Cellular microRNA miR-10a-5p inhibits replication of porcine reproductive and respiratory syndrome virus by targeting the host factor signal recognition particle 14

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Abstract
Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important viruses affecting the swine industry worldwide. MicroRNAs have recently been demonstrated to play vital roles in virus–host interactions. Our previous research on small RNA deep sequencing showed that the expression level of miR-10a increased during the viral life cycle. The present study sought to determine the function of miR-10a and its molecular mechanism during PRRSV infection. In the current study, the result of PRRSV infection inducing miR-10a expression was validated by quantitative reverse transcriptase PCR. Overexpression of miR-10a-5p using its mimics markedly reduced the expression level of intracellular PRRSV ORF7 mRNA and N protein. Simultaneously, overexpression of miR-10a-5p also significantly decreased the expression level of extracellular viral RNA and virus titres in the supernatants. These results demonstrated that miR-10a-5p could suppress the replication of PRRSV. A direct interaction between miR-10a-5p and signal recognition particle 14 (SRP14) was confirmed using bioinformatic prediction and experimental verification. miR-10a-5p could directly target the 3'UTR of pig SRP14 mRNA in a sequence-specific manner and decrease SRP14 expression through translational repression but not mRNA degradation. Further, knockdown of SRP14 by small interfering RNA also inhibits the replication of PRRSV. Collectively, these results suggested that miR-10a-5p inhibits PRRSV replication through suppression of SRP14 expression, which not only provides new insights into virus–host interactions during PRRSV infection but also suggests potential new antiviral strategies against PRRSV infection.

INTRODUCTION
Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important infectious diseases, resulting in severe reproductive failure in sows and respiratory syndromes in young pigs. The disease causes significant economic losses to the swine industry worldwide each year. The causative pathogen of PRRS is the PRRS virus (PRRSV), a small enveloped, linear, single positive-stranded RNA virus and a member of the order Nidovirales, family Arteriviridae, which causes persistent infection and immunosuppression [1]. It is very difficult for current vaccination strategies to effectively control PRRSV infection because of the high antigenic heterogeneity [2–4], the replication in lung alveolar macrophages [5] and the antibody-dependent enhancement of PRRSV [6]. Therefore, it is imperative to study PRRSV pathogenesis mechanisms so that more effective control measures can be developed.

microRNAs (miRNAs) are evolutionarily conserved, small, endogenous, noncoding RNAs that regulate gene expression at the post-transcriptional level either by inhibiting mRNA translation or by inducing mRNA degradation [7]. Growing evidence indicates that miRNAs can modulate virus replication directly as well as the host cell response to viral infection in a pro-viral or antiviral manner [8, 9]. It has been demonstrated that hepatitis C virus replication depends on a liver-specific miRNA, miR-122 [10]. Kaposi’s sarcoma-associated herpesvirus up-regulates miR-132 expression that, in turn, inhibits the expression of p300, a transcriptional co-activator,
to promote viral replication [11]. In previous research, using high-throughput sequencing technology, we demonstrated that miR-10a is involved in the host immune response to PRRSV in porcine alveolar macrophages (PAMs), because the expression level of miR-10a increased during the viral life cycle [12]. However, the function of miR-10a and its molecular mechanism during PRRSV infection remains unknown.

In the present study, we investigated the exact role of miR-10a-5p and its molecular mechanism during PRRSV infection. We found that overexpression of miR-10a-5p markedly inhibits PRRSV replication through suppression of signal recognition particle 14 (SRP14) protein expression in PAMs. These data not only provide new insights into virus–host interactions during PRRSV infection, but also suggest potential new antiviral strategies against PRRSV infection in the future.

RESULTS
PRRSV infection induces miR-10a-5p expression

Our previous research on small RNA deep sequencing showed that miR-10a-5p was induced by PRRSV infection [12]. To validate the result, miR-10a-5p levels in the PAMs were quantified by quantitative reverse transcriptase PCR (qRT-PCR) during PRRSV infection. As shown in Fig. 1, miR-10a-5p was up-regulated in PAMs infected with PRRSV as early as 2 h post-infection (p.i.), and then its expression increased gradually as the infection progressed. The abundance of miR-10a-5p in PAMs infected with PRRSV increased by 1.5-, 30-, 53-, 80- and 238-fold at 2, 6, 9, 12 and 24 h p.i. compared with uninfected controls, respectively. The results demonstrated that PRRSV indeed affects the expression level of miR-10a-5p.

miR-10a-5p suppresses the replication of PRRSV

PRRSV infection significantly increased the abundance of miR-10a-5p in PAMs, indicative of the potential function of miR-10a-5p in PRRSV biology. To investigate this, PAMs were transfected with miR-10a-5p mimics or negative control (NC) mimics 24 h before infection with PRRSV at an m.o.i. of 0.1. The expression of viral RNA and protein, and PRRSV production were analysed by qRT-PCR, Western blot and virus titres at the indicated time points. As shown in Fig. 2(a), transfection with miR-10a-5p mimics markedly reduced the expression level of PRRSV ORF7 mRNA, as exhibited by 95 and 95% decreased ORF7 expression at 12 and 24 h p.i. relative to the miR-NC transfection group, respectively. Similar results were observed when PRRSV N protein expression levels in cells were analysed (Fig. 2d). Transfection with miR-10a-5p mimics also resulted in markedly decreased PRRSV RNA levels, which decreased by 94 and 99% (Fig. 2b) at 12 and 24 h p.i. versus the miR-NC transfection group with a concomitant 1.33- and 1.5-log decrease at 12 and 24 h p.i. in supernatant virus titres when compared to the miR-NC mimic-treated group, respectively (Fig. 2c).

miR-10a-5p targets the 3'UTR of pig SRP14 mRNA directly

Our previous research on small RNA deep sequencing showed that the expression level of miR-10a-5p correlated inversely with that of SRP14 mRNA during PRRSV infection. Further, the interaction between miR-10a-5p and SRP14 (LOC100522176) was predicted [12]. To identify whether miR-10a-5p is complementary to 3'UTR of pig SRP14 mRNA, we performed in silico analysis using a number of bioinformatics tools. We found that there is one putative miR-10a-5p seed match site in the SRP14 3'UTR of pig (Fig. 3a).

To investigate whether miR-10a-5p could potentially decrease SRP14 expression by binding to the 3'UTR, a plasmid-based luciferase reporter assay was used to examine the effects of miR-10a-5p overexpression on reporter gene expression in cells. The effect of miR-10a-5p on reporter

![Fig. 1.](image-url)
gene expression was monitored by co-transfection of cells with miRNA mimics and a luciferase reporter containing the pig SRP14 3’UTR (psiCheck2-SRP14-3’UTR-WT). As shown in Fig. 3(c), overexpression of miR-10a-5p resulted in a 40% decrease in luciferase expression from psiCheck2-SRP14-3’UTR-WT. In contrast, co-transfection with miR-NC mimics had no effect. To investigate whether miR-10a-5p directly targets the 3’UTR of SRP14 mRNA, which contained the predicted seed match site for miR-10a-5p (Fig. 3a), the effect of mutating the seed sequence in the SRP14 3’UTR of pig (Fig. 3b) was examined. As expected, disruption of the seed sequence in plasmids containing the pig SRP14 3’UTR (psiCheck2-SRP14-3’UTR-MUT) ablated the ability of miR-10a-5p mimics to inhibit the expression of luciferase (Fig. 3c).

To confirm the above results, the mutant miR-10a-5p (miR-10a-5p-Mut) restoring base complementarity with mutated SRP14 3’UTR sequences (Fig. 3b) was generated, and its effect on WT and mutant SRP14 3’UTR reporter activity was examined. As shown in Fig. 3(c), miR-10a-5p-Mut mimics did not affect the luciferase expression from psiCheck2-SRP14-3’UTR-WT. In contrast, miR-10a-5p-Mut mimics significantly decreased the luciferase activity of psiCheck2-SRP14-3’UTR-MUT (Fig. 3c). These results demonstrated that miR-10a-5p could directly target the 3’UTR of pig SRP14 mRNA.

**miR-10a-5p down-regulates expression of SRP14 protein in PAMs**

To determine if overexpression of miR-10a-5p decreases SRP14 expression, PAMs were transfected with miR-10a-5p mimics or miR-NC mimics for 12 h followed by infection with PRRSV at an m.o.i. of 0.1, and the gene expression level of intracellular PRRSV ORF7 was evaluated by qRT-PCR at 0, 12 and 24 h p.i. (a). PRRSV RNA (b) and virus titres (c) in the supernatants were analysed by qRT-PCR and TCID\textsubscript{50} at 0, 12 and 24 h p.i., respectively. Results are expressed as means±SD of three independent experiments. Statistically significant values: ***P<0.001. (d) PAMs transfected with miR-10a-5p mimics or miR-NC mimics were infected with PRRSV and lysed at 12 and 24 h p.i. Expression levels of PRRSV N and α-tubulin were determined by Western blotting.
PAMs, and the effect on PRRSV replication was examined. PAMs were transfected with small interfering RNA (siRNA) targeting SRP14 (siSRP14) for 24 h followed by infection with PRRSV at an m.o.i. of 0.1. The expression of SRP14, viral RNA and protein, and PRRSV production were analysed by qRT-PCR, Western blot and virus titres at the indicated time points. The siRNA knockdown of SRP14 (Fig. 5a) and 0, 82 and 78 % reduction in PRRSV ORF7 mRNA (Fig. 5b) at 0, 12 and 24 h p.i. when compared with the siRNA negative control (siNC), respectively. Further, transfection with siSRP14 also resulted in markedly decreased PRRSV RNA levels in supernatants, which decreased by 61 and 81 % (Fig. 5c) at 12 and 24 h p.i. when compared to the siNC-treated group, respectively. Simultaneously, the expression of SRP14 protein was considerably reduced, and the

![Image](Fig. 3. miR-10a-5p targets the 3′UTR of pig SRP14. (a) The 3′UTR of pig SRP14 contains one binding site (highlighted) for miR-10a-5p. (b) Seven nucleotides in the seed binding site of pig SRP14 3′-UTR were replaced. Seven-nucleotide mutations were introduced to the seed target site of miR-10a-5p, and restoration of seed binding between mutant miR-10a-5p and mutant 3′UTR of SRP14 mRNA was shown. (c) Fifty nanograms of WT or MUT reporter plasmids was co-transfected with 20 nM miR-10a-5p mimics, miR-10a-5p-Mut mimics or miR-NC mimics into the HEK293FT cells. Reporter activities were determined 36 h post-transfection by dual-luciferase assays. Results are expressed as means±SD of three independent experiments. Statistically significant values: **P<0.01, ***P<0.001.)
Western blotting. by qRT-PCR at 24 h post-transfection. Data are expressed as mean±
SD of three independent experiments. (b) PAMs transfected with miR-10a-5p mimics or
miR-NC mimics were lysed at 24 h post-transfection, and the protein expression of SRP14 and α-tubulin was determined by Western blotting.

**DISCUSSION**

Host- or virus-encoded miRNAs have been shown to play vital roles in viral infections by regulating the expression of host or viral genes at the post-transcriptional level [8, 9, 13]. Host hsa-miR-200 miRNA family members repress immediate early protein 2 (UL122) of human cytomegalovirus by targeting the UL122 3’UTR [14]. miR-122 regulates hepatitis C virus translation and RNA synthesis by altering the balance of viral RNAs engaged in replication versus translation [15, 16]. Influenza A virus-generated virus small RNAs (vsRNAs) regulate the switch of influenza virus genome transcription to replication [17]. West Nile virus-encoded KUN-miR-1 facilitates virus replication by targeting the cellular GATA4 mRNA [18], while a dengue virus (DENV)-encoded DENV-vsRNA-5 inhibits viral replication by targeting the virus nonstructural protein 1 gene [19]. Recent reports showed that miRNAs also regulate PRRSV infection and replication. Host miR-181 inhibits PRRSV replication by targeting both viral genomic RNA and receptor CD163 [20, 21]. miR-26a and miR-30c inhibit PRRSV replication by modulating type I interferon responses [22–24], and miR-125b reduces PRRSV replication by negatively regulating the NF-kB pathway [25]. Our previous work demonstrated that PRRSV encodes a functional PRRSV-vsRNA1, which auto-regulates PRRSV replication by suppressing viral NSP2 gene expression [26], and host miR-24-3p and miR-22 promote PRRSV replication by targeting the host cytoprotective enzyme haem oxygenase-1 (HO-1) [27, 28]. In the present study, we demonstrated that host miR-10a-5p can inhibit PRRSV replication by targeting the host factor SRP14 (Figs 2 and 3). These findings suggest that miRNAs encoded by PRRSV and host indeed play important roles during PRRSV infection, which might be associated with persistent PRRSV infection. Previous studies have reported that miR-10 plays an important role during development and cancer [29]. miR-10a was up-regulated in primary hepatocellular carcinomas, colon cancer and nucleophosmin 1-mutated acute myeloid leukaemia [30–32], but it was down-regulated in chronic myeloid leukaemia and acute myeloid leukaemia [33, 34]. Loss of miR-10a could induce intestinal adenomas through collaborating with activated Wnt signalling in female mice [35]. miR-10 also regulated the angiogenic behaviour of zebrafish and human endothelial cells by promoting vascular endothelial growth factor signalling [36]. Our previous study [12] and this study (Fig. 1) demonstrated that infection of PAMs with PRRSV markedly induced miR-10a-5p, which provided conclusive support for the physiological relevance of miR-10a-5p in the cellular response to PRRSV infection. However, overexpression of miR-10a-5p inhibited PRRSV replication (Fig. 2), which might be associated with persistent PRRSV infection. PRRSV might employ miR-10a-5p to auto-regulate production of viral transcripts and orchestrate necessary viral protein expression patterns to establish persistent infection. vsRNA-5, a functional miRNA-like RNA encoded by DENV, also negatively regulates viral replication [19]. Another report has shown that MHV68-encoded miRNAs are important to establishing lifelong infection, as they facilitate viral latency within the key virus reservoir of memory B cells [37].

Gene expression could be regulated by miRNAs through recognition of complementary sequence target elements. Through site-directed mutant and restoring mutant, we demonstrated that miR-10a-5p targets the 3’UTR of pig SRP14 mRNA directly and in a sequence-specific manner (Fig. 3). Further, knockdown of SRP14 by siRNA inhibits the replication of PRRSV (Fig. 5). SRP14, a ubiquitous cytoplasmic ribonucleoprotein complex, is a universally conserved protein-targeting machine [38]. SRP14 and SRP9 proteins form a heterodimer, which arrests the elongation of the nascent chains and regulates the translational output in response to stress and viral infection [39, 40]. miRNAs down-regulate gene expression either by inhibiting miRNA translation or by inducing miRNA degradation [41, 42]. Our previous study showed that overexpression of miR-24-3p in MARC-145 cells significantly down-regulated both HO-1 mRNA and protein expression [27], which suggested that miR-24-3p might decrease HO-1 expression through both mRNA degradation.
and translation repression. However, overexpression of miR-10a-5p reduced only protein and not the mRNA expression level of SRP14 (Fig. 4), indicating that miR-10a-5p might decrease SRP14 expression through translational repression but not mRNA degradation. miR-377 and miR-217 were also shown to down-regulate HO-1 expression by translational repression and not mRNA degradation in both HEK 293 cells and primary HUVEC cells [43].

In summary, the present study demonstrated that infection of PRRSV-target cells with PRRSV induces miR-10a-5p expression. miR-10a-5p then binds to the functional sites in the 3′UTR of SRP14 and down-regulates SRP14 protein expression to repress PRRSV replication. These findings not only provide new insights into virus–host interactions during PRRSV infection, but also suggest potential new control measures for future PRRSV infection.

METHODS

Cells, viruses and reagents
MARC-145 cells derived from African green monkey kidney cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% FBS and 1% antibiotic–antimycotic (Life Technologies) at 37°C and 5% CO₂. PAMs were isolated from healthy 6-week-old crossbred weaned (Landrace × Yorkshire) PRRSV-negative pigs using a lung lavage technique as previously described [44] with minor modifications [27].

Synthetic dsRNAs that mimic endogenous miR-10a-5p when transfected into cells (miR-10a-5p), as well as synthetic miR-10a-5p containing specific site mutations (miR-10a-5p-mut), and siRNA targeting SRP14 (siSRP14) were synthesized by RiboBio.

qRT-PCR
Total RNA from PAMs or supernatant PRRSV RNA was isolated using the TRizol reagent and reverse transcribed using the Primescript RT Reagent kit (TaKaRa) according to the manufacturer’s instructions. Both random 6 mers and oligo dT primer were used for the RT reaction. Quantitative PCR (qPCR) was performed with a Step One Plus Real-Time PCR system (Applied Biosystems) and FastStart Universal SYBR Green Master (Roche), with the specific forward primer 5’-GGTTCTGTGGAGGGATTTGA-3’ and
the reverse primer 5′-CCCTTACTGTGCTGCTTTTG-3′ for SRP14, and with the specific forward primer 5′-AGATCATCGCACCACAAAAC-3′ and the reverse primer 5′-GACACAAATTGCGCCGCTACA-3′ for ORF7 of PRRSV qPCR, as described previously [45]. HPRT1 mRNA was used as an internal reference [46].

For detection of miR-10a-5p, the quantitative stem–loop RT-PCR method was designed as described previously with some modifications [47, 48]. Total RNA was reverse transcribed with the stem–loop RT primers 5′-CTCAACTGGTGCTGGAGTCGGCAATTCAGTTGAGTACCTGT-3′. The qPCR was performed using FastStart Universal SYBR Green Master (Roche) with the miR-10a-5p-specific forward primer 5′-ACACTCCAGCTGGGTACCCTGTA-3′ and the universal reverse primer 5′-CTCAACGTGATGTGAGTGATACCTGT-3′. The abundance of the U6 RNA served as an internal reference [26].

For detection of supernatant PRRSV RNA, a plasmid bearing a 372 bp fragment of the PRRSV ORF7 sequence was used to generate a standard curve. The standard curve was plotted from the results of parallel PCR performed on serial dilutions of standard DNA. RNA absolute quantities were calculated by normalization to the standard curve [27].

**Western blot analysis**

Western blotting was performed as described previously [45] with minor modifications. PAMs were lysed, and the cellular proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The PVDF membrane was probed with one of the following primary antibodies: rabbit anti-SRP14 polyclonal antibodies at a 1:2000 dilution or an anti-a-tubulin antibody at a 1:5000 dilution (Abcam). HRP-conjugated anti-mouse IgG at a 1:2000 dilution (Jackson) was used to label primary antibody binding. Immunolabelled proteins were visualized using ECL reagent (Pierce).

**Virus titration**

Virus progeny production was determined by titration as described previously [50] with minor modifications. MARC-145 cells were trypsinized and seeded in a 96-well plate 24 h before virus infection. Virus supernatants were prepared through serial dilutions, and 100 µl solution was added to each well in replicates of eight. Six days after infection, the TCID₉₀ was calculated by the Reed–Muench method.

**Computational prediction of miRNA–mRNA target**

miR-10a-5p targets were predicted by using the miRanda algorithm. miR-10a-5p binding sites in SRP14 3′UTR from Sus scrofa were predicted using RNAhybrid [51], TargetScan [52] and microRNA [53]. This prediction is based on an algorithm that determined the optimal complementarity between miRNAs and a given mRNA.

**Construction of psiCheck2 target luciferase reporters**

The 3′UTR of pig SRP14 (XM_003121602.5, 602 bp, position +422 to +1023) was amplified from cDNA in PAMs, subcloned into psiCheck2 vector (Promega) using NotI and XhoI enzymes and designated as psiCheck2-SRP14-WT.

To generate the miRNA target site mutant, mutation was introduced into the 3′UTR segments containing the miR-10a-5p potential binding site of SRP14. Then, this segment was synthesized and annealed into double strands, subcloned into the psiCheck2 vector (Promega) using NotI and XhoI enzymes and designated as psiCheck2-SRP14-MUT.

**Luciferase reporter assay**

The luciferase reporter assay was processed as described previously [27, 28, 46] with the following modifications. HEK293FT cells were co-transfected with 50 ng psiCheck2-SRP14-WT or psiCheck2-SRP14-MUT plasmid and 20 nM miR-10a-5p mimics or miR-10a-5p-MUT mimics or negative control mimics (miR-NC) using X-tremeGENE HP DNA Transfection Reagent (Roche). Thirty-six hours post-transfection, cells were lysed and luciferase expression was measured on a Synergy HT Multi-Mode Microplate Reader (BioTek) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

**Statistical analysis**

All experiments were performed with at least three independent experiments. Statistical significance was determined by Student’s t-test when only two groups were compared or by one-way ANOVA when more than two groups were compared. A P value of <0.05 was considered statistically significant.

**Funding information**

This research was supported by the National Natural Science Foundation of China (31472173), and the Agricultural Science and Technology Innovation Transformation Project in Shaanxi Province of China (NYKJ-2016-08).

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

All animal work was in strict accordance with the guidelines of the Institutional Animal Care and Use Committee and approved by the Animal Care and Use Committee of Northwest A&F University. The virus used in this study was a highly pathogenic PRRSV strain, GD-HD (GenBank ID: KP793736.1).

**References**


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