Autophagy sustains the replication of porcine reproductive and respiratory virus in host cells

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ABSTRACT

In this study, we confirmed the autophagy induced by porcine reproductive and respiratory syndrome virus (PRRSV) in permissive cells and investigated the role of autophagy in the replication of PRRSV. We first demonstrated that PRRSV infection significantly results in the increased double-membrane vesicles, the accumulation of LC3 fluorescence puncta, and the raised ratio of LC3-II/β-actin, in MARC-145 cells. Then we discovered that induction of autophagy by rapamycin significantly enhances the viral titers of PRRSV, while inhibition of autophagy by 3-MA and silencing of LC3 gene by siRNA reduces the yield of PRRSV. The results showed functional autolysosomes can be formed after PRRSV infection and the autophagosome–lysosome-fusion inhibitor decreases the virus titers. We also examined the induction of autophagy by PRRSV infection in pulmonary alveolar macrophages. These findings indicate that autophagy induced by PRRSV infection plays a role in sustaining the replication of PRRSV in host cells.

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of porcine reproductive and respiratory syndrome (PRRS) which is one of the most economically important infectious diseases for the pig industry worldwide (Garner et al., 2001; Neumann et al., 2005; Pejsak et al., 1997; Rossow, 1998). The virus has a positive-stranded genomic RNA packaged in an enveloped icosahedral viral capsid, and is classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) (Cavanagh, 1997). Members of genus *Arterivirus* replicate in the replication complexes which are double-membrane vesicles (DMVs) probably derived from paired endoplasmic reticulum membranes (Pedersen et al., 1999). Previous studies have suggested that the nonstructural proteins (Nsps) of arteriviruses play a crucial role in the formation of replication complexes (Pedersen et al., 1999; Snijder et al., 2001; van der Meer et al., 1998). However, it is unknown how the replication complexes for PRRSV generate and how the Nsps of PRRSV interact with host cells.

In eukaryocytes, autophagy is a widely existed conservative mechanism, which can transport long-lived cytoplasmic proteins and damaged organelles to lysosomes to degrade for maintaining the cellular homeostasis (Klionsky, 2007; Klionsky and Emr, 2000). During the autophagy process, long-lived proteins and damaged organelles are wrapped in autophagosomes, a kind of double-membrane vesicles which are considered as one of the hallmarks of autophagy; the matured autophagosomes fuse with lysosomes to form autolysosomes in which the content is degraded (Klionsky and Emr, 2000). Two ubiquitin-like molecules, the microtubule-associated protein 1 light chain 3 (LC3) and Atg12p, are necessary for the formation and maturation of autophagosome. LC3 is the most widely used molecular marker for monitoring autophagy (Kirkegaard et al., 2004; Klionsky et al., 2008; Ohsumi, 2001). The lysosome-associated membrane protein 1 (LAMP1), a marker of autolysosomes, is required for the fusion of autophagosomes and endosomal vesicles (Kirkegaard et al., 2004). The autophagy pathway is regulated under various GTPases, kinases and phosphatases. The mammalian target of rapamycin (mTOR) and the phosphatidylinositol 3-kinase (PI3K) regulate two mechanisms respectively, which are considered the best molecules to manipulate the early

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stage of autophagosome formation (Petiot et al., 2000; Ravikumar et al., 2004). Additionally, bafilomycin A1, a specific inhibitor of the vacuolar type H$^+$-ATPase (V-ATPase), is an efficient inhibitor for the fusion of autophagosomes and lysosomes (Yamamoto et al., 1998).

Autophagy is not only necessary for cellular homeostasis, but also plays an important role in other physiological and pathological processes (Levine and Kroemer, 2008; Mizushima, 2007), as well as in both adaptive immunity and innate immunity (Levine and Deretic, 2007; Menendez-Benito and Neefjes, 2007). In recent years, a number of studies have revealed the direct interaction between autophagy and viral infection. Although autophagy is considered as a central component of host defense machinery against bacterial, viral, and protozoa infections (Levine and Deretic, 2007; Schmid and Münz, 2007; Talloczy et al., 2006), some viruses have evolved mechanisms to escape autophagy of host cells (Chou and Roizman, 1994; Harrow et al., 2004; Ohsumi, 2001; Suhy et al., 2000), or may utilize autophagy for their own benefit of replication (Wong et al., 2008). Certain positive-stranded RNA viruses and cytoplasmic DNA viruses replicate in cytoplasmic membranes which display some hallmarks of autophagosomes such as double membrane and positive for the autophagy protein LC3 (Suhy et al., 2000). Autophagy induced ;by coxsackievirus B3 (CVB3) and encephalomycarditis virus (EMCV) infection can promote the viral replication (Wong et al., 2008; Zhang et al., 2011). In coronaviruses, it has been demonstrated that mouse hepatitis virus (MHV) can utilize part of autophagy to replicate or the autophagy mechanism to enhance their replication (de Haan and Reggiori, 2008; Prentice et al., 2004). However, other studies have suggested that the autophagy pathway or the component of autophagy was not essential for the replication of coronaviruses (Zhao et al., 2007; Cottam et al., 2011). More investigations need to be conducted to provide more evidence whether nidoviruses actually hijack the autophagy pathway for their own replication. In the present study, we demonstrated that autophagy can be induced in the permissive MARC-145 cells and the natural host cell—pulmonary alveolar macrophages (PAMs) upon PRRSV infection, and meanwhile investigated the role of autophagy in the replication of PRRSV.

Results

Autophagy is triggered in MARC-145 cells by PRRSV infection

To determine whether autophagy is induced in MARC-145 cells by PRRSV infection, the monolayer MARC-145 cells were inoculated with PRRSV and then subjected to TEM, confocal microscopy or western blotting assays at indicated timepoints. The cells were subjected to TEM and confocal microscopy at 72 h postinfection (pi). And the cells were harvested from 24 h pi to 96 h pi with the time interval of 24 h for western blotting analysis to detect LC3 so as to monitor the progress of autophagy. Meanwhile, the virus titers in supernatants of cell culture at all time-points were assayed to track the replication of PRRSV.

Complete mitochondria and endoplasmic reticulum structures were observed in mock-infected MARC-145 cells (Fig. 1A), while the PRRSV-infected MARC-145 cells exhibited massive double-membrane vesicles and single-membrane vesicles around the perinuclear region (termed as autophagosome-like vesicles) (Fig. 1B). By confocal microscopy analyses, it was indicated that the PRRSV-infected cell presented significant enhancement of LC3 signals with the fluorescence punctuates accumulation (Fig. 1G), while the mock-infected cell had weak LC3 signals without the visible fluorescence punctuates accumulation (Fig. 1D). In addition, no LC3 punctuates accumulation was observed in the uninfected cells. Moreover, as the LC3 signals of infected cells, the PRRSV N protein signals in the infected cells also presented fluorescence punctuates accumulation (Fig. 1F), and the fluorescence punctuates of N protein were highly colocalized with the fluorescence punctuates of LC3 (Fig. 1H). Nsp2 is necessary and sufficient to induce the formation of double-membrane structures during the formation of the arterivirus replication complex (Snijder et al., 2001). Thus Nsp2 can be used as a marker of replication units on some degree. By the confocal microscopy with Nsp2 antibodies and LC3 antibodies, perinuclear fluorescence punctuates colocalization was also found (Fig. 1L–N). These observations obviously indicate that a significant increase in the number of autophagosome-like vesicles exists in the cytoplasm of PRRSV-infected cells.

Before autophagy induction, the LC3 diffusely exits in the cytoplasm as the cytosolic form—LC3-I which is about 18 kDa. At the early stages of autophagosome formation, LC3-I is converted to LC3-II which is the phosphatidylethanolamine (PE) conjugated form with a molecular mass of approximately 16 kDa; then LC3-II specifically integrates into the autophagosome membranes (Kabeya et al., 2000). Therefore, the level of LC3-II in the total protein of cell is a reliable index to quantify the process of autophagy and the ratio of LC3-II to β-actin is regarded as an accurate index of autophagy (Klionsky et al., 2008). Compared with the protein bands of mock-infected cells (Fig. 1O), the MARC-145 infected cells by JXwn06 and HB-1/3.9 both presented significant band pattern transformation, showing the bands of LC3-I gradually decreased to vanish while the bands of LC3-II gradually increased (Fig. 1P and Q). As shown in Fig. 1R, from 24 h pi on, the difference of ratio of LC3-II/β-actin between the JXwn06 infected cells and the mock cells was significant ($p < 0.001$) and form 72 h pi on, the difference between HB-1/3.9 infected cells and the mock cells was also significant ($p < 0.001$) (Fig. 1O). Meanwhile, the titer of PRRSV reached the peak at 72 h pi as well (Fig. 1S). These results suggest there is a positive correlation between the replication of PRRSV and autophagy induction upon PRRSV infection. Taken together, our results indicate that autophagy is induced in MARC-145 cells upon PRRSV infection.

The replication of PRRSV in MARC-145 cells is enhanced by the induction of autophagy

To determine whether autophagy is an antiviral mechanism of host cells or utilized by PRRSV replication, we further investigated the effect of autophagy induction on the replication of PRRSV in MARC-145 cells. MARC-145 cells, prior to virus inoculation, were treated with rapamycin which can specifically induce autophagy pathway by directly inhibiting the action of mTOR (Blommaart et al., 1995; Kamada et al., 2000; Klionsky et al., 2005; Noda and Ohsumi, 1998). Cells were harvested at indicated time points and subjected to western blotting analysis. The intracellular viruses and viruses in supernatant of cell cultures at the corresponding time points were titrated, respectively.

Since no time shifting of virus replication after usage of rapamycin was detected, three time points with the same interval of virus replication were selected, which were at the early stage (24 h pi), at the peak of replication (72 h pi) and at the late stage (120 h pi) respectively. Compared with the mock-infected cells (Fig. 2A), the LC3 punctuates increased in the cells treated with rapamycin (Fig. 2B). As shown in Fig. 2C and D, the ratio of LC3-II/β-actin, which represents autophagy level of the infected cells treated with rapamycin, was higher than that of the infected cells treated by DMSO, and from 72 h pi on, a significant difference between the two groups was observed ($p < 0.05$). The intracellular titer of PRRSV in the infected cells treated with rapamycin was higher than that in the infected cells treated with DMSO (Fig. 2E), which showed a

significant difference ($p < 0.01$) from 72 h pi on. The virus titers in the supernatant had a similar tendency (Fig. 2F). In order to exclude the impact of rapamycin on cell viability, cell viability was tested after treatment with rapamycin, and the results indicated that there was no significant difference on the cell viability between treated cells and mock cells (Fig. 2G). These results suggest that autophagy enhance the replication of PRRSV.

The replication of PRRSV is reduced by the inhibition of autophagy

We further analyzed the effect of autophagy inhibition on the replication of PRRSV. The autophagy inhibitor—3-MA that suppresses the formation of autophagosomes without affecting protein synthesis is widely used for autophagy inhibition (Seglen and Gordon, 1982). LC3 is necessary for the formation and maturation
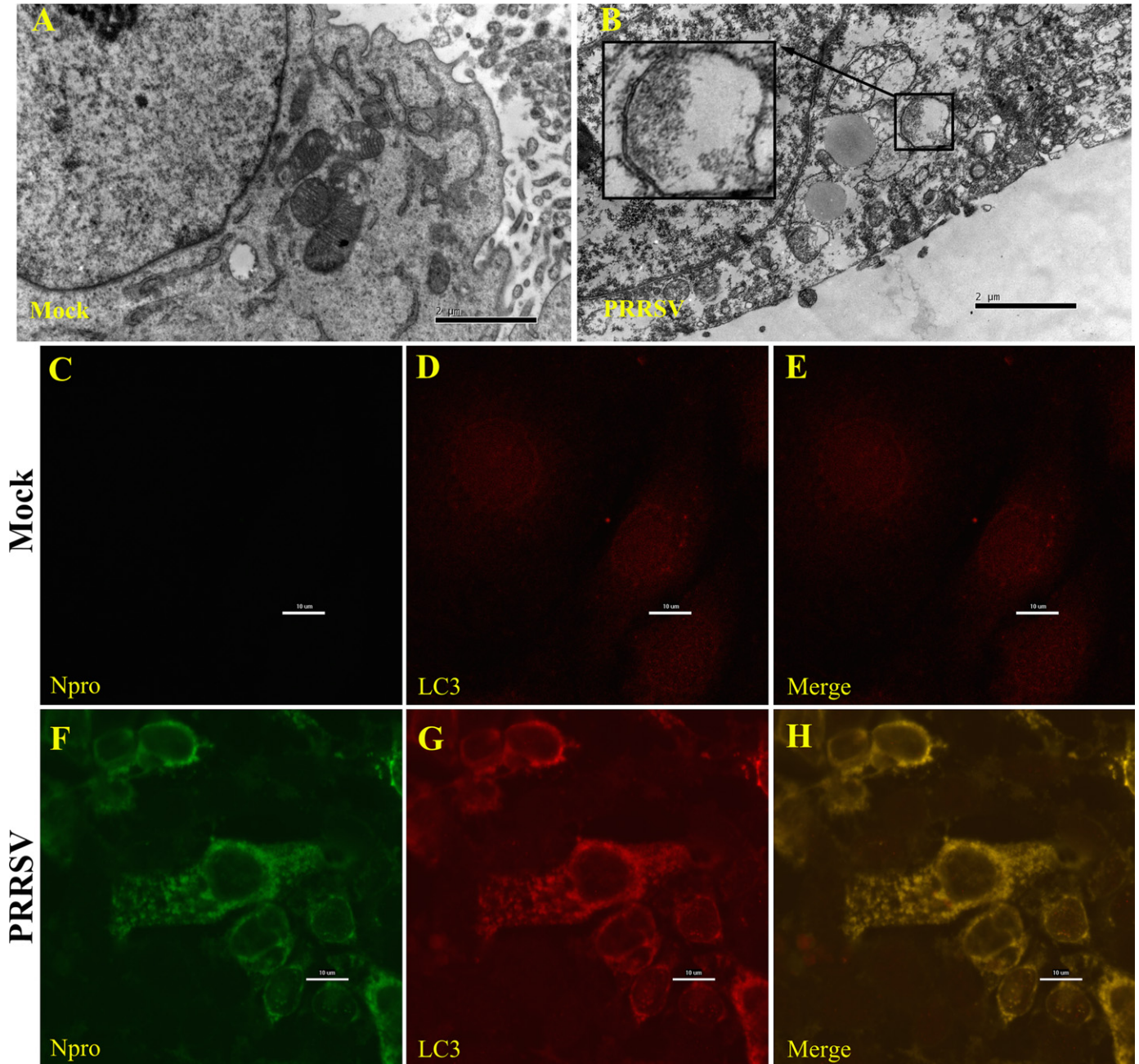
Fig. 1. PRRSV infection induces autophagy in MARC-145 cells: (A) and (B) TEM images of mock and infected MARC-145 cells. Cells were harvested at 72 h pi and processed and imaged as described in Materials and methods. Magnified areas in panel B are indicated by rectangles. (C–K) Confocal microscopy images of mock and infected MARC-145 cells. Cells were fixed at 72 h pi and stained with antibodies against PRRSV N protein (F) and LC3 (G) and then merge (H). Panels C–E are mock cells stained with antibodies against PRRSV N protein (C) and LC3 (D) and merge (E). Scale bars in panels A and B represent 2 μm, and in panel C–H represent 10 μm. Panels I–K are mock cells stained with antibodies against PRRSV Nsp2 protein (I) and LC3 (J) and merge (K). (L–N) Cells were fixed at 72 h pi and stained with antibodies against PRRSV Nsp2 (L) and LC3 (M) and then merge (N). (O–S) Results of western blotting assays and the virus one step growth curve. Mock cells (O), JXwn06 infected cells (P) and HB-1/3.9 infected cells (Q) were harvested at indicated time points and subjected to western blotting. Optical densities of each protein band were measured, and the optical density ratio of LC3-II/β-actin was calculated (R). Panel S is the one step growth curve of corresponding time points. Magnified areas in panel N are indicated by rectangles. Data are means ± standard deviations (error bars) from three independent trials. Asterisks mean a significant difference in LC3-II/β-actin value between infected cells and mock cells. The data were obtained in three independent experiments, and error bars mean ± SD of the three experiments (two-way ANOVA test; ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Scale bars in panels I–N represent 10 μm.
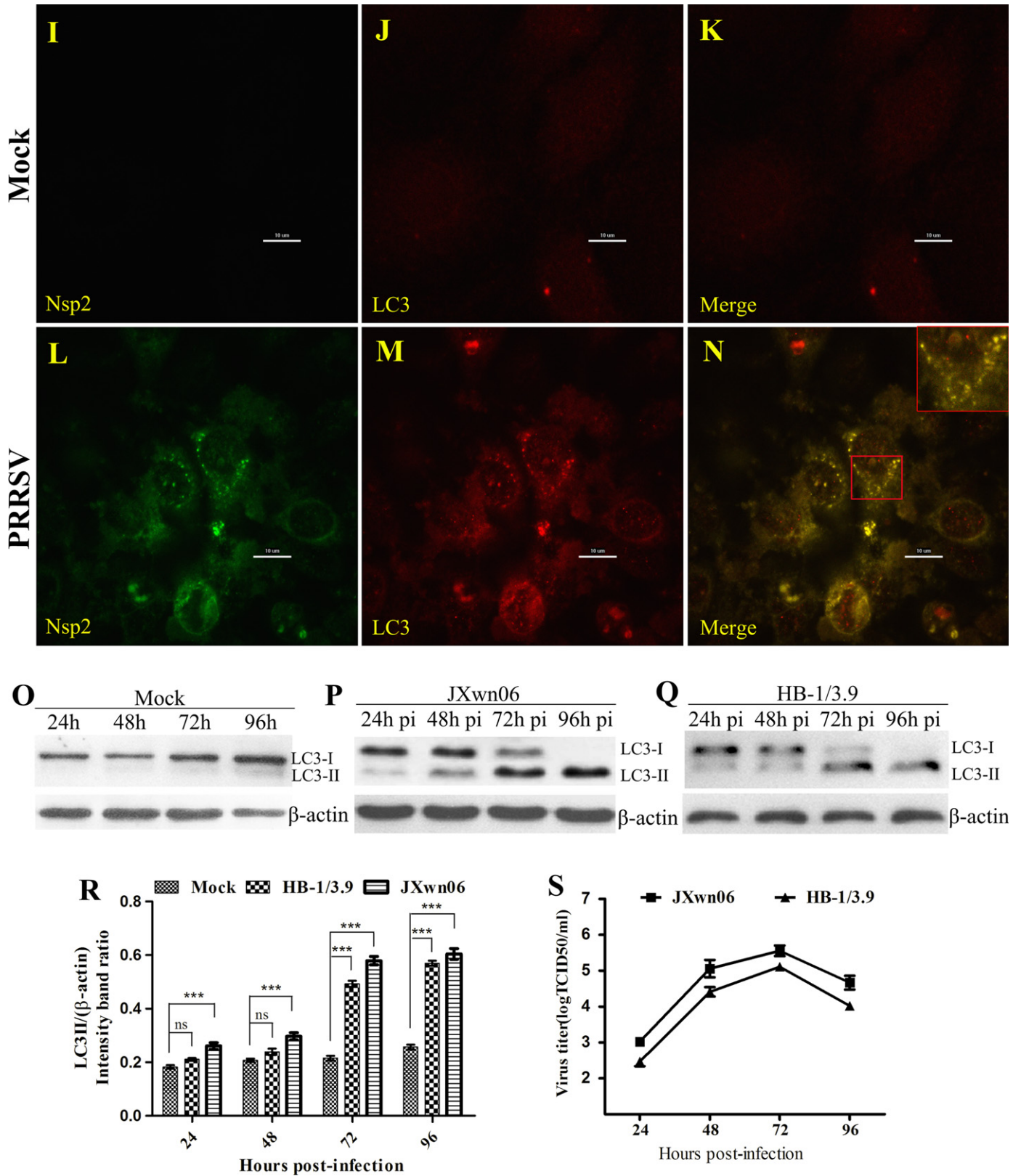
Fig. 1. (continued)

of autophagosome, therefore silencing LC3 will specifically block the autophagy pathway (Klionsky et al., 2008; Ohsumi, 2001).

MARC-145 cells were treated with 3-MA of different concentrations prior to infection and then inoculated with PRRSV. Since no time shifting of peak of virus replication after usage of 3-MA

was detected, three time points with the same interval of virus replication were selected, which were at the early stage (24 h pi), at the peak of replication (72 h pi) and at the late stage (120 h pi) respectively. As shown in Fig. 3, both the titers of intracellular viruses (Fig. 3A) and supernatant (Fig. 3B) viruses in 3-MA-treated
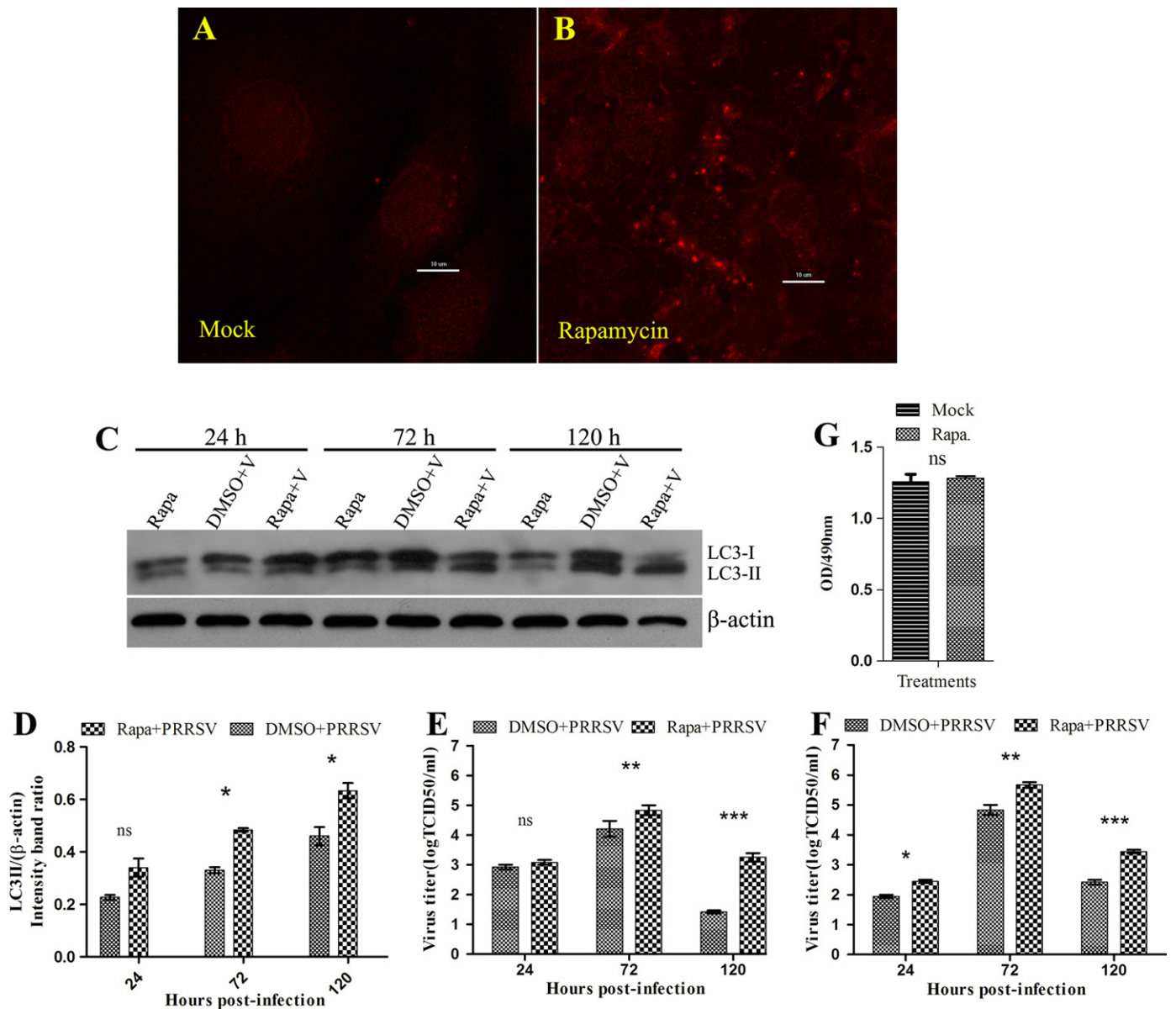
Fig. 2. Autophagy induction enhanced the replication of PRRSV. (A) and (B) Autophagy was induced by 1 μM of rapamycin. Image of immunofluorescence of mock MARC-145 cells stained by the antibodies against LC3 (A). MARC-145 cells were treated with 1 μM of rapamycin for 12 h, and then subjected to immunofluorescence staining with the antibodies to LC3 (B). Cells treated with 1 μM of rapamycin (Rapa), cells treated with DMSO for 3 h and infected with PRRSV of 0.05 MOI (DMSO+V) and cells treated with 1 μM of rapamycin for 3 h and infected with PRRSV of 0.05 MOI (Rapa+V) were harvested at the time points of 24 h pi, 72 h pi and 120 h pi and then subjected to Western blotting analysis (C). The Western blotting result was digitalized and optical density ratio of LC3-II/β-actin was calculated (D). The intracellular (E) and supernatant (F) virus titer of each time point was measured. (G) MARC-145 cells, seeded in 96-well plate for 48 h and treated with rapamycin or DMSO for 24 h, were added with 20 μl of CellTiter 96® AQueous One Solution each well for 4 h at 37 °C. The absorbance at 490 nm was recorded and compared of rapamycin treated cells and mock treated cells. The data were obtained in three independent experiments, and error bars mean ± SD of the three experiments (two-way ANOVA test; ns, $P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Scale bars in panels A and B represent 10 μm.

MARC-145 cells were reduced compared with those in the mock treated cells. Moreover, the inhibition effect of 3-MA was dose-dependent. When the cells were treated with 10 mM of 3-MA, the replication of PRRSV was almost completely suppressed. In order to exclude the impact of 3-MA on cell viability, cell viability was tested after treatment with different doses of 3-MA, and the results indicated that there was no significant difference on the cell viability between treated cells and mock treated cells (Fig. 3C). Meanwhile the LC3 gene was knocked down using siRNAs specific for LC3β silencing. Compared with scrambled siRNA-transfected cells, the LC3 level of the cells treated with LC3-specific siRNAs was notably depleted (Fig. 3D). At 24 h

post-transfection, MARC-145 cells were inoculated with PRRSV. After inoculation for 72 h, the total titers of PRRSV were measured. The total PRRSV titer of scrambled siRNA transfected group was significantly higher than the two siLC3 groups, moreover, the more efficient a siLC3 worked the more significantly lowered the PRRSV titer of this group than scrambled siRNA group (Fig. 3D and E). In order to exclude the impact of LC3 silencing on cell viability, cell viability was tested after treated with siLC3s, and the results indicated that there was no significant difference on the cell viability between siLC3s-treated cells and siSCR-treated cells (Fig. 3F). As a whole, our results further suggest that autophagy plays an important role in the replication of PRRSV.
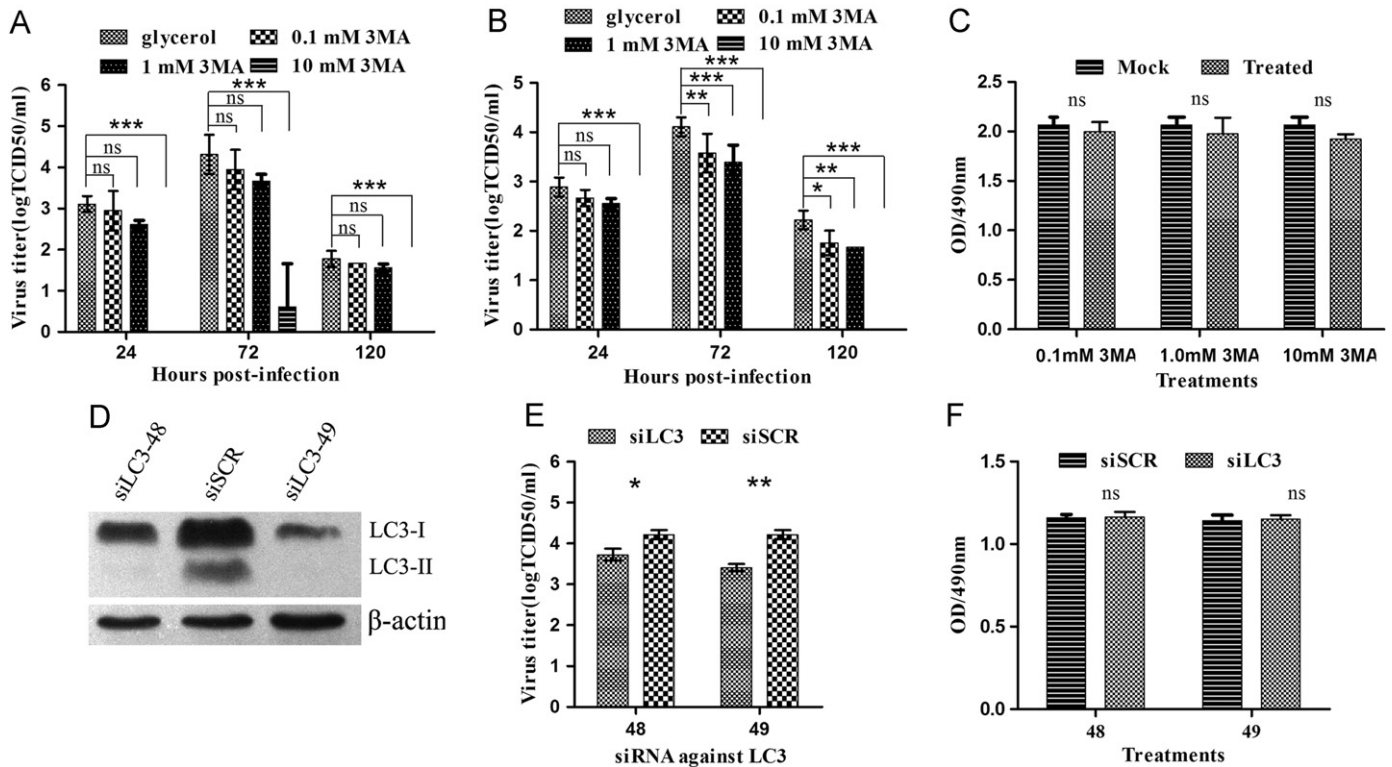
Fig. 3. Autophagy inhibition reduced the replication of PRRSV. MARC-145 cells were treated with different concentrations of 3-MA for 24 h and inoculated with PRRSV. Intracellular virus titers (A) and virus titers of supernatants (B) were measured. MARC-145 cells, seeded in 96-well plate for 48 h and treated with different doses of 3-MA for 24 h, were added with 20 μl of CellTiter 96® AQueous One Solution for each well for 4 h at 37 °C. The absorbance at 490 nm was recorded. The absorbance at 490 nm was recorded and which of treated groups and mock group was compared (C). MARC-145 cells cultivated for 48 h were transfected with 20 pmol of siRNAs specific for LC3 gene (siLC3-48 and siLC3-49) and scrambled siRNA (siSCR) using Lipofectamine 2000. At 24 h post-transfection, the depletion of the targeted gene was detected by Western blotting with antibodies against LC3 (D). At the same time, cells were inoculated with PRRSV and cultivated for another 72 h. The total PRRSV titers of each treatment group were measured (E). (F) MARC-145 cells, seeded in 96-well plate for 48 h and treated with siLC3s or siSCR for 24 h, were added with 20 μl of CellTiter 96® AQueous One Solution each well for 4 h at 37 °C. The absorbance at 490 nm was recorded and compared of siLC3s treated cells and siSCR treated cells. The data were obtained in three independent experiments, and error bars mean ± SD of the three experiments (two-way ANOVA test; ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

The degradation of late stage of autophagy pathway was not blocked by the replication of PRRSV and bafilomycin A1 treatment reduced PRRSV titer

We next conducted this set of experiments to investigate the mutual impact of the replication of PRRSV and the degradation function of late stage of autophagy pathway. In order to determine the impact of replication of PRRSV on the degradation function of late stage of autophagy pathway, MARC-145 cells were fixed and stained with antibodies against N protein and LAMP1 and subjected to confocal microscopy at a late stage time point of 120 h pi. LAMP1 is a maker of lysosomes and acquired for fusion of autophagosomes with endosomal vesicles, thus LAMP1 is an autolysosome maker as well (Kirkegaard et al., 2004). The result of confocal microscopy revealed punctuates accumulation of LAMP1 in the cytoplasm of the infected cells (Fig. 4E). The N protein of PRRSV was mainly distributed nearby the cell membrane (Fig. 4D). As shown in Fig. 4F, LAMP1 and N protein were completely colocalized. Then we detected the level of p62/SQSTM1 of PRRSV infected and mock infected MARC-145 cells. P62/SQSTM1 is a multifunctional protein serving as a link between LC3 and ubiquitinated substrates and specifically degraded by the autolysosomes, therefore it is considered a marker for autophagy-mediated protein degradation activity (Klionsky et al., 2008). For the mock infected cells, p62/SQSTM1 bands existed to the end (Fig. 4G). For the infected cells, the p62/SQSTM1 band rapidly decreased, and disappeared from 72 h pi on (Fig. 4H). The results of confocal microscopy and the detection of p62/SQSTM1 level indicated that PRRSV

were colocalized with autolysosomes and the replication of PRRSV enhanced the degradation function of late stage of autophagy pathway instead of inhibiting it.

On the other hand, we investigate the impact of the degradation function of late stage of autophagy pathway on the replication of PRRSV. MARC-145 cells were inoculated with PRRSV and treated with 100 nM of Bafilomycin A1, which inhibits the efficient degradation by inhibiting the autophagosome–lysosome-fusion (Klionsky and Emr, 2000; Yamamoto et al., 1998). Then the PRRSV were tittered. As the treatment time of bafilomycin A1 prolonged, both the titers of intracellular and supernatant viruses decreased significantly (Figs. 4I and J). In order to exclude the impact of bafilomycin A1 on cell viability, cell viability was tested after treated with bafilomycin A1 and the results indicated that there was no significant difference on the cell viability between treated cells and untreated cells (Fig. 4K). Meanwhile p62/SQSTM1 was also detected following treatment of bafilomycin A1 on PRRSV infected MARC-145 cells to evaluate the effect of bafilomycin A1 inhibition on degradation of late stage of autophagy pathway. The accumulation of p62/SQSTM1 was observed as treatment of bafilomycin A1 prolonged (Fig. 4L). Though bafilomycin may affect virus infection with an autophagy-unrelated process, this result at least showed a possibility that degradation function of late stage of autophagy pathway may be favor to the replication of PRRSV.

The results of this part suggested that the degradation function of late stage of autophagy pathway was not impacted by the replication of PRRSV and bafilomycin A1 treatment reduced PRRSV titer.
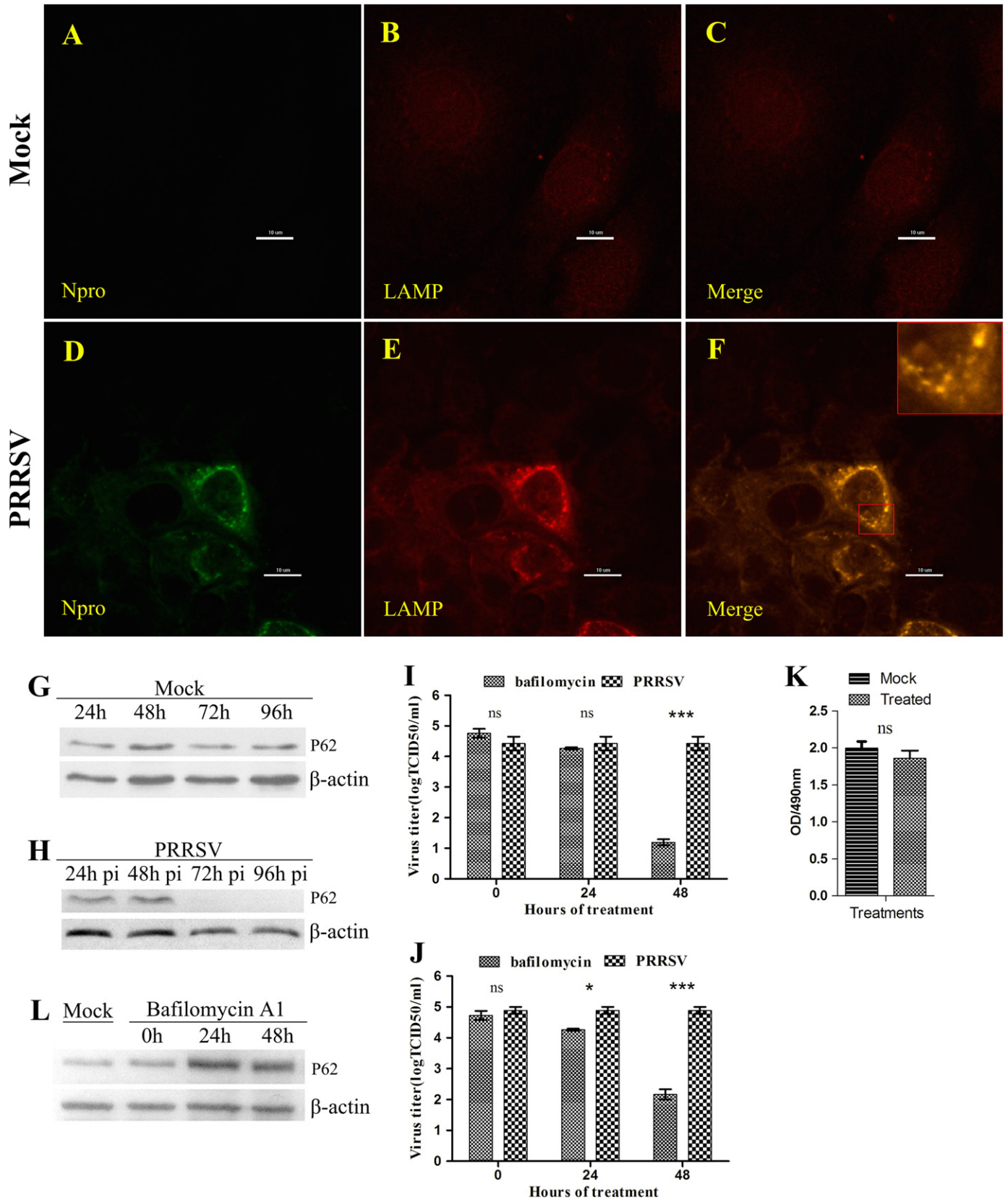
Fig. 4. Images for autolysosome formation and p62/SQSTM1 degradation. Panels (A)–(C) are the mock cells stained with antibodies against N protein, LC3 and merge. (D)–(F) Images of confocal microscopy of MARC-145 cells infected by PRRSV for 120 h and mock MARC-145 cells. Cells were fixed at 120 h pi and stained with antibodies against N protein of PRRSV (D) and LAMP1 (E) and merged (F). Magnified areas in panel F are indicated by rectangles. (G) and (H) Results of Western Blotting for p62/SQSTM1 detection. Mock cells (G) and PRRSV infected cells (H) were harvested at indicated time points and subjected to Western blotting analysis. Intracellular (I) and supernatant (J) virus titers at different time points by treatment with bafilomycin A1. MARC-145 cells, seeded in 96-well plate for 48 h and treated with bafilomycin A1 for 24 h, were added with 20 μl of CellTiter 96$^{\circledR}$ AQueous One Solution for each well for 4 h at 37 °C. The absorbance at 490 nm was recorded and which of treated groups and mock group was compared with χ^2 test (K). p62/SQSTM1 was detected following treatment of bafilomycin A1 on PRRSV infected MARC-145 cells (L). The data of panels I and J were obtained in three independent experiments, and error bars mean ± SD of the three experiments (two-way ANOVA test; ns, $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Scale bars in panels (A)–(F) represent 10 μm.
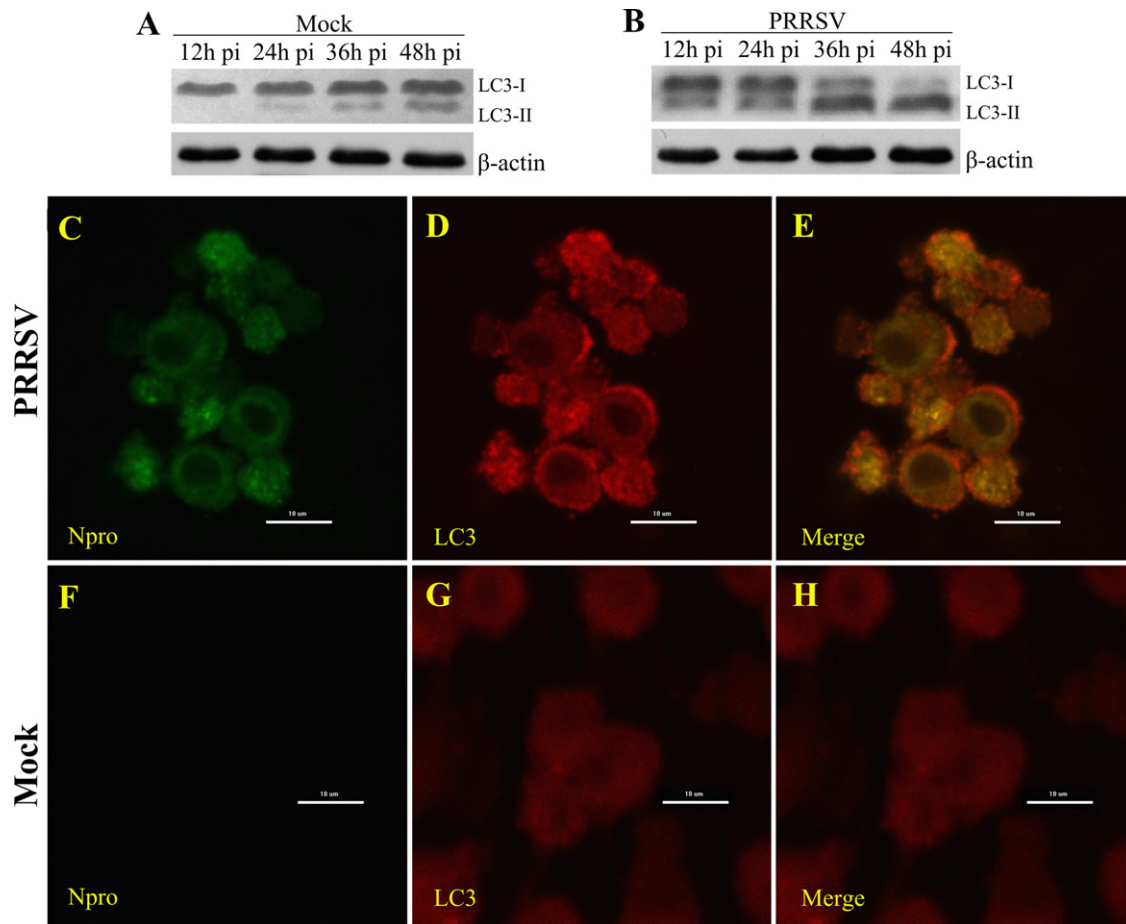
Fig. 5. Autophagy was induced in PAMs by PRRSV infection: (A) and (B) PAMs, prepared and maintained as described in Materials and methods, were inoculated with PRRSV; the total proteins were harvested at indicated time points, and then subjected to Western blotting analysis to detect the changes of LC3-I and LC3-II. Panel A is the result of Western blotting of uninfected PAMs, while panel B is the result of Western Blotting of infected PAMs. (C)–(E) Confocal microscopy images of infected PAMs. PAMs were fixed at 24 h pi and stained with antibodies against N protein of PRRSV (C) and LC3 (D). Panel E is the merged image of panels (C) and (D). Panels (F)–(H) are mock-infected PAMs stained with antibodies against N protein of PRRSV, LC3 and merge. Scale bars in panels (C)–(H) represent 10 μm.

Autophagy is induced in PAMs by PRRSV infection

PAMs are the natural host cells of PRRSV (Duan et al., 1997; Rossow et al., 1996), and PRRSV infection results in the destruction and function alterations of these cells (Molitor et al., 1997; Pol et al., 1997; Thanawongnuwech et al., 1997). We also conducted some of the experiments to analyze the relationship between autophagy and PRRSV infection using PAMs. The results by western blotting showed that the LC3-II bands changed lightly in the mock-infected PAMs (Fig. 5A); while in the PRRSV-infected PAMs, obvious band pattern transformation occurred, from the beginning the bands of LC3-I decreased and the bands of LC3-II increased, to the end almost all LC3 was of LC3-II form at 48 h pi (Fig. 5B). The change of LC3 was similar to the results in MARC-145 cells. Punctuates accumulation of LC3 in the infected PAMs was shown by the images of confocal microscopy (Fig. 5C and D). Moreover, the fluorescence punctuates of N protein were colocalized with the fluorescence punctuates of LC3 (Fig. 5E). Obviously, these results indicate that autophagy is induced in PAMs upon PRRSV infection.

Discussion

The experiments reported in this paper were performed in order to provide a better understanding of the interaction between PRRSV infection and host cells, with a particular focus on the effect of host autophagy on the replication of PRRSV. In recent years, a growing number of studies have demonstrated that the infection processes of viruses are closely related with autophagy of host cells. The autophagy pathway may play an antiviral role in host cells. In Sindbis virus, studies indicated that the autophagy protein Beclin 1 could reduce neuronal apoptosis and the neuronal expression of the Beclin 1 could decrease the virus titers in Central Nervous System (CNS) (Liang et al., 1998). In the case of herpes simplex virus type 1 (HSV-1), the antiviral effect of autophagy exists as well, which degraded the proteins of the virus (Talloczy et al., 2006). However, the viruses, such as HSV-1 and CVB3, in return evolved mechanisms to escape or hijack the autophagy pathway (Orvedahl et al., 2007; Wong et al., 2008). Recent studies indicated that autophagy-related proteins including Beclin-1, Atg4B, Atg5 and Atg12 were required for translation of incoming viral RNA at the initiation of hepatitis C virus (HCV) replication (Dreux et al., 2009), and dengue virus (DENV) infection could lead to an autophagy-dependent processing of lipid droplets and triglycerides to release free fatty acids, which is necessary for its efficient replication (Heaton and Randall, 2010). Obviously, these studies provide valuable implications for PRRSV research. In nidoviruses including coronaviruses and arteriviruses, they might successfully subvert host autophagy to their advantage. Certain nonstructural proteins (Nsps) of nidoviruses assemble together and anchor on DMVs to form the

viral RNA replication–transcription complex. Although the precise mechanism regarding the formation of the replication complexes has not been clarified yet, more and more evidence indicated that the membrane they anchored on resemble autophagosomes (Gosert et al., 2002; Snijder et al., 2001, 2006; van der Meer et al., 1998). A recent publication indicated the Nsp6 protein of infectious bronchitis virus (IBV), an avian coronavirus, could generate autophagosomes from the endoplasmic reticulum, but it did not show the relationship between that mechanism and the replication of IBV (Cottam et al., 2011). Direct experimental data is needed to confirm whether the DMVs on which virus replication complexes anchored are autophagosomes.

PRRSV, a member of genus arterivirus, is an important pathogen for pigs. Unveiling the autophagy phenomenon induced by PRRSV infection in host cells and the role of autophagy in the replication of PRRSV is helpful for us to understand the interaction between the virus and host cell and cell pathogenesis of PRRSV. In our present study, we used MARC-145 cells—the highly permissive cells of PRRSV as the model cell line to conduct all the experiments and further performed some of experiments in PAMs—the natural target cell. We first observed the presence of morphologically characteristic double-membrane vesicles in the PRRSV-infected MARC-145 cells. These vesicles were termed autophagosome-like vesicles in our paper although we do not have enough evidence to define them as autophagosomes, and the nature of the vesicles is needed to be confirmed. We further found both the N protein and the Nsp2 of PRRSV colocalized with the autophagy protein LC3 in the PRRSV-infected MARC-145 cells, and also the N protein of PRRSV colocalized with the autophagy protein LC3 in the PRRSV-infected PAMs by confocal microscopy. Meanwhile, we monitored the change of LC3 of both PRRSV-infected MARC-145 cells and PRRSV-infected PAMs by western blotting analyses. Our findings provide the evidence that PRRSV infection is able to induce autophagy in host cells.

Manipulation of host autophagy pathway can affect the replication of some viruses. In CVB3, the inhibition of autophagy by pharmacological compounds or interference of autophagy-critical-gene with target-specific siRNA can reduce the virus production, while the induction of autophagy promotes the virus replication (Wong et al., 2008). In order to further analyze the role of autophagy in the replication of PRRSV in MARC-145 cells, we conducted induction of autophagy by the autophagy inducer—rapamycin, and inhibition of autophagy by the inhibitor—3-MA and siRNA silencing of LC3 gene. Our results showed that the titers of PRRSV in MARC-145 cell were significantly increased resulting from autophagy induction with pharmacological compounds, whereas the inhibition of host autophagy with the inhibitor 3-MA reduced virus generation. Given 3-MA is a nucleoside analog, and it may have inhibitory effects on RNA replication, we then knocked down the LC3 gene with siRNAs to specifically block the autophagy pathway and assessed the impact of this treatment on PRRSV replication. Similar to the results of 3-MA treatment, the PRRSV titers following LC3 silencing were significantly lower. PRRSV replication was not completely stopped may be due to that LC3 gene was not totally silenced. Thus, the relationship between silencing efficiency and the virus titers indirectly indicated LC3 is helpful for PRRSV replication. Though the relationship between autophagy and the mechanism of replication of PRRSV and MHV may be different, a recent study have indicated that LC3-I is also required by MHV (Reggiori et al., 2010), a member of coronaviruses. Our results provide another evidence for that some nidoviruses might successfully take advantage of host autophagy (or components of autophagy) for their own replication. We tried to investigate whether the manipulation of autophagy affect the release of PRRSV, we measured both the titers in supernatant and intracellular viruses. Unfortunately, no apparent difference between supernatant and intracellular viruses was found,

which indicated that autophagy manipulating would not affect the release of PRRSV. It will be an interesting issue to investigate which part (s) or which gene (s) of autophagy pathway is (are) involved in the replication of PRRSV. Taken together, our data indicate that the autophagy pathway in host cells is likely hijacked by PRRSV to facilitate its replication.

Autolysosomes are the functional factor of this degradation pathway, and the degradation activity plays a critical role in protecting the cell against microbes. A recent report suggested that prevention of autophasosome–lysosome-fusion by silencing LAMP2 significantly increased the expression of CVB3 protein and virus titer (Wong et al., 2008). However, in our study, the inhibitior of autophasome–lysosome-fusion reduced the virus titers as the treatment of bafilomycin A1 prolonged. Our results were consistent with a prior paper which reported that bafilomycin A1 interfered with an early stage of virus infection (Kreutz and Ackermann, 1996). Though there are other possibilities that bafilomycin A1 affect PRRSV spread, the results indicated that the functional autolysosomes may be helpful for PRRSV generation. On the other side more explorations are needed to explain the mechanism.

PAMs are the primary target cells of PRRSV (Duan et al., 1997; Rossow et al., 1996), and they act in diverse biological functions. They also play critical role in both innate immunity and adaptive immunity in pigs. Previous study indicated that PRRSV infection could induce apoptosis of infected PAMs as well as periphery non-infected PAMs (Labarque et al., 2003). Understanding the cellular mechanism associated with function distortion of PRRSV-infected PAMs is helpful for controlling PRRS. Here we for the first time show that autophagy is induced in the PRRSV-infected PAMs. Additionally, emerging evidence has linked cellular autophagy with innate and adaptive immune programs. Eissa and colleagues reported that Toll-like receptors (TLRs) are able to trigger the formation of autophagosomes, and silencing TLR4 resulted in significant reduction in autophagosomes (Xu et al., 2008). Results of other groups demonstrated that IFN-γ and TNF-α which belong to Th1 cytokines can up-regulate autophagy, while Th2 cytokines such as IL-4 and IL-13 may counteract autophagy induction (Gutierrez et al., 2004; Singh et al., 2006). Another research group reported that the autophagy pathway may play an alternative role in the delivering cytosolic antigens for MHC-II (Brazil et al., 1997). It is also reported that if cellular damage is too extensive and apoptosis is suppressed, the autophagy pathway which initially functions as a cytoprotective mechanism may kill the cells (Boya et al., 2005). Considering the wide association of autophagy with other cellular pathways such as TLR pathway, antigen presentation pathway and apoptosis, our results open a new window for cracking the pathogenesis of PRRSV. These issues related to the autophagy induced by PRRSV infection are worthy to be further explored.

Recently, a paper indicated the similar viewpoints that, the PRRSV infection induced autophagy; induction autophagy increased the PRRSV titer; and inhibition of autophagy reduced the PRRSV titer (Chen et al., 2012). Our works have provided more data and in-depth analysis to confirm that PRRSV infection can induce autophagy in MARC-145 cells, and also investigated the induction of autophagy by PRRSV infection in PAMs, its natural host cells. Additionally, we further explored the relationship between the replication of PRRSV and the degradation function of late stage of autophagy pathway.

To summarize, our findings for the first time indicate that autophagy is triggered in host cells upon PRRSV infection and involved in the replication of PRRSV, implying that PRRSV can utilize the autophagic pathway to sustain their own replication in host cells. Our present study is helpful for further exploring the pathogenesis related to the interaction between PRRSV and host cells.

Conclusions

In this study, the results first indicate that autophagy is induced in MARC-145 cells upon PRRSV infection. Then the results suggest that on autophagy induction the PRRSV replication is enhanced, while reduced by the inhibition of autophagy. Next it is shown that the degradation function of late stage of autophagy pathway and the replication of PRRSV were beneficial to each other. Finally, our results indicate that autophagy is induced in PAMs upon PRRSV infection.

Materials and methods

Cells and virus

Cells of the MARC-145 line, a highly permissive for PRRSV replication, were used in this study. The cells were maintained in GIBCO^{TM} Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corporation, Auckland, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories Inc, South Logan, UT) at 37 °C, with 5% CO_2. Porcine pulmonary alveolar macrophages (PAMs) were prepared with 4-week-old specific-pathogen-free (SPF) piglets (Beijing Center for SPF Swine Breeding and Management), according to the methods previously described (Wensvoort et al., 1991; Zhang et al., 2009). The PAMs were maintained in RPMI-1640 medium (GIBCO, Invitrogen Corporation, CA) containing 10% FBS, 100 units/mL of penicillin, and 100 μg/ml of streptomycin at 37 °C, with 5% CO_2. The PRRSV stock used in this study was the fifth passage culture of PRRSV JXwn06, a high-virulence PRRSV strain and the fifth passage culture of HB-1/3.9, a low-virulence PRRSV strain (Zhou et al., 2009). The titers of the virus were determined as the tissue culture infective dose 50 (TCID$_{50}$) per milliliter by using the Reed-Muench method. Briefly, MARC-145 cells cultivated in 96-well plates were inoculated with the virus dilutions (100 μl/well) prepared by serial 10-fold dilution. After absorption for 1 h at 37 °C, the suspensions in the wells were removed, and DMEM with 10% FBS was added to the wells. Plates were incubated for an additional 72–96 h; virus titers were determined by the presence of a visible CPE.

Antibodies and reagent

Monoclonal antibodies (McAb) against N protein and nonstructural protein 2 (Nsp2) of PRRSV used in this study were prepared by our laboratory (Gu et al., 2000; Yan et al., 2007). Primary antibodies specific for LC3, p62/SQSTM1 and LAMP1, as well as anti-rabbit and anti-mouse secondary antibodies, were purchased from Sigma (Sigma, St. Louis, MO). The inducer—rapamycin, the inhibitor—3-methyladenine (3-MA) of autophagy, and the autophagosome–lysosome-fusion inhibitor—bafilomycin A1 were also purchased from Sigma.

Virus infection and cell treatment

MARC-145 cells cultured in 6-well cell culture cluster (Costar, Corning Inc., Corning, NY) were infected at a multiplicity of infection (MOI) of 0.05 in DMEM with 10% FBS. For autophagy induction, cells were treated with 1 μM of rapamycin dissolved in dimethyl sulfoxide for 3 h prior to virus infection. For autophagy inhibition experiments, cells were treated with different concentrations of 3-MA dissolved in glycerol for 24 h prior to virus inoculation. For autophagosome–lysosome-fusion inhibition, cells were treated with 100 nM of bafilomycin A1 at different time points following virus infection. PAMs were incubated for 12 h at 37 °C in 5% CO_2 in RPMI-1640 medium with 10% FBS, and the non-adherent cells were removed by gentle washing with RPMI-1640 medium prior to inoculation. Then, the cells were inoculated with 0.05 of MOI of PRRSV.

Cell viability test

Cell viability was assayed with CellTiter 96® AQueous One Solution Cell Proliferation Assay kits (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Approximately 1×10^5 MARC-145 cells per well were seeded in a 96-well cell plate and cultured for 48 h at 37 °C with 5% CO_2. Then, the medium was replaced with fresh medium containing 3-MA (0.1 mM, 1 mM or 10 mM) or bafilomycin A1 (100 nM) and incubated for 24 h. 20 μl of CellTiter 96® AQueous One Solution was added to each well and incubated for 4 h at 37 °C. The absorbance at 490 nm was recorded using a 96-well plate reader (Bio-Rad Laboratories, Inc.).

Confocal microscopy

Cells were plated in 96-well plates, then were infected with PRRSV or treated with reagents at 37 °C in DMEM containing 10% FBS. Following incubation of 1 h, the inoculums were removed and replaced with DMEM containing 10% FBS. At the indicated time points, the cells were washed with PBS (pH 7.4) and fixed with 95% pre-chilled ethanol for 15 min. For confocal microscopy experiments, all primary antibodies against the proteins of PRRSV were derived from mouse, and all primary antibodies against cellular autophagic proteins were derived from rabbits. The primary antibodies were diluted with PBS containing 5% FBS (pH 7.4), and were adsorbed for 1 h at 37 °C and then washed three times with PBS (pH 7.4). For each experiment group, the cells were stained with McAb to N protein plus anti-LC3 antibodies, McAb to Nsp2 plus anti-LC3 antibodies, or McAb to N protein plus anti-LAMP1 antibodies, respectively. The cells were then incubated for 1 h at 37 °C with a 1:100 dilution of anti-rabbit and/or anti-mouse secondary antibodies conjugated to tetraethyl rhodamine isothiocyanate (TRITC) or fluorescein isocyanate (FITC). Cells were washed three times with PBS (pH 7.4). Finally, the cells were immediately visualized at 488 nm and 543 nm under a Nikon TE-2000E confocal fluorescence microscope (Nikon Instruments Inc., Melville, NY).

Transmission electron microscopy (TEM)

The MARC-145 cells in flasks (Corning Inc., NY) were mock-infected or PRRSV-infected for 72 h. Then the cells were washed once with PBS (pH 7.4) and scraped. Following pelleted at 600g for 10 min, the cells were fixed with 2.5% glutaraldehyde and stored at 4 °C overnight. For subsequent processing, the cells were washed in 1 M PBS, post-fixed with 1% osmium tetroxide for 1 h at room temperature. Then the samples were dehydrated in a series of graded acetone, and embedded in Spurr's plastic resin, polymerized overnight. Ultrathin sections were cut with Leicauc 6i section cutter, and were stained with uranyl acetate and lead citrate and then examined and imaged under a JEM-1230 electron microscope.

Extraction of cellular proteins

Treated or infected cells at indicated time pointes were digested with trypsin. The cells were collected and washed with PBS (pH 7.4) three times, then centrifuged at 600g for 6 min at 4 °C. Ice-cold RIPA lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton x-100, 0.1% SDS, 1% Sodium Deoxycholate, 50 mM Sodium fluoride, 5 mM Sodium orthovanadate,

1 mM DTT] with protease inhibitor cocktail (Roche Molecular Biochemicals, Germany) was added to the cell pellets. Sample tubes were incubated on ice for 15 min, sonicated at 30% duty cycle with 3 output control for 10 pulses, and centrifugated at 16,000g for 20 min. The supernatant was transferred to fresh tubes and stored at −80 °C for western blotting.

Gene silencing with siRNA

Sets of siRNA used in this study for silencing LC3β were obtained from Ambion. Two days after seeding, the MARC-145 cells were transfected with 20 pmol of siRNA using Lipofectamine 2000 (Invitrogen Corporation, Auckland, NY). At 24 h post-transfection, experiments were conducted. The depletion of the targeted gene was detected by western blotting with antibodies against LC3. The same treatments were conducted with the scrambled siRNA as a negative control.

Western blotting

Cell lysate samples, diluted in RIPA buffer, were heated at 100 °C for 5 min and loaded on 20% (w/v) SDS-PAGE gels. Proteins were transferred to Polyvinylidene Fluoride membranes (Millipore, Bedford, MA) and blocked with 5% separated milk dissolved in PBS (pH 7.4) at 4 °C overnight. Membranes were reacted with primary antibodies and then with corresponding secondary antibodies conjugated to horseradish peroxidase. Protein bands were visualized with enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ) by the ECL plus Western Blot Detection System (Kodak, NY). Images were captured using GelDoc XR (Bio-Rad, Hercules, CA), and adjusted for total brightness and contrast with Adobe Photoshop CS3. The optical density of band was analyzed with Quantity One 1-D software (Bio-Rad, version 4.6.2).

Acknowledgments

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