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Short communication

Induction of autophagy enhances porcine reproductive and respiratory syndrome virus replication

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ABSTRACT

Autophagy is an evolutionarily conserved lysosome-dependent degradation pathway that acts in the maintenance of cellular homeostasis and plays important functions in viral replication and pathogenesis. In this study, we investigated the role of autophagy in the replication of porcine reproductive and respiratory syndrome virus (PRRSV), an agent that has caused devastating losses in the international swine industry since the late 1980s. Using protein quantification and microscopy, we observed that PRRSV infection results in LC3-I/II conversion, an increased accumulation of punctate GFP-LC3-expressing cells, and a higher number of autophagosome-like double-membrane vesicles in the cytoplasm of host cells. Inhibition of autophagy using 3-methyladenine (3-MA) or small interfering RNAs targeting ATG7 and Beclin-1 led to a significant reduction in PRRSV titers and protein expression. Conversely, induction of autophagy by rapamycin resulted in increased viral replication. These results demonstrate that PRRSV infection induces autophagy which, in turn, enhances viral replication efficiency.

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Autophagy is an evolutionarily conserved intracellular process that plays an important role in both the maintenance of cellular homeostasis and in cell survival under stress conditions (Levine and Klionsky, 2004). Mechanistically, autophagy is a lysosome-dependent degradation pathway for the degradation of long-lived proteins and damaged organelles in eukaryotic cells (Klionsky, 2007). Many intracellular and extracellular stresses, such as nutrient starvation, damaged organelles, unfolded protein aggregation and cell death, can induce the autophagic response. During cellular autophagy, cytoplasmic proteins or organelles are sequestered within characteristic double membrane vesicles (DMVs), termed the autophagosome, and shuttled to lysosomes. Matured autophagosomes eventually fuse with lysosomes to degrade and/or recycle their contents (Baehrecke, 2005). More than 30 specific genes have been identified to be involved in the autophagy pathway. In yeast, these are termed autophagy-related genes (ATGs) (Klionsky et al., 2003), and most ATGs are conserved between yeast and mammals.

Besides the physiological functions of autophagy, an increasing body of evidence indicates that autophagy functions in both antiviral and pro-viral capacities in the life cycles of a broad range of viruses (Kudchodkar and Levine, 2009). Autophagy can serve as an innate immune response to suppress viral infection (Schmid and Munz, 2007). For example, the cellular autophagy induced by inhibiting the PI3K/Akt signaling pathway during vesicular stomatitis virus (VSV) infection plays an important role in inhibiting VSV replication (Shelly et al., 2009). However, many viruses have evolved mechanisms to hinder autophagy in infected cells. For instance, herpes simplex virus type 1 (HSV-1) encodes ICP34.5 protein to block the induction of autophagy by binding Beclin-1 or via dephosphorylation of eIF2α (Orvedahl et al., 2007; Talloczy et al., 2002). Human cytomegalovirus (HCMV) infection can antagonize cellular autophagy by activating the mTOR signaling pathway (Klionsky, 2007). In addition, some viruses, including coxsackievirus B3, poliovirus, dengue virus, influenza A virus, and foot-and-mouth disease virus (Kirkegaard, 2009; Lee et al., 2008; O'Donnell et al., 2011; Wong et al., 2008; Zhou et al., 2009), can even utilize autophagy to promote their replication. These findings indicate that autophagy can exert positive or negative effects on viruses, highlighting the complexity of relationship between viruses and autophagy. Further elucidating the processes by which viruses interact with autophagy pathways is likely to lead to a better understanding of viral replication and pathogenesis.

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases in pigs worldwide, characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs (Neumann et al., 2005). The etiological agent, PRRS virus (PRRSV) belongs to the

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Nidovirales order, *Arteriviridae* family of positive-sense single-stranded RNA viruses (Cavanagh, 1997). Previous studies demonstrated that infection with mouse hepatitis virus (MHV), which is also grouped into the order *Nidovirales*, triggered cellular autophagy, and inhibition of autophagy inhibited MHV growth in murine embryonic stem cells (Prentice et al., 2004). However, another study showed that a component of the cellular autophagy, ATG5, was not required for replication and release of MHV in primary macrophages or low passage primary murine embryonic fibroblasts (Zhao et al., 2007). In addition, Cottam and co-workers (2011) found that avian coronavirus, infectious bronchitis virus (IBV), activated cellular autophagy, however, autophagy was not essential for IBV infection. These contradictory data highlights the necessity of additional investigations to determine if nidoviruses are indeed hijacking the autophagy machinery (de Haan and Reggiori, 2008). In this study, we investigated the role of cellular autophagy in PRRSV infection.

To determine whether PRRSV infection triggers cellular autophagy, we first examined the modification of LC3 (microtubule-associated protein, light chain 3), a hallmark of autophagy (Mizushima, 2004). The precursor form of LC3, LC3-I, is normally distributed in the cytoplasm in quiescent cells. However, once autophagy is triggered by a stimulus, LC3-I converts to its lipidated form, LC3-II, which localizes to both the inside and outside of phagophores. This conversion results in the protein migrating more rapidly in SDS–polyacrylamide gels (Kabeya et al., 2000). Thus, it is generally accepted that the ratio of LC3-II/LC3-I correlates well with the formation and the number of autophagosomes. In this study, Marc-145 cells were infected with PRRSV strain WUH3, a highly pathogenic North American type PRRSV (Li et al., 2009), at a MOI of 0.5. The infected cells were harvested at 12, 24 and 36 h post-infection (hpi) and Western blots were performed using an anti-LC3 monoclonal antibody (Cell Signaling) which recognizes both LC3-I and LC3-II. Cells pretreated with rapamycin for 12 h served as a positive control. Although little LC3-II could be detected in mock-infected cells, a significant amount of LC3-II was detected in cells infected with PRRSV strain WUH3. Furthermore, the expression of LC3-II increased with the progression of PRRSV infection (Fig. 1A), which was demonstrated by detecting the expression of PRRSV nonstructural protein 2 (Nsp2). Densitometric analysis of three independent experiments using gene tools (Syngene product version 3.06) also showed that the densitometry ratio of LC3-II to β-actin significantly increased at 24 and 36 h after PRRSV infection, compared to the mock-infected cells (Fig. 1B). These results indicated that the increased LC3-II expression is dependent on PRRSV infection.

Punctate accumulation of LC3, which indicates the recruitment of LC3-II to autophagic vacuoles, is also considered to be a hallmark characteristic of autophagy activation (Mizushima, 2004). To investigate LC3-II incorporation into autophagosomes, we constructed a GFP-tagged LC3 plasmid (pEGFP-LC3) by PCR amplifying the coding sequence of LC3 from HEK293 cells with specific primers LC3-F/LC3-R (Table 1). The amplified fragment was then inserted into a pEGFP-C1 vector (ClonTech), resulting in the fusion expression plasmid pEGFP-LC3. Marc-145 cells were transfected with the pEGFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen), followed by PRRSV infection 24 h later. At 24, 36 h after infection, cells were fixed with 4% formaldehyde. PRRSV-infected cells were detected with a mouse monoclonal antibody against PRRSV Nsp2 and Cy3-conjugated goat anti-mouse IgG (Sigma). Cells expressing LC3 were detected by the fluorescence of EGFP-LC3 fusion protein. Cell nuclei were counterstained with 0.01% 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Fluorescent images were examined under confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss). Cells pretreated with rapamycin for 4 h served as a positive control and cells transfected with pEGFP-LC3 without PRRSV infection were used as a negative control. As shown in Fig. 1C, many punctate EGFP-LC3 distribution were observed in rapamycin-pretreated cells (Fig. 1C), although little punctate EGPF-LC3 could be detected in mock-infected cells. Cells with both Nsp2-positive (PRRSV-infected cells) and EGFP-positive (pEGFP-LC3-transfected cells) showed punctate accumulation of LC3. However, no or rare punctate accumulation could be observed in cells with EGFP-positive/Nsp2-negative in the same image, indicating that punctate accumulation of EGFP-LC3 depends on PRRSV infection. The number of cells with punctate EGFP-LC3 location relative to all green fluorescent protein-positive cells was counted (100 EGFP-positive cells were counted in each sample) (Fig. 1D). Nearly 70% of EGFP-positive cells contained punctate EGPF-LC3 after PRRSV infection, indicating the formation of autophagosomes during PRRSV infection.

Electron microscopy is the technology of choice for identifying the morphology of autophagic compartments (Mizushima, 2004). To further confirm that PRRSV infection can induce cellular autophagy, PRRSV- or mock-infected cells were fixed in 2.5% glutaraldehyde for 20 min. Cells pretreated with rapamycin for 4 h served as a positive control. After three washes in PBS, cells were postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate, followed by dehydration in a graded series of acetone washes before being embedded in Agar 100 epoxy resin. Ultra-thin sections (60–70 nm) were cut and viewed on a Hitachi H-7650 transmission electron microscope (Hitachi Ltd., Tokyo, Japan). Autophagic vacuoles were rarely observed in the mock-infected cells (Fig. 2A), but abundant autophagosome-like DMVs (ca. 20 per cell) with a diameter of 300–1000 nm could be seen in PRRSV-infected cells (Fig. 2B–D) or rapamycin-treated cells (Fig. 2E–G). It is well known that nidoviruses infection also induce replication-associated autophagosome-like double-membrane vesicles with a diameter of 300 nm (Fang and Snijder, 2010; Knoops et al., 2008). A functional link between autophagic DMVs and coronavirus-induced replication-associated DMVs remains controversial (Cottam et al., 2011; de Haan and Reggiori, 2008; Knoops et al., 2008; Prentice et al., 2004; Reggiori et al., 2010; Zhao et al., 2007). It is possible that coronavirus-induced replication-associated DMVs are modified autophagosomes, or independent structures induced by assembly of viral proteins (Cottam et al., 2011). In our study, various DMVs with a diameter of 300–1000 nm could be observed in the same image, indicating that there co-existed the two different DMVs in PRRSV-infected cells.

We next asked whether autophagosome induction is a host antiviral response to PRRSV replication. To assess this, Marc-145 cells were pretreated with rapamycin (100 μM), a pharmacological reagent that induces cellular autophagy by inhibiting the mTOR pathway, for 3 h prior to PRRSV infection (MOI 0.5). The infected cells were cultured in the presence of rapamycin (100 μM) and collected at different time points for plaque assay to determine virus titers. To our surprise, induction of autophagy by rapamycin did not suppress PRRSV yield, but resulted in an almost threefold increase in viral titer at 24 and 36 h after infection (Fig. 3A). Thus, we speculated that PRRSV utilizes autophagy to promote its replication.

Table 1
Primers used in this study.

Primer	Sequence 5′ → 3′
LC3-F	TTTGAATTCCATGCCGTCGGAGAAGACCTTCAAG
LC3-R	TTTGGATCCTTACACTGACAATTTCATCCCGAACG
ATG7-F	TGTGAGTCGTCCAGGATTGG
ATG7-R	GCAAAACAGATACCATCAATTCCA
Beclin-1-F	TGTCACCATCCAGGAACTCA
Beclin-1-R	CTGTTGGCACTTTCTGTGGA
GAPDH-F	TCATGACCACAGTCCATGCC
GAPDH-R	GGATGACCTTGCCCACAGCC

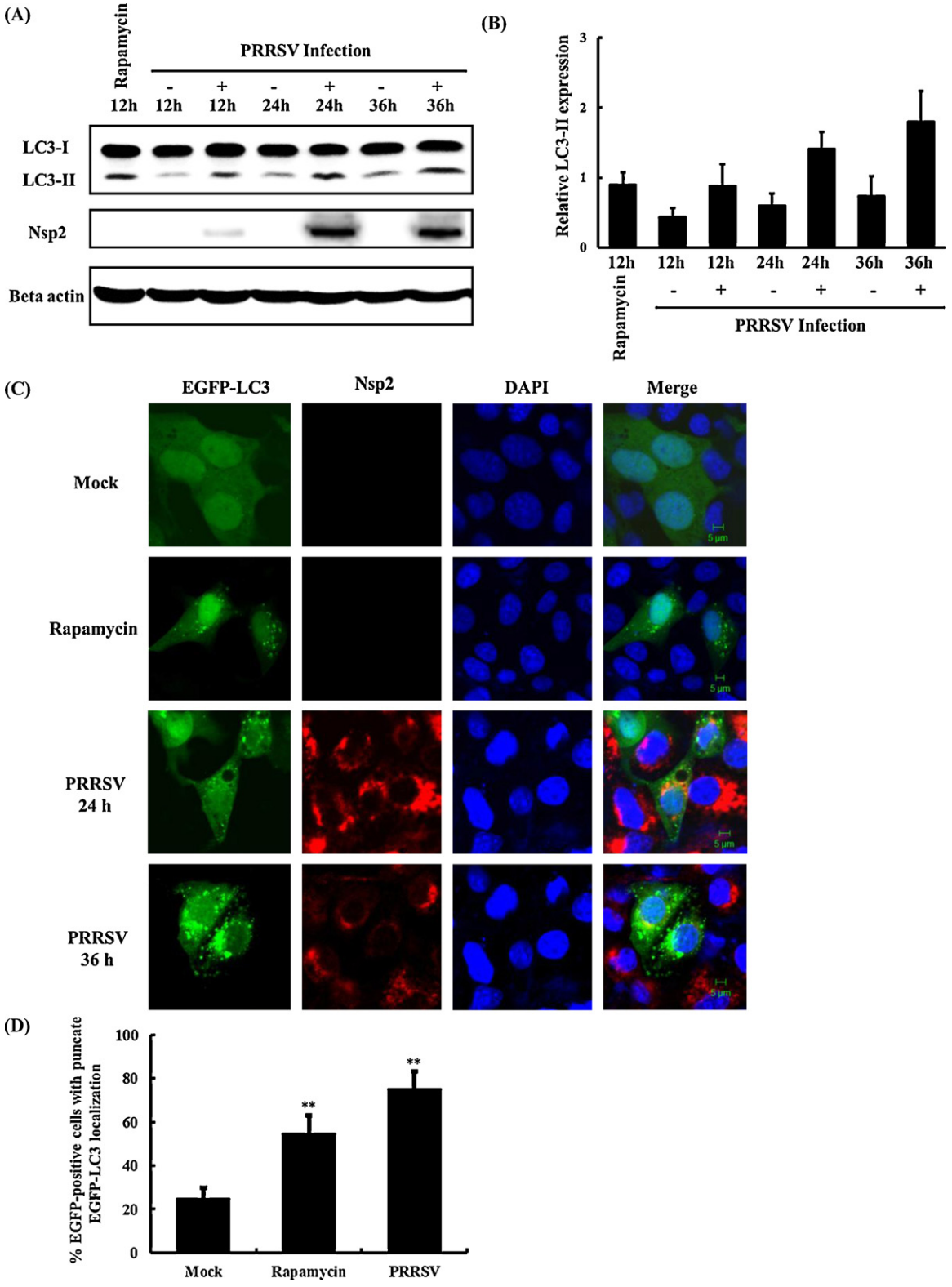

Fig. 1. Modification of LC3 protein during PRRSV infection. (A) Western blot analysis of the expression of LC3-II and LC3-I with the progression of PRRSV infection. Marc-145 cells were infected with PRRSV at a MOI of 0.5. The infected cells were collected at the indicated time points postinfection for Western blot analysis using a polyclonal antibody against LC3 or a monoclonal antibody against PRRSV Nsp2. β-Actin expression was used as a protein loading control. (B) Relative levels of LC3-II were estimated by densitometric scanning after normalization against β-actin and are shown as bar diagrams. Data represent means of three replicates. (C) The redistribution of EGFP-LC3 in PRRSV-infected cells. Marc-145 cells were transfected with plasmid pEGFP-LC3 expressing EGFP-LC3 fusion protein. Twenty-four hours later, the transfected cells were infected or mock-infected with PRRSV (MOI 0.5). At 24 or 36 h post-infection, cells were fixed and processed for indirect immunofluorescence assay using monoclonal antibodies against PRRSV Nsp2, followed by Cy3-conjugated goat anti-mouse IgG. Treatment with rapamycin was used as a positive control. Cell nuclei were counterstained with DAPI. Fluorescent images were examined under confocal laser scanning microscope. (D) The relative number of cells with punctate EGFP-LC3 location relative to all green fluorescent protein-positive cells was calculated. Data are expressed as mean ± SD results of three experiments. $^{**}P < 0.01$ as compared with the mock-infected group.

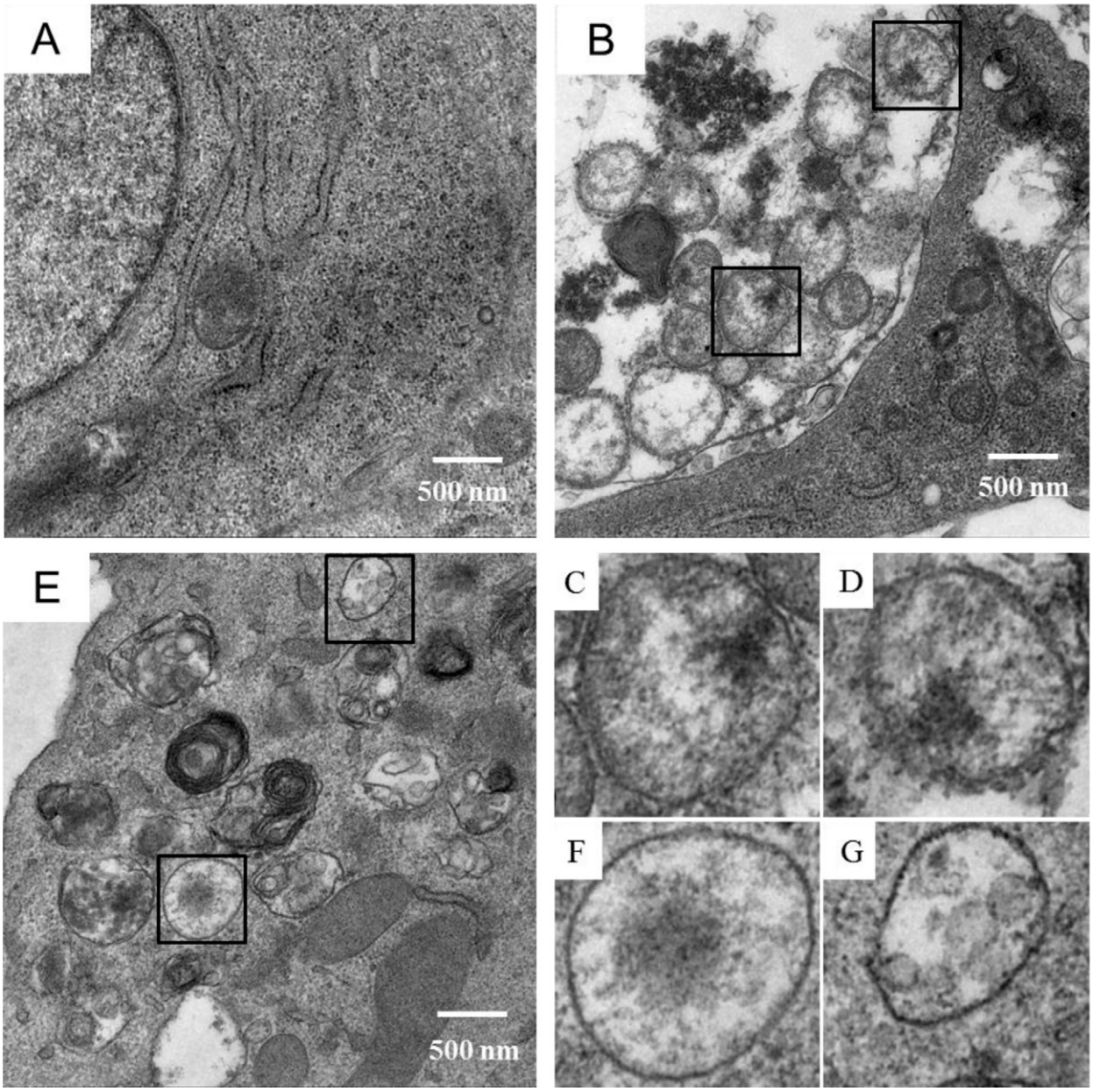

Fig. 2. PRRSV infection increases autophagosome-like vesicle formation. Marc-145 cells were mock-infected (A) or infected (B) with PRRSV for 36 h, followed by electron microscopy observation. Cells treatment with rapamycin (E) for 4 h was used as positive control. The higher-magnification images of autophagosome-like vesicle in PRRSV-infected (C, D) and rapamycin-treated (F, G) cells are shown.

To confirm this hypothesis, Marc-145 cells were pretreated with 3-methyladenine (3-MA), a pharmacological reagent which blocks autophagy by inhibiting class III phosphatidylinositide (PI) 3-kinase (PI3K) without affecting protein synthesis (Wu et al., 2010). We used this reagent to inhibit autophagy prior to PRRSV infection. Cells were cultured in the presence of 3-MA (5 mM) and collected at 12, 24, 36 h after infection. Virus titers were then determined by plaque assay. Inhibition of autophagy by 3-MA treatment resulted in a significant reduction of virus titer at all examined time points (Fig. 3B), supporting our hypothesis that PRRSV utilizes autophagy to promote its replication.

To extend these studies, we performed RNA interference (RNAi) experiments to knockdown the expression of two critical genes of the autophagy pathway: ATG7 and Beclin-1. ATG7 is a key autophagy gene, encoding a protein E1-like enzyme that is part of the ubiquitin-like pathways essential to the formation of autophagic vacuoles (Kim et al., 1999). It has been shown that deletion of ATG7 results in lower autophagosome formation and impaired starvation-induced bulk degradation of proteins and organelles in mice (Komatsu et al., 2005). Beclin-1, the orthologous Atg6 of yeast, is a key component of the Class III PI3K complex, which plays an important role in the initiation of the autophagosome formation (Backer, 2008; Cao and Klionsky, 2007). Gene knockdown studies indicate that in plants, slime molds, nematodes, fruit flies, mice, and humans Beclin-1 is a conserved essential component of autophagy (Levine and Klionsky, 2004). siRNA molecules targeting ATG7, Beclin 1 and a scrambled control were obtained from Santa Cruz. To confirm the silencing efficiency of the siRNAs, Marc-145 cells were transfected and total cellular RNA was extracted using TRIzol reagent (Invitrogen) 24 h later. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using primers for ATG7 (ATG7-F/ATG7-R),

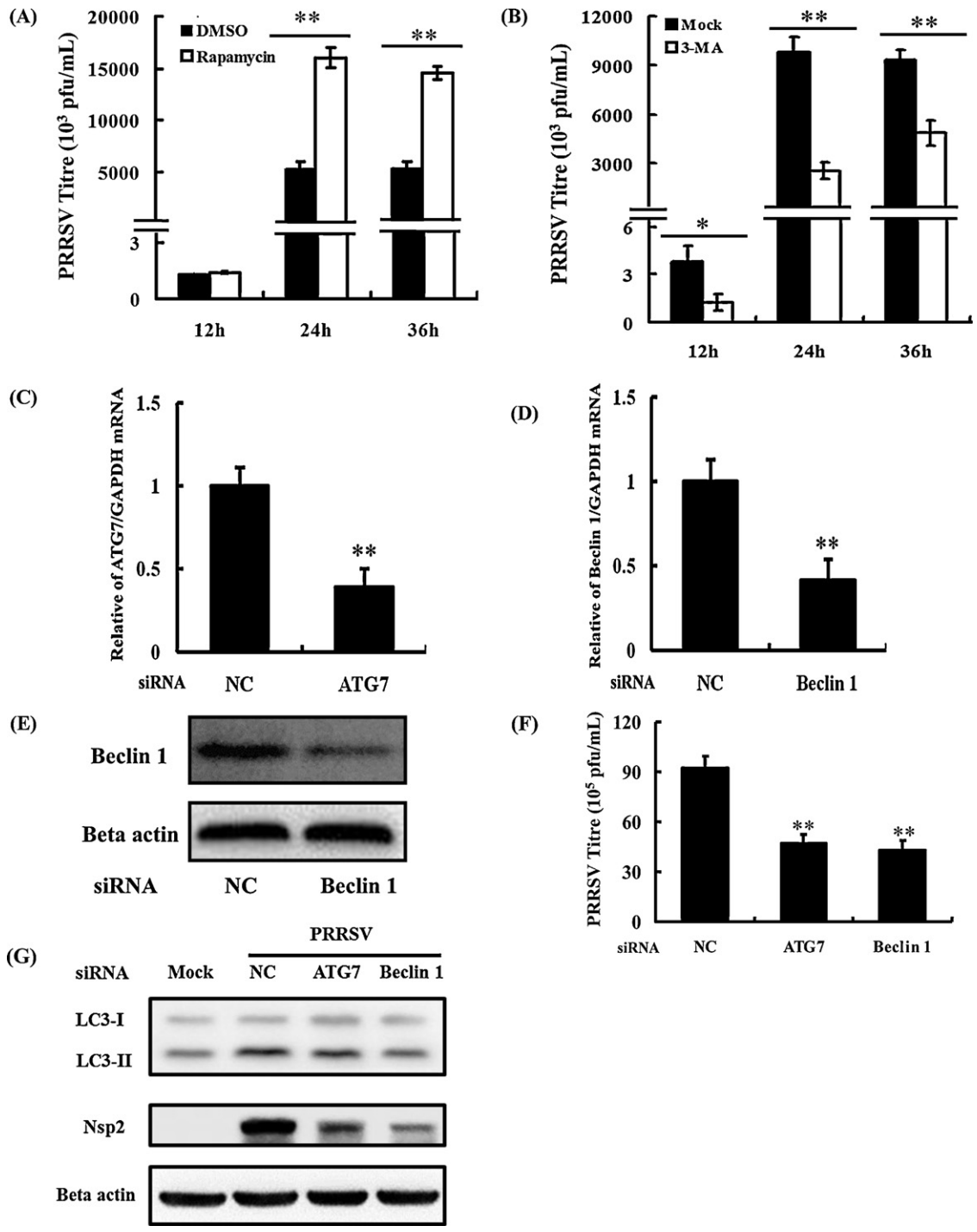

Fig. 3. Induction of cellular autophagy enhances PRRSV replication. (A, B) Marc-145 cells were pretreated with rapamycin (A) or 3-MA (B) for 3 h prior to PRRSV infection. The infected cells were collected at the indicated time points for plaques assay to determine viral titers. (C, D) Silencing efficiency of siRNA targeting ATG7 (C) or Beclin-1 (D). Marc-145 cells were transfected with the indicated siRNA. Twenty-four hours later, qRT-PCR was performed to analyze the mRNA expression of ATG7 or Beclin-1. The relative mRNA expression was obtained compared to β-actin. (E) Western blot analyzes the silencing efficiency of Beclin-1 siRNA. (F) Knockdown of ATG7 or Beclin-1 promotes PRRSV replication. Marc-145 cells were transfected with siRNAs targeting ATG7 or Beclin-1 for 24 h, followed by PRRSV infection. At 36 h post-infection, cells were collected to determine viral titers by plaque assay. (G) Knockdown of ATG7 or Beclin-1 reduces the expression of LC3-II and PRRSV Nsp2. Marc-145 cells were similarly treated as in (F). At 36 h post-infection, cells were harvested to detect the expression of Nsp2 and LC3 by Western blot. All data are expressed as mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$.

Beclin-1 (Beclin-1-F/Beclin-1R) and GAPDH (GAPDH-F/GAPDH-R) (Table 1). Compared with the control siRNA, the siRNAs targeting ATG7 (Fig. 3C) and Beclin-1 (Fig. 3D) significantly suppressed mRNA expression of the corresponding target. The silencing efficiency of the siRNA targeting Beclin-1 was also confirmed in the protein level by Western blot analysis with Beclin-1 specific polyclonal antibody (Sigma) (Fig. 3E). To further investigate PRRSV replication after knockdown of ATG7 or Beclin-1, Marc-145 cells were transfected with 100 nM siRNA targeting ATG7, Beclin-1, or control. At 24 h post-transfection, cells were infected with PRRSV (MOI 0.5) and collected 36 h later to determine virus titers by plaque assay. Knockdown of ATG7 or Beclin-1 significantly decreased PRRSV yield (Fig. 3E). To confirm the reduction of virus titer was suffered by the inhibition of cellular autophagy, we detected the expression

of LC3-II by Western blot. As shown in Fig. 3G, cells transfected with siRNA targeting ATG7 or Beclin-1 significantly decreased LC3-II expressions. Furthermore, the expression of PRRSV Nsp2 was also reduced. There were some disputes about the link of cellular autophagy and the replication of coronavirus, including MHV, IBV, and SARS-CoV (Cottam et al., 2011; de Haan and Reggiori, 2008; Prentice et al., 2004; Reggiori et al., 2010; Zhao et al., 2007). Furthermore, we also found that treatment with rapamycin or 3-MA did not affect the replication of transmissible gastroenteritis virus (TGEV), a porcine coronavirus (unpublished data). However, our present experimental observations using pharmacological agent 3-MA or rapamycin, together with the results from silencing ATG7 and Beclin-1, clearly demonstrated that induction of cellular autophagy increased PRRSV replication.

In summary, this report constitutes the first demonstration that PRRSV infection triggers the formation of autophagosomes, and that this might be a strategy employed by the virus to enhance its replication. Future studies should focus on the mechanisms by which PRRSV induces autophagy and utilizes the pathway to promote its replication. In addition, Shrivastava and colleagues recently showed that hepatitis C virus (HCV) induces autophagy in infected cells to enhance its replication. Significantly, they found that inhibition of IFN expression during HCV infection is triggered by cellular autophagy (Shrivastava et al., 2011), indicating that there is relationship between autophagy and IFN production. PRRS is an also immunosuppressive disease, and it has been shown that PRRSV can enhance its replication by inhibiting IFN-β (Calzada-Nova et al., 2011; Miller et al., 2004; Murtaugh et al., 2002; Yoo et al., 2010). Whether this mechanism is triggered by cellular autophagy should also be investigated.

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