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# Polyamine regulation of porcine reproductive and respiratory syndrome virus infection depends on spermidine-spermine acetyltransferase 1

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ABSTRACT

Like obligate intracellular parasites, viruses co-opt host cell resources to establish productive infections. Polyamines are key aliphatic molecules that perform important roles in cellular growth and proliferation. They are also needed for the successful multiplication of various viruses. Little is known about the effects of polyamines on Arteriviridae infections. Here, porcine reproductive and respiratory syndrome virus (PRRSV), an economically prominent porcine virus, was used to investigate virus–polyamine interactions. We found that PRRSV infection significantly downregulated the levels of cellular polyamines. Using an inhibitor or specific short interfering RNAs (siRNAs) of ornithine decarboxylase 1, a key anabolic enzyme involved in the classical de novo biosynthesis of polyamines, we found that polyamine depletion abrogated PRRSV proliferation, and this effect was recoverable by adding exogenous spermidine and spermine, but not putrescine to the cells, suggesting that the host inhibits polyamine biosynthesis to restrict PRRSV proliferation. Further analysis revealed that the expression level of spermidine-spermine acetyltransferase 1 (SAT1), a catabolic enzyme that reduces spermidine and spermine levels, was upregulated during PRRSV infection, but conversely, SAT1 had an inhibitory effect on PRRSV reproduction. Our data show that polyamines are important molecules during PRRSV-host interactions, and polyamines and their biosynthetic pathways are potential therapeutic targets against PRRSV infection.

## 1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), which causes porcine reproductive and respiratory syndrome (PRRS), is an enveloped, positive sense, single-stranded RNA virus, and pigs are the only known hosts. PRRSV contains at least ten open reading frames (ORFs) in its genome, of which, ORF1a and ORF1b encode non-structural proteins (nsps), and ORF2a-ORF2b-ORF3-ORF4-ORF5/ORF5a-ORF6-ORF7 encode eight structural proteins; namely, GP2, E, GP3, GP4, GP5a, GP5, M, and N (Dokland, 2010). PRRS, which typically manifests as reproductive disorders in sows, death in newborn pigs, and respiratory diseases in young pigs, is considered one of the costliest swine diseases worldwide, and leads to tremendous economic losses in the pig farming industry (Lunney et al., 2016). The high genetic diversity and variability in PRRSV results from frequent mutation and viral recombination events, and has been an obstacle to developing an effective vaccine against it (Kappes and Faaberg, 2015). Therefore, studying virus–host interactions is important for the discovery of novel antiviral targets against various PRRSV genotypes.

Polyamines are small positively charged aliphatic molecules with two or more amine groups. Because polyamines are able to bind nucleic acids and proteins, and are involved in the regulation of a wide variety of cellular processes, such as nucleic acid metabolism, transcription, translation, chromatin packaging, member fluidity and protein-RNA interactions, their significance in host cellular proliferation is clear. Viruses rely on host resources to produce progeny viruses; therefore, it is not surprising that polyamines also support the multiplication of viruses, including DNA viruses and RNA viruses belonging to various families. Multiple mechanisms are involved in the polyamine-associated regulation of viral infections, e.g., neutralizing negative charges on nucleic acids to facilitate viral genome packaging (Sun et al., 2010, stimulating the activity of viral polymerases (Kenyon et al., 2001; Moussatche, 1985; Wallace et al., 1981), and regulating viral translation and transcription (Mounce et al., 2016b)). Conversely, the level and activity of polyamines

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are modulated by viruses; for instance, polyamine synthesis is stimulated by human cytomegalovirus (HCMV) (Clarke and Tyms, 1991), while infection with herpes simplex viruses (HSV) restricts polyamine synthesis (McCormick and Newton, 1975).

Polyamine types differ across organisms. In eukaryotes, three biogenic molecules, including putrescine, spermidine and spermine, are considered polyamines (Michael, 2016b). Multiple enzymes are implicated in the maintenance of polyamine homeostasis by regulating the synthesis and degradation of polyamines. With polyamine synthesis, ornithine decarboxylase 1 (ODC1) decarboxylates arginine-derived ornithine into putrescine, which is converted into spermidine and spermine in turn by various spermidine synthases, including spermidine synthase and spermine synthase, to add aminopropyl groups. With polyamine catabolism, the acetyl groups are added to spermine and spermidine by spermidine-spermine acetyltransferase 1 (SAT1), followed by polyamine oxidases (PAOX) such that putrescine is finally formed. Additionally, spermine oxidase (SMO) mediates the conversion of spermine to spermidine (Michael, 2016a; Mounce et al., 2017). Conversely, some of these enzymes, such as ODC1 antizyme (OAZ1), SAT1, and S-adenosylmethionine decarboxylase (SAMDC), are regulated by the levels and activities of polyamines through a feedback mechanism. For example, OAZ1 regulates ODC1 activity in a polyamine-dependent manner, and high levels of spermidine and spermine antagonize the translation of SAMDC, while increasing the activity and expression levels of SAT1 (Law et al., 2001; Matsufuji et al., 1995; Pegg, 2008).

Virus–polyamine interactions have been investigated for over 60 years, and drugs antagonizing polyamine synthesis are thought to be potentially safe and efficient antiviral agents against numerous viruses (Firpo and Mounce, 2020). However, how polyamines and *Arteriviridae* family members (e.g., equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV) and PRRSV) relate to each other remains unclear. Here, we chose PRRSV as a representative of the *Arteriviridae* family and found that infection with PRRSV was likely to decrease polyamine levels by improving SAT1 expression. Moreover, the dependence of PRRSV on polyamines to establish a productive infection was demonstrated using agents that reduce polyamine levels, supporting their potential application as antivirals for PRRSV.

## 2. Materials and methods

### 2.1. Cells, virus, reagents and siRNAs

PK-15$^{CD163}$ cells are pig kidney (PK-15) cells with stable expression of the PRRSV CD163 receptor, making them permissive for infection with PRRSV (Wang et al., 2013). The PK-15$^{CD163}$ cells, which were gifted by En-min Zhou (Northwest A&F University, China), were kept in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA). Primary porcine alveolar macrophages (PAMs), the main target cells of PRRSV *in vivo*, were isolated from PRRSV-negative pigs by bronchoalveolar lavage and cultured in RPMI 1640 medium (HyClone, Logan, UT, USA). PRRSV strain WUH3 (GenBank Accession No. HM853673), a highly pathogenic PRRSV (Li et al., 2010), was amplified in PK-15$^{CD163}$ cells and PAMs. Next, the cells and supernatant were frozen together at −80 °C. The progeny viruses were released via three freezing-thawing.

Difluoromethylornithine (DFMO; D193), polyamine mixture (1000×; P8483), individual polyamines [spermidine (S0266), spermine (55513), putrescine (51799), putrescine dihydrochloride (P7505), spermidine trihydrochloride (S2501), and spermine tetrahydrochloride (S2876)], and dansyl chloride (D-2625) were purchased from Sigma-Aldrich (Burlington, MA, USA). DFMO was dissolved in sterile water at a concentration of 100 mM and the individual polyamines were separately dissolved in sterile water at concentrations of 1 M, and all were stored at −20 °C. Dansyl chloride, which was dissolved in acetone at a concentration of 5 mg/ml, was prepared before use. DENSpm (0468) from TOCRIS Bioscience (Bristol, UK) was dissolved in sterile water at a concentration of 100 mM, and stored at −20 °C.

**Table 1**
The sequences of ODC1-specific siRNAs used in this study.

| Gene name | siRNA sequence (sense 5'-3') | siRNA sequence (anti-sense 5'-3') |
|---|---|---|
| siRNA-1 | CCUUCACGCUUGCAGUUAATT | UUAACUGCAAGCGUGAAGGTT |
| siRNA-2 | GCACCUGAGAUGGCUCAAATT | UUUGAGCCAUCUCAGGUGCTT |

The siRNAs targeting pig ODC1 or negative control (NC) siRNA were each transfected at a final concentration of 50 nM using jetPRIME® (Polyplus-transfection®) according to the manufacturer's instructions. The siRNA sequences used here were listed in Table 1.

### 2.2. Cytotoxicity assays

The cytotoxicity of DFMO was evaluated using a 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 20 μL/well of MTT (5 mg/mL) was added to DFMO-treated PK-15$^{CD163}$ cells in 96-well plates, and the plates were incubated for 4 h before the MTT reagent was removed and replaced with 150 μL/well of dimethyl sulfoxide. The plates were gently shaken for 10 min to completely dissolve the formazan precipitate. Cytotoxicity was determined at an absorbance of 570 nm.

As PAM is a type of semi-suspended cells, CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega, Madison, WI, USA) was used to analyze the cytotoxicity of DENSpm in the PAMs. Briefly, 100 μL of CellTiter-Glo reagent was added to the DENSpm-treated PAMs, and the plates were shaken for 2 min. The PAMs were incubated for another 10 min, and cytotoxicity was measured on a 1450 MicroBeta TriLux instrument (PerkinElmer, MA, USA).

### 2.3. Western blot assays

Cells were lysed with lysis buffer (Beyotime, Shanghai, China) supplemented with a protease inhibitor cocktail (Beyotime), and denatured in 5 × sodium dodecyl sulfate (SDS) loading buffer by boiling at 95 °C for 10 min. Equal amounts of the cell lysis preparations were subjected to SDS polyacrylamide gel electrophoresis. The gel-bound proteins were electroblotted onto PVDF membranes (Millipore, Darmstadt, Germany). The monoclonal antibody (mAb) against the PRRSV nucleocapsid (N) protein was made in our laboratory. The mAb against β-actin (AC026) was obtained from Abclonal Technology (Wuhan, China). The polyclonal antibody against ODC1 was purchased from Proteintech (Wuhan, China).

### 2.4. Plaque assays

Cells were seeded in 6-well plates, cultured until about 90% confluence, and then infected with serial 10-fold dilutions of the PRRSV samples. After 1 h, the cells were washed twice with phosphate-buffered saline (PBS), and 1.6% low melting-point agarose and an equal volume of 3% DMEM mixed together was added to them. Cells were cultured at 37 °C for about 48 h, and then stained with neutral red dye at 37 °C for 1 h. After removing excess dye, the number of plaques was determined from three independent experiments.

### 2.5. RNA extraction and real-time reverse-transcriptase quantitative PCR (RT-qPCR) assays

Total RNA was extracted using TRIzol reagent (Invitrogen). The RNA was reverse-transcribed into cDNA by the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The qPCR experiments were performed in triplicate using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7500 real-time PCR system (Applied Biosystems). The primers used in this study were shown in Table 2.

**Table 2**
The primer sequences for qPCR used in this study.

| Gene name | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|---|---|---|
| β-actin | CTTCCTGGGCATGGAGTCC | GGCGCGATGATCTTGATCTTC |
| ODC1 | TGTACTGACCCCGAGACCTT | GGGTTGATGACACCGGTGAT |
| SAT1 | CATGGATTGGCAAACTGTTG | TCCATCCCTCTTCACTGGAC |
| ISG56 | AAATGAATGAAGCCCTGGAGTATT | AGGGATCAAGTCCCACAGATTTT |
| PRRSV nsp9 | ACCCTAGGACCTGTGAAC | GGCGAGTAACTTAGGAGATG |

### 2.6. Extraction of cellular polyamines

Cells in 10-cm cell culture dishes ($10^6$–$10^7$ cells/dish) were dislodged by scraping and collected into a 1.5 ml tube via centrifugation (4 °C, 12000 rpm/min, 1 min), followed by two washes with 1 ml of pre-cooled PBS. Each sample was sonicated in 250 μL of 2% (v/v) perchloric acid at 4 °C, stored (4 °C, 24 h), and centrifuged (~1500 ×g/min at 4 °C, 30 min). Finally, 200 μL of each supernatant was carefully collected and stored at 4 °C for future chromatographic analysis.

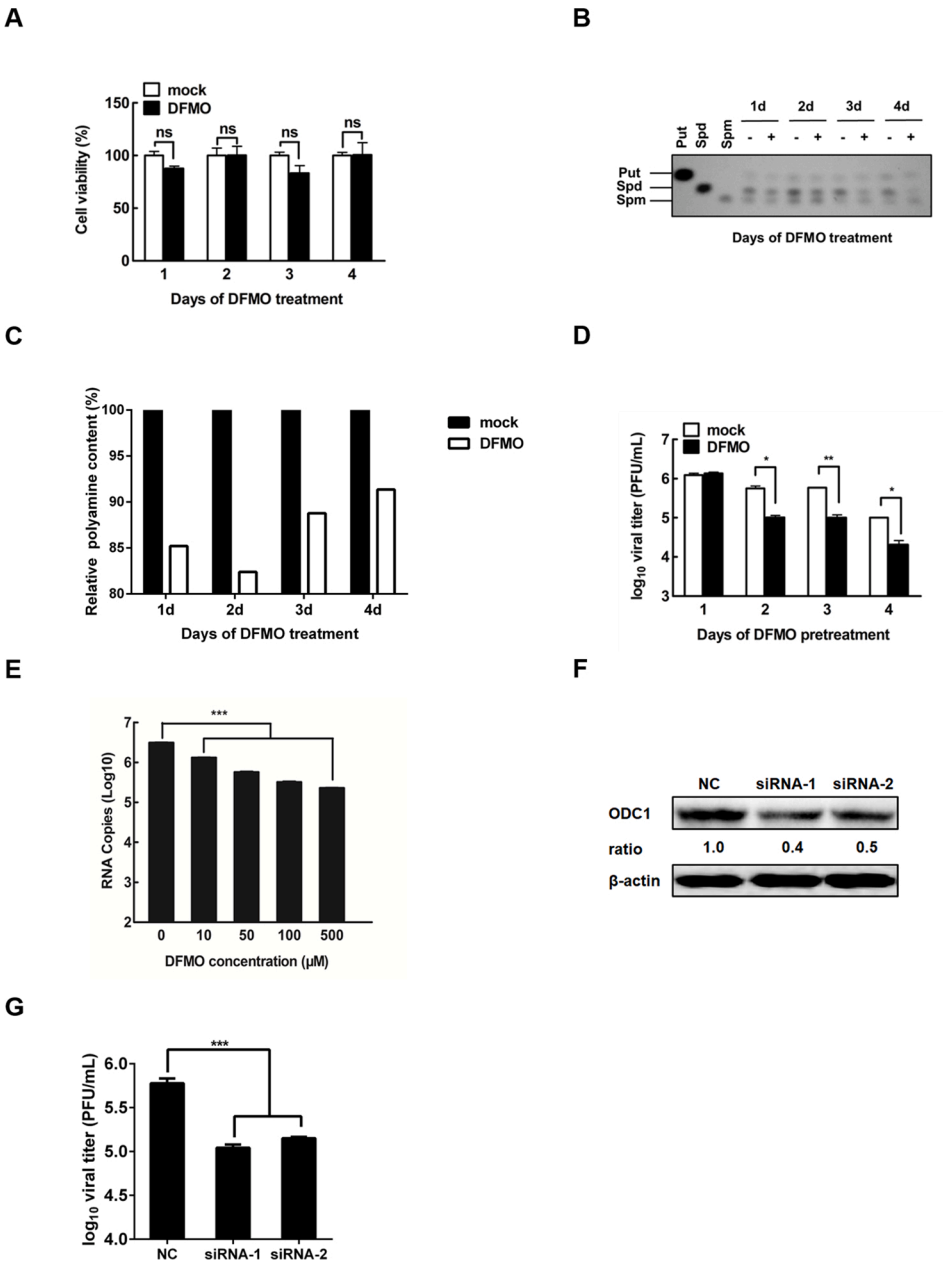


**Fig. 1. Polyamine depletion inhibits PRRSV proliferation. (A-B)** PK-15$^{CD163}$ cells were incubated or mock-incubated with 500 μM DFMO for 1, 2, 3 and 4 d. The MTT assay was then performed to determinate the cytotoxicity of DFMO **(A)**, and TLC was used to evaluate polyamine levels (Put: putrescine dihydrochloride; Spd: spermidine trihydrochloride; Spm: spermine tetrahydrochloride) at the indicated times **(B)**. **(C)** The relative polyamine content in Fig. 1B was analyzed using ImageJ software. The value of mock-treated cells in each group was set as 100%. **(D)** PK-15$^{CD163}$ cells were pretreated or mock-treated with DFMO (500 μM) for the number of days indicated, and then infected with PRRSV (MOI = 0.5) for 36 h. PRRSV titers were determined with a plaque assay. **(E)** PK-15$^{CD163}$ cells were pretreated with various concentrations of DFMO (0, 10, 50, 100, and 500 μM) for 2 d, and then infected with PRRSV (MOI = 0.5) for 36 h. PRRSV RNA copy numbers were determined by real-time RT-qPCR assay. **(F)** PK-15$^{CD163}$ cells were transfected with ODC1-specific siRNAs and NC siRNA for 36 h, then the expression levels of ODC1 were determined by western blot assay to evaluate the silencing efficiency of ODC1-specific siRNAs. (**G**) PK-15$^{CD163}$ cells were transfected with ODC1-specific siRNAs and NC siRNA for 36 h, then the cells were infected with PRRSV (MOI = 0.5). At 36 h post infection, the titers of PRRSV were determined with a plaque assay. The ratio of ODC1/β-actin was analyzed using ImageJ software. Data are expressed as the means and standard deviations from three independent experiments. *, $0.01 \leq p < 0.05$; **, $0.001 \leq p < 0.01$; ***, $p < 0.001$. ns: no significance.

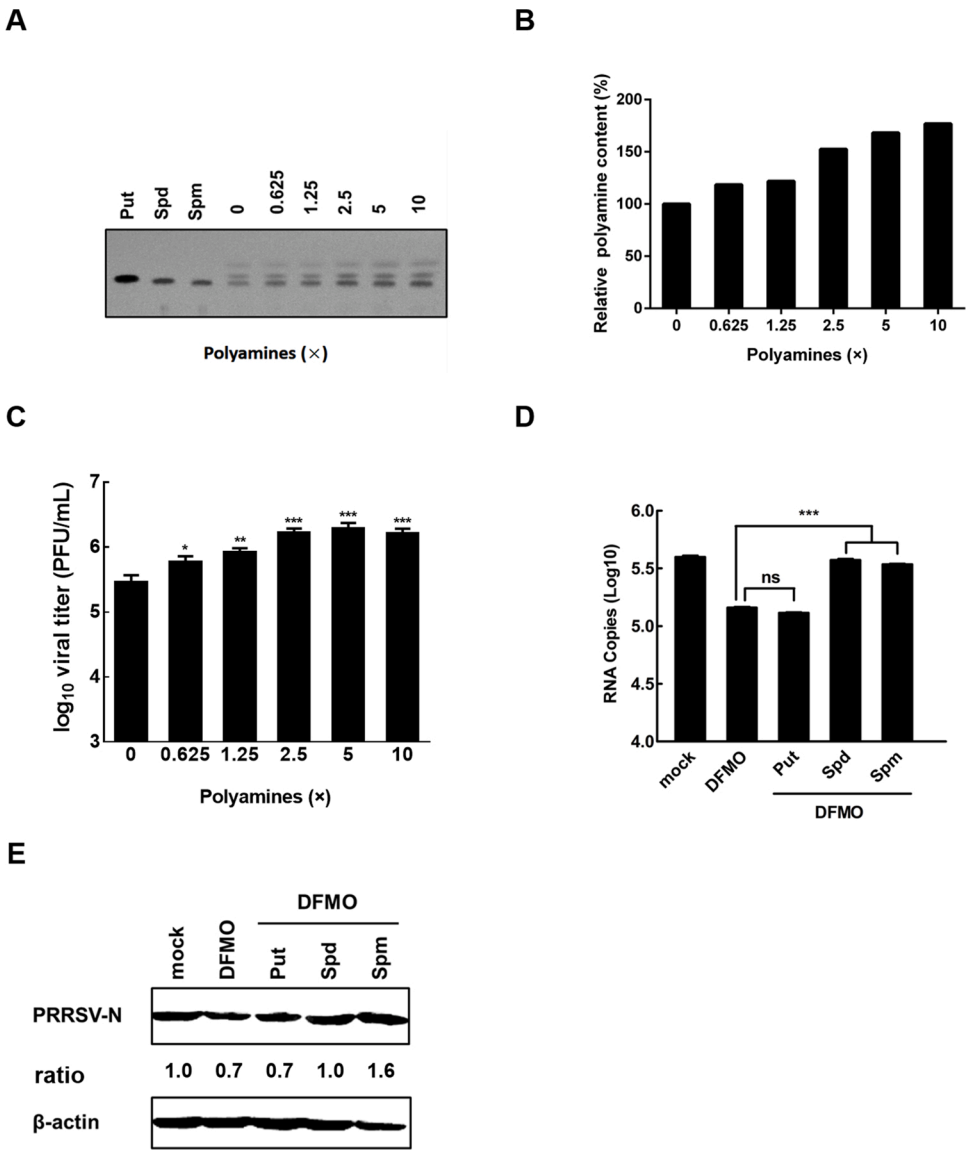


**Fig. 2. Spermidine and spermine benefit the proliferation of PRRSV. (A)** DFMO (500 μM) was added into PK-15$^{CD163}$ cells, after incubation for 2 d, DFMO was replaced with exogenous polyamines at different concentrations (0, 0.625×, 1.2×, 2.5×, 5× and 10×), and incubated for 1 d. Subsequently, the polyamine levels were detected using TLC. **(B)** The relative polyamine content in Fig. 2A was analyzed using ImageJ software. The value of mock-treated group was set as 100%. **(C)** PK-15$^{CD163}$ cells were pretreated with DFMO and then incubated with exogenous polyamines as described in (A). Then cells were infected with PRRSV (MOI = 0.5) for 36 h in the presence of exogenous polyamines. The PRRSV titers were assessed with a plaque assay. **(D-E)** PK-15$^{CD163}$ cells were pretreated or mock-pretreated with DFMO for 2 d. Then DMFO was discarded, and the cells were incubated or mock-incubated with individual polyamines, including putrescine (Put), spermidine (Spd) and spermine (Spm), each at a concentration of 10 μM for another 1 d, followed by the infection with PRRSV (MOI = 0.5) for 36 h in the presence of individual polyamine, respectively. Real-time RT-qPCR and western blot assays were conducted to evaluate the PRRSV RNA copy numbers **(D)** and PRRSV-N protein expression levels **(E)**, respectively. β-actin was used as the loading control. The ratio of PRRSV-N/β-actin was analyzed using ImageJ software. Data are expressed as the means and standard deviations from three independent experiments. *, $0.01 \leq p < 0.05$; **, $0.001 \leq p < 0.01$; ***, $p < 0.001$. ns: no significance.

### 2.7. Dansylation and thin-layer chromatography (TLC)

Polyamines were detected using TLC as described by Madhubala et al. (Madhubala, 1998). Briefly, twice the volume (400 μL) of dansyl chloride to sample volume was added to each sample, the negative control (2% perchloric acid), and external standards (putrescine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride, each at a concentration of 10 μM). Aliquots (200 μL) of a supersaturated sodium carbonate solution were added, followed by vortexing. The resultant mixtures were stored in the dark at room temperature (RT) for 16 h. Proline solution (100 μL volume; 150 mg/mL dissolved in sterile water and prepared before use) was added and incubated for 30 min in the dark to remove excess dansyl chloride. The dansyl amides were then extracted using 500 μL of toluene by vortexing the tubes at RT. The aqueous phases were separated from the toluene phases in the samples after centrifugation.

The final dansyl amides were applied in small spots to the TLC plate (silica gel matrix), and the plate was exposed to ascending chromatography in a glass tank saturated with cyclohexane-ethylacetate solvent (ratio 2:3) for 1.5–2 h in the dark, with the distance between the solvent front and the end mark set at 2.5–5 cm. Finally, the plate was air dried in the dark and visualized by UV exposure.

### 2.8. Statistical analysis

GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was used for data analysis using an unpaired t-test (two-tailed).

## 3. Results

### 3.1. Polyamine depletion antagonizes PRRSV proliferation

To explore the effect of polyamines on PRRSV infection, DFMO, a suicide ODC1 inhibitor, was used to inhibit polyamine synthesis. First, an MTT assay was conducted to test the cytotoxicity of DFMO. Because previous studies showed that incubation with DFMO at 500 μM for 2–4 days (d) can completely deplete intracellular polyamine (Lee et al., 2019), we treated PK-15$^{CD163}$ cells with the same concentration of DFMO for different time periods (1, 2, 3 and 4 d), and no significant cytotoxicity was observed (Fig. 1A). The results of the chromatographic analysis showed that DFMO (500 μM) treatment could deplete polyamine levels in PK-15$^{CD163}$ cells (Fig. 1B and C). Furthermore, when the PK-15$^{CD163}$ cells were pretreated with DFMO and infected with PRRSV at a multiplicity of infection (MOI) of 0.5, the plaque assay results revealed that DFMO (500 μM) treatment for 2–4 d strongly decreased

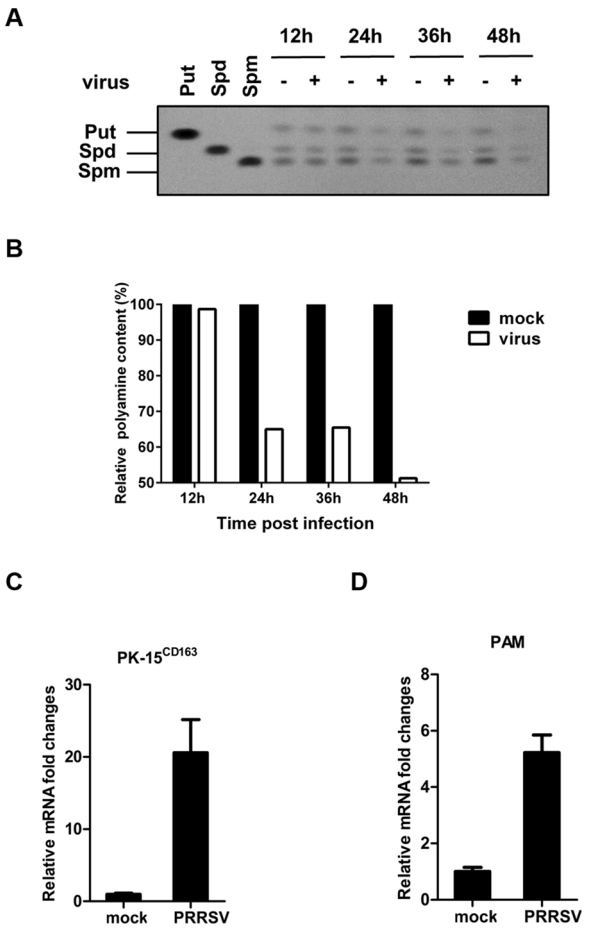


**Fig. 3. PRRSV infection decreases intracellular polyamine levels . (A)** PK-$15^{CD163}$ cells were infected with PRRSV (MOI = 0.5) for 12, 24, 36 and 48 h. TLC was then used to determinate polyamine levels. Put: putrescine dihydrochloride; Spd: spermidine trihydrochloride; Spm: spermine tetrahydrochloride. **(B)** The relative polyamine content in Fig. 3A was analyzed using ImageJ software. The value of mock-treated cells in each group was set as 100%. **(C-D)** PK-$15^{CD163}$ cells and PAMs were infected with PRRSV (MOI = 0.5) for 36 h, and real-time RT-qPCR assays were conducted to evaluate the relative mRNA levels of SAT1. The results represent the means and standard deviations from three independent experiments.

the PRRSV titers (Fig. 1D). DFMO also significantly reduced the number of PRRSV RNA copies in a dose-dependent manner (Fig. 1E), suggesting that polyamine depletion antagonizes PRRSV proliferation.

Previous study indicated that siRNA-mediated ODC1 knockdown downregulated polyamine biosynthesis (Zhu et al., 2012), thus we further detected the effect of polyamine depletion on PRRSV infection using ODC1-specific siRNAs. The knockdown efficiency of siRNAs was evaluated through western blot assay (Fig. 1F). Then ODC1-specific siRNA-transfected PK-$15^{CD163}$ cells were infected with PRRSV (MOI = 0.5), and plaque assay was conducted to test the titers of PRRSV. As shown in Fig. 1G, knockdown of ODC1 inhibited PRRSV proliferation compared with cells transfected with the NC siRNA, which further demonstrated that polyamine depletion exhibited inhibitory effect on PRRSV infection.

### 3.2. Spermidine and spermine promote PRRSV infection

To further confirm the role played by polyamines in the multiplication of PRRSV in cells, the effect of exogenous polyamines was investigated. DFMO-pretreated PK-$15^{CD163}$ cells, the polyamines of which were depleted, were treated with a mixture of exogenous polyamines at different concentrations for 1 d, and the polyamine levels were determined through TLC. The results indicated that addition of exogenous polyamines dose-dependently upregulated intracellular polyamine levels, suggesting that the cells could ingest exogenous polyamines (Fig. 2A and B). One day after adding the exogenous polyamines, PK-$15^{CD163}$ cells were infected with PRRSV (MOI = 0.5) for 36 h, and PRRSV titers were evaluated using a plaque assay. As shown in Fig. 2C, the PRRSV titers increased in line with the dose of exogenous polyamines provided.

Because the mixture of exogenous polyamines contains spermidine, spermine and putrescine, the DFMO-pretreated PK-$15^{CD163}$ cells were separately treated with the individual polyamines and then infected with PRRSV (MOI = 0.5) to determine which of them is required for PRRSV infection. The results of the real-time RT-qPCR assay and western blot assay revealed that both spermidine and spermine, but not putrescine, facilitated the proliferation of PRRSV (Fig. 2D-E).

### 3.3. SAT1 mediates the polyamine reduction during PRRSV infection

To investigate the polyamine content in PRRSV-infected cells, PK-$15^{CD163}$ cells in 10-cm cell culture dishes were infected with PRRSV (MOI = 0.5), and the cellular extracts were collected at the indicated time points (12, 24, 36, and 48 h) and analyzed by TLC to determine the polyamine levels. The results of the chromatographic analysis indicated that the levels of polyamines began to decrease at 24 h post PRRSV infection (Fig. 3A and B), implying the possibility that it is a novel antiviral strategy of host cells to reduce polyamine levels in PRRSV-infected cells.

To preliminarily explore the potential mechanism by which host cells restrict polyamine levels during PRRSV infection, the expression level of spermidine-spermine N1-acetyltransferase (SAT1), a key catabolic enzyme that acts to antagonize polyamine biosynthesis (Pegg, 2008), was tested using real-time RT-qPCR assay. As illustrated in Fig. 3C and D, the mRNA levels of SAT1 in both PRRSV-infected PAMs and PK-$15^{CD163}$ cells were upregulated, which might induce the convert of spermidine-spermine into putrescine to reduce polyamine levels.

### 3.4. SAT1 disrupts PRRSV multiplication

SAT1 was reported as a novel IFN stimulated gene (ISG) (Mounce et al., 2016b), and we also demonstrated that treatment with IFN-α induced ISGs production in PAMs (Fig. 4A). In addition, PRRSV is an IFN-sensitive virus; therefore, we speculated that the induction of SAT1 expression is likely to inhibit PRRSV infection. Thus, N1, N11-diethylnorspermine (DENSpm), an inducer of SAT1, was used here to evaluate the role of SAT1 in PRRSV multiplication. First, the cytotoxicity of DENSpm at different concentrations ($10^2$, $10^3$, $10^4$, and $10^5$ nM) in PAMs was assessed and no obvious cytotoxicity was observed (Fig. 4B). We then evaluated the effect of DENSpm on SAT1 and polyamines. The results of the real-time RT-qPCR assay and chromatographic analysis revealed that $10^4$ and $10^5$ nM DENSpm markedly upregulated the levels of SAT1 mRNA and downregulated the levels of spermine, with no obvious influence of DENSpm on SAT1 and polyamines at concentrations of $10^2$ and $10^3$ nM (Fig. 4C and D), indicating that DENSpm worked well in PAMs. Next, DENSpm-pretreated PAMs were infected with PRRSV (MOI = 0.5) for 36 h, the titer of PRRSV was evaluated using a plaque assay, and the expression level of PRRSV-N protein was examined by western blotting. Fig. 4 (E and F) shows that DENSpm disrupts PRRSV proliferation at concentrations of $10^4$ and $10^5$ nM, but not at $10^2$ and $10^3$ nM, a finding in line with the effects of DENSpm on SAT1 and polyamines. Thus, it appears that the expression level of SAT1 was increased in the PRRSV-infected cells to downregulate the levels of spermidine and spermine, which finally antagonized the ability of PRRSV to proliferate.

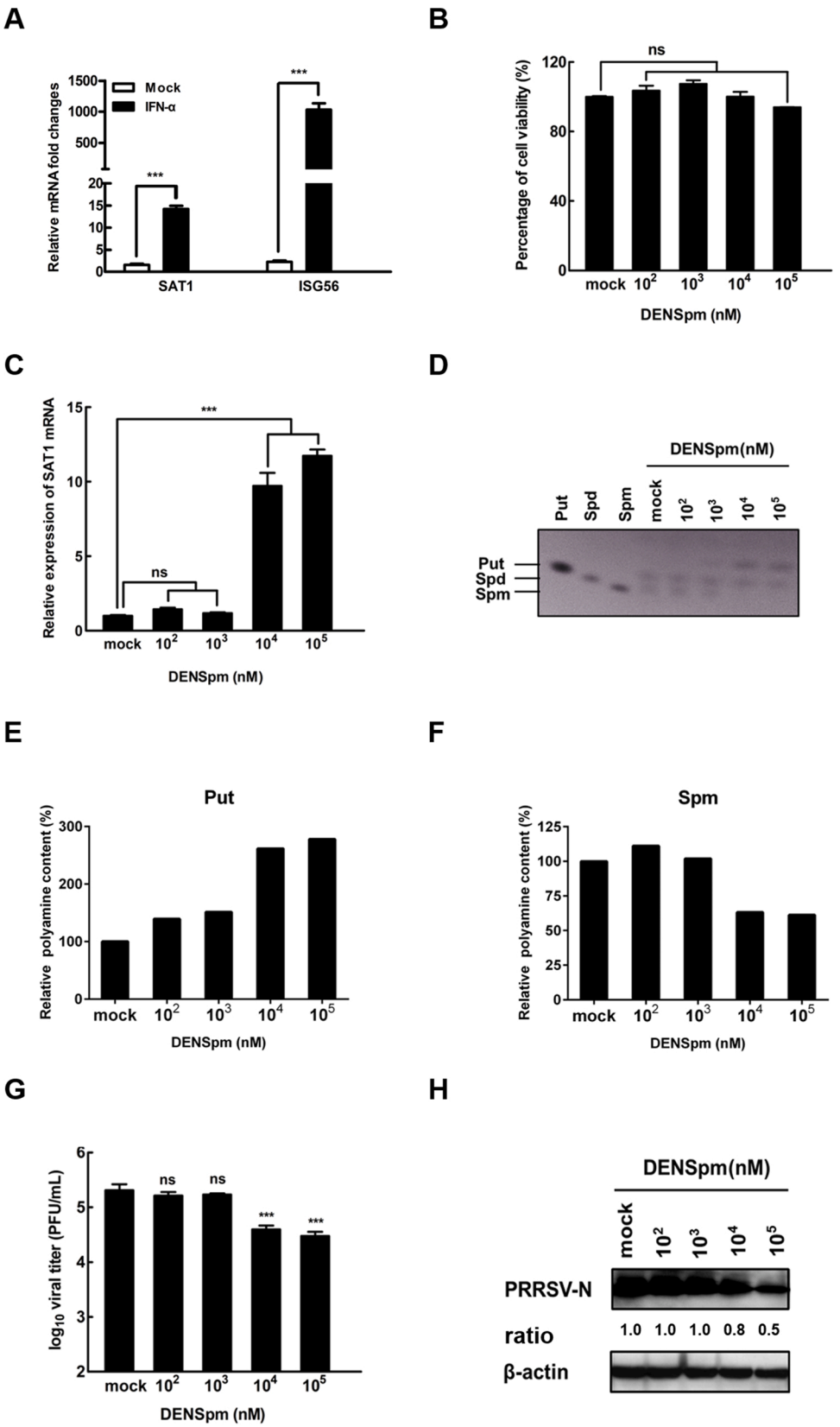


**Fig. 4. SAT1 restricts the proliferation of PRRSV. (A)** PAMs were treated with 1000 U/ml of IFN-α for 8 h, and the relative mRNA expression levels of SAT1 and ISG56 were determined by real-time RT-qPCR assays. ISG56 acted as the positive control. **(B-D)** PAMs were incubated or mock-incubated with DENSpm at different concentrations ($10^2$, $10^3$, $10^4$, and $10^5$ nM) for 16 h. The cytotoxicity of DENSpm was then evaluated using the CellTiter-Glo® luminescent cell viability assay **(B)**. The relative expression level of SAT1 mRNA was determined by real-time RT-qPCR assay **(C)**, and the polyamine levels were assessed using TLC **(D)**. Put: putrescine dihydrochloride; Spd: spermidine trihydrochloride; Spm: spermine tetrahydrochloride. **(E-F)** The relative content of polyamines (**E**: putrescine; **F**: spermine) in Fig. 4D was analyzed using ImageJ software. The value of mock-treated group was set as 100%. **(G-H)** PAMs were preincubated or mock-incubated with DENSpm at different concentrations ($10^2$, $10^3$, $10^4$, and $10^5$ nM) for 16 h, and then infected with PRRSV (MOI = 0.5) for another 36 h. PRRSV titers were evaluated with a plaque assay **(G)**, and the expression level of PRRSV-N protein was tested by western blotting **(H)**. β-actin was used as the loading control. The ratio of PRRSV-N/β-actin was analyzed using ImageJ software. Data are expressed as the means and standard deviations from three independent experiments. ***, $p < 0.001$. ns: no significance.

## 4. Discussion

It is common for viruses to alter the cellular metabolism to acquire the metabolites they need to successfully establish their infections. Polyamines are abundant, multifunctional biomolecules. That these molecules have intimate relationships with viruses (e.g., bacteriophages, plant viruses, and mammalian viruses) has been a known fact since the 1950s. Viruses rely on polyamines to enhance their infections via various mechanisms such as facilitating viral entry, and supporting the transcription, translation, packaging and synthesis of the viral

genome, viral binding to host cells, and regulating the activity of viral proteins. As the first reported mammalian virus associated with polyamines, herpes simplex virus (HSV-1) incorporates spermine and spermidine into its nucleocapsid and envelope, respectively (Gibson and Roizman, 1971). Additionally, presumably due to the binding activity of polyamines to nucleic acids, polyamine depletion impairs RNA replication of zika virus (ZIKV) and chikungunya virus (CHIKV) (Mounce et al., 2016b), and the translation of hepatitis C virus (HCV), ZIKV, and CHIKV (Masalova et al., 2017; Mounce et al., 2016b). The role played by polyamines in viral genome transcription has also been reported, such as in Marburgvirus and in Ebola virus (Olsen et al., 2018; Olsen et al., 2016). The activity of viral polymerases and proteases, specifically HCV, HSV-1 and Coxsackievirus B3 (CVB3), was shown to be regulated by polyamines as well (Dial et al., 2019; Korovina et al., 2012; Wallace et al., 1980). Interestingly, polyamines also play a role in the attachment stage of CVB3 during its infection (Kicmal et al., 2019).

Because polyamines perform vital roles in viral infections, their depletion disrupts the proliferation of numerous viruses from different families, such as *Herpesviridae* (HSV-1 and HCMV), *Togaviridae* (Semliki forest virus, Sindbis virus, and CHIKV), *Flaviviridae* (dengue virus serotype 1, Japanese encephalitis virus, Yellow fever virus, and ZIKV), *Picornaviridae* (enterovirus A71, poliovirus, and CVB3), *Bunyaviridae* (Rift Valley fever virus and La Crosse virus), *Rhabdoviridae* (rabies virus and Vesicular stomatitis virus) and *Coronaviridae* (Middle East respiratory syndrome coronavirus [MERS-CoV]) (Mastrodomenico et al., 2019; Mounce et al., 2016a; Mounce et al., 2016b; Tuomi et al., 1980; Tyms and Williamson, 1982). Here, we investigated the impact of polyamines on PRRSV, an *Arteriviridae* family member. We found that polyamine depletion inhibited the reproduction of PRRSV, which could be rescued by the addition of exogenous polyamines, indicating that polyamines function during infection with PRRSV. Interestingly, both *Coronaviridae* (MERS-CoV) and *Arteriviridae* (PRRSV), which are classified within the order *Nidovirales*, are characterizable by each having a 3′-coterminal nested set of subgenomic mRNAs, suggesting the possibility that viruses belonging to the other families within *Nidovirales*, such as the *Roniviridae* and *Mesoniviridae* families, infect host cells in a polyamine-dependent manner. Notably, some other viruses such as severe acute respiratory syndrome coronavirus (SARS-CoV), MERS-CoV, and novel coronavirus (SARS-CoV-2) are *Coronaviridae* family members within the order *Nidovirales*, which highlights the potential positive role for polyamines in these viruses.

Because viruses are more sensitive to polyamine depletion than host cells, polyamine depletion is considered a novel broad-spectrum antiviral strategy for selective clinical control of viral diseases with minimal cytotoxicity (Li and MacDonald, 2016; Mounce et al., 2016b). Thus, the antiviral potential of drugs targeting various steps in polyamine metabolism pathways for decreasing the levels and activity of polyamines, most of which have been developed as promising anticarcinogens (Seiler, 2003), are the focus of investigation. One of the best known is DFMO (also called eflornithine), which is approved by the US Food and Drug Administration to treat African sleeping sickness, having found that it acts as a safe and efficient anticancer and chemoprevention agent against several cancers such as gliomas, nonmelanoma skin cancers and colon cancer, with low toxicity (Alexiou et al., 2017). The antiviral effects of DFMO on various viruses have also been demonstrated (Masalova et al., 2017; Mounce et al., 2016a; Mounce et al., 2016b; Tuomi et al., 1980), suggesting that DFMO is a potential broad-spectrum antiviral drug. Other polyamine-targeted agents, including DENSpm (an inducer of SAT1), MDL 72757 (an inhibitor of PAOX) and other drugs regulating the hypusination of eIF5A, such as N1-guanyl-1,7-diamine-heptane, ciclopirox, and deferiprone, are promising antivirals as well (Fedorova et al., 2019; Mounce et al., 2016a; Olsen and Connor, 2017; Olsen et al., 2018; Olsen et al., 2016). Here, we have shown DFMO or DENSpm administration caused polyamine depletion, and also exhibited antiviral activities against PRRSV, implying their potential as candidate polyamine-targeted agents for the future development of safe and efficient antivirals for PRRSV, and even SARS-CoV, MERS-CoV, SARS-CoV-2, individually, or in combination with other antiviral drugs.

Viruses use diverse strategies to manipulate polyamine metabolism, and while most viruses regulate polyamine levels by interfering with the expression and activity of polyamine metabolic enzymes, for instance, the genome of *Paramecium bursaria* chlorella virus-1 encodes the entire polyamine biosynthetic pathway (Baumann et al., 2007). Epstein-Barr virus restricts SAT1 expression resulting in downregulated concentrations of acetylspermidine (Shi et al., 2013), whereas in HSV-1 the expression of SAMDC, one of the polyamine synthetases, is upregulated (Greco et al., 2005). Another virus, HCMV, enhances the activity of ODC1, thereby facilitating polyamine uptake (Isom, 1979). Conversely, in cells harboring a full-length HCV replicon, the polyamine levels were found to decrease by manipulating the levels of ODC, SAT1, and SMO, a catabolic enzyme of polyamines (Smirnova et al., 2017). In the present study, we found that polyamine levels fell after the cells had been infected with PRRSV as well, presumably resulting from the downregulated expression of SAT1. However, how PRRSV establishes an infection under polyamine depletion, which is adverse to its multiplication, is unclear. Given that (*i*) polyamine depletion induces the phosphorylation of the eukaryotic initiation factor 2α (eIF2α) and reduces phosphorylation of the translation repressor protein eIF4E-binding protein (4E-BP) (Miller-Fleming et al., 2015), subsequently leading to a translational shutdown, and (*ii*) PRRSV induces translational shutdown by regulating the phosphorylation of eIF2α and 4E-BP as well (Li et al., 2018), we speculate that, oppositely, PRRSV has evolved a novel immune evasion strategy whereby the virus decreases the polyamine level in its host cells to cause translation inhibition of host proteins, including the host restriction factors that suppress viral infections. This allows PRRSV to finally escape the antiviral effects of the host restriction factors, an idea requiring further investigation.

## 5. Conclusion

We have shown that polyamines, including spermidine and spermine, facilitate PRRSV infection, while in response, host cells develop countermeasures to decrease polyamine levels by upregulating the expression of SAT1. The anti-PRRSV effects of agents that inhibit polyamine biosynthesis or induce polyamine catabolism, such as DFMO and DENSpm, support the possibility that polyamine metabolism is a novel potential target for the further development of antivirals against PRRSV.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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