Porcine Reproductive and Respiratory Syndrome Virus Utilizes Nanotubes for Intercellular Spread

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ABSTRACT

Intercellular nanotube connections have been identified as an alternative pathway for cellular spreading of certain viruses. In cells infected with porcine reproductive and respiratory syndrome virus (PRRSV), nanotubes were observed connecting two distant cells with contiguous membranes, with the core infectious viral machinery (viral RNA, certain replicases, and certain structural proteins) present in/on the intercellular nanotubes. Live-cell movies tracked the intercellular transport of a recombinant PRRSV that expressed green fluorescent protein (GFP)-tagged nsp2. In MARC-145 cells expressing PRRSV receptors, GFP-nsp2 moved from one cell to another through nanotubes in the presence of virus-neutralizing antibodies. Intercellular transport of viral proteins did not require the PRRSV receptor as it was observed in receptor-negative HEK-293T cells after transfection with an infectious clone of GFP-PRRSV. In addition, GFP-nsp2 was detected in HEK-293T cells cocultured with recombinant PRRSV-infected MARC-145 cells. The intercellular nanotubes contained filamentous actin (F-actin) with myosin-associated motor proteins. The F-actin and myosin IIA were identified as coprecipitates with PRRSV nsp1 β , nsp2, nsp2TF, nsp4, nsp7-nsp8, GP5, and N proteins. Drugs inhibiting actin polymerization or myosin IIA activation prevented nanotube formation and viral clusters in virus-infected cells. These data lead us to propose that PRRSV utilizes the host cell cytoskeletal machinery inside nanotubes for efficient cell-to-cell spread. This form of virus transport represents an alternative pathway for virus spread, which is resistant to the host humoral immune response.

IMPORTANCE

Extracellular virus particles transmit infection between organisms, but within infected hosts intercellular infection can be spread by additional mechanisms. In this study, we describe an alternative pathway for intercellular transmission of PRRSV in which the virus uses nanotube connections to transport infectious viral RNA, certain replicases, and certain structural proteins to neighboring cells. This process involves interaction of viral proteins with cytoskeletal proteins that form the nanotube connections. Intercellular viral spread through nanotubes allows the virus to escape the neutralizing antibody response and may contribute to the pathogenesis of viral infections. The development of strategies that interfere with this process could be critical in preventing the spread of viral infection.

For many enveloped viruses, entry into a host cell is primarily through the binding of cellular receptors and subsequent endocytosis of the viral particle into the cells. The fusion of envelope with the endosomal membrane releases viral capsid into the cytosol of the infected cell (reviewed in reference 1). However, for some enveloped viruses, alternative pathways for cell-to-cell transmission have been described (reviewed in references 2 to 4). One emerging model proposes that some viruses can use long, filamentous intercellular connections (nanotubes) as a means to transport infectious viral materials to neighboring naive cells. Previously, intercellular nanotubes have been described as nanotubes, tunneling nanotubes, and bridging conduits (5–8; reviewed in reference 9). The fundamental feature of the intercellular nanotube is a long membrane-bound extension that connects two neighboring cells and can also link multiple cells together to form complex cellular networks (6). Nanotubes are 50 to 200 nm in diameter and can span several cell distances. These structures are primarily composed of filamentous actin (F-actin) and also contain myosin as a motor to drive the movement of organelles or other cargo into neighboring cells (6, 9). Intercellular nanotubes offer cellular communication over long distances, particularly for transporting relatively large cellular materials (10).

In this study, we investigated whether porcine reproductive and respiratory syndrome virus (PRRSV) utilizes intercellular nanotubes as an alternative pathway to spread infection. PRRSV is an enveloped, positive-sense, single-stranded RNA virus. The viral genome is about 15 kb in length. The 5' two-thirds of the viral genome encodes two large replicase polyproteins, pp1a and pp1ab, which are proteolytically processed into at least 14 functional nonstructural proteins (nsp1 to nsp12, with nsp1 autocleaved into nsp1 α /nsp1 β and nsp7 autocleaved into nsp7 α /nsp7 β) (reviewed in reference 11). Recently, two novel proteins,

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TABLE 1 List of antibodies used in this study

Antibody group and name	Protein target ^b	Predicted molecular mass (kDa)	Reference(s) or source
PRRSV antibodies			
MAb 123-128	nsp1β	23.0	22
pAb nsp2TF	nsp2TF (C terminus; epitope CFLKVGKVSAGDLV)	110.1	12, 13
pAb nsp2	nsp2 (C terminus; epitope NGLKIRQISKPSGG)	129.4	This study; 32
MAb 140-68	PLP2	129.4	13
MAb 69-267	nsp4	21.0	This study
MAb 108-16	nsp7	28.6	This study
MAb 101-48	nsp8	4.9	This study
MAb NI37	GP4	19.5	21
MAb 21-79	GP5	22.4	This study
MAb SDOW17	N	13.6	20
MAb 14-126	N	13.6	This study
Cellular protein antibodies			
MAb ab8226	F-actin (beta-actin)	42	Abcam
MAB ab55456	Nonmuscle myosin IIA	215 (heavy chain)	Abcam
pAb HPA001644	Nonmuscle myosin IIA	215 (heavy chain)	Sigma
pAb sc-20800	SV40 large T antigen of HEK-293T cells	94	Santa Cruz

^a PRRSV strain SD95-21.
^b MABs were produced in mouse; pAbs were produced in rabbit.

nsP2TF and nsp2N, were found to be expressed in the nsp2-coding region through a $-2/-1$ ribosomal frameshifting mechanism (12, 13). The 3' end of the viral genome encodes envelope proteins (GP2a, E, GP3, GP4, GP5, ORF5a, and M) and also nucleocapsid (N) protein that encapsulates the genomic RNA (reviewed in reference 14). PRRSV has a very restricted tropism for host cells. Among many different cell lines tested, only the African green monkey kidney cell line MA-104 and derivatives such as MARC-145 are fully permissive to PRRSV infection *in vitro* (15). In previous studies, PRRSV receptor-mediated viral entry into host cells has been studied extensively (reviewed in reference 16). It was reported that PRRSV particles gain entry into host cells through standard clathrin-mediated endocytosis. Following endosome acidification and membrane fusion, the viral genome is released into the cytosol where viral transcription and replication occur (17, 18). In this study, we found that PRRSV also uses intercellular nanotubes for transporting the infectious viral materials (viral RNA, certain replicases, and certain structural proteins) into the cytosol of a neighboring cell. This route of viral transmission involves the interaction of certain viral proteins with cytoskeleton proteins. More importantly, intercellular transport of viral materials was still detected in the presence of virus-neutralizing antibodies, which provides a new insight into mechanisms of immune evasion and viral pathogenesis.

MATERIALS AND METHODS

Cells and viruses. Vero-76, HEK-293T, BHK-21, and MARC-145 cells were maintained in minimum essential medium (Gibco) supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml streptomycin). Porcine alveolar macrophages (PAMs) were collected by lung lavage of a 9-week-old PRRSV-naïve pig using a method described previously (19). (The pig experiment was performed according to protocols approved by the Institutional Animal Care and Use Committee of Kansas State University.) Macrophages were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin. Cells were maintained at 37°C with 5% CO₂. The PRRSV isolate SD95-21 (GenBank accession number KC469618) was used for subsequent experiments. The green fluorescent protein (GFP)-tagged recom-

binant PRRSV (GFP-PRRSV), constructed in this study (see below), was used for tracking PRRSV infection in real-time live cells.

Antibodies and probes. Table 1 lists polyclonal and monoclonal antibodies used in this study. Antibodies for detecting PRRSV proteins, including monoclonal antibody (MAb) 123-128 (anti-nsp1β), MAb 140-68 (anti-nsp2 N terminus), MAb NI37 (anti-GP4), MAb SDOW17 (anti-N), and a rabbit antiserum (polyclonal antibody [pAb]; anti-nsp2TF) specific to the C-terminal peptide of nsp2TF were described previously (12, 13, 20–22). The pAb (anti-nsp2) specific to the C-terminal epitope (NGLKI RQISKPSGG) of nsp2 was produced by GenScript. MAb 21-79 (anti-GP5) was generated by immunizing BALB/c mice with a truncated GP5 recombinant protein (containing amino acids 31 to 61 and 128 to 200 of GP5), while MAb 69-267 (anti-nsp4), MAb 108-16 (anti-nsp7), MAb 101-48 (anti-nsp8), and MAb 14-126 (anti-N) were produced by immunizing mice with individual full-length nsp4, nsp7, nsp8, and N recombinant proteins as the antigens, respectively. Detailed experimental procedures for MAb production were described previously (23, 24). Anti-beta-actin (also reacts with F-actin, as indicated by the vendor) and anti-nonmuscle myosin IIA MABs were obtained from Abcam, and rabbit antiserum to myosin IIA was purchased from Sigma. The Alexa Fluor 555 phalloidin for staining the F-actin and 4',6-diamidino-2-phenylindole (DAPI) for staining the nucleus were purchased from Invitrogen. Polyclonal antibody sc-20800 (anti-simian virus 40 [SV40] large T antigen) and anti-mouse IgG were purchased from Santa Cruz.

Plasmids and transfections. The plasmid (pCMV-SD95-21-GFP) for expression of GFP-PRRSV was constructed by inserting the GFP gene into a pSD95-21 full-length cDNA infectious clone (25) in which the nsp2 hypervariable region encoding amino acids 324 to 434 was replaced with the GFP gene (GenBank accession number AAB02574) to express a GFP-nsp2 fusion protein. To obtain recombinant GFP-PRRSV, BHK-21 cells were seeded in a 35-mm glass bottom dish (MatTek) and transfected with plasmid DNA of pSD95-21-GFP. Recombinant viruses were recovered using a previously described method (26). For detection of GFP-nsp2 transport through nanotubes, HEK-293T cells were seeded in six-well plates and transfected with plasmid DNA of pCMV-SD95-21-GFP. Transfection was performed using HD-FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals).

Viral RNA detection. Stellaris fluorescence *in situ* hybridization (FISH) probes were designed and generated by Biosearch Technologies.

Stellaris RNA FISH Probe Designer was used to analyze the RNA coding region of the PRRSV N gene. The set of probes containing 43 CAL594-labeled specific probes was generated, which covers 100% of the nucleotides of the N gene of PRRSV strain SD95-21. To detect the expression of PRRSV RNA, fixed cells were hybridized with the FISH probe set according to the manufacturer's instructions. Briefly, MARC-145 cells were infected with PRRSV at a multiplicity of infection (MOI) of 0.1 in 35-mm glass-bottom dishes (MatTek). Cells were fixed at 18 h postinfection (hpi) with the fixation buffer (3.7% formaldehyde solution in nuclease-free phosphate-buffered saline [PBS]) and then permeabilized with 70% ethanol for 2 h at 4°C. After the ethanol was discarded, wash buffer A (20% Stellaris RNA FISH wash buffer A and 1% deionized formamide in nuclease-free water) was added, and samples were incubated for 5 min at room temperature. Within a humidified chamber, 100 μ l of the hybridization buffer that contained RNA-detecting probe and anti-N MAb SDOW17 (1:2,000 dilution) was dispensed onto the cells. After 4 h of incubation in the dark at 37°C, 1 ml of wash buffer A plus 1:500-diluted goat anti-mouse pAb conjugated with Alexa Fluor 488 (Jackson ImmunoResearch) was added as the secondary antibody (Ab). The petri dishes were then incubated in the dark at 37°C for an additional 1 h. The cell nuclei were counterstained with 1 μ g/ml DAPI (Invitrogen). After samples were washed with buffer B (Stellaris RNA FISH wash buffer B), the coverslips were removed from the glass-bottom dishes using removal fluid (MatTek) and mounted on the section slides with Prolong (Invitrogen). Confocal microscopy was performed with an LSM 880 instrument (Zeiss).

Immunofluorescence assays and live-cell microscopy. MARC-145 cells or PAMs were grown on glass-bottom 35-mm cell culture dishes (MatTek). MARC-145 cells were infected with PRRSV at an MOI of 0.1 or mock infected with infection medium (Dulbecco's modified Eagle's medium [DMEM] containing 2% horse serum and 100 μ g/ml streptomycin). Alternatively, PAMs were infected with PRRSV at an MOI of 1 or mock infected with the infection medium. At 12 hpi, cells were fixed with 4% paraformaldehyde (PFA) for 10 min, permeabilized with 0.5% Triton X-100 for 10 min, and then blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. To detect PRRSV proteins, a specific MAb at a concentration of 1:1,000 was used, as we described previously (12, 21, 23). To detect filamentous actin (F-actin) or myosin IIA, cells were stained with Alexa Fluor 594-conjugated phalloidin (Molecular Probes) or anti-myosin IIA rabbit pAb (Sigma). After 1 h of incubation at 37°C, cells were washed with PBS, and a secondary antibody, Alexa Fluor 488 AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch) or Alexa Fluor 594 AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch), was added at a concentration of 1:250 in PBS. After cells were incubated at room temperature for 1 h, they were washed in PBS and then mounted onto glass slides using Prolong Gold with DAPI (Invitrogen). Slides were left to set at 4°C in the dark. For live-cell movies, infected MARC-145 cells or transfected HEK-293T cells were set into an open cultivation system of the Zeiss confocal microscope and maintained in warm DMEM buffered with HEPES. The live-cell chamber was mounted on a heated stage to maintain the culture at 37°C. Immunostained and live cells were imaged with an LSM 880 Zeiss confocal microscope (Zeiss). Collected images were processed using Zen 2 and Adobe Photoshop CS3.

Cocultivation of MARC-145 and HEK-293T cells. MARC-145 cells were infected with recombinant GFP-PRRSV (MOI of 1). At 12 hpi, cells were washed with PBS and trypsinized, and 2×10^4 infected MARC-145 cells were mixed with 2×10^5 HEK-293T cells. The mixed cells were seeded on a 35-mm glass-bottom cell culture dish. As controls, the GFP-PRRSV-infected MARC-145 cells and HEK-293T cells were also cultured separately. After 36 h of cocultivation, cells were fixed with 4% PFA for 10 min, permeabilized with 0.5% Triton X-100 for 10 min, and then blocked with 1% BSA in $1 \times$ PBS for 30 min at room temperature. To differentiate HEK-293T cells from MARC-145 cells, cells were stained with pAb sc-20800 (Santa Cruz) that recognizes the SV40 large T antigen in HEK-293T

cells. To detect PRRSV nsp2 protein, MAb 140-68 was used at a concentration of 1:1,000. After 1 h of incubation at 37°C, cells were washed with PBS, and secondary antibodies, Alexa Fluor 488 AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch) and Alexa Fluor 594 AffiniPure goat anti-Rabbit IgG (Jackson ImmunoResearch), were added at concentrations of 1:250 in PBS. After incubation at room temperature for 1 h, cells were washed in $1 \times$ PBS. Immunostained cells were imaged with an LSM 880 Zeiss confocal microscope (Zeiss). Collected images were processed using Zen 2 and Adobe Photoshop CS3.

IP and SDS-PAGE. Whole-cell lysates of infected or transfected cells were suspended in Pierce immunoprecipitation (IP) lysis buffer. To reduce nonspecific background, cell lysates were precleared with preimmune rabbit serum or nonspecific mouse ascites. Protein A/G Plus magnetic beads (Pierce) and specific MAb were added to precleared cell lysates. After samples were incubated overnight at 4°C, immune complexes were washed three times with wash buffer and one time with ultrapure water. After samples were boiled in $2 \times$ Laemmli sample buffer for 5 min, proteins were separated on an 8 to 16% SDS-PAGE gradient gel (Invitrogen).

Western blotting. Western blotting was performed as we described previously (12, 23). The membrane was probed with a protein-specific MAb or pAb. IRDye 680-conjugated goat anti-rabbit Ab and/or IRDye 800CW-conjugated goat anti-mouse Ab (Li-Cor Biosciences) was used as the secondary antibody. Imaging of the blot was performed using an Odyssey infrared imaging system (Li-Cor Biosciences).

Mass spectrometry. Immunoprecipitation was performed as we described previously (12). Myosin and actin were coimmunoprecipitated from PRRSV-infected MARC-145 cells using anti-GP5 MAb. Proteins from coimmunoprecipitations (co-IPs) were separated on an 8 to 16% SDS-PAGE gradient gel and stained with Coomassie brilliant blue G-250 (Bio-Rad). The gel was destained, and protein bands at the predicted sizes of myosin and actin were excised. In-gel trypsin digestion and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis (Bruker Daltonics Ultraflex II) were performed at the Biotechnology and Proteomics Core Facility at Kansas State University. Mass spectra were searched against a Swiss-Prot protein database and analyzed by mMass software (<http://www.mmass.org>).

Neutralization assays. Immune serum from PRRSV-infected pigs was initially used in a standard PRRSV-neutralizing assay as described previously (27). As a control, serum sample from uninfected pigs was included in the assay. Briefly, a 2-fold dilution of the serum sample was prepared in a 96-well plate (100 μ l/well). PRRSV SD95-21 (200 50% tissue culture infective doses [TCID₅₀]; 100 μ l/well) was added to mix with serum, and the mixture was incubated for 1 h at 37°C. After incubation, the serum-virus complex was transferred onto confluent MARC-145 cells that were plated 2 to 3 days ahead. At 18 hpi, cells were fixed using acetone-methanol (1:1 ratio) at -20°C for 30 min. Fixed cells were stained with PRRSV N protein-specific MAb, and Alexa Fluor 488 AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch) was used as a secondary antibody. Fluorescent foci of infected cells were observed and counted using a phase-contrast fluorescence microscope. Virus titers were interpreted as the number of fluorescent focus units per milliliter (FFU/ml). The serum dilution that caused greater than 90% inhibition of virus infection was used in subsequent experiments to determine its effect on intercellular spreading of the virus. PRRSV SD95-21 (200 TCID₅₀) was first added onto confluent MARC-145 cells. After 3 h of incubation at 37°C to allow virus entry into cells, an identified serum sample with a known virus-neutralizing titer was added onto the PRRSV-infected cells. At 6, 12, 24, or 36 hpi, cells were fixed, stained with specific antibodies, and analyzed by a phase-contrast fluorescence microscope. Cell culture supernatant was harvested at 6, 12, 24, and 36 hpi, and virus titers were determined by virus titration on MARC-145 cells.

Drugs and virus replication inhibition assay. The actin inhibitor cytochalasin D (Sigma) and the myosin II inhibitor ML7 (Sigma) were dissolved in dimethyl sulfoxide (DMSO). Cytochalasin D and ML7 were

stored as 10 mM stocks at -20°C in aliquots for single use. For virus replication inhibition assays, confluent MARC-145 cells in glass-bottom 12-well tissue culture plates (MatTek) were pretreated with compounds at a concentration of 0 μM or 5 μM . After a 30-min incubation at 37°C , the cells were infected with 200 TCID₅₀ of PRRSV. After 1 h of incubation, the infected cells were washed twice with $1\times$ PBS, and fresh medium containing the compounds was added. The cells were fixed and stained at 24 hpi, and the supernatant was collected for virus titration. PRRSV titers were determined by fluorescent-focus assay, as we described previously (28). Cell viability was determined using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions.

RESULTS

Intercellular nanotubes contain PRRSV proteins and RNA. In previous studies of influenza virus and retrovirus transmission, viral membrane and nucleocapsid proteins were found to be associated with intercellular nanotubes (7, 29, 30). To investigate the spreading of viral materials through intercellular nanotubes during PRRSV infection, MARC-145 cells were first infected with PRRSV strain SD95-21. Mock-infected cells were included as a control (data not shown). At 12 hpi, cells were fixed, permeabilized, and immunostained using a MAb against GP5 or N protein. Since F-actin and myosin are known to be involved in the formation of intercellular nanotubes, cells were also doubly stained for F-actin or myosin IIA to visualize nanotubes. Using confocal microscopy, contiguous, long F-actin- and myosin IIA-containing intercellular nanotubes were clearly visualized connecting two neighboring cells. PRRSV infection promoted the intercellular nanotube formation. There was a 6.5-fold increase in the number of nanotubes in infected cells at 12 hpi compared to the level observed in noninfected cells (data not shown). In PRRSV-infected MARC-145 cells, GP5 was detected as punctate structures spreading through the nanotubes, while N appeared to be inside the nanotubes (Fig. 1A to D). Since PAMs are the primary host cells for PRRSV, we also examined PRRSV-infected PAMs. The abundance of nanotubes in PAMs was lower than that in MARC-145 cells, which could be related to differences in cell morphology and density. However, where nanotubes connecting PAMs were present, PRRSV GP5 and N were detected in association (Fig. 1E and F). These results suggested that, in addition to influenza virus and retroviruses (7, 29, 30), PRRSV could also transport viral materials through intercellular nanotube connections. We next determined which viral proteins could be transported through the nanotubes in MARC-145 cells. A panel of available polyclonal and monoclonal antibodies (Table 1) was used in immunofluorescence confocal microscopy. Intercellular nanotubes were detected containing PRRSV nsp1 β , nsp2, nsp2TF, nsp4, nsp7, and nsp8 in infected cells (Fig. 2A to F and H to M). In all cases, the viral proteins were in highly localized puncta. Interestingly, few nanotubes were observed containing GP4. Under the 10 microscopic fields of view that we searched, we found only one nanotube that contained GP4; most of the nanotubes that we detected lacked GP4 although they connected two neighboring infected cells (Fig. 2G and N). To further confirm this result, we repeated the experiment, and cells were doubly stained with anti-nsp2 pAb and anti-GP4 MAb. As a comparison, other cells were doubly stained with anti-nsp2 pAb and anti-GP5 MAb. Nanotubes connecting two infected cells could be easily detected under each microscopic field of view, and they all contained nsp2 and GP5 (Fig. 2O and P); however, most of them did not contain GP4 (Fig. 2O). Again, we

FIG 1 Detection of intercellular nanotubes containing PRRSV GP5 and N protein. (A to D) MARC-145 cells were infected by PRRSV strain SD95-21 at an MOI of 0.1 and fixed at 12 hpi. (E and F) Porcine alveolar macrophages were infected at an MOI of 1 with PRRSV and fixed at 12 hpi. The fixed cells were immunostained for GP5 (green; A, B, and E) or N protein (green; C, D, and F) together with cytoskeleton protein of F-actin (red; A and C) or myosin IIA (red; B, D, E, and F). Images were taken by a confocal microscope (LSM 880; Zeiss). Scale bars, 10 μm (merged images) and 5 μm (zoomed images, right column).

FIG 2 Intercellular nanotubes contain viral proteins in PRRSV-infected cells. MARC-145 cells were infected by PRRSV strain SD95-21 at an MOI of 0.1 and fixed at 12 hpi. (A to N) The fixed cells were immunostained for nsp1 β (A and H), nsp2TF (B and I), nsp2 (C and J), nsp4 (D and K), nsp7 (E and L), nsp8 (F and M), or GP4 (G and N) together with F-actin (A to G) or myosin IIA (H to N). Viral proteins were labeled with green fluorescence, and cytoskeleton proteins were labeled with red fluorescence. (O to Q) Double staining of nsp2 with GP4 (O and Q) or GP5 (P). The PRRSV nsp2 was labeled with green fluorescence, and GP4 or GP5 was labeled with red fluorescence. Pictures were taken by a confocal microscope (LSM 880; Zeiss). Scale bars, 10 μ m (merged images) and 5 μ m (zoomed images, right column).

found only two nanotubes containing both nsp2 and GP4 within 10 microscopic fields of view (Fig. 2Q).

Previous studies showed that arterivirus replicase proteins associated with genomic RNAs to form replication and transcrip-

tion complexes (RTCs), the viral replicative machinery inside the host cell (reviewed in reference 11). Thus, we suspected that PRRSV may transport entire RTCs through intercellular nanotubes. We performed fluorescence *in situ* hybridization to deter-

FIG 2 continued

mine whether PRRSV RNA might be within nanotubes. Using a set of PRRSV N gene-specific FISH probes labeled with Cal-594, viral RNA was indeed found within nanotubes (Fig. 3A). Since viral N proteins are associated with genomic RNA to form nucleocapsids, N proteins were also immunostained and imaged. N proteins were indeed found to be colocalized with the viral RNA in the nanotubes (Fig. 3B to D), suggesting that nucleocapsid proteins transport with viral RNA through intercellular nanotubes.

Cell-free virions are not required for intercellular spread of PRRSV infection through nanotubes. To determine whether intercellular nanotube connections could serve as an alternative pathway to transfer infections between cells, we analyzed PRRSV spread in the presence of PRRSV-neutralizing antibodies. Initially, a standard virus-neutralizing assay was used to determine the neutralizing antibody titer of a swine immune serum. As a control, a negative serum sample from uninfected pigs was included in the analysis. The result showed that the swine immune serum at a titer of 1:4 completely blocked the virus infection (Fig. 4A), indicating that the swine serum blocked the initial virus entry into the host cells. To determine whether the virus could use an alternative pathway to spread the infection, we first infected cells with the virus in the absence of swine serum and then added 1:4 swine immune serum at 3 hpi. At 6, 12, 24, and 36 hpi, cells were immunostained with anti-N MAb. At 6 hpi, single, fluorescent infected cells were observed at frequencies equivalent to those of the control cultures receiving negative serum. In general, it takes about 12 h for PRRSV to complete a cycle of replication and release progeny particles. In the presence of the virus-neutralizing serum, few small foci with intercellular nanotube connections were observed at 12 hpi; larger foci with more nanotubes were observed at 24 and 36 hpi, indicating that the viruses were continuing spread from cell to cell in the presence of neutralizing antibody (Fig. 4B). To confirm that neutralizing antibody remained through the time course, cell culture supernatants were inoculated onto fresh MARC-145 cells. Culture

FIG 3 Detection of PRRSV RNA in the intercellular nanotube connections. MARC-145 cells were infected by the PRRSV SD95-21 strain at an MOI of 0.1 and fixed at 12 hpi. (A) Viral RNA was detected by fluorescence *in situ* hybridization of RNA using a CAL594-labeled PRRSV N gene RNA FISH probe (red). (B) PRRSV N protein was immunostained using anti-N MAb SDOW17 (green). (C) Cell nucleus is stained with DAPI (blue). (D) The colocalized foci are readily visible in the nanotubes (yellow). Images were taken by a confocal microscope (LSM 880; Zeiss). Scale bar, 20 μ m.

supernatants containing swine immune serum did not generate any infections, while parallel control supernatants containing preimmune serum generated infection, with the virus titer reaching 2.5×10^6 FFU/ml (Fig. 4C).

The data shown in Fig. 4 suggested that there are two modes of PRRSV spread, through extracellular virus particles and through nanotubes. The presence of neutralizing antibody in the cell culture supernatant restricted the extracellular virus spread, leaving the nanotube connections to spread the infection (Fig. 4). To confirm this, live-cell movies were taken to visualize the real-time movement of viral proteins. To track protein movement, we used a recombinant PRRSV (GFP-PRRSV) that expresses GFP-tagged nsp2. The experiment conducted to obtain the data shown in Fig. 4 was repeated using GFP-PRRSV and was performed in the presence of virus-neutralizing antibody. At 24 hpi, infected cells were analyzed using a live-cell imaging system. The movie was taken with 30-s frames over a 70-min time course. GFP-nsp2 was clearly observed moving through an intercellular nanotube into another neighboring cell (see Movie S1 in the supplemental material). Selected frames from the live-cell movie are presented in Fig. 5A, in which the yellow arrows and inset squares indicate the position of GFP-nsp2 as it moves from the lower cell into the upper cell. As a comparison, GFP-PRRSV-infected cells maintained without PRRSV-neutralizing antibody were also included in the analyses (Fig. 5B; see also Movie S2). Similar results were obtained under both cell culture conditions.

PRRSV cell receptors are not required for intercellular spread of infection through nanotubes. Cell-free virus infections

FIG 4 Intercellular spreading of PRRSV infection in cells with the presence of virus-neutralizing antibody. (A) Standard virus-neutralizing assay. Immune serum from PRRSV-infected pigs was serially diluted and incubated with the virus for 1 h; the virus-antibody complex was then added on the MARC-145 cells. At 12 hpi, cells were stained with anti-N MAb, and virus foci were counted under the fluorescence microscope. The percentage of viral growth reduction was calculated in comparison to the result from cells treated with negative-control serum. (B) Cell-to-cell spreading of the viruses under the existence of virus-neutralizing antibody. MARC-145 cells were infected with PRRSV SD95-21. At 3 hpi, the 1:4-diluted immune serum or negative-control serum was added onto the infected cells. At 6, 12, 24, or 36 hpi, cell culture supernatant was harvested, and cells were fixed and stained with anti-N MAb. Images were taken by a confocal microscope (LSM 880; Zeiss). Scale bar, 50 μ m. (C) Virus titers in the harvested cell culture supernatant from the experiment described in panel B. Virus titer was determined by counting the virus foci under the fluorescence microscope, and the result was interpreted as the number of fluorescent focus units (FFU) per ml. NS, negative-control serum from a noninfected pig; PS, immune serum from a PRRSV-infected pig.

require host cell receptors (reviewed in reference 16). To determine whether nanotube-mediated transfer of PRRSV requires receptors, we transfected the full-length cDNA infectious clone of GFP-PRRSV, pSD95-21-GFP, into HEK-293T cells and then monitored virus spread. HEK-293T cells do not express the PRRSV receptor and cannot be infected by PRRSV particles, but they can support PRRSV replication once the viral genome is transfected into the cells. At 24 h posttransfection, cell culture supernatant was harvested, and the virus titer was determined by fluorescent-focus assay. The result confirmed that infectious viral progenies were produced in transfected cells, with the virus titer reaching 2×10^4 FFU/ml. Transfected cells were analyzed by a live-cell imaging system. GFP-nsp2 was clearly observed moving through an intercellular nanotube into another neighboring HEK-293T cell (Fig. 5C; see Movie S3 in the supplemental material). Of note, GFP-nsp2 appeared to be moving more slowly in HEK-293T cells than in MARC-145 cells (compare Movies S1, S2, and S3 in the supplemental material). In the nanotubes that we observed, it took about 16 min for the GFP-nsp2 particle to move from one MARC-145 cell to another, while 67 min was required for the GFP-nsp2 particle to move from one HEK-293T cell to another.

To further confirm our result, we determined whether viral components could transfer from infected MARC-145 cells to un-

infected, PRRSV receptor-negative HEK-293T cells. A MARC-145 and HEK-293T cell cocultivation experiment was performed. MARC-145 cells were first infected with GFP-PRRSV (Fig. 6A) and then mixed at 12 hpi with naive HEK-293T cells. After 36 h of cell cocultivation, cells were fixed and visualized by confocal microscopy. Since GFP fluorescence becomes dim after cell fixation, we used anti-nsp2 MAb to detect the expression of GFP-nsp2 in the infected cells (Fig. 6, first column). To differentiate the HEK-293T cells from MARC-145 cells, we used rabbit antiserum that specifically recognizes SV40 large T antigen of HEK-293T cells (Fig. 6, second column). Notably, nanotubes were observed connecting infected MARC-145 cells with HEK-293T cells, and GFP-nsp2 was detected in the nanotube connection and in the target HEK-293T cells (Fig. 6C). In this particular image, the infection was transferred from the MARC-145 cell to a fairly distant HEK-293T cell.

Cytoskeleton proteins are involved in intercellular transportation of viral proteins. Given that the cytoskeleton proteins F-actin and myosin are present in intercellular nanotube structures, we determined whether nanotube-associated viral proteins interact with the cytoskeleton proteins. The membrane proteins of several other viruses were previously reported to interact with myosin (8, 31). Interaction of PRRSV GP5 and myosin was initially analyzed by immunoprecipitation. Lysates of PRRSV-in-

FIG 5 Live-cell images demonstrating intercellular transport of PRRSV GFP-tagged nsp2 through nanotubes. (A) GFP-PRRSV-infected cells maintained in culture medium containing PRRSV-neutralizing antibody. (B) GFP-PRRSV-infected cells maintained in regular cultural medium (no PRRSV-neutralizing antibody). (C) HEK-293T cells were transfected with a PRRSV full-length cDNA infectious clone, pCMV-SD95-21-GFP. At 24 h postinfection (A and B) or transfection (C), cells were analyzed using a live-cell image system of a confocal microscope (LSM 880; Zeiss). In both infected MARC-145 cells and transfected HEK-293T cells, the dot-like GFP-nsp2 proteins were visualized as moving through an intercellular nanotube connection (arrows) into the cytoplasm of a neighboring cell. Insets show a zoomed area of interest that contains the GFP-nsp2 proteins. The specific nanotube shown between MARC-145 cells is about 20 μm in length, while the specific nanotube shown between HEK-293T cells is about 12 μm in length. Scale bar, 10 μm .

infected MARC-145 cells were harvested at 36 hpi, and viral proteins were immunoprecipitated using anti-GP5 MAb and then separated by SDS-PAGE. Protein bands were detected by Coomassie brilliant blue staining. Excluding the two bands of 50 and 25 kDa of the MAb heavy and light chains, the other two prominent bands with apparent masses close to 250 kDa and 50 kDa were subjected to MALDI-MS analysis. The band close to 250 kDa was identified as nonmuscle myosin heavy chain IIA (myosin IIA; predicted molecular mass of 215 kDa), while the band close to 50 kDa was identified as F-actin (predicted molecular mass of 42 kDa) (Fig. 7A). Subsequently, we determined whether the PRRSV proteins present in the nanotubes (Fig. 1 and 2) also interacted with myosin and F-actin. A panel of specific antibodies recognizing PRRSV nsp1 β , nsp2, nsp2TF, nsp4, nsp7, nsp8, GP4, GP5, and N protein were used in IP and Western blot analysis. Immunoprecipitated proteins from PRRSV-infected cells were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against myosin IIA and F-actin. Western blotting confirmed that myosin IIA and F-actin coprecipitated with GP5, but they were not detected in the coprecipitate of GP4 (Fig. 7B). The specificity of the experimental condition was further confirmed using anti-mouse IgG as a control. Subsequently, myosin IIA and F-actin were also detected as coprecipitates with N, nsp1 β , nsp2, nsp2TF, nsp4, nsp7, and nsp8 (Fig. 7B and C). This result is consistent with the results shown in Fig. 1 and 2, in which GP5, N, nsp1 β , nsp2, nsp2TF, nsp4, nsp7, and nsp8 were associated with nanotubes.

To verify the specific interaction of PRRSV proteins with myosin IIA and F-actin, co-IP was performed in PRRSV-infected cells using the antibody against myosin IIA. Western blot analysis using PRRSV protein-specific antibodies detected GP5, N, nsp1 β , nsp2, and nsp4 coprecipitated with myosin IIA (Fig. 7D, E, G, H, and J). Again, GP4 was not detected (Fig. 7F). In addition, nsp2TF, nsp7, and nsp8 were also not specifically detected although they were detected in the nanotubes and although their specific MAbs were able to pull down the myosin in the co-IP experiment (Fig. 7B).

Since F-actin and myosin are within nanotube structures and since myosin IIA was identified to interact with certain viral proteins, we further determined whether disrupting the structure of F-actin and myosin IIA could block the intercellular nanotube pathway for cell-cell spreading of the virus. PRRSV-infected cells were treated with ML7, cytochalasin D, or solvent control dimethyl sulfoxide (DMSO). ML7 is a specific inhibitor of myosin light-chain kinase, regulating myosin IIA function. Cytochalasin D depolymerizes actin by binding to F-actin, which causes breakage of the actin filaments. Using confocal microscopy, PRRSV-infected cells that were treated with ML7 or cytochalasin D showed significantly fewer (80% less) intercellular nanotube connections than those that were treated with DMSO, and few viral clusters were observed at 24 dpi (Fig. 8A). In the presence of either inhibitor, cell viabilities were not affected at the tested concentration (5 μM for ML7 and cytochalasin D) (Fig. 8B), but viral titers were reduced by several logs (2.56-log reduction for ML7; 1.93-log

FIG 6 Inter-cellular transport of the GFP-nsp2 protein in cocultured MARC-145 and HEK-293T cells. (A) GFP-PRRSV-infected MARC-145 cells were fixed and stained with anti-nsp2 MAb 140-68 and anti-SV40 T antigen pAb sc-20800. (B) GFP-PRRSV-infected HEK-293T cells were fixed and stained with anti-nsp2 MAb 140-68 and anti-SV40 T antigen pAb sc-20800. (C) GFP-PRRSV-infected MARC-145 cells were trypsinized at 12 hpi and mixed with naïve HEK-293T cells. At 36 h postcultivation, cells were fixed and stained with anti-nsp2 MAb 140-68 and anti-SV40 T antigen pAb sc-20800. The nsp2 was labeled with green fluorescence, and SV40 large T antigen was labeled with red fluorescence. Pictures were taken by a confocal microscope (LSM 880; Zeiss). Scale bar, 10 μ m.

reduction for cytochalasin D) in comparison to the titer of the DMSO-treated control (Fig. 8C).

DISCUSSION

The primary pathway for cell-to-cell spread of the PRRSV virus, an enveloped RNA virus, involves assembly, maturation, and release from virus-producing cells and attachment, entry, and disassembly in virus target cells. These processes require abundant cellular resources and also demand that the virus particles evade extracellular host defensive components. In this study, we found that PRRSV can use intercellular nanotube connections as an alternative pathway for cell-to-cell spread. Utilizing this pathway to directly access the cytoplasm of a naïve cell presents an efficient spreading route that bypasses many of the otherwise critical assembly, budding, and cell entry steps.

Inside the host cell cytoplasm, the genomic RNA and replicase-associated RTC constitute the infectious core components that elicit a productive infection. Thus, intercellular transport of viral genomic RNA and replicase proteins is sufficient for rapid spreading of the infection. In PRRSV-infected cells, confocal images showed that PRRSV replicase proteins nsp1 β , nsp2, nsp4, nsp7, and nsp8 were associated with intercellular nanotubes. The presence of PRRSV nsp2 in an intercellular connection was reported previously (32), but the mechanism by which this protein arrived in the connection and its relevance to virus spread were unknown at that time. In our study, the movement of viral proteins from cell to cell was documented with live-cell movies, in which GFP-tagged nsp2 was transported from one cell to another in GFP-PRRSV-infected cells. This process was further demonstrated to take place between unrelated MARC-145 and HEK-293T cells.

Since nsp1 β , nsp2, nsp4, nsp7, and nsp8 are components of the viral RTC, the data indicate that PRRSV transports RTC components through intercellular nanotubes. PRRSV proteins nsp9 to nsp12 are also assumed to be part of the RTC. The nsp9 protein is generated by a -1 programmed ribosomal frameshift (PRF) at the ORF1a/1b junction, resulting in the nsp9 product that contains nsp8 in its N terminus. This means that the anti-nsp8 MAb could also recognize nsp9; however, future study using an nsp9 C terminus-specific antibody is needed to confirm the presence of nsp9 in the nanotube connections. Due to lack of specific antibodies to nsp1 α , nsp5-nsp6, and nsp10 to nsp12, whether these replicase-associated proteins are transported through the nanotube connections needs to be determined in the future. Furthermore, double-membrane vesicles (DMVs) were previously reported to be associated with the RTC of arterivirus (33); it is possible that DMVs are transported with the viral materials. In mouse CAD cells, vesicles of lysosomal origin carrying prion proteins are actively transferred through intercellular nanotubes (34). In HIV type 1 infections, viral material-containing endosome cargoes were transported through nanotubes to neighboring uninfected macrophages (8). The involvement of cellular vesicles in nanotube transport of viral materials is currently under active investigation in our laboratories. Notably, the novel PRRSV -2 PRF product, nsp2TF, was also observed to associate with intercellular nanotubes but was not colocalized with the other nanotube-associated nsps (data not shown). This is consistent with our previous findings in which nsp2TF and nsp2 are not colocalized in infected cells (12). Therefore, there may be multiple independent mechanisms for nsp association with nanotubular components. In addition, it

FIG 7 Coprecipitation of PRRSV proteins with cytoskeleton proteins F-actin and myosin IIA. (A to C) PRRSV-infected (+) MARC-145 cell lysates or mock-infected (–) cell lysates were used for immunoprecipitation using PRRSV protein-specific MAb as indicated on the top of each panel. Anti-mouse IgG (α -mIgG) was used as a control. The immunoprecipitated proteins were separated by 8 to 16% Tris-glycine gradient gels (A) and immunoblotted by anti-myosin IIA pAb and anti-F-actin mAb (B and C). (D to L) PRRSV-infected MARC-145 cell lysates (+) or mock-infected (–) cell lysates were used for immunoprecipitation using anti-myosin IIA pAb. The immunoprecipitated proteins were separated by 8 to 16% Tris-glycine gradient gels and immunoblotted by PRRSV protein-specific MAb as indicated on the bottom of each panel. IP, immunoprecipitation; WB, Western blotting. Arrows in panels A to C indicate F-actin (42 kDa) and nonmuscle myosin heavy chain IIA (215 kDa). Arrows in Panels D, E, G, H, and J indicate the target protein, as shown at the bottom of each panel.

is worth noting here that some of the GFP-tagged nsp2 that we observed could be GFP-nsp2TF or GFP-nsp2N since a $-2/-1$ PRF in the nsp2 region generated $\sim 20\%$ of nsp2TF and $\sim 7\%$ nsp2N, respectively (12).

Theoretically, viral membrane proteins typically needed for virus cell egress and entry might be dispensable for virus transport through intercellular nanotubes. Recent studies suggested that PRRSV minor glycoproteins (GP2a, GP3, and GP4) are the primary determinants of host cell binding and may also be involved in membrane fusion and entry (35, 36). Using the PRRSV GP4-specific MAb, few nanotubes were detected containing GP4, which supports the notion that these membrane proteins may not be required for spreading the infection using the intercellular nanotube pathway. In contrast, significant amounts of GP5 proteins were detected associated with intercellular nanotubes. It was

reported that a GP5/M protein heterodimer of arterivirus plays an important role in viral envelope formation (37–39). GP5 and M have been proposed to be the driving force for virus budding, which may be involved in the formation of the highly curved edges of the membrane (reviewed in reference 40). Our confocal microscopic images showed that GP5 proteins appeared as cell surface membrane-bound structures around the infected cells as well as on the intercellular nanotubes. The result made us suspect that GP5 could be integrated into the extended cell surface membrane during the formation of intercellular tunnels (nanotubes), in which GP5 becomes a component of the nanotube for transporting the other viral infectious core materials. It is unknown whether GP5 is directly involved in the transporting process of viral materials or is a more passive component of nanotubes. The unavailability of the anti-M protein antibody and also antibodies

FIG 8 Inhibitors of F-actin and myosin IIA affect the intercellular spread of PRRSV. (A) Confluent MARC-145 cell monolayers were pretreated with ML7 or cytochalasin D (Cyto D) compound at a concentration of 5 μ M or 0 μ M. Nontreated cells (NT) were used as a control. After a 30-min incubation at 37°C, the cells were infected at an MOI of 0.1 with PRRSV. After 1 h of incubation, the infected cells were washed with PBS, and fresh medium containing cytochalasin D or ML7 compound was added. At 24 hpi, cells were fixed and immunostained with PRRSV N protein-specific MAb. Images in columns 1 and 3 were taken using a 20 $\times$ objective lens (scale bar, 50 μ m). The inset at the bottom right corner of each image shows a $\times$ 40 zoomed region of interest (ROI). Scale bar for the inset, 10 μ m. (B) Effect of cytochalasin D or ML7 on the viability of the MARC-145 cells compared to the viability of DMSO-treated (0 μ M) or nontreated (NT) control cells. Graphs show the results (mean and standard deviation) of a representative experiment ($n = 3$). (C) Viral growth inhibition efficiency of the myosin IIA inhibitor ML7 or F-actin inhibitor cytochalasin D. Virus titration was conducted using cell culture supernatant collected from the experiment in shown in panel A. Virus titer was determined by counting the virus foci under the fluorescence microscope, and the result was interpreted as fluorescent focus units (FFU) per milliliter. Statistical significance between the groups was determined by a one-way analysis of variance test using GraphPad InStat Prism (version 5.0), and a P value of < 0.01 (***) was considered statistically significant.

to GP2a, E, and GP3 prevented us from analyzing the association of these proteins with nanotubes. Future studies are needed to determine whether M and other membrane proteins play a role in the intercellular spreading of the virus through nanotube connections. In addition, it will be interesting to know whether one or more viral membrane proteins promote nanotubular extension from a cell and fusion with a neighboring cell. Our data showed that PRRSV receptors are not required for nanotubular virus transport, which suggests that viral fusion proteins may not be needed to establish the nanotubular connections. Future in-depth analyses are needed to determine whether PRRSV infection and certain viral protein(s) promote nanotube formation.

Using the FISH method, viral RNA and N protein were observed to be largely colocalized within the nanotubes. This result is expected since viral genomic RNA is required to generate the next cycle of viral replication. As discussed above, viral genomic RNA is packaged with N proteins to form the nucleocapsid. Transporting the N protein-bonded RNA from cell to cell could be a mechanism for protecting the integrity of viral RNA during the transportation process. It still needs to be elucidated whether the assembled nucleocapsid was transported or whether only unassembled proteins were transported.

Intercellular nanotubes are composed of F-actin, and myosin is known as a motor protein binding to F-actin (reviewed in refer-

ences 41 and 42). Myosin is encoded by a multigene family and expressed as multiple isoforms (43). Previously, myosin Va was reported to facilitate the movement of organelles along intercellular nanotubes (reviewed in reference 9). Recently, myosin X was reported to play an important role in the formation of functional intercellular nanotubes within neuronal CAD cells (44). The findings of our study suggest that myosin IIA could be driving the transportation of PRRSV core infectious materials through the intercellular nanotube connections. In live-cell movies, we noticed that the movement of GFP-nsp2 within nanotubes proceeded by leaps rather than by gradual transitions, a pattern which is consistent with motor protein-assisted cargo transport. Our co-IP results showed that myosin IIA can be coprecipitated with PRRSV nsp1 β , nsp2, nsp4, GP5, and N, suggesting that these viral proteins may directly or indirectly bind to myosin IIA during transport. Confocal microscopy also detected PRRSV nsp2TF, nsp7, and nsp8 in the intercellular nanotubes, and using nsp2TF-, nsp7-, and nsp8-specific antibodies, myosin IIA could be coprecipitated from infected cells. However, using myosin IIA-specific antibody, these viral proteins were not coprecipitated. This could be caused by lower protein expression levels, the insensitivity of our assay, or other unrevealed mechanism(s). Future studies are needed to determine the mechanisms of interaction between GP5 and other viral proteins with cytoskeleton proteins; the detailed intercellular nanotube transporting process needs to be eluci-

dated, including the process of how the viral materials are initially loaded, transported through the nanotubes, and unloaded in the target cells.

The use of intercellular nanotube connections as alternative pathways for viral cell-to-cell transmission could contribute to the pathogenesis of viral infection. Notably, PRRSV-neutralizing antibodies could block initial virus entry into the host cells but could not interfere significantly with cell-to-cell transmission through intercellular nanotube connections. Previous studies also reported that viral spread through intercellular connections allows many viruses, including influenza A virus and HIV, to evade neutralizing antibody response (7, 45). Neutralizing antibodies that effectively inhibit cell-free HIV from infection are less effective or fail entirely to inhibit cell-to-cell transmission of the virus (45). In a previous study (46), passive transfer of PRRSV-neutralizing antibodies (at 1:8 titer) to young weaned pigs blocked viremia in blood but could not prevent virus replication in peripheral tissues, and viral loads reached the levels similar to the level in control pigs. These pigs, similar to the control pigs, still excreted infectious viruses to sentinel animals. We speculate that cell-to-cell transmission of PRRSV through intercellular nanotubes could take place in peripheral tissues, a process that is resistant to neutralizing antibodies. This may contribute to viral persistence in peripheral tissues. Future studies are needed to elucidate the extent to which nanotubular intercellular transmission contributes to PRRSV spread *in vivo* and whether this process is involved in viral pathogenesis. If this mechanism is truly central to the pathogenesis of PRRSV infection and persistence, the development of inhibitors that directly block this process would be critical to prevent the spread of viral infections.

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