Role of Zinc metalloprotease (ZMPSTE24) on Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) replication in vitro

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1 **Role of Zinc metalloprotease (ZMPSTE24) on Porcine Reproductive and**
2 **Respiratory Syndrome Virus (PRRSV) replication *in vitro***

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30 **Abstract**

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

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42 *Key words: virus restriction factors, ZMPSTE24, Porcine Reproductive and*
43 *Respiratory Syndrome Virus (PRRSV)*

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59 Restriction factors such as interferon stimulated genes (ISGs) inhibit
60 viruses at various stages of their life cycle [14]. Many enveloped viruses enter
61 host cells by receptor mediated endocytosis. Triggered by acidic pH within
62 endosomes, the viral fusion peptide undergoes conformational change and
63 facilitates virus fusion with endosome membrane and uncoating. Several pH
64 sensitive viruses that depend on low pH for fusion have been shown to be strongly
65 inhibited by IFITM3 [15]. IFITM3 is localized in the early or late endosomes and
66 impedes virus-host membrane fusion, thereby blocking release of the genome [1,
67 3]. The restriction factor ZMPSTE24 works in cooperation with the IFITM3
68 protein to restrict entry of several enveloped viruses [5, 10, 16]. ZMPSTE24 acts
69 as a downstream effector of IFITM3 [5, 10]. The ZMPSTE24 protein is
70 constitutively expressed and localized to inner nuclear membrane [2]. It is
71 important in the processing of precursor prelamin A into lamin A in the nuclear
72 lamina [2]. Defect in ZMPSTE24 has been shown to enhance accumulation of
73 prelamin A [4]. Currently, there are very limited studies exploring the mechanism
74 by which it restricts virus entry including its interaction with the IFITM proteins
75 and IFITM independent virus restriction.

76 PRRSV is an enveloped, positive sense, single stranded RNA virus
77 belonging to the family *Arteriviridae* within the order *Nidovirales* [12]. PRRSV
78 causes severe respiratory disease in young pigs and reproductive failure in sows
79 [12]. Upon attachment and internalization, PRRSV enters into the early endosome
80 in clathrin-coated vesicles and virus-endosome membrane fusion leads to virus
81 uncoating [17, 19]. The role of IFITM3 in restricting PRRSV replication has been
82 recently reported [18]. However, very little is known whether ZMPSTE24 also
83 restricts PRRSV replication. In this present study, we investigated the role of

84 ZMPSTE24 in PRRSV replication and examined whether ZMPSTE24 restricts
85 PRRSV entry into cells.

86 To investigate the effect of ZMPSTE24 on PRRSV replication, we first
87 employed the ZMPSTE24 overexpression system. ZMPSTE24 overexpression has
88 been shown to significantly reduced influenza A virus (IAV) infection in A549
89 cells [5]. Briefly, MARC-145 cells cultured in a 12-well plate were transfected
90 with 1.25 µg per well of either pCMV-3Tag-8 control vector or FLAG-tagged
91 ZMPSTE24 cloned into pCMV-3Tag-8 (ZMPSTE24-FLAG) plasmid [5] using
92 Lipofectamine 3000 Transfection Kit (Invitrogen). Cells were incubated in
93 complete medium (DMEM with 10% FBS and 1% penicillin-streptomycin) for 72
94 h. Then, PRRSV 23983 was added to each well at an MOI of 1. Cells and
95 supernatant were harvested 24 h later. Western blot analysis was done using the
96 following primary antibodies: anti-Flag mouse monoclonal 1° Ab against flag
97 tagged ZMPSTE24 (9A3) (Cell Signaling Technology, cat. no. 8146S) at 1:1000
98 dilution, anti-PRRSV N monoclonal Ab SR-30 at 1:300 dilution, and mouse
99 monoclonal anti-beta actin Ab (Sigma-Aldrich, catalog# A2228) at 1:5000
100 dilution. The membrane was then incubated with Goat anti-Mouse IRDye®
101 conjugated 2° Ab (LI-COR, catalog# 926-32210) diluted to 1:5000 and observed
102 using LI-COR Odyssey Infrared Imaging System. As shown in Fig.1A,
103 ZMPSTE24 protein was detected in cells transfected with pCMV-3Tag-8 plasmid
104 expressing ZMPSTE24-FLAG but not in vector control pCMV-3Tag-8
105 transfected cells. A lower level of expression of PRRSV N protein was detected in
106 ZMPSTE24 overexpressing cells as compared to the vector control cells (Fig.
107 1A). Fifty-percent tissue culture infectious dose (TCID₅₀) assay was performed to
108 determine the infectious PRRSV 23983 titer in the supernatant [9]. An average of
109 146-fold decrease (2.16 log reduction) in virus titer in ZMPSTE24 expressing

110 cells was observed (Fig. 1B). To further confirm the antiviral activity of
111 ZMPSTE24, MARC-145 cells were transfected with 0.6 µg of each plasmid in a
112 24-well plate and infected with PRRSV 23983 for 24 h, as described earlier. All
113 wells were fixed with 80% acetone and incubated with 1:80 diluted FITC
114 conjugated anti-PRRSV Ab specific to nucleocapsid (N) protein (SDOW-17) at
115 37°C for 1 h. The images were captured at 10x magnification using an Olympus
116 IX70 Inverted-Microscope (Epi-fluorescence and bright field).
117 Immunofluorescence staining showed fewer virus positive staining (green) in
118 ZMPSTE24 overexpressing cells as compared to the vector control transfected
119 cells (Fig. 1C). To quantify the relative abundance of PRRSV infection, cells were
120 harvested and analyzed further by flow cytometry. A significant reduction in
121 mean fluorescence intensity ($p < 0.05$) was observed in ZMPSTE24
122 overexpressing cells as compared to the vector control cells at 24 hpi (Fig. 1D).
123 To test if cell viability played a role in ZMPSTE24 mediated virus restriction,
124 cytotoxicity assay was performed. MARC-145 cells cultured in a 96-well plate
125 were transfected with 0.3 µg of either the vector control pCMV-3Tag-8 or
126 ZMPSTE24-FLAG for 72 h. Each well was treated with 10 µl of CCK-8 solution
127 (Sigma, cat. no. 96992), then incubated at 37°C for 3 h, and absorbance was
128 measured at 450 nm using a Synergy 2 plate reader (Bio-Tek). No significant
129 difference ($p > 0.05$) in cell viability between vector control and ZMPSTE24
130 transfected cells was observed (Fig. 1E). Overall, the results suggest that
131 overexpression of ZMPTSE24 reduces PRRSV replication.

132 To further confirm the role of ZMPSTE24 in restricting PRRSV
133 replication, we employed silencing RNA mediated knockdown of endogenous
134 ZMPSTE24 approach. Briefly, MARC-145 cells cultured in a 12-well plate were
135 transfected with either negative control siRNA (Ambion, cat. no. AM4642) or

136 ZMPSTE24 siRNA (Life technologies, siRNA ID# s20067) at a final
137 concentration of 2 pmoles/ μ l for 72 h using Lipofectamine RNAi max reagent
138 (Invitrogen). The cells in each well were then infected with PRRSV 23983 at an
139 MOI of 1 for 24 h. Total RNA was extracted from cells and qRT-PCR was
140 performed to validate the ZMSPTE24 gene knockdown efficiency and to quantify
141 PRRSV gene transcript. Primer sequences specific to beta-actin, PRRSV N, and
142 ZMPSTE24 genes used in the study are available upon request. Mean fold change
143 in gene expression was calculated using the $\Delta\Delta$ CT method [11]. Beta-actin was
144 used as a reference gene for comparing the fold change between two treatment
145 groups [11]. As shown in Fig. 2A, an average of 74% knockdown of endogenous
146 ZMPSTE24 gene was achieved. An average of 1.2-fold increase in PRRSV N
147 gene transcript relative to the silencing control was observed (Fig. 2B).
148 Surprisingly, virus titers in the supernatants of ZMPSTE24 knockdown cells as
149 quantified by TCID₅₀ assay [9] showed a slight decrease relative to control
150 silenced cells and an average of 2.5-fold decrease was observed (Fig. 2C). To test
151 if siRNA induced silencing affected cell viability, MARC-145 cells were
152 transfected with either negative control siRNA or ZMPSTE24 siRNA (2
153 pmoles/ μ l) for 72 h and subjected to the cytotoxicity assay. There was no
154 significant difference ($p > 0.05$) in cell viability between negative control siRNA
155 and ZMPSTE24 siRNA transfected groups (Fig. 2D). Overall, results showed that
156 silencing of endogenous ZMPSTE24 does not appear to have a significant impact
157 on PRRSV replication. This observation could be due to the knockdown
158 efficiency (74%) achieved in our hands, while previous studies used cells derived
159 from gene knockout animals [5]. Additionally, it is possible that transient
160 silencing of ZMPSTE24 may not mimic the cells from gene knockout mice [5, 8].

161 Previous studies showed that ZMPSTE24 and IFITM3 work together to
162 block entry of various enveloped viruses including IAV and arenaviruses [5, 16].
163 To examine whether overexpression of ZMPSTE24 restricts PRRSV entry,
164 MARC-145 cells cultured in two 8-chamber slides were transfected with 0.3 µg of
165 each plasmid using the procedure as described earlier. PRRSV 23983 at an MOI
166 of 4 was added to all wells. Two slides were fixed in 3.7% formaldehyde at either
167 3 or 24 hpi and then incubated with 0.2% Triton x-100. Then slides were
168 incubated overnight with mouse monoclonal anti-Flag primary antibody (9A3)
169 (Cell Signaling Technology, cat. no. 8146S) at 1:1600 dilution. The slides were
170 then incubated for 1 h with Alexa fluor 647 conjugated goat anti-mouse IgG 2° Ab
171 (Abcam) at 1:200 dilution and then with anti-PRRSV FITC-SDOW-17. Cells
172 were observed using Olympus Fluoview FV1200 Laser Scanning Confocal
173 Microscope at 40x magnification. There were no significant differences ($p > 0.05$)
174 in the number of PRRSV positive cells or total viral RNA copies between the
175 vector control and ZMPSTE24 over-expressing cells at 3 hpi (Fig. 3A and 3B).
176 qRT-PCR showed approximately 900-fold change in ZMPSTE24 mRNA
177 transcript in ZMPSTE24 overexpressing cells compared to control vector,
178 confirming the successful transfection and over-expressing of ZMPSTE24 (Fig.
179 3C). Our results are in indirect contrast to an earlier study which showed a
180 dramatic lower number of IAV virus positive cells compared to the vector control
181 as early as 2 hpi and persisted until 8 hpi [5]. This difference could be due to
182 different virus entry mechanisms or distinctive replication efficiency of each virus
183 in the respective cell culture model systems.

184 PRRSV completes one infectious cycle in about 8 h [18]. We observed
185 that PRRSV colocalized with the early endosome marker EEA1 at 3 hpi, but most
186 disappeared from early endosome at 6 hpi (data not shown). Very few PRRSV

187 colocalize with LAMP-1, a marker of late endosome/lysosome, during the first 6
188 hpi. Our data is consistent with one previous study showing colocalization of
189 PRRSV with EEA1 but not with the late endosomal marker [17]. However, a
190 more recent study showed colocalization of PRRSV with late endosome marker
191 [18]. Therefore, the existing data remain controversial regarding whether PRRSV
192 enters cells through later endosomes. We observed extensive colocalization
193 between ZMPSTE24 and PRRSV at 3 hpi (Fig. 3D), suggesting virus entry into
194 early endosomes was not disturbed by ZMPSTE24 overexpression. However,
195 little or no colocalization between PRRSV and ZMPSTE24 was observed at 24
196 hpi (Fig. 3D), suggesting that restriction of PRRSV replication in cells with
197 ZMPSTE24 overexpression likely occurred after 3 hpi. It is known that IFITM3
198 and ZMPSTE24 work together to trap enveloped virus in the endolysosomal
199 compartment for degradation [5, 10]. Future studies on colocalization of PRRSV
200 with late endosome/lysosome marker (LAMP-1) in ZMPSTE24 overexpressing
201 cells may reveal more information on whether PRRSV is also being degraded in
202 late endosome/lysosome.

203 We observed a difference in the morphology of virus infected cells
204 between the cells not expressing ZMPSTE24 and the cells over-expressing
205 ZMPSTE24. Specifically, an increased number of cell surface protrusions was
206 observed in the vector control group at 3 hpi (data not shown). One possible
207 explanation is that ZMPSTE24 overexpression may suppress the formation of cell
208 surface protrusions mediated by small GTPase such as Rho [6, 7, 13]. Future
209 investigation may clarify if ZMPSTE24 also interferes with PRRSV intercellular
210 spread. Overall, the data suggest that overexpression of ZMPSTE24 does not
211 significantly impact virus entry and may limit virus replication via a post-entry
212 step. Although ZMPSTE24 inhibited several RNA and DNA viruses requiring pH

213 dependent fusion, it failed to inhibit murine leukemia virus [5]. Alternatively,
214 ZMPSTE24 may also bind to other substrates for its antiviral activity [10]. The
215 detailed mechanism by which overexpression of ZMPSTE24 restricts PRRSV
216 replication needs to be examined in future studies.

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

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239 **Author Contributions**

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

244

245 **Compliance with Ethical Standard:**

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252 **Conflict of Interest:** The authors declare no conflict of interest.

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344 **Figure legends**

345 **Fig. 1** Over-expression of ZMPSTE24 in MARC-145 cells reduces PRRSV
346 replication. Standard deviation and mean of three replicates are shown for each
347 graph. **A:** Western blot analysis showing the expression of ZMPSTE24 in cells
348 transfected with ZMPSTE24 expressing plasmid. Lanes 1-3 were transfected with
349 pCMV-3Tag-8 vector control (V) while wells 4-6 were transfected with pCMV-
350 3Tag-ZMPSTE24-FLAG (Z). Beta-actin was used as a loading control. The
351 expression of PRRSV N protein is shown. **B:** Virus titers in the supernatants of
352 PRRSV infected cells at 24 hpi. * indicates $p < 0.05$. **C:** Immunofluorescence
353 staining of virus-infected cells at 24 hpi. Top panel shows the bright field image
354 and the lower panel shows PRRSV positive staining. **D:** Flow cytometry analysis
355 of virus-infected cells at 24 hpi. * indicates $p < 0.05$. **E:** CCK-8 assay was used to
356 compare cell viability between vector control and ZMPSTE24 containing plasmid
357 transfected cells. No significant difference ($p > 0.05$) was observed.

358 **Fig. 2** Silencing of endogenous ZMPSTE24 slightly affects PRRSV replication.
359 Standard deviation and mean of three replicates are shown for each graph. **A:**
360 qRT-PCR showed an average ZMPSTE24 knockdown efficiency of 74%. **B:** The
361 relative fold of change in viral RNA copies in ZMPSTE24 siRNA transfected
362 cells compared to control silencing RNA transfected cells. No significant
363 difference ($p > 0.05$) was observed between the two groups. **C:** TCID₅₀ assay
364 showing virus titers in the supernatants of ZMPSTE24 silencing cells as compared
365 to the control silencing cells. No significant difference between the two groups
366 was observed ($p > 0.05$). **D:** Cell viability was not significantly ($p > 0.05$) affected
367 by the transient silencing of ZMPSTE24.

368 **Fig. 3** Over-expression of ZMPSTE24 does not affect virus entry into MARC-145
369 cells. **A:** Quantitative analysis of PRRSV positive cells in ZMPSTE24 expressing

370 cells and vector control cells at 3 hpi. Percentage of PRRSV positive cells was
371 determined by dividing the green (FITC-SDOW-17) staining cells by total number
372 of cells (DAPI stained, blue) in each microscopic field. The average and standard
373 deviations of five different microscopic fields are shown. The percentage of red
374 staining (Alexa 647, ZMPSTE24 expressing cells) that is positive for PRRSV was
375 determined similarly. Red and green co-staining cells were counted and divided
376 by the total number of red staining cells. No significant difference ($p > 0.05$) was
377 observed between vector control and ZMPSTE24 transfected group. **B:** The
378 relative fold change in viral RNA copies in ZMPSTE24 transfected cells when
379 compared to vector control at 3 hpi as determined by qRT-PCR. Standard
380 deviation and mean of three replicates are shown. **C:** qRT-PCR showing the
381 relative fold increase in ZMPSTE24 mRNA level in ZMPSTE24 transfected cells
382 compared to vector control cells. Standard deviation and mean of three replicates
383 are shown. **D:** Colocalization of PRRSV N protein with exogenous ZMPSTE24.
384 Representative images show the extensive colocalization of PRRSV N with
385 ZMPSTE24 at 3 hpi and little or no colocalization of PRRSV N protein with
386 ZMPSTE24 at 24 hpi. Green: PRRSV N protein. Red: Flag-tagged ZMPSTE24.
387 Blue: DAPI nuclei.

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396 **Fig. 1A**

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