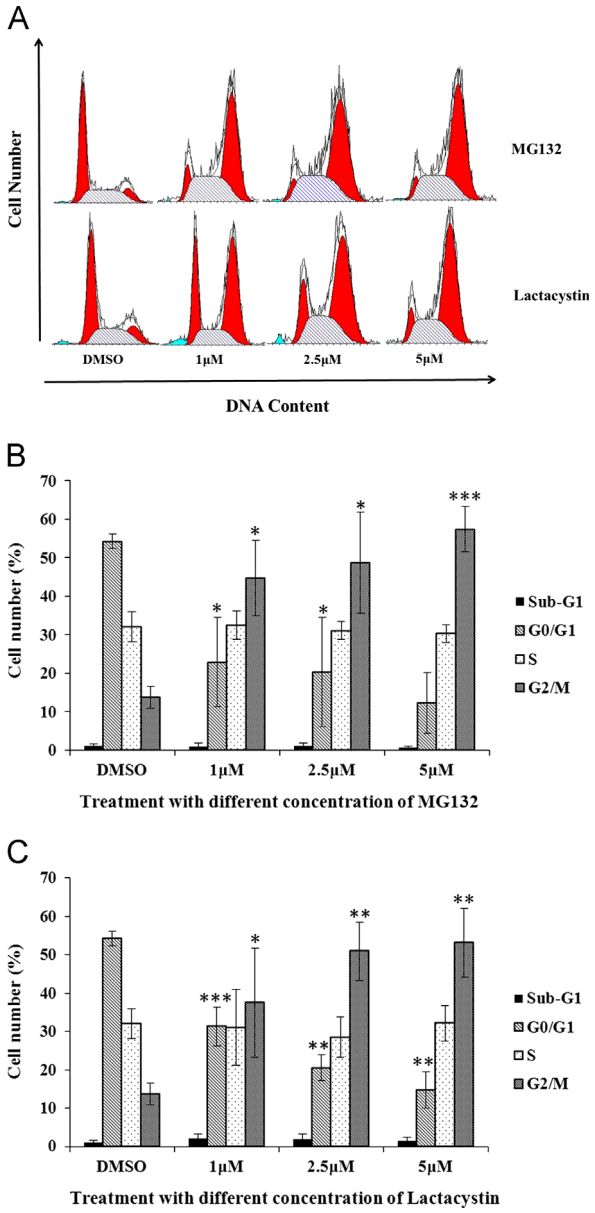

Fig. 3. Effects of proteasome inhibitors on the cell cycle distribution in PK15 cells. (A) Flow cytometry analysis of cells through PI staining. Percentages of the MG132- (B) and lactacystin- (C) treated PK15 cells in each phase of the cell cycle.

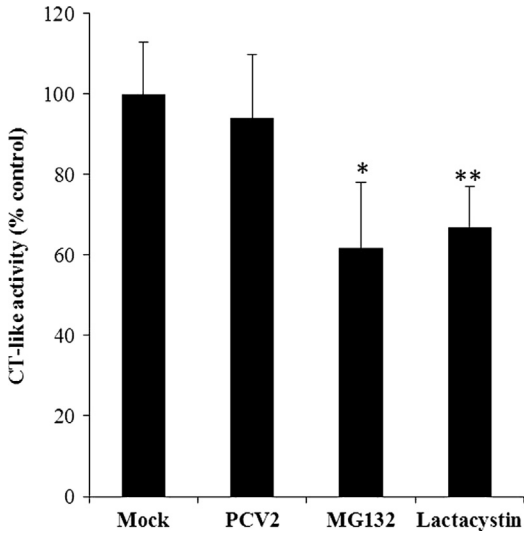

Fig. 4. Proteasome activity in PCV2-infected PK15 cells. PK15 cells were infected with PCV2 or DMEM, and cells treated with MG132 and lactacystin were used as the positive controls. The cell lysates were collected, and the proteasomal activity was measured using the fluorogenic proteasome substrate SLLVY-AMC (Merck).

2006). Proteasome inhibitors reduce viral RNA synthesis and capsid protein expression of Coxsackievirus B3 infection (Si et al., 2008), and appear to not only impair entry but also RNA synthesis and subsequent protein expression of different coronavirus (Raaben et al., 2010). Ubiquitin-mediated proteolysis is required for the transcriptional activation of synthetic herpes simplex viral VP16 transcription activators (Zhu et al., 2004). Ubiquitin ligase Nedd4 can interact with the Gag protein of human T-cell leukemia virus type 1 (HTLV-1) and that this interaction plays a critical role in HTLV-1 budding (Sakurai et al., 2004). MG132 affects Influenza A virus (IAV) infection at a postfusion step (Widjaja et al., 2010). Tat, a potent transactivator encoded by the human immunodeficiency virus type 1 (HIV-1), is regulated by ubiquitination to stimulate its transcriptional properties (Bres et al., 2003). In the present study, we found that the proteasome inhibitors significantly inhibited the viral transcription and protein expression. Replication of the PCV2 requires the Rep protein complex which consists of 2 viral proteins Rep and Rep′, and Rep′ is a spliced form of the Rep mRNA (Faurez et al., 2009). Thus, it was not surprising that treatment of PCV2-infected cells with proteasome inhibitors reduced viral transcription as well as protein expression.

Proteasome inhibitors regulate the cell cycle progression. In the present study, the inhibitors were found to arrest the cell cycle progression of PK15 cells at the G2/M phase, which is consistent with the results of a previous study (Zanotto-Filho et al., 2010). PCV2 replication starts from the entry of the single-stranded DNA genome into the nucleus, which resulted in their inclusion in the daughter nuclei at the end of mitosis. Viral replication is initiated by the binding of rep protein complex to the stem loop structure and depends on the cellular DNA polymerase expressed during the S phase (Tischer et al., 1987). Although the proteasome inhibitors have no effect on the S phase at the early infection stage, the cell cycle is a complex process that involves numerous regulatory proteins. G2/M arrest inhibits cell cycle progression, which may influence the entry of the PCV2 genome into the nucleus and the expression of cellular enzymes. These findings indicate that proteasome inhibitors regulate the replication of PCV2 likely in a cell cycle-dependent manner.

Ubiquitination and proteasome degradation are important processes regulated by the UPS (Ciechanover, 1994). The findings obtained in the present study, which demonstrate that PCV2 infection appears to have no effect on the proteasomal activity and that the knockdown of ubiquitin reduces the viral titer, highlight the possibility that the PCV2 replication are modulated via protein ubiquitination. The ubiquitination of cellular proteins is required for the effective replication of Coxsackievirus B3 (Si et al., 2008). In addition, Liu et al. demonstrated that the ORF3 protein of PCV2 decreases the expression of pPirh2, an E3 ubiquitin ligase, and increases the expression of p53 (Liu et al., 2007). The interaction between ubiquitination and viral infection requires further study.

In conclusion, we have shown that proteasome inhibitors (MG132 and lactacystin) reduce PCV2 infectivity, as demonstrated by decreases in the number of viral genomic copies, viral protein expression, and RNA transcription, at the early viral infection stage. In addition, the role of the UPS in PCV2 replication may be associated with protein ubiquitination. Our results suggest that the UPS may be a novel antiviral target against PCV2.

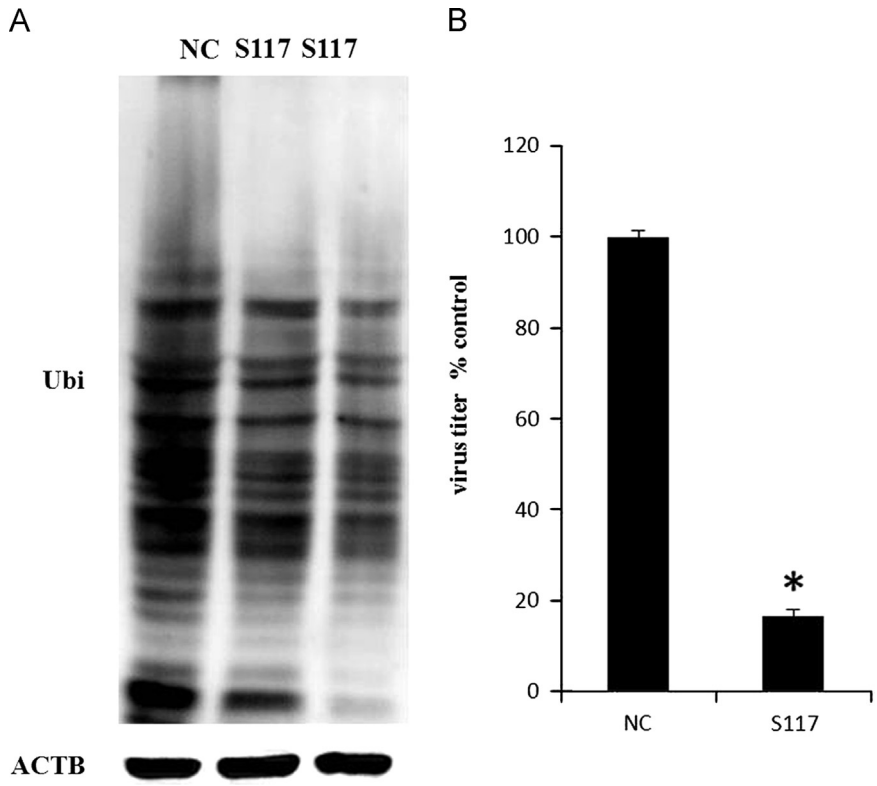

Fig. 5. Knockdown of ubiquitin expression by siRNA reduces PCV2 DNA copies. PK15 cells were transiently transfected with the ubiquitin siRNA or NC RNA and then infected with PCV2. The cell lysates were collected at 12 hpi. (A) A western blot assay was performed using anti-ubiquitin and anti-ACTB antibodies. (B) The supernatants were collected at 12 hpi, and the viral DNA copies were determined by absolute quantitative real-time PCR. $^{*}P < 0.05$ compared to the number of viral DNA copies in the NC-transfected cells.

Materials and methods

Virus and cells

The PK15 cell line, free of PCV, was maintained in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% heat-inactivated newborn calf serum (NCS) at 37 °C in a humidified 5% CO_2 incubator. PCV2 (GI: 225322671), which was originally isolated from a pig farm in the Hubei Province of China, was cultured on PK15 cell lines.

Virus infection

PK15 cells were seeded in cell culture plates, grown to 50–60% confluence, and then infected at a multiplication of infection (MOI) of 10 with PCV2 or mock-infected with DMEM for 1 h in serum-free DMEM. The cells were then washed with PBS and cultured in DMEM supplemented with 2% NCS. For the inhibition experiments, PK15 cells were infected with PCV2 and incubated with DMEM containing the inhibitors at the designed time.

Cell viability assay

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-2H-tetrazolium bromide) assay was performed to determine the cell viability as previously described (Li et al., 2012). Briefly, the cells were inoculated on a 96-well plate and incubated with different concentrations of the inhibitors. The cells were then incubated with MTT solution for 4 h and collected. The absorbance was measured at a wavelength of 490 nm using an ELISA reader.

Quantitative real-time PCR

The total viral DNA from the PCV2-infected PK15 cells was extracted using a Virus DNA Extraction Kit (Omega). To quantify the viral DNA copies, absolute quantitative real-time PCR was performed using the FastStart Universal Probe Master (Rox) (Roche) in a total volume of 20 μL on an ABI prism 7500 (Applied Biosystems). The cycle conditions were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For the standard curve, serial dilutions of a plasmid that was constructed by inserting the PCV2 gene (GI: 225322671) into the pMD™18-T vector (TAKARA) were used to quantify the virus genomic copy number (Yang et al., 2012).

Relative quantitative real-time RT-PCR was performed to evaluate the RNA transcription of ORF2. The total cell RNA at 12 hpi was extracted using the Trizol RNA extraction reagent (Invitrogen). The primers, which are shown in Table 1, were designed using the Beacon Designer 7.9 analysis software. The RNA samples were treated with DNase I for 60 min at 37 °C. The cDNA was produced by the reverse transcription (RT) of 1 μg of RNA using random primers (Roche) according to the manufacturer's protocols. Quantitative real-time PCR was performed using a total volume of 20 μL on a LightCycler 480 (Roche) instrument according to the instructions provided by the manufacturer of the FastStart Universal SYBR Green Master (Roche). The cycle conditions were 30 s at 95 °C and 40 cycles of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s (Cheng et al., 2012). Each sample was run in triplicate. The sample was normalized to DMSO-treated PK15 cells as the reference sample, and GAPDH or β-actin were used as the reference gene.

Western blotting

The PK15 cells were lysed in a lysis buffer containing 50 mM Tris–HCl (pH 6.8), 10% glycerol, and 2% SDS. The whole cell lysate

Table 1
The specific primers of relative quantitative real-time RT-PCR.

Genes	Forward primer(5′–3′)	Reverse primer(5′–3′)
ORF2	ACCGTTACCGCTGGAGAA	TTGACTGTGGTTCGCTTGATAG
β-actin	TGGCACCACACCTTCTACA	ATCTTCTCACGGTTGGCTTTG
GAPDH	TGCCAACGTGTCGGTTGT	TGTCATCATATTTGGCAGGTTT

extracts were then diluted in 5 × sample buffer and boiled for 10 min. Each sample was subjected to 12% SDS-PAGE and then transferred to nitrocellulose membranes (Millipore). After blocking with 1% (w/v) BSA-TBST (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween-20) for 2 h at room temperature, the membranes were incubated with primary antibodies to ORF2 (prepared in our laboratory), ubiquitin (ubi, Bioss), and β-actin (ACTB, Beyotime) at room temperature for 2 h and then with HRP-conjugated secondary antibodies. The membranes were then visualized using the SuperSignal West Pico Luminal kit (Pierce).

Cell cycle analysis by flow cytometry

The cell cycle distributions and sub-G1 cell populations were measured by staining the DNA with PI as previously described (Han et al., 2009). Briefly, the inhibitor- or DMSO-treated PCV2-infected PK15 cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol at −20 °C. After washing with PBS, the cells were resuspended in PBS containing 20 μg/mL RNase A and 50 μg/mL PI and incubated at room temperature for 30 min in the dark. The nuclear DNA content was examined using a FACS Calibur system (BD Biosciences, USA) with the Cell Quest software. The results were representative of at least three independent experiments.

Ubiquitin siRNA transfection

The PK15 cells were grown to 30–50% confluence and then transiently transfected with ubiquitin-specific siRNA using Lipofectamine 2000 according to the manufacturer suggested protocol. A negative control (NC) siRNA was used as a control. The silencing efficiency was detected by immunoblot analyses using an anti-ubiquitin antibody. Fifteen hours after transfection, the cells were infected with PCV2 as previously described.

Statistical analysis

The results are presented as the means ± standard deviations (SD). The statistical analysis was performed using SPSS Statistics 17.0. Differences with a *P* value less than 0.05 were considered significant, and differences with a *P* value less than 0.01 were considered highly significant.

Acknowledgments

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