Modulation of CD163 Expression by Metalloprotease ADAM17 Regulates Porcine Reproductive and Respiratory Syndrome Virus Entry

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ABSTRACT

As a consequence of their effects on ectodomain shedding, members of the A disintegrin and metalloprotease (ADAM) family have been implicated in the control of various cellular processes. Although ADAM family members are also involved in cancer, inflammation, and other pathologies, it is unclear whether they affect porcine reproductive and respiratory syndrome virus (PRRSV) infection. Here, we demonstrate for the first time that inhibition of ADAM17 enhances PRRSV entry in Marc-145 and porcine alveolar macrophages (PAMs). We also demonstrate that the inhibition of ADAM17 upregulates membrane CD163 expression, a putative PRRSV receptor that is exogenously expressed in BHK-21 and endogenously expressed in Marc-145 and PAMs. Furthermore, overexpression of ADAM17 induced downregulation of CD163 expression and a reduction in PRRSV infection, whereas ablation of ADAM17 expression using specific small interfering RNA resulted in upregulation of CD163 expression with a corresponding increase in PRRSV infection. These ADAM17-mediated effects were confirmed with PRRSV nonpermissive BHK-21 cells transfected with CD163 cDNA. Overall, these findings indicate that ADAM17 downregulates CD163 expression and hinders PRRSV entry. Hence, downregulation of ADAM17 particular substrates may be an additional component of the anti-infection defenses.

IMPORTANCE

ADAM17 is one of the important membrane-associated metalloproteases that mediate various cellular events, as well as inflammation, cancer, and other pathologies. Here, we investigate for the first time the role of the metalloprotease ADAM17 in PRRSV infection. By using inhibitor and genetic modification methods, we demonstrate that ADAM17 negatively regulate PRRSV entry by regulating its substrate(s). More specifically, ADAM 17 mediates the downregulation of the PRRSV cellular receptor CD163. The reduction in CD163 expression represents another component of the anti-infection response initiated by ADAM17.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-strand RNA virus in the family *Arteriviridae* and the order *Nidovirales* (1). Based on genetic differences, PRRSV has been divided into European strains (EU; represented by Lelystad) and North American strains (NA; represented by VR2332) (2, 3). Porcine reproductive and respiratory syndrome (PRRS) is characterized by an acute viral infection that leads to respiratory problems in growing pigs and reproductive failure in sows. In China, a highly pathogenic strain of PRRSV (HP-PRRSV) was isolated and identified in 2006. HP-PRRSV, which has a 30-amino-acid depletion in nonstructural protein 2 (nsp2), has caused great economic losses for the swine industry (4, 5).

PRRSV has a tropism for cells of the monocytic lineage such as porcine alveolar macrophages (PAMs). To date, researchers have identified two essential viral receptors that mediate PRRSV entry and uncoating; these receptors are sialoadhesin (CD169 or Siglec-1) and the scavenger receptor CD163 (6–8). In primary macrophages, these two receptors are expressed at high level, which explains why PAMs are highly susceptible to PRRSV infection. In addition, MA-104 (African green monkey kidney cell) and its derivative Marc-145 are well-characterized cell lines that have been used to sustain PRRSV in culture. CD163 has been identified as a key molecule in PRRSV entry into Marc-145 cells even though these cells do not belong to the monocyte-macrophage lineage (7). Several groups have reported that the expression of CD163 alone in nonpermissive cells leads to high titers of progeny virus (7,

9–13), which indicates that CD163 plays a major role in PRRSV entry.

As noted earlier, CD163 belongs to the scavenger receptor cysteine-rich superfamily (14). The expression of CD163 is tightly regulated by a variety of factors and is downregulated by proinflammatory cytokine tumor necrosis factor alpha (TNF- α) and the cross-linking of Fc γ receptor (15, 16). Previous reports revealed that a disintegrin and metalloprotease 17 (ADAM17) mediates human CD163 downregulation and production of the soluble form of CD163 (17–19). As a member of the metalloprotease family, ADAM17 is the best-studied ADAM sheddase (20); ADAM sheddases can cleave various cell surface proteins, typically at a juxtamembrane site, resulting in the downregulation of membrane proteins expression and the release a soluble form of ectodomain fragment (21, 22). The list of cell surface proteins known to be cleaved by ADAM17 is still growing, and most of its substrates, including TNF- α , TNF-RI, TNF-RII, and L-selectin (23,

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TABLE 1 Primers used for PCR in this study

Primer ^a	Sequence (5'–3')
ADAM17-F1*	CTTGGTACCATGAGGCGGCGTCTCCTCATCCTGA
ADAM17-R1*	GTGCTCGAGTTACTTGTCTGTCATCGTCTTTGTAG TCGTGATGGTGATGGTGATGCACACAGT
CD163-F*	CTGGGTACCATGGACAACTCAGAATGGTG
CD163-R*	GTGCTCGAGTTATTGTACTTCAGAGTGGTCTCC
ADAM17-F2†	GCACAGGTAATAGCAGTGAGTGC
ADAM17-R2†	CACACAATGGACAAGAATGCTG
GAPDH-F†	GAGTCAACGGATTTGGTCTG
GAPDH-R†	GGTGCCATGGAATTTGCCAT

* *, The Flag tag (underlined) was fused at the carboxyl terminus of ADAM17 by PCR and was cloned at the KpnI (boldface) and XhoI (italics) sites in the pCAGGS vector; †, primers used for relative quantitative PCR.

24), play important roles in modulating inflammation, indicating that ADAM17 has a critical role in host immune response. In spite of the central role ADAM17 in many biological processes, how this metalloprotease governs PRRSV infection remains unstudied.

Here, we examined the role of ADAM17 on PRRSV infection. We demonstrate that ADAM17 directly reduces PRRSV entry as a consequence of its proteolytic activity. ADAM17 downregulated the exogenous and endogenous expression of CD163, a PRRSV putative receptor, in different cells. Ablation of ADAM17 expression using a specific inhibitor or small interfering RNA duplexes reduced CD163 downregulation and enhanced PRRSV infection, whereas overexpression of ADAM17 increased CD163 downregulation and suppressed PRRSV infection. Taken together, these data provide direct evidence that the metalloprotease ADAM17 regulates PRRSV entry by modifying the expression of membrane CD163.

MATERIALS AND METHODS

Cells and viruses. The following cells were maintained in Dulbecco minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher): Marc-145, a monkey kidney cell line subcloned from MA-104; BHK-21, a cell line derived from baby Syrian hamster kidney; and 293T. Primary porcine alveolar macrophages (PAMs) were freshly harvested from 6-week-old specific-pathogen-free (SPF) pigs and were maintained in DMEM with 10% heat-inactivated FBS and penicillin-streptomycin. The animal experiment was approved by the Harbin Veterinary Research Institute and performed in accordance with animal ethics guidelines and approved protocols. The animal ethics committee approval number is Heilongjiang-SYXK-2006-032. All cells were cultured in a humidified atmosphere at 37°C and 5% CO₂. The type II PRRSV strain HuN4 (GenBank accession number EF635006), which is a highly pathogenic PRRSV strain that was isolated in China, was grown and titrated in Marc-145 cells as described before (25) and was stored at –80°C.

Immunofluorescence assay (IFA) and viral plaque assay. After various treatments, the cell monolayer was washed with phosphate-buffered saline (PBS) and inoculated with PRRSV HuN4 at a multiplicity of infection (MOI) of 0.1 for 60 min at 37°C with rocking. After removal of the inoculum, the cell monolayer was washed twice with PBS and covered with the medium. At 24 h postinoculation, the cells were washed twice with PBS, fixed in 33.3% acetone for 30 min at room temperature, and dried. Fixed cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-PRRSV N protein monoclonal antibody (MAb) SDOW17 (Rural Technologies, USA) for 30 min at 37°C. After the cells were washed three times with PBS at 5-min intervals, the fluorescence was visualized with an Olympus inverted fluorescence microscope equipped with a camera, and the number of susceptible cells was counted. A plaque assay was performed as previously described with slight modifications

TABLE 2 Sequences of sense strands of double-stranded RNA used to ablate specific protein expression in Marc-145 cells

Double-stranded RNA	Target	Sense strand sequence (5'–3')
1#-siRNA	ADAM17	GGAUGUAAUUGAACGAUUU
2#-siRNA	ADAM17	GAGAGUACAACUACAAUUU
3#-siRNA	ADAM17	CUGGUUACAACUCAUGAAU
Control siRNA	Unrelated	UUCUCCGAACGUGUCACGUTT

(26). Typically, Marc-145 cells cultured in a 6-well-plate until 90% confluence were inoculated with PRRSV HuN4 at an MOI of 0.1. After 60 min of incubation, cell monolayers were washed and then overlaid with an equivalent volume of 2× DMEM containing 10% FBS and 3.0% low-melting-temperature agarose (Cambrex, Rockland, ME). When the agarose overlay solidified, the plates were inverted and moved to a humidified incubator at 37°C and 5% CO₂. When plaques had fully developed, the plates were stained with crystal violet (5% [wt/vol] in 20% ethanol).

Transfection of ADAM17 and CD163. The genes of ADAM17 and CD163 were amplified from mouse and porcine macrophages cDNA, respectively, and cloned into the pCAGGS vector (Addgene, USA). The Flag tag (DYKDDDDK) was fused at the carboxyl terminus of ADAM17 by PCR using the primers listed in Table 1. The nucleotide sequences of the plasmids encoding ADAM17 and CD163 were determined to ensure that the correct clones were used in the study. Marc-145 or BHK-21 cells were transfected with 2 µg of target plasmid pCAGGS/ADAM17, pCAGGS/CD163, and vector control pCAGGS using X-tremeGENE transfection reagent (Roche). At 24 h posttransfection, the cells were inoculated with PRRSV HuN4 at an MOI of 0.1, and the infection of PRRSV was detected by IFA as described above. Also at 24 h posttransfection, the cells were lysed in RIPA lysis buffer (Beyotime, Nantong, China) for Western blot analysis of the expression of ADAM17 or CD163.

Metalloprotease ADAM17 inhibition assay. To investigate the role of ADAM17 in altering PRRSV entry by regulating viral cellular receptors, such as CD163 and CD169, we used batimastat (BB94; Sigma-Aldrich, St. Louis, MO) (27) as a metalloprotease ADAM17 inhibitor. The cytotoxicity effect of BB94 was determined by trypan blue staining with three replicates for each inhibitor concentration (2, 5, 10, 20, and 50 µM). Concentrations of <10 µM were nontoxic (see Results), and cells were therefore incubated with 2 µM BB94 for 24 h before they were inoculated with PRRSV HuN4 at an MOI of 0.1. PRRSV infection was examined by IFA and plaque assay as described above. In addition, the surface expression level of CD163 or CD169 on some cells was assessed using specific antibodies by flow cytometry. For BHK-21 cells, the cells were preincubated with BB94 for 24 h and then transfected with pCAGGS/CD163. At 24 h posttransfection, CD163 expression on the cell surface was detected by flow cytometry. In addition, at 24 h posttransfection of pCAGGS/CD163, BHK-21 cells were inoculated with PRRSV at an MOI of 0.1, and PRRSV infection was assessed by IFA. To rule out the effect of intracellular inhibition of BB94 and to ensure that the metalloprotease ADAM17 alters PRRSV entry rather than PRRSV replication, a Marc-145 monolayer was inoculated with PRRSV at an MOI of 0.1 for 60 min and then incubated in a medium containing the ADAM17 inhibitor BB94. At 24 h postinoculation, the infection of PRRSV was detected by IFA. For all experiments, cells were treated with the appropriate amount of dimethyl sulfoxide (DMSO; carrier) for mock treatments.

To confirm ADAM17 function, small interfering RNA (siRNA) duplexes were introduced to knock down ADAM17 expression. Marc-145 cells were grown to ~60% confluence in an antibiotic-free growth medium and transfected with siRNA duplexes (ON-TARGET-plus SMART-pools siRNA oligonucleotides; Sigma-Aldrich) using Dharmafect-2 transfection reagent (Thermo Scientific) according to the manufacturer's instructions. Cells were separately transfected with each of three siRNA duplexes targeted to ADAM17 and with control siRNA duplexes targeted to a scramble sequence (Table 2) at concentrations of 25 and 100

TABLE 3 Effect of BB94 on survival rates of three cell types

Cell type	Treatment	Mean cell survival rate (%) $\pm$ SD at various concn of BB94				
		2 μ M	5 μ M	10 μ M	20 μ M	50 μ M
Marc-145	BB94	95.40 $\pm$ 1.78	95.23 $\pm$ 0.70	88.43 $\pm$ 7.60	87.77 $\pm$ 2.42	86.03 $\pm$ 0.29
	DMSO	95.27 $\pm$ 2.87	97.90 $\pm$ 1.08	93.13 $\pm$ 1.77	91.87 $\pm$ 1.33	91.73 $\pm$ 0.42
PAM	BB94	100	100	93.23 $\pm$ 2.00	86.07 $\pm$ 2.15	76.00 $\pm$ 5.69
	DMSO	100	100	100	93.60 $\pm$ 2.33	86.63 $\pm$ 0.90
BHK-21	BB94	97.73 $\pm$ 0.25	97.07 $\pm$ 0.93	97.37 $\pm$ 1.07	89.63 $\pm$ 1.05	82.83 $\pm$ 1.42
	DMSO	98.23 $\pm$ 0.96	98.30 $\pm$ 0.56	98.93 $\pm$ 0.73	95.43 $\pm$ 1.07	93.20 $\pm$ 1.04

nM. At 24 h after transfection, cell lysates were prepared and assayed for specific gene silencing by real-time PCR and Western blot. In some experiments, PRRSV infection at an MOI of 0.1 was performed 24 h posttransfection.

SDS-PAGE and Western blotting. Cells were detergent lysed as previously described (28). Typically, samples were separated by SDS-PAGE under reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 2% bovine serum albumin in PBS for 60 min and then incubated with a primary antibody for 60 min. After they were washed three times with PBS plus 0.05% Tween 20, the membranes were incubated with IRDye-conjugated secondary antibody (Li-Cor Biosciences, Lincoln, NE) diluted in washing buffer for 60 min. Membranes were washed as described above and then scanned and analyzed with an Odyssey instrument (Odyssey infrared imaging system; Li-Cor Biosciences) according to the manufacturer's instructions. For the detection of ADAM17 expression, the PVDF membrane was probed with rabbit polyclonal antibody to ADAM17 (Abcam, Cambridge, MA). The blotting antibody for CD163 was raised in rabbit and stocked in our laboratory. The Mab M2 (Sigma-Aldrich, St. Louis, MO) was used to detect the expression of Flag-tagged proteins. Anti- β -actin Mab (C4) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow cytometry. Flow cytometric analyses were performed on a FACSAria instrument (BD Biosciences) as described previously (29). Allophycocyanin (APC) conjugated mouse anti-human CD163 Mab (BioLegend) detects CD163 expressed on Marc-145 cells. Mouse anti-pig CD163 and anti-pig CD169 MAbs were purchased from AbD Serotec. Mouse anti-PRRSV N protein Mab SDOW17 labeled with FITC (SDOW17-F) was purchased from Rural Technologies. For intracellular PRRSV detection, cells were pretreated with a fixation/permeabilization kit following the manufacturer's protocol (eBioscience) and were then stained with SDOW17-F. Isotype-matched negative control MAbs were used to evaluate levels of nonspecific staining. Typically, 10,000 labeled cells were analyzed. All samples were analyzed using FlowJo 8.7 (Tree Star) and FACS Diva (BD Biosciences).

Quantitative reverse transcription-PCR (RT-PCR). At 24 h posttransfection of siRNA, total RNA was extracted from cells by using an RNeasy RNA purification kit (Qiagen) to generate cDNA with oligo(dT) primers using Superscript II reverse transcriptase (Invitrogen, USA). Quantitative RT-PCRs were conducted in triplicate using SYBR premix Ex Taq (TaKaRa) and 10 μ M concentrations of specific primers (Table 1). Relative quantification was performed by the cycle threshold ($\Delta\Delta C_T$) method (30). Briefly, C_T values were normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA (GAPDH; internal standard), and the ΔC_T was determined by the formula $\Delta C_T = C_T(\text{ADM17}) - C_T(\text{GAPDH})$. The fold change was determined by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T(1\#-\text{siRNA to } 3\#-\text{siRNA}) - \Delta C_T(\text{siRNA}_{\text{Control}})$.

Statistical analysis. Values are expressed as means $\pm$ the SD. The data were analyzed with Student *t* test in Excel. A *P* value of <0.05 was considered significant.

RESULTS

A metalloprotease inhibitor enhances the production of PRRSV progeny. To investigate the role of metalloproteases in PRRSV infection, we used the metalloprotease inhibitor batimastat (BB94) (31). The cytotoxic effects of BB94 were examined by trypan blue staining on two types of PRRSV permissive cells, Marc-145 cells and primary porcine alveolar macrophages (PAMs), as well as on PRRSV nonpermissive BHK-21 cells. No significant toxicity was evident for any of the three types of cells at BB94 concentrations $< 10 \mu\text{M}$ (Table 3). Therefore, Marc-145 cells were treated with 2 μM BB94 for 24 h before they were inoculated with PRRSV HuN4 at an MOI of 0.1; PRRSV production was assessed with a plaque assay. The metalloprotease inhibitor BB94 significantly increased the production of PRRSV progeny (Fig. 1A). We also used IFA to evaluate the effects of the inhibitor on viral infection, and the results again showed that BB94 enhanced viral infection (Fig. 1B). The intracellular effect of BB94 was examined by adding BB94 to Marc-145 cells after PRRSV incubation; the result showed that BB94 did not modify PRRSV replication in comparison to the mock control (Fig. 1C). In other words, modulation of metalloprotease activity might alter PRRSV infection efficiency at the stage of viral entry but not at the other stage of viral life cycle. These findings indicate that a proteolytic cleavage event, most likely ADAM17-mediated ectodomain shedding, regulates the expression level of cellular surface protein-like receptor(s) and affects viral entry.

ADAM17 suppresses PRRSV entry. Having founded that PRRSV entry is enhanced by the metalloprotease inhibitor BB94, we next attempted to identify the metalloprotease involved. Because ADAM17, one of the most important metalloproteases, is known to have a wide spectrum of substrates, we examined the effect of ADAM17 on PRRSV HuN4 entry by transient-overexpression assay. As indicated by IFA, the number of PRRSV-positive cells was lower in Marc-145 cells transfected with pCAGGS/ADAM17 than in the pCAGGS vector control (Fig. 2A), which suggests that PRRSV entry is suppressed by constitutively activated ADAM17. We also confirmed the overexpression of ADAM17 in Marc-145 by Western blot analysis of cell lysate from transfected cells with the anti-Flag antibody. As shown in Fig. 2B, it revealed that specific ADAM17 protein band presented in pCAGGS/ADAM17-transfected Marc-145 cells.

To further determine the involvement of ADAM17 in PRRSV entry, we next used siRNA duplexes to reduce the endogenous expression of ADAM17 in Marc-145 cells. When Marc-145 cells were separately transfected with each of three siRNA duplexes

FIG 1 Role of metalloproteases in PRRSV entry. (A) Plaque assay for PRRSV production. Marc-145 cells were treated with 2 μ M metalloprotease inhibitor BB94 for 24 h before PRRSV HuN4 inoculation, and monolayers were stained with crystal violet at 3 days postinoculation. Plaque assay results are expressed as the means $\pm$ the standard deviations (SD) of at least three independent experiments. *, $P < 0.05$. (B and C) Immunofluorescence assay to detect PRRSV infection. Marc-145 cells were treated with DMSO or BB94 for 24 h before they were inoculated with PRRSV HuN4 (B); Marc-145 cells were first inoculated with PRRSV HuN4 and then incubated in the medium containing DMSO or BB94 (C). At 24 h after inoculation, the cell monolayer was fixed and examined for PRRSV infection by IFA with FITC-conjugated MAb SDOW17. The PRRSV-positive cells were counted in three independent experiments, and values are means $\pm$ the SD. *, $P < 0.05$. The P value was calculated using Student t test.

against ADAM17, the ADAM17 mRNA level was significantly decreased in each case relative to levels in cells transfected with control siRNA (Fig. 2C). The results also showed that the knockdown efficiency of the siRNA duplex was dose-dependent and that efficiency was higher with the 2#-siRNA than with the other two (Fig. 2C). Western blot analysis of detergent lysates collected from cells transfected with ADAM17-specific 1#- or 2#-siRNA duplexes revealed a significant reduction in the level of ADAM17 protein relative to the levels obtained with control siRNA and mock-transfected cells (Fig. 2D). Therefore, 2#-siRNA duplex at 100 nM was used for subsequent experiments unless otherwise stated. At 24 h after transfection of siRNA duplexes, Marc-145 cells were

inoculated with PRRSV HuN4, and infection was assessed by IFA after an additional 24 h. The results showed that the number of cells infected with PRRSV was significantly greater in the ADAM17-specific siRNA transfection group than in the control siRNA group ($P < 0.05$) (Fig. 2E). Overall, these results indicate that the metalloprotease ADAM17 suppresses PRRSV entry, suggesting that ADAM17-mediated ectodomain shedding of its putative substrate(s) might be involved in this regulatory mechanism.

Membrane-associated CD163 is regulated by ADAM17. CD163 has been identified as a possible cellular receptor for PRRSV in the PRRSV permissive cell line Marc-145 (7). Research-

FIG 2 ADAM17 suppresses PRRSV entry. (A) The number of PRRSV-positive cells was decreased by ADAM17 overexpression. Marc-145 cells were transfected with pCAGGS/ADAM17 or pCAGGS vector for 24 h before they were inoculated with PRRSV HuN4. At 24 h after inoculation, the cell monolayer was fixed, and PRRSV-infected cells were counted. (B) Detergent lysates from equivalent cell numbers of Marc-145 overexpressing ADAM17 or empty vector were subjected to reducing SDS-PAGE and immunoblotting with antibodies to Flag or β -actin (loading control). (C) ADAM17 knockdown efficiency as indicated by real-time PCR. Marc-145 cells were transfected with siRNA duplexes against monkey ADAM17 or control siRNA. The knockdown efficiency of ADAM17-specific siRNA duplexes at 100 nM was >60%, as indicated by the relative levels of ADAM17 mRNA to GAPDH mRNA. (D) Detergent lysate from ADAM17-specific siRNA transfected Marc-145 cells was subjected to reducing SDS-PAGE and immunoblotting with antibodies to ADAM17 or β -actin (loading control). (E) ADAM17-specific siRNA enhances PRRSV infection. Marc-145 cells were transfected with 2#-siRNA duplexes or control siRNA at 100 nM for 24 h before they were inoculated with PRRSV HuN4. At 24 h postinoculation, the cell monolayer was fixed, and the number of PRRSV-positive cells was determined by FITC-conjugated MAb SDOW17. In panels A, C, and E, representative values from three separate experiments are shown, and each value represents the mean $\pm$ the SD of three separate experiments. *, $P < 0.05$. The P value was calculated using a Student t test.

ers recently showed that ADAM17 is required for the induction of CD163 ectodomain shedding by phorbol 12-myristate 13-acetate (PMA) in human cells (19, 32). Therefore, we hypothesized that constitutive ADAM17 activity modifies PRRSV entry by regulating the expression level of its putative substrate CD163. In support of this hypothesis, BB94 substantially was used and we found that BB94 reduced ADAM17 constitutive activity and upregulated expression of CD163 on Marc-145 cells (Fig. 3A).

To further determine the involvement of ADAM17, the effect of ADAM17 overexpression on CD163 downregulation was examined. As shown in Fig. 3B, the expression level of CD163 was lower in Marc-145 cells that contained pCAGGS/ADAM17 and overexpressed ADAM17 than in cells that contained the pCAGGS vector control. To obtain direct evidence that ADAM17 plays an important role in CD163 downregulation, we introduced an ADAM17-specific siRNA. As shown in Fig. 3C and D, the constitutive downregulation of CD163 by endogenous ADAM17 was better attenuated by ADAM17 siRNA at a concentration of 100 nM than at 25 nM. Western blot analysis showed that the ADAM17-specific siRNA reduced endogenous ADAM17 expression in Marc-145 (Fig. 2D). These data revealed that the metalloprotease ADAM17 is involved in the downregulation of membrane CD163 expression level in Marc-145, suggesting that the regulatory effect of ADAM17 on PRRSV entry in Marc-145 is triggered by the modification of the expression level of the ADAM17 substrate CD163.

ADAM17 is expressed on PAMs and leads to alteration of PRRSV entry. PAM is the major primary cell type that is infected by PRRSV *in vivo*. CD163, a specific marker of macrophages, is a putative PRRSV receptor on PAMs and is closely associated with PRRSV entry (7, 33, 34). Based on previous findings, we hypothesized that ADAM17 regulates PRRSV entry on PAMs as well. First, we assessed ADAM17 expression in freshly isolated PAMs by Western blotting. As shown in Fig. 4A, a specific ADAM17 protein band was detected from the detergent lysate collected from PAMs and also from 293T cells.

To determine whether ADAM17 plays a role in PRRSV entry in PAMs, we treated PAMs with the ADAM17 inhibitor BB94 before inoculation with PRRSV HuN4. Consistent with the findings obtained with Marc-145, the percentage of PRRSV-infected PAMs was increased by BB94 treatment (Fig. 4B), indicating that ADAM17 is involved in PRRSV entry in PAMs as well.

Because CD169 is another PRRSV receptor on PAMs (6, 35, 36), flow cytometry was used to quantify the expression levels of CD169 on PAMs. After PRRSV infection, we did not observe a downregulation of CD169 on PAMs, and BB94 treatment did not upregulate CD169 expression (Fig. 4C), suggesting that CD169 is not the major factor involved in the alteration of PRRSV entry by ADAM17.

Previous reports showed that porcine CD163 is efficiently downregulated by an unknown protease upon cell activation and PRRSV infection (12). Here, we found that PRRSV HuN4 induced the downregulation of CD163 expression on PAMs and that the downregulation of CD163 following PRRSV infection was blocked by the ADAM17 inhibitor BB94 (Fig. 4D). Porcine CD163 has putative cleavage sites that are similar to those in the juxtamembrane region of human CD163 (Fig. 4E), indicating that porcine CD163 may be cleaved by ADAM17 at the same location. Taken together, the findings described here reveal that the activity of ADAM17 on PAMs is stimulated upon PRRSV infection and

that such activity results in the downregulation of its putative substrate CD163 and in the suppression of PRRSV entry. When ADAM17 activity is impaired, downregulation of its putative substrate CD163 is reduced and the upregulated expression of CD163 on PAMs results in an increase in PRRSV entry.

Downregulation of CD163 by ADAM17 is critical for PRRSV entry. Researchers previously reported that expression of porcine CD163 alone in BHK-21 (a PRRSV nonpermissive cell) confers susceptibility to PRRSV infection (7). To further determine whether CD163 downregulation mediated by ADAM17 reduces PRRSV entry, we first used CD163-specific primers to clone the full-length CD163 from PAMs into the pCAGGS vector (Table 1). Next, we transiently expressed porcine CD163 cDNA in BHK cells by transfection with pCAGGS/CD163. Western blot analysis of detergent extracts collected from BHK-CD163 revealed the presence of an ~130-kDa polypeptide, which corresponds to the mature porcine CD163 protein (Fig. 5A). These pCAGGS/CD163 transfected cells showed susceptibility to PRRSV infection compared to pCAGGS vector control (Fig. 5B). We then determined whether downregulation of CD163 by ADAM17 was associated with reduced PRRSV entry. We observed that the number of PRRSV-infected cells was significantly higher in the transfected cells treated with BB94 than in the mock control (Fig. 5C). Flow cytometry showed that the expression level of CD163 in the transfected cells was significantly increased by BB94 treatment (Fig. 5D). These data indicate that the regulation of membrane CD163 expression level by ADAM17 is a critical factor for PRRSV entry.

DISCUSSION

Emerging evidence indicates that ectodomain shedding by different cells plays a critical role in numerous human diseases, including cancer, autoimmune diseases, and inflammation. Ectodomain shedding modulates the activity of various cytokines, growth factors, and their receptors, as well as adhesion molecules (22, 37–40). In the present study, we report for the first time that the metalloprotease ADAM17 helps regulate PRRSV entry *in vitro*. We also report that ADAM17 downregulates the expression level of membrane CD163, which is a PRRSV major receptor, from the surface of PRRSV permissive cells such as Marc-145 and PAM.

We and others have previously shown that ADAM17 is involved in the ectodomain shedding of various cell surface proteins during cell activation and apoptosis, indicating that ADAM17 has a role in controlling almost every cellular function (41–44). Thus, a few research groups began to observe relationships between ADAM17 and viral infection. Dolnik et al. found that ADAM17 is involved in Ebolavirus glycoprotein shedding (45). Others reported that ADAM17, when activated by the spike protein of SARS CoV, cleaves the virus's receptor angiotensin-converting enzyme 2 (ACE2) and affects virus entry and pathogenesis (46–49). Whether ADAM17 plays a role in PRRSV infection, however, was unclear before the present study. Here, our data indicate that ADAM17 activity reduces PRRSV infection at the stage of viral entry rather than any other stage of viral life cycle (Fig. 1). Our results and those of others suggested that ADAM17 modulates protein expression on the cell surface. As a consequence, we suspected that ADAM17 might induce the downregulation of the cellular receptor(s) during PRRSV infection, resulting in the reduction in PRRSV entry. To test this hypothesis, we used two genetic approaches. The first approach involved ADAM17 overexpression; overexpression had been extensively used to assess the

FIG 3 ADAM17-mediated downregulation of CD163 in Marc-145 cells. (A) Marc-145 cells were treated with BB94 or carrier DMSO for 24 h. All cells were then stained for surface expression of CD163 with APC-conjugated anti-human CD163 MAb, and staining was detected by flow cytometry. (B) Marc-145 cells were transfected with pCAGGS/CD163 or pCAGGS vector control for 24 h before flow cytometry assay was used to detect cellular surface expression of CD163. (C and D) Marc-145 cell were transfected with 1# and 2# ADAM17-specific siRNA duplexes for 24 h as indicated. Relative CD163 surface expression levels were determined by flow cytometry. The concentration of siRNA duplexes was 25 nM (C) and 100 nM (D). The x-axis = $\log_{10}$ fluorescence. Nonspecific antibody labeling was determined by using the appropriate isotype negative-control antibodies, as indicated. All values are representative of three independent experiments.

FIG 4 The metalloprotease ADAM17 reduces PRRSV entry in PAMs. (A) ADAM17 expression was measured on PAMs. Detergent lysates collected from PAMs and 293T were directly subjected to reducing SDS-PAGE and immunoblotting with rabbit anti-ADAM17 polyclonal antibody (upper panel). Detergent lysates were also immunoblotted with rabbit negative-control antibody (lower panel). (B) ADAM17 activity reduces PRRSV entry. PAMs were inoculated with PRRSV HuN4 in the presence of BB94 or carrier DMSO for 24 h. The percentage of PRRSV-positive PAMs was determined by flow cytometry and analyzed by FlowJo 8.7. The values from three independent experiments are expressed as means $\pm$ the SD. *, $P < 0.05$. The P value was calculated using Student t test. (C and D) The effect of ADAM17 on PRRSV entry depended on CD163 but not on CD169. PAMs were inoculated with PRRSV HuN4 in the presence of BB94 or DMSO for 24 h. Relative CD169 or CD163 surface expression levels, as indicated, were determined by flow cytometry. Negative control antibody staining of untreated cells is indicated as isotype. The x-axis = $\log_{10}$ fluorescence. All values are representative of at least three independent experiments using PAMs isolated from separate SPF pigs. (E) Alignment of the extracellular juxtamembrane regions of human, gorilla, and porcine CD163. Dashes indicate gaps introduced to maximize homology. Gray shading indicates the putative CD163 cleavage site (19).

FIG 5 Reduction of CD163 expression by ADAM17 reduces PRRSV entry. (A) Detection of porcine CD163 expression in BHK-21 cells. BHK-21 cells were transfected with pCAGGS/CD163 or pCAGGS vector for 24 h. Cell lysates were subjected to reducing SDS-PAGE before immunoblotting with antibodies to porcine CD163 or β -actin (loading control). (B) The number of PRRSV-positive cells on transfected BHK-21 cells expressing CD163 was increased by treatment with an inhibitor of the metalloprotease ADAM17. BHK-21 cells expressing CD163 were inoculated with PRRSV HuN4 for 24 h before they were fixed and stained with FITC-conjugated MAb SDOW17. The numbers of PRRSV-positive cells in three independent experiments are expressed as means $\pm$ the SD. *, $P < 0.05$. The P value was calculated using a Student t test. (D) Expression of CD163 on the surface of BHK-21 cells was assessed by flow cytometry. BHK-21 cells were transfected with pCAGGS/CD163 or vector control for 24 h in the presence of BB94 or DMSO. Relative CD163 surface expression levels in BHK-21 were determined by flow cytometry, as indicated. The x-axis = $\log_{10}$ fluorescence. Nonspecific antibody labeling was determined using the appropriate isotype negative-control antibody, as indicated. Values are means of three independent experiments.

function of particular proteins (42). Overexpression of ADAM17 decreased the number of PRRSV-infected cells (Fig. 2A). In the second approach, specific siRNAs were used to knock down ADAM17 expression. Knockdown of ADAM17 expression enhanced the number of PRRSV-positive cells. Hence, our findings indicate that, in addition to contributing to cell activation and apoptosis, ADAM17 is also involved in resistance to PRRSV entry.

PRRSV is one of the most serious pathogens threatening the swine industry worldwide. The major cell type directly destroyed by PRRSV infection *in vivo* is PAM. To date, PAM is also the only primary cell type supporting PRRSV infection *in vitro*. Here, we report for the first time that PAMs express ADAM17 (Fig. 4A) and that the treatment of PAM with an ADAM17 inhibitor increases PRRSV infection efficiency (Fig. 4B), which is consistent with the results obtained with Marc-145. These findings indicate that the metalloprotease ADAM17 might affect PRRSV entry by regulating the expression level of PRRSV cellular receptor(s). As noted ear-

lier, two essential PRRSV receptors have been identified in primary macrophages, and these are CD169 and scavenger receptor CD163. CD169 is responsible for the internalization of the PRRSV virion (6, 50), and CD163 is involved in PRRSV entry and uncoating (51). Therefore, we examined the effect of the ADAM17 inhibitor BB94 on the expression level of both receptors in PAMs. Although BB94 treatment of PAMs did not affect CD169 expression level, BB94 treatment upregulated CD163 expression level. These data indicate that the upregulation of CD163 but not of CD169 facilitates PRRSV entry when ADAM17 activity is specifically inhibited.

In humans, CD163 is cleaved from the cell membrane by a proteolytic shedding mechanism in response to PMA, activation of Toll-like receptors such as Fc γ receptor cross-linking, and inflammatory stimuli such as lipopolysaccharide; the cleavage produces soluble CD163 (sCD163) (16–18). Recently, Etzerodt et al. showed that ADAM17 mediates the ectodomain shedding of hu-

man CD163 (19, 32) and that CD163 cleavage is species-specific because the CD163 juxtamembrane region in certain nonprimates lacks the substrate motif (¹⁰⁴⁴Arg-Ser-Ser-Arg). Here, our data show that Marc-145, a cell line derived from a primate, has a high level of CD163 expression, which can be downregulated by activated ADAM17 (Fig. 3). To rule out the effect of sCD163 on PRRSV infection, the cell culture medium was removed, and the cell monolayer was washed with PBS before viral inoculation for all PRRSV infection experiments as described in Materials and Methods. These data indicate that the modification of ADAM17 activity affects PRRSV entry by shifting the CD163 expression level on the Marc-145 cell surface.

Because a previous report (12) and the current report indicate that CD163 expression level decreases upon PRRSV infection, it seems that ADAM17 activity is directly stimulated by viral infection. In contrast to the juxtamembrane sequence of CD163 in primates, the juxtamembrane sequence of CD163 in swine is similar to that in monkeys and humans, and contains the CD163 cleavage motif (¹⁰⁴²Arg-Ser-Ser-Phe) (Fig. 4E). This suggests that the cleavage of porcine CD163 by ADAM17 might reduce PRRSV entry efficiency. It is also possible, however, that the effect of ADAM17 on PRRSV entry of PAMs might not be limited to the cellular receptor CD163. Previous reports showed that nontarget cells (BHK-21, PK-15, and CHO-K1) expressing CD163 are infected by VR2332, the North American type of PRRSV (52). In the present study, BHK-21 transfected with porcine CD163 cDNA were infected by PRRSV (Fig. 5B). Using this cell, we confirmed that ADAM17 can suppress PRRSV entry by downregulation of membrane CD163 expression level (Fig. 5C and D). These results indicate that ADAM17 is further activated by viral infection and that ADAM17 downregulates CD163, the putative receptor of PRRSV, and thereby suppresses PRRSV entry.

In addition to providing insight into a mechanism by which cells resist PRRSV infection, the present results could have practical applications. Besides the membrane-associated CD163, a soluble form of CD163 is present in plasma. Recently, the concentration of sCD163 has been identified as a useful biomarker for diseases such as sepsis, leukemia, and AIDS (53–56). Because CD163 is expressed primarily on macrophages and sCD163 is mainly secreted from macrophages, sCD163 might be regarded as a specific marker of macrophage function. Further studies are needed to test this hypothesis. Since membrane-associated CD163 plays a critical role in PRRSV entry, the CD163 molecule might be a useful target for preventing or reducing PRRSV infection. Also, Kowal et al. reported that CD163 has a strong anti-inflammatory potential, which suggests that CD163 plays an important role in immune response when pigs are exposed to PRRSV infection (57). Therefore, the combined use of ADAM17 and sCD163 may be useful in enhancing the porcine anti-PRRSV response.

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