Role of zinc metalloprotease (ZMPSTE24) in porcine reproductive and respiratory syndrome virus (PRRSV) replication in vitro

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Abstract
The transmembrane zinc metalloprotease ZMPSTE24 works in cooperation with interferon-induced transmembrane protein 3 (IFITM3) to restrict entry of several enveloped viruses. We investigated the role of ZMPSTE24 in porcine reproductive and respiratory syndrome virus (PRRSV) replication. ZMPSTE24 overexpression significantly reduced PRRSV replication in MARC-145 cells. Interestingly, knockdown of endogenous ZMPSTE24 did not significantly impact virus replication. There was no significant difference in the percentage of PRRSV-positive cells and viral RNA copies at 3 hours postinfection (hpi) between cells transfected with ZMPSTE24-FLAG and the vector control. Our results suggest that ZMPSTE24 overexpression may restrict PRRSV replication at a post-entry step.

Restriction factors such as interferon-stimulated genes (ISGs) inhibit viruses at various stages of their life cycle [14]. Many enveloped viruses enter host cells by receptor-mediated endocytosis. Triggered by the acidic pH within endosomes, the viral fusion protein undergoes a conformational change and facilitates virus fusion with the endosome membrane and uncoating. Several pH-sensitive viruses that depend on low pH for fusion have been shown to be strongly inhibited by IFITM3 [15]. IFITM3 is localized in the early or late endosomes and impedes virus-host membrane fusion, thereby blocking release of the genome [1, 3]. The restriction factor ZMPSTE24 works in cooperation with the IFITM3 protein to restrict entry of several enveloped viruses [5, 10, 16]. ZMPSTE24 acts as a downstream effector of IFITM3 [5, 10]. The ZMPSTE24 protein is constitutively expressed and localized to the inner nuclear membrane [2]. It is important in the processing of the precursor prelamin A to lamin A in the nuclear lamina [2]. A defect in ZMPSTE24 has been shown to enhance accumulation of prelamin A [4]. Currently, there are very limited studies exploring the mechanism by which it restricts virus entry, including its interaction with the IFITM proteins and IFITM-independent virus restriction.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-sense, single-stranded RNA virus belonging to the family Arteriviridae within the order Nidovirales [12]. PRRSV causes severe respiratory disease in young pigs and reproductive failure in sows [12]. Upon attachment and internalization, PRRSV enters into the early endosome in clathrin-coated vesicles, and virus-endosome membrane fusion leads to virus uncoating [17, 19]. The role of IFITM3 in restricting PRRSV replication has been reported recently [18]. However, very little is known about whether ZMPSTE24 also restricts PRRSV replication. In this present study, we investigated the role of ZMPSTE24 in PRRSV replication and examined whether ZMPSTE24 restricts PRRSV entry into cells.

To investigate the effect of ZMPSTE24 on PRRSV replication, we first employed a ZMPSTE24 overexpression system. ZMPSTE24 overexpression has been shown to significantly reduce influenza A virus (IAV) infection in A549 cells [5]. Briefly, MARC-145 cells cultured in a 12-well plate were transfected with 1.25 µg per well of either pCMV-3Tag-8 control vector or FLAG-tagged ZMPSTE24 cloned into pCMV-3Tag-8 (ZMPSTE24-FLAG) plasmid [5] using a Lipofectamine 3000 Transfection Kit (Invitrogen). Cells were incubated in complete medium (Dulbecco’s modified...
but not in the vector control pCMV-3Tag-8-transfected cells expressing ZMPSTE24-FLAG. ZMPSTE24 protein was detected in cells transfected with pCMV-3Tag-8 plasmid using Odyssey Infrared Imaging System. As shown in Fig. 1A, the IRDye-conjugated secondary Ab (LI-COR, catalog no. 926-32210) diluted to 1:15000 and observed using a LI-COR Odyssey Infrared Imaging System. As shown in Fig. 1A, ZMPSTE24 protein was detected in cells transfected with the pCMV-3Tag-8 plasmid expressing ZMPSTE24-FLAG but not in the vector control pCMV-3Tag-8-transfected cells. Fifty-percent tissue culture infectious dose (TCID₅₀) assay was performed to determine the infectious PRRSV SD-23983 titer in the supernatant [9]. An average decrease of 146-fold (2.16 log reduction) in virus titer was observed in ZMPSTE24-expressing cells (Fig. 1B). To confirm the antiviral activity of ZMPSTE24, MARC-145 cells were transfected with 0.6 μg of each plasmid in a 24-well plate and infected with PRRSV SD-23983 for 24 h as described earlier. All wells were fixed with 80% acetone and incubated at 37°C for 1 h with 1:80-diluted FITC-conjugated anti-PRRSV Ab specific for the nucleocapsid (N) protein (SDOW-17). The images were captured at 10x magnification using an Olympus IX70 inverted microscope (epifluorescence and bright field). Immunofluorescence staining showed fewer virus-positive-staining (green) cells among the ZMPSTE24-overexpressing cells when compared to the vector control transfected cells (Fig. 1C). To determine the relative abundance of PRRSV infection, cells were harvested and analyzed further by flow cytometry. A significant reduction in mean fluorescence intensity (p < 0.05) was observed in ZMPSTE24-overexpressing cells when compared to the vector control cells at 24 hpi (Fig. 1D). To test if cell viability played a role in ZMPSTE24-mediated virus restriction, a cytotoxicity assay was performed. MARC-145 cells cultured in a 96-well plate were transfected with 0.3 μg of either the vector control pCMV-3Tag-8 or ZMPSTE24-FLAG for 72 h. Each well was treated with 10 μl of CCK-8 solution (Sigma, cat. no. 96992) and incubated at 37°C for 3 h, and absorbance was measured at 450 nm using a Synergy 2 plate reader (Bio-Tek). No significant difference (p > 0.05) in cell viability was observed between the vector control and ZMPSTE24-transfected cells (Fig. 1E). Overall, these results suggest that overexpression of ZMPSTE24 reduces PRRSV replication.

To confirm the role of ZMPSTE24 in restricting PRRSV replication, we employed silencing-RNA-mediated knockdown of endogenous ZMPSTE24. Briefly, MARC-145 cells cultured in a 12-well plate were transfected with either negative control siRNA (Ambion, cat. no. AM4642) or ZMPSTE24 siRNA (Life Technologies, siRNA ID# s20067) at a final concentration of 30 nM for 72 h using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). The cells in each well were then infected with PRRSV SD-23983 at an MOI of 1 for 24 h. Total RNA was extracted from cells, and qRT-PCR was performed to determine the ZMPSTE24 gene knockdown efficiency and to quantify the PRRSV gene transcript. Primer sequences specific to the beta-actin, PRRSV N, and ZMPSTE24 genes used in the study are available upon request. The mean fold change in gene expression was calculated using the ΔΔCT method [11]. Beta-actin was used as a reference gene for comparing the fold change between two treatment groups [11]. As shown in Fig. 2A, an average of 74% knockdown of the endogenous ZMPSTE24 gene was achieved. An average of 1.2-fold increase in PRRSV N gene transcript relative to the silencing control was observed (Fig. 2B). Surprisingly, virus titers in the supernatants of ZMPSTE24 knockdown cells, as quantified by TCID₅₀ assay [9], showed a slight decrease relative to control silenced cells. The virus titers in the control groups were approximately 1.8-fold higher than in the ZMPSTE24 knockdown group (Fig. 2C). To test if siRNA-induced silencing affected cell viability, MARC-145 cells were transfected with either negative control siRNA or ZMPSTE24 siRNA (30 nM) for 72 h and subjected to a cytotoxicity assay. There was no significant difference (p > 0.05) in viability between cells transfected with negative control siRNA and those transfected with ZMPSTE24 siRNA (Fig. 2D). Overall, the results showed that silencing of endogenous ZMPSTE24 does not appear to have a significant impact on PRRSV replication. This observation could be due to the relatively low knockdown efficiency (74%) achieved in our hands, while previous studies used cells derived from gene-knockout animals [5]. Additionally, it is possible that transient silencing of ZMPSTE24 is not sufficient to mimic the cells from gene-knockout mice [5, 8].

Previous studies showed that ZMPSTE24 and IFITM3 work together to block entry of various enveloped viruses, including IAV and arenaviruses [5, 16]. To examine whether overexpression of ZMPSTE24 restricts PRRSV entry, MARC-145 cells cultured in two 8-chamber slides were transfected with 0.3 μg of each plasmid using the procedure described above. PRRSV SD-23983 at an MOI of 4 was added to all wells for 3 h, while an MOI of 1 was added to all wells for 24 h. Two slides were fixed in 3.7% formaldehyde at either 3 or 24 hpi and then incubated with 0.2% Triton X-100. The slides were then incubated overnight with mouse monoclonal Anti-FLAG primary antibody (9A3) (Cell Signaling Technology, cat. no. 8146S) at a 1:1600 dilution, followed by incubation for 1 h with Alexa Fluor 647–conjugated goat anti-mouse IgG secondary Ab (Abcam) at a 1:200 dilution.
Fig. 1 Overexpression of ZMPSTE24 in MARC-145 cells reduces PRRSV replication. The standard deviation and mean of three replicates are shown for each graph. (A) Western blot analysis showing the expression of ZMPSTE24 in cells transfected with the ZMPSTE24 expression plasmid. Lanes 1-3 were transfected with pCMV-3Tag-8 vector control (EV), while lanes 4-6 were transfected with pCMV-3Tag-ZMPSTE24-FLAG (Z). Beta-actin was used as a loading control. (B) Virus titers in the supernatants of PRRSV-infected cells at 24 hpi. *, p < 0.05. (C) Immunofluorescence staining of virus-infected cells at 24 hpi. The top panel shows a bright field image, and the lower panel shows PRRSV-positive staining. (D) Flow cytometry analysis of virus-infected cells at 24 hpi. *, p < 0.05. (E) CCK-8 assay to compare cell viability between vector control and ZMPSTE24-containing plasmid-transfected cells. No significant difference (p > 0.05) was observed.
dilution, and then with anti-PRRSV FITC-SDOW-17. Cells were observed using an Olympus Fluoview FV1200 Laser Scanning Confocal Microscope at 40x magnification. There were no significant differences (p > 0.05) in the number of PRRSV-positive cells or total viral RNA copies between the vector control and ZMPSTE24-overexpressing cells at 3 hpi (Fig. 3A and B). qRT-PCR showed an approximately 900-fold change in the level of the ZMPSTE24 mRNA transcript in ZMPSTE24-overexpressing cells compared to the vector control, confirming the successful transfection and overexpression of ZMPSTE24 (Fig. 3C). Our results are in indirect contrast to an earlier study that showed a dramatically lower number of IAV-virus positive cells compared to the vector control, which was observable as early as 2 hpi and persisted until 8 hpi [5]. This difference could be due to different virus entry mechanisms or differences in the replication efficiency of each virus in the respective cell culture model systems.

PRRSV completes one infectious cycle in about 8 h [18]. We observed that PRRSV colocalized with the early endosome marker EEA1 at 3 hpi, but most of the viral antigen had disappeared from early endosome at 6 hpi (data not shown). A few PRRSV particles colocalized with LAMP-1, a marker of the late endosome/lysosome, during the first 6 hours. Our data are consistent with one previous study showing a strong colocalization of PRRSV with EEA1 but not much with the late endosomal marker [17]. However, a more recent study showed colocalization of PRRSV with a late endosome marker [18]. Therefore, the existing data remain controversial regarding whether PRRSV enters cells through late endosomes. We observed extensive colocalization between ZMPSTE24 and PRRSV at 3 hpi (Fig. 3D), suggesting that virus entry into early endosomes was not disturbed by ZMPSTE24 overexpression. However, little or no colocalization between PRRSV and ZMPSTE24 was observed at 24 hpi (Fig. 3D), suggesting that restriction of PRRSV replication in cells with ZMPSTE24 overexpression likely occurred after 3 hpi. It is known that IFITM3 and ZMPSTE24 work together to trap enveloped viruses in the endolysosomal compartment for degradation [5, 10]. Future studies on colocalization of PRRSV with late endosome/lysosome marker (LAMP-1) in ZMPSTE24-overexpressing cells may provide more information on whether PRRSV is also degraded in late endosomes/lysosomes.
Fig. 3 Overexpression of ZMPSTE24 does not affect virus entry into MARC-145 cells. (A) Quantitative analysis of PRRSV-positive cells among ZMPSTE24-expressing cells and vector control cells at 3 hpi. The percentage of PRRSV-positive cells was determined by dividing the green (FITC-SDOW-17) stained cells by the total number of cells (DAPI stained, blue) in each microscopic field. The average and standard deviation of five different microscopic fields are shown. The percentage of red stained cells (Alexa 647, ZMPSTE24-expressing cells) that were positive for PRRSV was determined in a similar manner. Red and green co-stained cells were counted and divided by the total number of red-stained cells. No significant difference ($p > 0.05$) was observed between the vector control and the ZMPSTE24-transfected cells. (B) The relative fold change in viral RNA copies in ZMPSTE24-transfected cells when compared to vector control at 3 hpi as determined by qRT-PCR. The standard deviation and mean of three replicates are shown. (C) qRT-PCR showing the relative fold increase in the ZMPSTE24 mRNA level in the ZMPSTE24-transfected cells compared to vector control cells. The standard deviation and mean of three replicates are shown. (D) Colocalization of the PRRSV N protein with exogenous ZMPSTE24. Representative images show extensive colocalization of PRRSV N with ZMPSTE24 at 3 hpi and little or no colocalization of PRRSV N with ZMPSTE24 at 24 hpi. Green, PRRSV N protein; red, FLAG-tagged ZMPSTE24; blue, DAPI-stained nuclei.
We observed a difference in the morphology of virus-infected cells not expressing ZMPSTE24 and those overexpressing ZMPSTE24. Specifically, an increased number of cell-surface protrusions was observed in the vector control group at 3 hpi (data not shown). One possible explanation is that ZMPSTE24 overexpression may suppress the formation of cell-surface protrusions mediated by a small GTPase such as Rho [6, 7, 13]. Future investigation may clarify whether ZMPSTE24 also interferes with intercellular spread of PRRSV. Overall, the data suggest that overexpression of ZMPSTE24 does not significantly impact virus entry and may limit virus replication via a post-entry step. Although ZMPSTE24 has been shown to inhibit several RNA and DNA viruses that require pH-dependent fusion, it failed to inhibit murine leukemia virus [5]. Alternatively, ZMPSTE24 may also bind to other substrates for its antiviral activity [10]. The detailed mechanism by which overexpression of ZMPSTE24 restricts PRRSV replication needs to be examined in future studies.

In conclusion, overexpression of ZMPSTE24 reduced PRRSV infection in MARC-145 cells. There was no significant difference in the percentage of cells positive for PRRSV or the number of viral RNA copies in ZMPSTE24-overexpressing cells when compared to the vector control cells at 3 hpi. This suggests that ZMPSTE24 may inhibit PRRSV replication at a post-entry step. The role of endogenous ZMPSTE24 in PRRSV replication seems to be limited in our experimental model system and needs to be investigated further in future studies.

Author contributions XW designed the study, analyzed the data, and wrote the manuscript. PK conducted experiments, analyzed the results, and wrote the manuscript. SA conducted experiments and analyzed data. EN and SL provided reagents/analytic tools. MH contributed to confocal microscopy and analysis.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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