Autophagy and apoptosis play significant roles in PRRSV infection and replication. However, the interaction between these 2 processes in PRRSV replication is still far from being completely understood. In our studies, the exposure of MARC-145 cells to PRRSV confirmed the activation of autophagy and subsequent induction of apoptosis. The inhibition of autophagy by 3-methyladenine (3-MA) caused a significant increase in PRRSV-induced apoptosis, showing a potential connection between both mechanisms. Moreover, we observed an increase in Bad expression (a pro-apoptotic protein) and Beclin1 (an autophagy regulator) in virus-infected cells up to 36h. Co-immunoprecipitation assays showed the formation of Bad and Beclin1 complex in PRRSV infected cells. Accordingly, Bad co-localized with Beclin1 in MARC-145 infected cells. Knockdown of Beclin1 significantly decreased PRRSV replication and PRRSV-induced autophagy, while Bad silencing resulted in increased autophagy and enhanced viral replication. Furthermore, PRRSV infection phosphorylated Bad (Ser112) to promote cellular survival. These results demonstrate that autophagy can favor PRRSV replication by postponing apoptosis through the formation of a Bad-Beclin1 complex.

Introduction

The swine industry suffers huge economic losses through porcine reproductive and respiratory syndrome virus (PRRSV) that causes reproductive failure, respiratory distress, high morbidity and mortality in pigs of all ages. Despite years of extensive research in pathogenesis of PRRSV, the host factors and host species/cells involved are poorly understood. The two evolutionarily conserved biological processes, autophagy and apoptosis, are essential for development, tissue homeostasis and their imbalance causes disease. Autophagy is recognized both as a unique cell death pathway and as an adaptation to various stresses that supports cell growth and survival. On the other hand, apoptosis is widely regarded as a major mechanism of programmed cell death.1 There are several proofs to believe that the fate of cells is differentially affected by autophagy and apoptosis, which can antagonize or assist each other under certain circumstances. Autophagy may acts as an adaptive response to suppress cell death, but can also stimulate autophagic cell death.2,3 Moreover, several important signaling pathways such as mTOR, DAPK, Beclin1 and caspases, mediate the complex cross-talk between autophagy and apoptosis that further enlightens its implications in disease and pathogen interactions. Furthermore, it has been reported that autophagy and apoptosis regulate virus-host interactions, where the virus-induced apoptotic cell death is inhibited by autophagy, while apoptosis-associated signal activation shuts off the autophagic process. A similar case is that of HTLV-1 Tax protein-induced autophagy, which contributes to protect human astrogliaoma cells from death receptor-mediated apoptosis.4 Another study demonstrates a significant increase in apoptotic cell death in autophagy-deficient cells infected with influenza A virus (IAV) that was exacerbated by the virus-encoded protein M2.5 However, in some cases autophagy acts as a process of programmed cell death where autophagy-relevant proteins may participate in inducing apoptosis. This has been elucidated by Beclin1 protein that can promote autophagosome initiation. Silencing of Beclin1 protein expression resulted in a decreased percentage of early and late apoptotic cells in the late stage of ECTV-MOS infection in L929 cells.6 Additionally, the results of Espert et al. showed that 3-MA, an inhibitor of autophagy, totally inhibited Env-mediated cell death, and autophagy was also a prerequisite for Env-induced activation of the apoptotic pathway through CXCR4.7

It has been demonstrated that HP-PRRSV has the capacity to exploit autophagy and apoptosis for its replication.8,9 However, interpreting the functional interaction between autophagy and apoptosis that service PRRSV replication can greatly improve our understanding of the pathogenesis of PRRSV infection. Therefore, in present study cell autophagy and apoptosis were induced in MARC-145 cells at different
stages of viral life cycle that showed autophagy can postpone apoptotic cell death induced by PRRSV infection. Moreover, we demonstrated that PRRSV-induced autophagy in MARC145 cells was regulated by Bad and Beclin1 interaction through phosphorylation.

**Materials and Methods**

**Cells and viruses**

African green monkey kidney cell line, MARC-145 cells that was cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37 °C under a 5% CO2 atmosphere were infected with PRRSV strain WUH3, a highly pathogenic North American type PRRSV (a gift from State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, PR China). Virus titers were determined as the tissue culture infectious dose 50 (TCID50) per milliliter by using the Reed-Muench method.

**Quantitative PCR**

MARC-145 cells grown in 24-well plates were infected with PRRSV or mock-infected at a multiplicity of infection (MOI) of 1. Total cellular RNA was extracted at the indicated time points using TRIzol reagent (Invitrogen) and cDNA was synthesized by using Superscript III reverse transcriptase kit (Invitrogen, CA). Real-time RT-PCR was performed using SYBR Green Real Time PCR Master Mix (Toyobo Biologics, Osaka, Japan) in the LightCycler 480 (Roche Molecular Biochemicals). Individual transcripts in each sample were assayed three times. The PCR conditions were as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 60°C and 40 s at 72 °C. The levels of indicated molecules were determined by evaluating the threshold cycle (Ct) of target gene after normalizing against the Ct value of β-actin dependent on the delta delta cycles to threshold (ΔΔCt) method. Specific primers used for quantitative PCR (Q-PCR) assays are listed in Table 1.

**Western blot analysis**

Cells were harvested in lysis buffer containing protease inhibitor cocktails and the protein concentration was determined using the Bradford assay. Lysed cells were electrophoresed on 10% or 15% SDS-PAGE gels. Proteins were transferred to Polyvinylidene Fluoride membranes (BioRad) and blocked with 5% dried skimmed milk and then incubated with corresponding antibodies at 4°C overnight. Membranes were reacted with corresponding secondary antibodies conjugated to hors eradish peroxidase (HRP), goat anti-rabbit-HRP (1:5000; Cell Signaling Technology (CST)), or goat anti-mouse-HRP (1:5000; BOSTER, China), and finally visualized by the addition of the SuperSignal West Pico chemiluminescent substrate (Pierce). Quantification of band density was done using ImageJ with normalization to the β-actin signal or GAPDH. Abundance of interested proteins in various treatments was expressed relative to that under mock conditions. Primary antibodies were as follows: rabbit anti-LC3B (1:1000; Sigma-Aldrich), rabbit anti-p62 (1:1000; Sigma-Aldrich), rabbit anti-Beclin1 (1:1000; CST), anti-Bad (1:1000, GeneTex), anti-Bcl-xl (1:1000, GeneTex), mouse anti-PRRSV NSP2 (a gift from State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, PR China) rabbit anti-β-actin (1:3000, CST) or mouse anti-GAPDH (1:3000,proteintech).

**Table 1. Primers of genes for qPCR.**

| Gene | Primer sequences | Annealing temperature |
|------|------------------|-----------------------|
| Bad  | 5’GGTGACCCGAGATGTTGCCGCTC3’ | 60°C |
|      | 5’CAGGCACTTGGAAGGTACG3’ |
| Beclin1 | 5’GGTGAGGGATGGAAGGCTG3’ | 60°C |
|      | 5’TGGGGCTGTGGTAAGTAATGG3’ |
| β-actin | 5’AGCAAGACGAGTACGCGAT3’ | 60°C |
|      | 5’CAAGAAAGGTTGAAGCTACT3’ |

**Nuclear morphological observation**

Cells were plated in 24-well tissue culture plates for 24 h and infected with PRRSV for 12, 24, 36 and 48h. At the indicated time points, the cells were fixed 4% paraformaldehyde for 10 min at room temperature, permeated with 0.1% Triton X-100 for another 10 min at room temperature and stained with Hoechst 33258 (1 mg/ml of phosphate-buffered saline; PBS) at 37°C for 15 min. The nuclear morphology was observed with an inverted fluorescent microscope (Olympus, Tokyo, Japan), after the cells were washed with PBS three times.

**Flow cytometry analysis**

MARC-145 cells, pretreated with rapamycin (500 nM), 3-MA (10 mM) or DMSO (control) for 4 h, were infected with PRRSV at an MOI of 1. The infected cells were collected and washed with PBS three times. The cells were incubated with AnnexinV APC and stained with 7-ADD for 15 minutes at room temperature. The percentage of apoptotic cells was analyzed using FAC Scan flow cytometer with CellQuest pro software (BD Biosciences, San Jose, CA). The annexin V-positive/7ADD-negative cells were considered to be apoptotic cells at the early period, annexin V-positive/7ADD-positive cells were considered to be apoptotic cells at the later period.

**Gene silencing with siRNA**

SiRNA oligonucleotides with specificity for Beclin1 and Bad and non-targeting siRNA (siCtrl) were synthesized from GenePharma (Shanghai, China). MARC-145 cells were grown to 60–70% confluence in six-well cell culture plates and then transiently transfected with indicated siRNA by using siRNA-Mate transfection regent (GenePharma) according to the manufacturer’s instructions. Knockdown efficiencies were detected by western blot analysis. In addition, 24h after transfection with siRNA, the cells were infected with PRRSV and collected at 24hpi and 36hpi, for immunoblotting analysis or for the detection of autophagosomes.
Confocal fluorescence microscopy
Mock or infected MARC145 cells were fixed 4% paraformaldehyde (PFA) for 10 min before permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 1% bovine serum albumin in PBS for 1 h and then incubated for 1 h on ice with primary Abs (rabbit anti-LC3, CST). After washing, cells were stained for 1 h on ice with goat anti-rabbit FITC antibody (BOSTER) diluted 1:200. The nucleus was stained with 4′-6-diamidino-2-phenylindole (DAPI). The fluorescence was observed under a confocal fluorescence microscope. In another experiment for the colocalization of proteins, the cells were stained with mouse anti-Beclin1 plus rabbit anti-Bad antibody and 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 rhodamineisothiocyanate (TRITC) or fluorescein isocyanate (FITC). The nucleus was stained with DAPI and cells were washed three times with PBS. The co-localization of Bad and Beclin1 was observed at 488 nm and 543 nm under a confocal fluorescence microscope.

Co-immunoprecipitation (Co-IP) assay
Mock-treated or virus-infected cells were lysed with RIPA buffer (Beijing Dingguo Biotechnology Co. LTD) supplemented with protease inhibitor complex. Cell lysates were centrifuged at 13,000 rpm for 15 min at 4°C, and soluble protein was incubated with anti-Beclin1 antibody overnight at 4°C. The immune complexes were captured with Protein G-agarose beads (Beyotime Biotechnology, China), followed by incubation at 4°C for 1 h. The immunoprecipitates were then washed three times with lysis buffer, boiled with 2× protein loading dye and Western blotting was then performed according to standard protocols.

Transmission electron microscopy (TEM)
MARC-145 cells were either mock infected or infected with PRRSV at MOIs of 1 for 6, 12, 24, 36 and 48 h, respectively. Cells were pelleted by centrifugation (800rpm for 10 min) and then fixed with 2.5% glutaraldehyde overnight at 4°C. After being washed with 0.1M PBS, the cells were post-fixed with 1% osmium tetroxide for 1 h at room temperature and washed again. After dehydration and resin embedding, the cells were ultrathin sectioned with LKB—V section cutter, and were stained with uranyl acetate and lead citrate. High resolution images were taken with Hitachi H-600 transmission electron microscope.

Statistical analysis
All statistical analyses were performed by one-way ANOVA and Student’s t-test using a SPSS 16.0 software package (SPSS Inc., version 16.0). The data were expressed as the mean ± standard deviation (SD) from at least three independent experiments.

Results
Cells autophagy and apoptosis involved in PRRSV infection
The possibility of complex cross-talk between autophagy and apoptosis makes us investigate the relationship between PRRSV induced autophagy and apoptosis, in which we first assessed whether these two processes could be co-induced in PRRSV-infected MARC-145 cells. For this purpose, MARC-145 cells were infected with highly pathogenic American type PRRSV strain WUH3 at MOI of 1, where LC3 puncta or LC3-II formation was measured by Immunofluorescence or Western blot respectively. LC3-I, the cytosolic form of Microtubule-associated protein 1A/1B-light chain 3 (LC3) have to conjugate to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is localized on autophagosomes during its formation, therefore the accumulation of LC3-II is regarded as a marker of autophagy. 10 A significant increase in punctate LC3 was seen in PRRSV-infected cells when compared to uninfected cells (Fig. 1A). An increase in the levels of LC3-II in PRRSV infected cells was also detected significantly until 36 h post infection (hpi) with observed declined LC3-II levels at 48hpi, when compared with uninfected cells by protein gel blot assay (Fig. 1B-C). Meanwhile, as the expression of p62 protein is related with autophagic flux, 11 we also measured p62 protein levels in PRRSV infected cells which shows a continuous decline till 36 hpi with an increase at 48hpi (Fig. 1B-D), indicating that PRRSV enhanced autophagic flux as onset of the early events in virus infection. The results from Hoechst 33258 staining showed that PRRSV infected MARC-145 cells exhibit characteristics of apoptosis such as cell nucleus cleavage and chromatin condensation in a time-dependent manner (Fig. 1E).

Autophagy is triggered earlier than PRRSV-induced cell apoptosis
The requirement of host cells by virus to effectively reproduce and infect their next host is already known. Some previous researches have shown that autophagy and anti-apoptosis are stimulated by PRRSV in the early infection stage and are required for PRRSV replication. 8,9 Whether PRRSV can delineate the temporal relationship between autophagy and apoptosis to facilitate PRRSV replication and viral progeny production needs to be determined. An increase was observed in autophagic-like vesicles formation after 12 hpi and reached its peak at 36 hpi with a gradual disappearance till 48hpi (Fig. 2A). Further quantitative analyses also demonstrated the change of autophagosome formation (Fig. 2B). Moreover, we also observed that there was no significant change in membrane structure, mitochondria or nucleus at 12h post PRRSV infection compared to uninfected cells, while a series of apoptotic phenomenon, such as nuclear fragmentation, cytoplasmic shrinkage and clusters of vacuoles in the cytoplasm were observed in PRRSV-infected MARC-145 cells at 24, 36 and 48hpi. Meanwhile, we found the expression of anti-apoptosis protein Bcl-xl was increased from 24hpi to 36hpi and then began to decrease at 48hpi, which might correlate to increased apoptotic cell death. The switch between autophagy and apoptosis was not clearly defined. The above results in combination conclude that PRRSV-induced autophagy did not coincide with apoptosis appearance and the promotion of PRRSV
replication might be due to the autophagic anti-apoptotic function or inhibition of cell apoptosis.

**Autophagy inhibition accelerates apoptotic death of MARC-145 cells in PRRSV infection**

As autophagy and apoptosis that exhibit different roles for cell death are triggered by PRRSV infection to regulate viral replication, it was very critical to investigate the relationship between cell autophagy and apoptosis induced by PRRSV at the early stages of viral life cycle. Autophagy process was inhibited using 3-MA, an autophagy inhibitor, and RNAi for beclin1 that is required for initiating the autophagosome formation. The result showed in Figure 3A-B that the inhibition of autophagy by 3-MA increased the number of apoptotic cells compared to uninfected and infected MARC-145 cells by flow cytometry analysis with Annexin V-APC/7ADD whereas cells treated by Rapamycin, an autophagy activator, did not affect cell viability. In addition, when cells were transfected with Beclin1 siRNA or control siRNA (NC) a significant decrease of Beclin1 at protein levels was observed, knockdown of Beclin1 also led to the same result in inducing the cleavage of caspase3 (Fig. 3C-D). Our results suggested that the inhibition of autophagy strengthened cell apoptosis induced by PRRSV, and Beclin1 participated in the interplay between autophagy and apoptosis during the early stages of the PRRSV live cycle in MARC-145.

**PRRSV upregulates Bad and Beclin1 expression**

Both Bad and Beclin1 are the Bcl2 homology 3 (BH) domain-only protein and they have also been determined to relate with viral replication. To study whether Bad and Beclin1 are involved in PRRSV infection, we examined their regulation at the mRNA and protein level. We observed that the expression of...
Bad mRNA increased with the progression of PRRSV infection, while PRRSV infection progressively stimulated Beclin1 expression from 6hpi to 36hpi that it declined thereafter at 48hpi (Fig. 4A). Western blot analysis of Bad protein did not expose significant changes in its expression level during PRRSV infection in MARC-145 cells, in contrast, PRRSV infection robustly increased Beclin1 protein expression with maximal production at 36hpi (Fig. 4B-C). However, intracellular distribution of Bad protein in control and infected MARC-145 cells varied as determined by fluorescence microscopy (Fig. 4D). Non-infected MARC-145 cells release diffused localization of Bad. Meanwhile, the localization of Bad was almost strongly transferred into the
cytoplasmic virus replication in MARC-145 cells at 36h hpi. The different distribution of Bad suggested its involvement in PRRSV replication. Together, these results indicate that upregulation of Beclin1 expression and the intracellular distribution of Bad protein mediated by PRRSV infection may participate in autophagy-inhibited cell apoptosis and PRRSV replication.

Bad regulated PRRSV-induced autophagy through interacting with Beclin1

It has been shown that Bad, a pro-apoptotic protein, participates in nutrient starvation-induced activation of autophagy, but not rapamycin-induced autophagy. However, the cross-talk between Bad and autophagy induction during PRRSV infection remains unknown. We found that Bad knockdown in PRRSV-infected MARC-145 cells by RNAi significantly led to the increased expression of LC3 as compared with mock-infected MARC-145 cells, when silencing of Bad decreased the cleavage of caspase3 (Fig. 5A-B). Additionally, the punctate accumulation of LC3 also had a significant increase under Bad knockdown; while the silencing of Beclin1 reduced the punctate of LC3 (Fig. 5C). Together with these results, we demonstrated that Bad could affect cell autophagy induced by PRRSV.

To further investigate how Bad regulates PRRSV-induced autophagy, co-immunoprecipitation analysis or immunofluorescence assay for the Bad and Beclin1 interaction was performed that showed PRRSV infection promoted the association between Bad and beclin1, while the association of Beclin1 and Bax, another pro-apoptotic effector protein, was inhibited although there was no change in the interaction between Beclin1 and Bcl2 in PRRSV infected cells (Fig. 6A-B) another pro-apoptotic effector protein, indicating that Beclin1-Bcl2 dissociation may not be important for PRRSV-mediated autophagy induction and Bad may interact with Beclin1 to regulate autophagy. Our results above showed that the knockdown of Bad was able to increase PRRSV-induced autophagy, but it has been proved that pro-apoptotic Bad inhibited the interaction between Beclin1 and the anti-apoptotic proteins thus causing an induced autophagy, hence we speculated that PRRSV could utilize Bad phosphorylation that inactivated its pro-
Figure 4. PRRSV infection regulated the expression of Bad and Beclin1 and intracellular distribution of Bad. (A) Total RNA was isolated from mock or PRRSV infected MARC-145 cells at a MOI of 1 at different time points and subjected to qPCR to analyze Bad and Beclin1 expression. β-actin was used as an internal control. (B) Cell lysates were analyzed for Beclin1 and Bad expression by Western blotting. The blot was reacted with anti-β-actin antibody for comparison of protein load. (C) The fold changes of protein are presented after normalizing with internal control and were arbitrarily set as 1. Data presented were representative of three independent experiments. (D) Fluorescence microscopy images showing immunofluorescence staining for Bad (green), PRRSV NSP2 (red) and DAPI (blue) in mock cells and PRRSV-infected MARC-145 cells.
Figure 5. (For figure legend, see page 106).
apoptotic function to activate Beclin1-dependent autophagy. Western blot assay revealed an increase of p-Bad (S112) up to 36hpi (Fig. 6C), suggesting that Bad phosphorylation plays a critical role in PRRSV-induced autophagy, although Bad phosphorylation in other sites and its role for affecting Beclin1 binding needs to be detected.

Discussion

Apart from the role of autophagy and apoptosis in host immune responses against viral infections, these have been evolutionarily exploited by some infectious pathogens to benefit their own replication. Research evidence suggests that influenza A virus increases LC3-II protein expression to induce the formation of autophagosomes by regulating the AKT-TSC2-mTOR signaling pathway. In addition, M2, HA, and NS1 proteins of influenza A virus contributed to induce cell autophagy \(^{19,20}\); and influenza A virus infection inhibited apoptosis at the early stage of infection and induced the apoptotic cell deaths at the late stage of infection for its own replication. \(^{21,22}\)

The interaction between autophagy and apoptosis has been shown to involve in influenza A virus infection. Influenza A virus-infected apoptotic cell deaths were significantly enhanced in the autophagy-deficient cells; on the other hand, influenza A virus encodes PB1-F2 that could indirectly suppress autophagy by regulating M2 and promoting apoptosis at the terminal stage of infection. \(^{23,24}\)

PRRSV, a highly mutable virus, causes acute respiratory disease and reproduction failure in pigs with complex mechanisms of pathogenesis, resulting in unavailability of reliable vaccines and lesser success for preventing PRRSV infection. Recently, a number of evidences have shown that PRRSV replication was able to be regulated by autophagy and apoptosis in order to promote viral replication through its replication complex or regulating mitochondria-mediated pathway and so on. \(^{25,26}\) Therefore, the understanding of the molecular processes contributing to autophagy and apoptosis and investigation of the mechanisms mediating the cross-talk between autophagy and apoptosis in PRRSV infection are necessary.

In this present paper, we determined that cells autophagy was induced in MARC-145 cells upon infection with PRRSV, while cell apoptosis could be induced at the later stage of viral infection, indicating that there is a time difference between PRRSV-induced autophagy and apoptosis. Moreover, our results also showed decreased autophagosomes and increased chromatin condensation and nuclear fragmentation during the late infection phase (48h post-infection), and the expression of LC3 \(^{yy}\) and anti-apoptotic protein Bcl-xl significantly decreases at 48hpi. It has been previously reported that Autophagy sustains PRRSV replication and it stimulates anti-apoptotic pathways at the early stage of infection where PRRSV proteins are involved in apoptotic inhibition. \(^{27,28}\) It may provide a potential mechanism where PRRSV exploits autophagy induction to delay apoptotic cell death for PRRSV replication. To claim the role of autophagy in PRRSV-regulated apoptosis in MARC-145 cells, we carried out the inhibition of autophagy using the inhibitor 3-MA and Beclin1 siRNA to investigate the level of apoptosis. The results in our study demonstrated that inhibition of autophagy increased the level of apoptotic cells and the cleavage of caspase3, suggesting that inhibition of autophagy affects PRRSV-induced apoptosis.

The BH3-only pro-apoptotic protein Bad promotes cell death by binding and inactivating the survival function of Bcl-xl and BCL2, \(^{2,29}\) while Bad phosphorylation inactivates its apoptotic function through binding to 14-3-3 scaffold proteins or activation of Bad kinases including ERK1/2 and Akt signaling pathway. \(^{30-33}\) In addition, Bad overexpression promoted the autophagy level in the absence of nutrient starvation, whereas Bad depletion had no or little effect on rapamycin-induced autophagy. \(^{34}\) Another report showed that rapamycin enhanced phosphorylation of Bad at serine 112 and 136 in human lung cancer cells to enhance Bad/14-3-3 interaction and reduces Bad/Bcl-XL binding, \(^{35}\) suggesting that Bad can also contribute to regulate cell autophagy in various conditions.

Our results show that PRRSV infection upregulates Bad and Beclin1 expression at the transcriptional level, while the expression of Bad protein reveals changes during the PRRSV replication cycle in MARC-145 cells. The punctate accumulation at the round of PRRSV replication site in the cytosol, compared with mock-infection cells where Bad was diffusely localized in the mitochondrial membrane, consistent with the research. \(^{36}\) These results raise a question as to whether Bad is involved in PRRSV-induced autophagy. The knockdown assays and immunofluorescent staining utilized in the present study demonstrate the increase in expression of LC3-II and punctate accumulation of LC3 by Bad knockdown in PRRSV infected cells as compared to SiBeclin1 (SiNC) treated cells which shows that Bad is able to regulate PRRSV-induced autophagy in MARC-145 cells. It has been shown that Beclin1 is an autophagy protein with a BH3-only domain and can participate in regulating autophagy and apoptosis, \(^{37,39}\) we observed co-immunoprecipitation between endogenous Beclin1 and Bcl2 or Bad in PRRSV infected MARC-145 cells where Bad was found to co-localize with Beclin1 through confocal microscopy analysis, which suggests that not only the dissociation of Beclin1/Bcl2 is important for PRRSV-induced autophagy but Bad and Beclin1 complex may also play a role in PRRSV infection. In addition, the interaction between Bax and Beclin1 reduced in PRRSV infection may lead to reveal that Bax is able to reduce autophagosome formation. \(^{40,41}\) Our results also showed that Bad phosphorylation (S112) could be enhanced during PRRSV infection. Previous reports have shown

Figure 5. (See previous page) Bad regulated PRRSV-induced autophagy in MARC-145 cells. (A) The knockdown efficiency of Beclin 1 protein was detected in untreated control cells (MOCK), control RNAi-treated cells (NC), and beclin 1-silenced cells (sibad-1 or sibad-2) 48h after transfection by protein gel blot analysis. The β-actin as control was used to confirm equal loading of protein. (B) The silencing of Bad reduced the cleavage of caspase3 while increasing the expressing of LC3\(^{yy}\). (C) Confocal fluorescence microscopy analysis of LC3 punctate and PRRSV replication in Bad and Beclin1 knockdown. The wild type cells (MOCK) and RNAi-transfected cells (SiNC, sibad and sibeclin1) were infected with PRRSV at MOI of 1. At 36h post-infection, cells were fixed and permeabilized. LC3 was stained with rabbit anti-LC3 antibody (green) and PRRSV NSP2 was with stained mouse anti-NSP2 antibody (red). The nuclei were stained with DAPI (blue).
that pro-survival Bcl2 family members affect autophagy only indirectly by inhibiting Bax and Bak, indicating that Bcl2 and/or Bcl-xl that directly bind to the BH3 domain of Beclin1 may not inhibit autophagy; on the other hand, Bad phosphorylation has a pro-survival function and reduces the binding of Bad and BCL2/Bcl-xl. PRRSV infection triggers Bad phosphorylation and results in regulating the interaction between Bad and Beclin1. Akt and ERK1/2 have been reported to function as physiologic Bad kinases and were required for PRRSV infection, indicating that the interaction among Bad, Beclin1, Akt and ERK1/2 signaling pathway plays an important role for PRRSV infection.

In summary, our data demonstrate that autophagy can delay PRRSV-induced apoptosis in MARC-145 cells and Bad can participate in regulating PRRSV-induced autophagy via interaction with Beclin1. Our findings have established that PRRSV induces autophagy, which in turn affects Bad expression and or phosphorylation to promote PRRSV replication. Our understanding as to whether PRRSV regulates Bad phosphorylation in other sites and how Bad phosphorylation affects Beclin1 and PRRSV-induced autophagy is still insufficient. Further investigation on the cross-talk between Bad, autophagy and PRRSV will greatly improve our understanding of the immune escape of PRRSV that might provide insights for the development of novel antiviral therapies against PRRSV infection.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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