Arch Virol (2014) 159:2091–2096
DOI 10.1007/s00705-014-2016-4

BRIEF REPORT

Role of phosphatidylinositol 3-kinase (PI3K) and Akt1 kinase in porcine reproductive and respiratory syndrome virus (PRRSV) replication

Xiuqing Wang · Hanmo Zhang · Alex M. Abel · Alan J. Young · Liji Xie · Zhixun Xie

Received: 29 July 2013 / Accepted: 2 February 2014 / Published online: 18 February 2014

Abstract We have previously reported that inhibition of phosphatidylinositol 3-kinase (PI3K) reduces porcine reproductive and respiratory syndrome (PRRSV) replication. Here, we further investigate the mechanism by which PI3K inhibition affects virus replication and the role of Akt1 kinase in virus replication. We found that PI3K inhibition reduced viral gene transcription by approximately 1.5-fold. Accordingly, viral protein synthesis was significantly reduced by PI3K inhibition. Interestingly, cells overexpressing the dominant negative mutant Akt1 exhibited a significant reduction in viral gene transcription compared to cells overexpressing the constitutively active Akt1. Viral protein synthesis was also enhanced in cells overexpressing the constitutively active Akt1. Overall, our data show that both PI3K and Akt1 play a role in viral gene expression, leading to an increase in virus replication.

Keywords Porcine reproductive and respiratory syndrome virus (PRRSV) · Phosphatidylinositol-3-kinase (PI3K) · Akt1 · Viral gene transcription · Viral protein synthesis

H. Zhang contributed equally to the work.

X. Wang (✉) · H. Zhang · A. M. Abel
Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57007, USA
e-mail: xiuqing.wang@sdstate.edu

A. J. Young
Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD 57007, USA

L. Xie · Z. Xie
Guangxi Veterinary Research Institute, 51 Youai North Road, Nanning, Guangxi 530001, People's Republic of China

Viruses are obligate intracellular pathogens and absolutely depend on the host cells' machineries for their survival and reproduction. Thus, it is not surprising that all viruses, including genetically simple RNA viruses, have evolved elaborate mechanisms to manipulate the host's various signaling pathways for their own benefit. One of the relatively well-characterized pathways that a number of viruses have been shown to utilize to enhance their replication and pathogenesis is the phosphatidylinositol 3-kinase (PI3K)-dependent Akt (PI3K/Akt) pathway.

The PI3K/Akt pathway is involved in many cellular processes, such as cell proliferation, differentiation, and survival [6]. PI3K can be activated by the binding of autophosphorylated tyrosine kinase receptors or non-receptor tyrosine kinases to the Src homology (SH2) domain of its regulatory/adaptor subunit p85. Activated PI3K phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP3), which serves as a second messenger by recruiting Akt to the plasma membrane [4, 6, 9]. Akt is subsequently phosphorylated by phosphoinositide-dependent kinases 1 and 2 (PDK1 and PDK2). Activated Akt can further activate a number of different signal transduction proteins to regulate a variety of cellular functions. For example, activated Akt can activate Rac1, which is involved in vesicular trafficking and endocytosis activities by modulating actin polymerization. Activated Akt can activate anti-apoptotic signal proteins such as Bcl-2 and inhibit pro-apoptotic proteins such as p53 and caspases to promote cell survival. Activated Akt can also activate Ras/Raf/MEK/Erk signal pathways to promote protein synthesis, cell proliferation, and differentiation processes. Akt can activate glycogen synthesis through inhibition of GSK-3, protein synthesis via mTOR, and downstream factors such as 4E-BP1 [6]. Overall, the PI3K/Akt pathway contributes significantly to cellular functions.

A number of DNA and RNA viruses are capable of activating the host's PI3K/Akt pathway. The role of PI3K/Akt in viral entry and/or early replication processes has been reported for many viruses [3, 8, 9, 17–19]. For example, the PI3K/Akt pathway has been shown to control the cellular entry of Ebola virus by activating Rac1, a Rho family GTPase, and its downstream effectors [17]. Others have suggested a role of PI3K activation in directing virus trafficking to late endocytic compartments for the uncapping and virus replication process [3]. Furthermore, PI3K/Akt activation appears to be critical for the efficient transcription and translation of viral genes of influenza A virus, vaccinia virus, and other viruses [9, 18, 19].

Porcine reproductive and respiratory syndrome virus (PRRSV) is a plus-sense, single-stranded RNA virus with a genome length of approximately 15 kb. PRRSV is a member of the family *Arteriviridae* in the order *Nidovirales*. The primary target cells for PRRSV are alveolar macrophages and dendritic cells [12, 21]. PRRSV replicates rapidly in these susceptible cells and completes its initial replication cycle in approximately 8–12 h. Virus replication in susceptible cells invariably causes apoptosis of infected cells via both intrinsic and extrinsic pathways as well as oxidative stress [10, 14, 21]. Recent studies suggest that PRRSV activates the PI3K/Akt pathway during early infection to possibly extend cell survival to facilitate virus replication [10, 13, 22]. However, one recent study showed that PI3K inhibition did not affect PRRSV replication in MARC-145 cells [23]. Here, we sought to further investigate the role of PI3K and Akt1 in viral gene transcription, viral protein synthesis, and virus replication in MARC-145 cells.

MARC-145 cells grown in DMEM + GlutaMAX (Invitrogen) medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin were used throughout the study. PRRSV-23983 was grown in MARC-145 cells, and the titer was determined using the $TCID_{50}$/ml assay. To determine whether PI3K inhibition affects viral gene transcription, we pretreated MARC-145 cells with a PI3K-specific inhibitor, LY294002 (Cell Signaling Technologies), at a concentration of 50 μM or the same volume of DMSO for 1 h prior to PRRSV infection. The dose of LY294002 we used was not toxic to the cells but efficiently inhibited PI3K, as evidenced by the Akt phosphorylation profile (data not shown). At 24 h postinfection, cells were harvested and subjected to total RNA extraction. Real-time RT-PCR was performed to quantify the viral gene transcript after normalization with the beta-actin gene as described previously [11]. Primers for the beta-actin gene and the PRRSV glycoprotein 4 (Gp4), membrane protein (M), and nucleocapsid (N) genes were designed using the PrimerQuest ^{SM} program at Integrated DNA Technologies (IDT Inc., Coralville, IA) based on the beta-actin sequence (GenBank accession no. CB018065) and the PRRSV-2332 sequence (GenBank accession no. AY150564). Primer sequences are available upon request. Each sample was run in duplicate, and experiments were repeated at least three times. Fold changes were calculated based on the ΔΔCt method as described previously [11] using the DMSO-treated group as a reference. As shown in Figure 1A, an average of an approximately 1.5-fold decrease in PRRSV Gp4 gene transcript was observed in the LY294002-treated groups compared to the DMSO-treated groups ($p > 0.05$).

To further investigate whether viral protein synthesis is also reduced by PI3K inhibition, we pretreated MARC-145 cells with a PI3K specific inhibitor, LY294002, at a concentration of 50 μM or the same volume of DMSO for 1 h prior to PRRSV infection. At 24 h postinfection, cells were harvested, and cell lysates were used in western blot analysis to detect viral protein expression. PRRSV-positive serum samples were used as a primary PRRSV-specific polyclonal antibody. HRP-labeled anti-swine IgG was used as a secondary antibody conjugate to detect PRRSV proteins. The expression of beta-actin detected by a monoclonal antibody to beta-actin (Sigma) and HRP labeled anti-mouse IgG was included as a loading control. Proteins were visualized by ECL detection (GE Biosciences) and detected by using the Bio-Rad ChemiDoc XRS Imaging System (Bio-Rad). Image analysis was performed using QuantityOne analysis software (Bio-Rad). Fold changes were calculated after normalization with beta-actin using the DMSO treatment group as a reference. The results showed that PI3K inhibition deceased viral membrane (M) protein synthesis compared to DMSO-treated cells by an average of 2.6-fold after normalization with beta-actin protein (Fig. 1B). A similar reduction in viral nucleocapsid protein (N) expression was observed (data not shown). Since PI3K inhibition reduced the cell proliferation rate (data not shown), we speculate that the reduced viral gene transcription and translation could possibly be due to the decreased cell proliferation as a result of PI3K inhibition. We have also observed that PI3K inhibition significantly reduced the intracellular virus titer compared to that of the DMSO group (Fig. 1C, $p < 0.05$).

Since Akt is one of the downstream targets of PI3K that is known to be involved in virus replication, we investigated whether manipulation of Akt1 directly affects viral gene transcription and translation. To accomplish this, we obtained a set of plasmids that express either a wild-type Akt1 (pLNCX HA Akt1, Addgene plasmid # 9004), a dominant negative mutant Akt1 (pLNCX HA Akt1 K179M, Addgene plasmid # 9007), or a constitutively active Akt1 (pLNCX myr HA Akt1, Addgene plasmid # 9005). All plasmids were gifts from Dr. William Sellers [16]. Briefly, 4×10^5 MARC-145 cells were plated in each

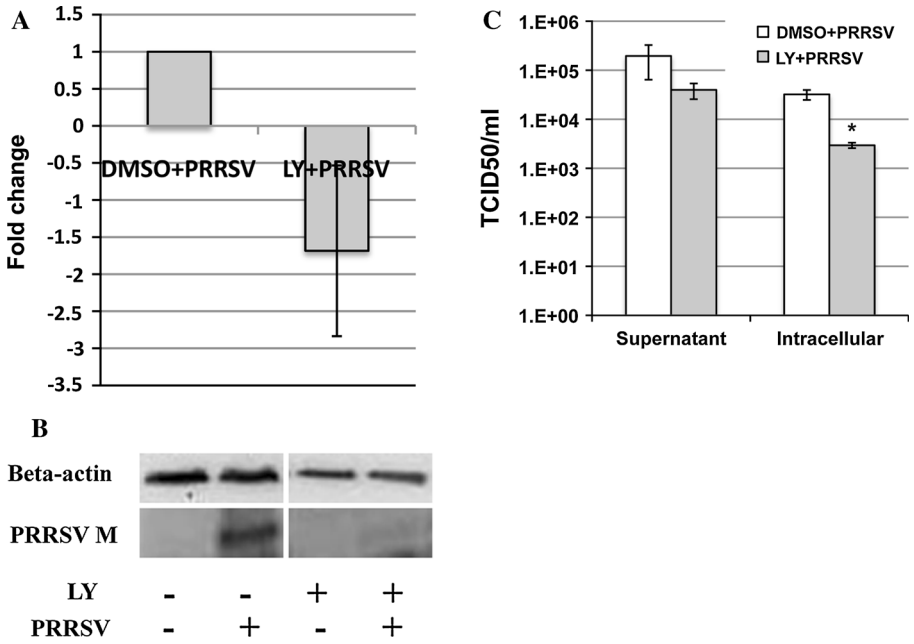

Fig. 1 PI3K inhibition reduces viral gene transcription, translation, and virus replication. **A**. The amount of glycoprotein 4 (Gp4) gene transcript in the presence or absence of LY294002, a PI3K specific inhibitor, was determined by real-time RT-PCR after normalization with the housekeeping gene, beta-actin. The average fold change in six individual experiments and its standard deviation are shown. **B**. Western blot analysis of viral protein synthesis in LY-treated cells. LY, LY294002 treatment group; DMSO, DMSO treatment group. One representative western blot is shown. The experiment was repeated three times, and similar results were obtained. **C**. Intracellular and supernatant virus titers in the LY294002- and DMSO-treated groups. The averages and standard deviations of three experiments are shown. * indicates a significant difference in intracellular virus titers between the DMSO- and LY294002-treated groups ($p < 0.05$)

well of a 6-well plate the night before transfection. Cells were transfected with pLNCX HA Akt1, pLNCX HA Akt1 K179M or pLNCX myr HA Akt1 using Lipofectamine 2000 transfection reagent (Invitrogen). At 24 h post-transfection, cells were infected with 1 MOI of PRRSV-23983. At 24 h postinfection, cells were harvested and subjected to total RNA extraction. Real-time RT-PCR was performed to quantify the viral gene transcript after normalization with the beta-actin gene. Fold changes were calculated based on the ΔΔCt method as described previously [11] using the group transfected with the wild-type Akt1 plasmid as a reference. As shown in Fig. 2A, cells expressing the dominant negative mutant Akt1 K179M had a significant decrease in viral Gp4 gene transcription compared to the ones expressing the dominant active myr HA Akt1 ($p < 0.01$). A similar level of decrease in viral M and N gene transcription was observed in cells expressing the dominant negative mutant Akt1 K179M (data not shown). An average of 1.88-fold increase in viral Gp4 gene transcription was observed in the myr HA Akt1 group compared to the wild-type HA Akt1 group ($p > 0.05$). A comparable level of viral M and N gene transcription was increased in the myr HA Akt1 group (data not shown). Overall, the results suggest that Akt1 plays a much larger role than PI3K in regulating viral gene transcription. Since PI3K has multiple targets, it is possible that some of its targets may have opposing effects on viral gene transcription. Alternatively, Akt1 may be modulated by other kinases in addition to PI3K. This needs to be investigated further in future studies.

To further determine whether manipulation of Akt1 affects viral protein synthesis, we transfected MARC-145 cells with pLNCX HA Akt1, pLNCX HA Akt1 K179M, or pLNCX myr HA Akt1. At 24 h after transfection, cells were infected with PRRSV-23983 at an MOI of 1. Viral protein synthesis was examined at 24 h after virus infection by western blot analysis using PRRSV-positive serum. For detection of Akt and phosphorylated Akt, a HA-tag-specific mouse monoclonal antibody (Sigma) and a phospho-Akt (Ser473) rabbit antibody (Cell Signaling Technologies) were used as the primary antibodies followed by detection with IRDye® 800CW-conjugated goat anti-mouse IgG and IRDye® 680 donkey anti-rabbit IgG (Li-Cor Biosciences), respectively. Images were scanned and captured using an Odyssey Li-Cor Scanner (Li-Cor Biosciences). Image analysis was performed using QuantityOne analysis software (Bio-Rad). Fold changes were calculated after normalization with beta-actin using wild-type Akt1 as a reference. As shown in Fig. 2B, cells overexpressing the constitutively activated Myr-Akt1 showed a significant increase in their p-Akt/Akt ratio ($p < 0.01$) and enhanced viral protein synthesis compared to the dominant negative

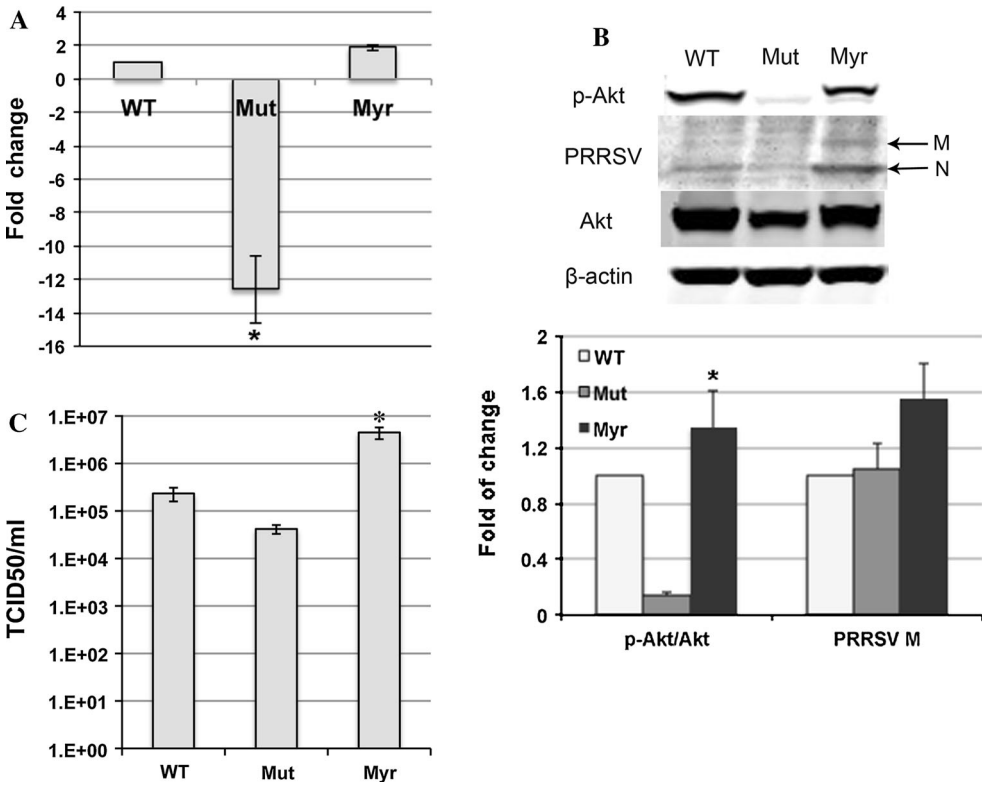

Fig. 2 Akt1 exhibits a great impact on viral gene transcription but has little effect on viral protein synthesis. **A**. Overexpression of dominant negative mutant Akt1 significantly reduced viral gene transcription. The amount of glycoprotein 4 (Gp4) gene transcript in cells expressing wild-type Akt1 (pLNCX HA Akt1), mutated Akt1 (pLNCX HA Akt1 K179M), and the myristoylated Akt1 (pLNCX myr HA Akt1) was determined by real-time RT-PCR after normalization with the housekeeping gene, beta-actin. The average fold changes of three individual experiments and their standard deviations are shown. WT, cells transfected with a plasmid containing wild-type Akt1 (pLNCX HA Akt1); Mut, cells transfected with a plasmid containing a mutated Akt1 (pLNCX HA Akt1 K179M); Myr, cells transfected with a plasmid containing the myristoylated Akt1 (pLNCX myr HA Akt1). * indicates a significant difference between the Mut and Myr groups ($p < 0.01$). **B**. Overexpression of constitutively activated Akt1 enhances viral protein synthesis. One representative western blot is shown. The experiment was repeated three times, and similar results were obtained. * indicates a significant difference in the pAkt/Akt ratios between the Myr and Mut groups and the Mut and WT groups ($p < 0.01$). **C**. Overexpression of constitutively activated Akt1 promotes virus replication in cells. Virus titers of the supernatants at 24 h after virus infection were determined using the $TCID_{50}$ assay. The averages and standard deviations of three experiments are shown. * indicates a significant difference between Myr and WT and between Myr and Mut ($p < 0.05$)

mutant Akt1 ($p > 0.05$). Surprisingly, only a small change in viral protein synthesis was observed between the wild-type Akt1 and dominant negative mutant Akt1 groups despite the significant difference in their p-Akt/Akt ratios ($p < 0.01$). We also examined the virus titers in the supernatants of the three treatment groups. As expected, the virus titer in cells expressing constitutively active Akt1 was significantly higher than in transfected cells expressing mutant and wild-type Akt1 ($p < 0.05$). Cells expressing the wild-type Akt1 showed significantly higher virus titers than cells expressing mutant Akt1 ($p < 0.05$) (Fig. 2C).

Viruses have evolved to manipulate the host's various signaling pathways for their own survival and reproduction in host cells. Simultaneously, host cells sense the danger posed by viruses by a variety of means and mount a multitude of innate defense mechanisms against viruses. The delicate balance between the two ultimately determines the fate of viruses and their host cells. PRRSV is a small RNA virus with a non-segmented, plus-sense RNA genome. It efficiently infects and destroys both alveolar macrophages and dendritic cells with little or no type I interferon induction [1, 5, 15, 21]. This suggests that PRRSV must have evolved strategies to manipulate the host's signaling pathways for its own survival and replication and to antagonize the host's innate defense mechanisms, such as type I interferon production. Although several recent studies have examined the role of viral nonstructural proteins in inhibiting the transcription and/or induction of type I interferon by monitoring the promoter activity of interferon-beta in cell culture systems that overexpress viral nonstructural proteins [2, 7, 20], very little is known about the interaction between PRRSV and the host's various signaling pathways in its naturally susceptible cells and how these interactions may ultimately affect virus

replication and the host's defense mechanisms, such as type I interferon production. We and other researchers have recently shown that PRRSV modulates the PI3K/Akt pathway [13, 22, 23]. However, the role of PI3K and Akt in PRRSV replication is controversial. In the present study, we further explored the effect of PI3K inhibition and Akt1 on viral gene transcription, viral protein synthesis, and virus replication. Our data suggest that PI3K inhibition significantly reduces viral protein synthesis (Fig. 1). Viral gene transcription was also reduced by PI3K inhibition. In contrast, Akt1 appears to play a significant role in viral gene transcription since cells overexpressing the dominant negative mutant Akt1 exhibited a significant decrease in viral gene transcription compared to cells overexpressing the constitutively active Akt1 (Fig. 2A). Enhanced viral protein synthesis was also observed in cells overexpressing the constitutively active Akt1. Surprisingly, a small change in viral protein synthesis was observed between cells overexpressing the dominant negative Akt1 and wild-type Akt1 (Fig. 2B). It is clear that PI3K and Akt1 exhibit different patterns in modulating PRRSV gene expression. Future studies are needed to further understand the relationship between the two kinases during PRRSV infection. Overall, our data suggest that both PI3K and Akt1 regulate viral gene expression, leading to increased virus replication.

An earlier study showed that PI3K inhibition had little effect on PRRSV replication [23]. This discrepancy in the results could be due to different cell culture conditions or different virus strains or different doses of PI3K-specific inhibitor used in the two studies. In the earlier study [23], the researchers used serum-starved MARC-145 cells, a highly virulent PRRSV strain, and a lower concentration (10 μM) of LY294002. Additionally, in the earlier study, the results were not verified using cells overexpressing mutated Akt1 and constitutively active Akt1. Taken together, we have presented evidence for the role of PI3K and Akt1 in facilitating the PRRSV replication process. Our future studies will be focused on elucidating the detailed mechanisms by which PI3K and Akt1 modulate viral gene expression and type I interferon induction during PRRSV infection.

Acknowledgements We thank Dr. Eric Nelson for providing PRRSV-positive pig serum and FITC-SDOW17 antibody. This study was supported by grants from USDA NRI (2009-35204-050790) and by the South Dakota Agricultural Experiment Station.

References

1. Albina E, Carrat C, Charley B (1998) Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. J Interf Cytokine Res 18:485–490
2. Beura LK, Sarkar SN, Kwon B, Subramaniam S, Jones C, Pattnaik AK, Osorio FA (2010) Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. J Virol 84:1574–1584
3. Brabec M, Blaas D, Fuchs R (2006) Wortmannin delays transfer of human rhinovirus serotype 2 to late endocytic compartments. Biochem Biophys Res Commun 348:741–749
4. Briand G, Barbeau B, Tremblay M (1997) Binding of HIV-1 to its receptor induces tyrosine phosphorylation of several CD4-associated proteins, including the phosphatidylinositol 3-kinase. Virology 228:171–179
5. Buddaert W, Van Reeth K, Pensaert M (1998) In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV). Adv Exp Med Biol 440:461–467
6. Cantley LC (2002) The phosphoinositide 3-kinase pathway. Science 296:1655–1657
7. Chen Z, Lawson S, Sun Z, Zhou X, Guan X, Christopher-Hennings J, Nelson EA, Fang Y (2010) Identification of two autocleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. Virology 398:87–97
8. Chugh P, Bradel-Tretheway B, Monteiro-Filho CM, Planelles V, Maggirwar SB, Dewhurst S, Kim B (2008) Akt inhibitors as an HIV-1 infected macrophage-specific anti-viral therapy. Retrovirology 5:11
9. Cooray S (2004) The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction in virus survival. J Gen Virol 85:1065–1076
10. Costers S, Lefebvre DJ, Delputte PL, Nauwynck HJ (2008) Porcine reproductive and respiratory syndrome virus modulates apoptosis during replication in alveolar macrophages. Arch Virol 153:1453–1465
11. Guo X, Rosa AJ, Chen DG, Wang X (2008) Molecular mechanisms of primary and secondary mucosal immunity using avian infectious bronchitis virus as a model system. Vet Immunol Immunopathol 121:332–343
12. Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Andrews JJ, Lum MA, Rathje JA (1996) Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 33:159–170
13. Huo Y, Fan L, Yin S, Dong Y, Guo X, Yang H, Hu H (2013) Involvement of unfolded protein response, p53 and Akt in modulation of porcine reproductive and respiratory syndrome virus-mediated JNK activation. Virology 444:233–240
14. Lee SM, Kleiboeker SB (2007) Porcine reproductive and respiratory syndrome virus induces apoptosis through a mitochondria-mediated pathway. Virology 365:419–434
15. Miller LC, Laegreid WW, Bono JL, Chitko-McKown CG, Fox JM (2004) Interferon type I response in porcine reproductive and respiratory syndrome virus-infected MARC-145 cells. Arch Virol 149:2453–2463
16. Ramaswamy S, Nakamura N, Vazquez F, Batt DB, Perera S, Roberts TM, Sellers WR (1999) Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc Natl Acad Sci USA 96:2110–2115
17. Saeed MF, Kolokoltsov AA, Freiberg AN, Holbrook MR, Davey RA (2008) Phosphoinositide-3 kinase-Akt pathway controls cellular entry of Ebola virus. PLoS Pathog 4:e1000141
18. Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y (2007) Effect of the phosphatidylinositol 3-kinase/Akt pathway on influenza A virus propagation. J Gen Virol 88:942–950
19. Soares JA, Leite FG, Andrade LG, Torres AA, De Sousa LP, Barcelos LS, Teixeira MM, Ferreira PC, Kroon EG, Souto-Padron T, Bonjardim CA (2009) Activation of the PI3K/Akt

pathway early during vaccinia and cowpox virus infections is required for both host survival and viral replication. J Virol 83:6883–6899
20. Song C, Krell P, Yoo D (2010) Nonstructural protein 1alpha subunit-based inhibition of NF-kappaB activation and suppression of interferon-beta production by porcine reproductive and respiratory syndrome virus. Virology 407:268–280
21. Wang X, Eaton M, Mayer M, Li H, He D, Nelson E, Christopher-Hennings J (2007) Porcine reproductive and respiratory syndrome virus productively infects monocyte-derived dendritic cells and compromises their antigen-presenting ability. Arch Virol 152:289–303
22. Zhang H, Wang X (2010) A dual effect of porcine reproductive and respiratory syndrome virus replication on the phosphatidylinositol-3-kinase-dependent Akt pathway. Arch Virol 155:571–575
23. Zhu L, Yang S, Tong W, Zhu J, Yu H, Zhou Y, Morrison RB, Tong G (2013) Control of the PI3K/Akt pathway by porcine reproductive and respiratory syndrome virus. Arch Virol 158(6):1227–1234