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MicroRNA-30c Modulates Type I IFN Responses To Facilitate Porcine Reproductive and Respiratory Syndrome Virus Infection by Targeting JAK1

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Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important pathogen and has evolved several mechanisms to evade IFN-I responses. We report that a host microRNA, miR-30c, was upregulated by PRRSV via activating NF-κB and facilitated its ability to infect subject animals. Subsequently, we demonstrated that miR-30c was a potent negative regulator of IFN-I signaling by targeting JAK1, resulting in the enhancement of PRRSV infection. In addition, we found that JAK1 expression was significantly decreased by PRRSV and recovered when miR-30c inhibitor was overexpressed. Importantly, miR-30c was also upregulated by PRRSV infection in vivo, and miR-30c expression corresponded well with viral loads in lungs and porcine alveolar macrophages of PRRSV-infected pigs. Our findings identify a new strategy taken by PRRSV to escape IFN-I–mediated antiviral immune responses by engaging miR-30c and, thus, improve our understanding of its pathogenesis. ***The Journal of Immunology*****, 2016, 196: 2272–2282.**

Interferon-I is fundamental in antiviral innate immunity, as well as in the mounting of adaptive immunity (1, 2). Upon viral infection, mammalian cells initiate pattern recognition receptor signaling to recognize pathogen-associated molecular patterns and then activate IFN regulatory factors (IRFs) and NF-κB to induce IFN-I production (3, 4). Binding of IFN-I to its receptor leads to the phosphorylation of Janus kinases, JAK1 and TYK2, which subsequently phosphorylate STAT1 and STAT2. These activated STAT molecules, together with IRF9, form a complex, IFN-stimulated gene (ISG) factor 3, and then translocate into the nucleus to bind the IFN-stimulated response element (ISRE) to initiate the transcription of ISGs, which are executors in the clearance of viral pathogens (5–8). The importance of IFN-I is emphasized by the considerable number of viruses that antagonize IFN-I production or signaling for their successful replication (9).

MicroRNAs (miRNAs) are a class of evolutionarily conserved small (~22 nt) noncoding RNAs that have emerged as key post-transcriptional modulators of gene expression and actively participate in modulating innate and adaptive immune responses and host–pathogen interactions (10–12). miRNAs are produced by all multicellular organisms and some viruses, and they act through binding to a partially complementary region in the target mRNA. Typically, this interaction is achieved through base pairing of the seed sequence of an miRNA and the 3′ untranslated regions (UTRs) of an mRNA (13, 14). Compelling evidence proved that miRNAs of viral and cellular origin can help viruses evade host immune responses by targeting vital components in the host immune system (15–17). It is not surprising that viruses can take advantage of host miRNAs that act as suppressors of IFN-I production to generate a suitable environment for their replication. Such miRNAs include the well-characterized miR-146a (18–20), miR-21 (21), and miR-576-3p (22). The crucial antiviral effects of IFN-I are dependent on the activation of the JAK-STAT pathway (7). miR-155 is a well-studied host miRNA and inhibits vesicular stomatitis virus infection by regulating IFN-I signaling through targeting SOCS1, a negative regulator of JAK1 (23). Another report described that a viral miRNA, TTV-tth8-miR-T1 encoded by a human torque teno virus, is capable of inhibiting IFN-I signaling by targeting N-myc (and STAT) interactor (24). Given the important roles of IFN-I signaling in antiviral responses, it is important to identify miRNAs that can regulate IFN-I signaling.

Porcine reproductive and respiratory syndrome is one of the most important diseases in the swine industry, causing great economic loss since its first report in the United States in 1987 (25–27). The etiological agent of the disease is porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped positive-strand

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Abbreviations used in this article: GAS, IFN-γ–activated site; HP-PRRSV, highly pathogenic PRRSV; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE, IFN-stimulated response element; miRNA, microRNA; MOI, multiplicity of infection; N, nucleocapsid; NC, negative control mimic; PAM, porcine alveolar macrophage; p-miR-30c-2, porcine primary miR-30c-2 promoter; poly(I:C), polyinosinic-polycytidylic acid; pri-miR-30c-1, primary miR-30c-1; pri-miR-30c-2, primary miR-30c-2; PRRSV, porcine reproductive and respiratory syndrome virus; qRT-PCR, quantitative real-time PCR; si-JAK1, JAK1 siRNA; siRNA, small interfering RNA; SPF, specific pathogen free; UTR, untranslated region.

RNA virus that belongs to the Arteriviridae family (28). During PRRSV infection, IFN-I production and signaling are severely crippled (29, 30). Recently, several reports showed that many miRNAs are altered by PRRSV, raising the possibility that they are involved in its pathogenesis (31, 32). However, it remains elusive whether host miRNAs are used by PRRSV in the evasion of innate immune responses.

In this study, we found that a host miRNA, miR-30c, could be upregulated by PRRSV infection through activating the NF-κB signal pathway. Subsequently, we demonstrated that miR-30c enhanced PRRSV infection through inhibition of the IFN-I signaling pathway by targeting JAK1. Importantly, miR-30c was significantly increased by PRRSV infection in vivo, and a positive correlation was observed between miR-30c production and viral loads in the lungs and porcine alveolar macrophages (PAMs) of PRRSV-infected pigs.

Materials and Methods

Ethics statement

All animal trials in this study were performed according to the guidelines of the Beijing Laboratory Animal Welfare and Ethics of the Beijing Administration Committee of Laboratory Animals and were approved by the Beijing Association for Science and Technology (approval ID SYXK [Beijing] 2007-0023). The animal studies also complied with the China Agricultural University Institutional Animal Care and Use Committee guidelines (ID: SKLAB-B-2010-003) and were approved by the Animal Welfare Committee of China Agricultural University.

Cells and viruses

Marc-145 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. CRL-2843 cells were grown in RPMI 1640 medium with 10% FBS. PAMs were obtained by lavaging the lungs of 6–8-wk-old specific pathogen–free (SPF) pigs, as described previously (33, 34), and maintained in RPMI 1640. Peritoneal macrophages were obtained by lavaging the peritoneal cavity of SPF pigs with RPMI 1640. All cells were cultured in a humidified incubator with 5% CO_2 at 37°C. JXwn06, a highly pathogenic PRRSV (HP-PRRSV) strain, and VR-2332, a prototypical North American type 2 isolate strain, were used. For viral titer assays, PRRSV was serially diluted 10-fold to infect PAMs or Marc-145 cells in 96-well plates (~5×10^4 cells), and its infection was determined by immunofluorescent staining to detect PRRSV nucleocapsid (N) protein at 72 h postinfection. Titrated viruses were preserved at −80°C temperature until use.

Reagents and Abs

Polyinosinic-polycytidylic acid [poly(I:C)] was purchased from InvivoGen. Recombinant porcine IFN-α (*Escherichia coli* derived) was a gift from Dr. Wenjun Liu (Chinese Academy of Sciences). Recombinant porcine IFN-β was purchased from Kingfisher Biotech, and recombinant porcine IFN-γ was purchased from R&D Systems. miRNA mimics, miRNA inhibitors, and small interfering RNA (siRNA) listed in Table I were synthesized by GenePharma. NF-κB inhibitor (BAY11-7082), JNK inhibitor (SP600125), and MAPK (p38) inhibitor (SB203580) were purchased from Enzo Life Sciences. PI3K inhibitor (LY294002) and MEK (Erk) inhibitor (PD98059) were purchased from Cell Signaling Technology. TBK1 inhibitor (BX-795), JAK1 inhibitor (ruxolitinib), and STAT1 phosphorylation inhibitor (fludarabine) were purchased from Selleck Chemicals. Abs against phospho-IκB, JAK1, phospho-STAT1, and STAT1 were purchased from Cell Signaling Technology. Rabbit antiserum against PRRSV GP5 was prepared in the laboratory of W.-h.F. Anti–β-actin Ab was purchased from Sigma. Goat anti-mouse and anti-rabbit secondary Abs were purchased from Santa Cruz Biotechnology. Abs were visualized using ECL reagent (CW Biotech), following the manufacturer's instructions.

Transfection and quantitative real-time PCR

HiPerFect transfection reagents (QIAGEN) were applied to transfect small RNAs, as described in the manufacturer's instructions. Cotransfection of small RNAs with DNA constructs was conducted using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. For quantitative real-time PCR (qRT-PCR) analysis of mRNA or miRNA expression, total RNAs were extracted, reverse transcribed, and amplified, as described previously (35). For miRNA analysis, the reverse transcription primer for miR-30 was 5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN-3′. The relative expression levels of miRNAs were normalized to U6. Previously described primers were used for mRNA analysis of porcine MX1, ISG15, PRRSV ORF7, and GAPDH (35, 36). Other specific primers for qRT-PCR analysis are listed in Table II. Their expressions were normalized to GAPDH. All qRT-PCR experiments were done in triplicate.

Immunofluorescence assay

Cells were fixed in cold methanol-acetone (1:1) for 10 min at 4°C, washed with PBS, and then blocked with 10% goat serum in PBS for 30 min. Immunofluorescence assay was performed by incubating with anti-PRRSV N protein mAb SDOW17 (1:10,000; Rural Technologies) or an isotype-control Ab for 1 h at room temperature, followed by washing with PBS. Cells were then incubated with FITC-conjugated goat anti-mouse IgG Ab (1:2000; Jackson ImmunoResearch) for 1 h at 37°C, and the expression of N protein was examined using fluorescence microscopy.

Western blot

To analyze the levels of PRRSV structural protein GP5 and host phospho-STAT1, STAT1, and JAK1 proteins, cells were lysed with RIPA (CW Biotech) supplemented with a protease inhibitor mixture (Roche). Proteins were separated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in PBS with Tween-20 (0.025% Tween-20) for 1 h at room temperature, and then incubated with anti-GP5 (1:5000), anti–phospho-IκB, anti–phospho-STAT1, anti-STAT1, or anti-JAK1 Abs (1:1000) for 1 h at room temperature. The membranes were then incubated with HRP-conjugated anti-rabbit secondary Ab (1:5000) for 1 h at room temperature. β-actin (1:5000) was used as a loading control. Proteins were visualized by chemiluminescence.

Plasmid construction and luciferase assays

To verify that JAK1 is a target of miR-30c, the predicted target site in the porcine JAK1 3′UTR (297 bp) was amplified and inserted into the C terminus of the firefly luciferase gene in the pGL3-control vector (Promega). A mutant vector was constructed by mutating five seed nucleotides using a site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. NF-κB, ISRE, IRFs responding plasmids, and IFN-β promoter vector were constructed as described previously (35, 36). A sequence containing three copies of the IFN-γ–activated site (GAS) promoter element from the IRF1 gene, 5′-CTGATTTCCCCGAAATGA-3′, was inserted into pGL6 to construct the GAS promoter vector. Porcine IFN-α promoter and porcine primary miR-30c-2 promoter (p-miR-30c-2; a fragment ~3 kb upstream from the primary miR-30c-2 [pri-miR-30c-2] coding sequences) vectors were generated by inserting sequences into the N terminus of firefly luciferase gene in the pGL3-basic vector. NF-κB site mutant vectors of p-miR-30c-2 were made by deleting either or both of the two predicated NF-κB binding sites using the site-directed mutagenesis kit. The luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. The pRL-TK vector expressing the *Renilla* luciferase gene was used as a normalization control. The expression constructs of porcine JAK1-ORF and JAK-ORF-UTR were generated using pcDNA3.1(+) (Life Technologies). The expression construct of human p65 was kindly provided by Dr. Jun Tang (China Agricultural University). All primers are listed in Table II.

Animal experiments

Ten 4-wk-old Large White-Dutch Landrace SPF pigs were randomly divided into two groups. Six pigs were inoculated intranasally with 1.5 ml HP-PRRSV strain JXwn06 ($10^{5.2}$ $TCID_{50}$/ml). PRRSV-infected pigs were euthanized at 7 d postinfection, and lungs were collected and processed with liquid nitrogen and stored at −80°C for RNA isolation. Four pigs were inoculated intranasally with PBS as controls. To obtain PAMs from HP-PRRSV–infected pigs, six 4-wk-old pigs were randomly divided into two groups. Three pigs were inoculated intranasally with JXwn06 (1.5 ml; $10^{5.2}$ $TCID_{50}$/ml), and three pigs were inoculated intranasally with PBS as controls. PRRSV-infected pigs were euthanized at 5 d postinfection, and PAMs were lavaged from the lungs for RNA isolation.

Table I. Sequences of miRNA mimics, inhibitors, and siRNA of JAK1

Small RNA	Sequence (5′→3′)
miR-30a	UGUAAACAUCCUCGACUGGAAG
miR-30b	UGUAAACAUCCUACACUCAGCU
miR-30c	UGUAAACAUCCUACACUCUCAGC
miR-30d	UGUAAACAUCCCCGACUGGAAGCU
miR-30e	UGUAAACAUCCUUGACUGGAAGCU
miR-30c-mut	UGUAUGUAUCCUACACUCUCAGC[a]
miR-146a	UGAGAACUGAAUUCCAUGGGUU
miR-155	UUAAUGCUAAUUGUGAUAGGGG
si-JAK1	CGGAUGAGGUUCUAUUUCA
miR-30c inhibitor	GCUGAGAGUGUAGGAUGUUUACA
miR-155 inhibitor	CCCCUAUCACAAUUAGCAUUAA

[a]Mutated sequences are underlined.

Statistical analysis

GraphPad Prism (GraphPad, San Diego, CA) was used to analyze our data. Differences were analyzed using the Student *t* test and were considered statistically significant at $p < 0.05$.

Results

miR-30c is upregulated by PRRSV through activating NF-κB

To investigate whether any of the host miRNAs are involved in PRRSV infection, we screened the miRNAs that were significantly upregulated or downregulated during PRRSV infection and then focused on the miR-30 family (31, 32). The porcine miR-30 family contains five members (miR-30a–e). Previous studies suggested that miR-30c is subject to LPS stimulation (37), and miR-30b was shown to be a negative regulator of immune responses to enhance IL-10 and NO production through targeting Notch1 (38). To investigate whether miR-30 family members can be regulated by PRRSV, a time-course assay was performed to assess the expression of five miR-30 family members in PAMs infected with HP-PRRSV strain JXwn06 (multiplicity of infection [MOI] = 1), inoculated with heat-inactivated JXwn06 (HI-JXwn06; 70°C for 60 min), or mock infected. Our results showed that miR-30c was increased at 12 h postinfection and peaked at 24 h postinfection (~3.8-fold induction) (Fig. 1A), whereas the expression of other miR-30 family members showed no significant alteration (Supplemental Fig. 1A, 1B). The expression of miR-30c in cells inoculated with HI-JXwn06 was not altered (Fig. 1A), suggesting that the increased miR-30c is dependent on PRRSV replication.

To further verify the induction of miR-30c by PRRSV infection, we analyzed the expression of its two primary transcripts: primary miR-30c-1 (pri-miR-30c-1) and pri-miR-30c-2, which are transcribed separately from two different genomic regions. We demonstrated that only pri-miR-30c-2 was significantly upregulated and reached a significant increment to ~4.8-fold at 24 h after JXwn06 infection. However, pri-miR-30c-1 was not induced (Supplemental Fig. 1C). To validate the upregulation of pri-miR-30c-2 by PRRSV, pri-miR-30c-2 promoter was transfected into Marc-145 cells, followed by JXwn06 infection. The activity of the promoter was enhanced by PRRSV infection in a dose-dependent manner, and it increased ~2.2- and 3.5-fold at MOIs of 0.1 and 1, respectively (Fig. 1C).

Next, to determine which transcription factor(s) is responsible for the regulation of pri-miR-30c-2, Marc-145 cells were treated for 1 h with the inhibitors of the key signaling molecules, including NF-κB (BAY11-7082), MEK (PD98059), PI3K (LY294002), p38 (SB203580), JNK (SP600125), TBK1 (BX-795), and JAK1 (ruxolitinib), and then were infected with JXwn06 at an MOI of 0.1. Our results showed that the NF-κB inhibitor exerted an inhibitory effect on the activation of the pri-miR-30c-2 promoter in a dose-dependent manner (Fig. 1D, Supplemental Fig. 1D), suggesting that NF-κB is involved in the induction of pri-miR-30c-2 by PRRSV. To verify the involvement of NF-κB in the regulation of pri-miR-30c-2 production, we analyzed the pri-miR-30c-2 pro-

Table II. Primers used in qRT-PCR analysis and plasmid construction

Primers	Sequence (5′→3′)
qRT-PCR primers	
pri-miR-30c-1-F	CCGTAGTGCGTGTAAACATC
pri-miR-30c-1-R	TGCTGGGTGAAGAGACTGT
pri-miR-30c-2-F	AAACATCCTACACTCTCAG
pri-miR-30c-2-R	CAGTCCATTTCTCTTTAGC
JAK1-F	ACCACACCCTGGAAGTTCAAAG
JAK1-R	CGCTGTCGATGCCCTCAC
IRF1F	GTGTCACCCATGCCCTCTAC
IRF1R	TAAGCAGGTACCCCTTCCCA
miR-30a-*d*-e-F	GCTGTAAACATCCTCGACTGGAAG
miR-30b-F	CGCTGTAAACATCCTACACTCAGCT
miR-30c-F	CGTGTAAACATCCTACACTCTCAGC
Uni-miR-R primer	GCGAGCACAGAATTAATACGACTCAC
Clone and mutation primers	
JAK1-3′UTRwt-F	GACTCTAGAATGAAATGGGGTCTATGCA
JAK1-3′UTRwt-R	GACTCTAGAGTGTTGGTAGTAGGTCTG
JAK1-3′UTRmut-F	CGGCCCAGTTCCATCCCGAGATATTTGGGATAGGGTACATACGTC
JAK1-3′UTRmut-R	TGGTGCTTGATGTCAACATAAACTTGACGTATGTACCCTATCCCA
JAK1-ORF(-UTR)-F	CCAGTGTGGTGGAATTCATGGCTTTTTGTGCTAAAATGAGGAGC
JAK1-ORF-R	GCCCTCTAGACTCGAGGTTGGGGAGGAGGACTTGAAACA
JAK1-ORF-UTR-R	GCCCTCTAGACTCGAGGCATGTGAAGTCTCTAGTACATTTATTTG
p-miR-30c-2-F	TACGCGTGCTAGCCCGGGCTCGAGGAGACTGCTTGTGATGCGG
p-miR-30c-2-R	CCAACAGTACCGGAATGCCAAGCTTCTGAGAGTGTAGGATGTTT
p-miR-30c-2-mutS1-F	ATTCCTGCTGGAAAGGCCTAGGCCCCCATGTACAACTTCTCCCCG
p-miR-30c-2-mutS1-R	GCTGGGGTTAGGGTTAGGCTGAGCTCGGGGAGAAGTTGTACATGG
p-miR-30c-2-mutS2-F	GTCTTTGGTCTTTTTAGGGCCGCACCCATGGCATAGGCTAGGGGT
p-miR-30c-2-mutS2-R	GGCCTGTAGCAACAGCTCAGATTAGACCCCTAGCCTATGCCATGG
GAS-F	TCGACTGATTTCCCCGAAATGACTGATTTCCCCGAAATGACTGATTTCCCCGAAATGA
GAS-R	GATCTCATTTCGGGGAAATCAGTCATTTCGGGGAAATCAGTCATTTCGGGGAAATCAG

moter using the PROMO (39) program and found two putative NF-κB binding sites. To examine whether these putative NF-κB binding sites are critical for pri-miR-30c-2 production, we constructed three reporter plasmids, in which either or both of the two binding sites were deleted (Fig. 1E). We transfected p65, one component of NF-κB into CRL-2843 cells to activate the NF-κB pathway and found that overexpression of p65 significantly activated the pri-miR-30c-2 promoter; alternatively, deletion of the NF-κB binding site 1 efficiently repressed activation of the promoter by p65 (Fig. 1F) or by PRRSV infection (Fig. 1G), suggesting that NF-κB binding site 1 is critical for the upregulation of pri-miR-30c-2 by PRRSV. NF-κB can be activated by PRRSV infection (40). To investigate whether the activation of NF-κB is coincident with miR-30c induction, we performed a time-course analysis to determine the activation of NF-κB in PRRSV-infected cells. Our results showed that NF-κB was activated as expected, and its activation corresponded well with the induction of mature miR-30c and primary miR-30c (Fig. 1H). Taken together, these results suggest that PRRSV infection upregulates miR-30c, primarily through the NF-κB signal-transduction pathway.

miR-30c enhances PRRSV replication and inhibits IFN-I signaling

To test whether miR-30c can affect PRRSV replication, PRRSV infection assays were performed in PAMs transfected with miR-30c mimics or miR-30c inhibitor. PRRSV infection was examined by

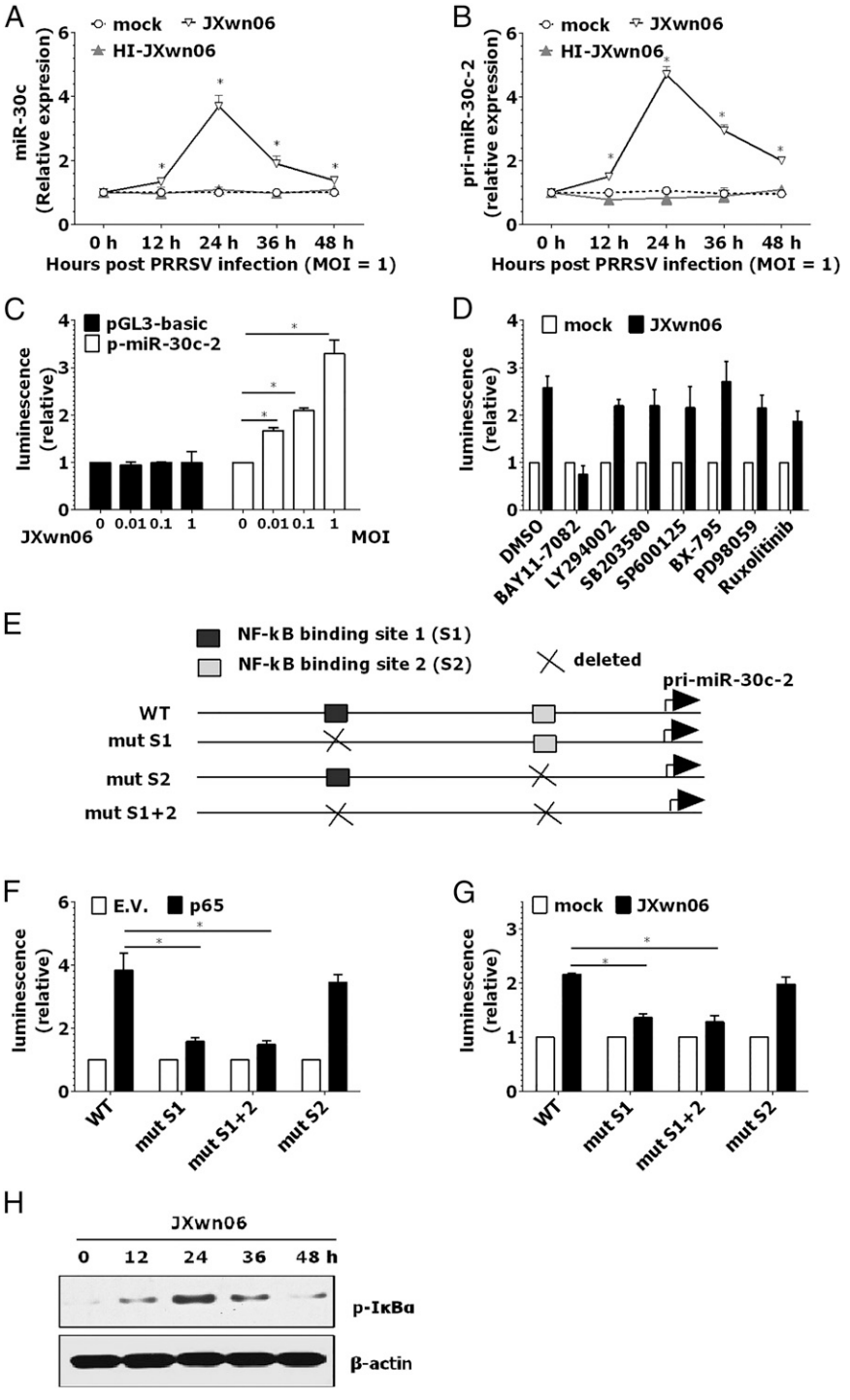

FIGURE 1. miR-30c is upregulated by PRRSV through an NF-κB–dependent mechanism. (**A**–**D**) miR-30c is upregulated by PRRSV. qRT-PCR analysis of miR-30c (A) and pri-miR-30c-2 (B) was performed in PAMs inoculated with JXwn06 or HI-JXwn06 at an MOI of 1 for the indicated times. (C) After cotransfection of 1 μg pGL3-basic or p-miR-30c-2 along with pRL-TK (20 ng) into Marc-145 cells for 6 h, cells were infected with JXwn06 at an MOI of 0.01, 0.1, or 1. At 48 h postinfection, luciferase activities were analyzed. (D) NF-κB is responsible for inducing miR-30c by PRRSV. After cotransfection of p-miR-30c-2 vector along with pRL-TK into Marc-145 cells for 6 h, cells were treated with DMSO (1 μM), NF-κB inhibitor (BAY11-7082; 5 μM), MEK inhibitor (PD98059; 10 μM), PI3K inhibitor (LY294002; 5 μM), p38 inhibitor (SB203580; 5 μM), JNK inhibitor (SP600125; 10 μM), TBK1 inhibitor (BX-795; 1 μM), or JAK1 inhibitor (ruxolitinib; 0.5 μM) for 1 h and infected with JXwn06 (MOI = 0.1). Forty-eight hours later, cells were harvested for luciferase assay. (**E**–**G**) NF-κB binding site 1 in pri-miR-30c-2 promoter is critical for pri-miR-30c-2 induction. (E) Putative NF-κB binding sites in pri-miR-30c-2 and its mutants with either (mut S1 or mut S2) or both (mut S1+2) of the two predicted sites deleted. (F) CRL-2843 cells were cotransfected with wild-type (WT) or mutant vectors (1 μg) along with pRL-TK (20 ng) and 0.5 μg p65 expression plasmid or empty vector (E.V.) for 48 h and then harvested for luciferase assay. (G) Marc-145 cells were cotransfected with WT or mutant vectors along with pRL-TK for 6 h and then infected with JXwn06 (MOI = 0.1). At 48 h postinfection, a luciferase assay was performed. Data in (A)–(G) represent three independent experiments (mean ± SD). (**H**) PAMs were infected with PRRSV (MOI = 1) for the indicated times, and cells were harvested to examine p-IκBα using Western blot analysis. Results from one representative experiment with similar results obtained in three independent experiments are shown. $*p < 0.05$.

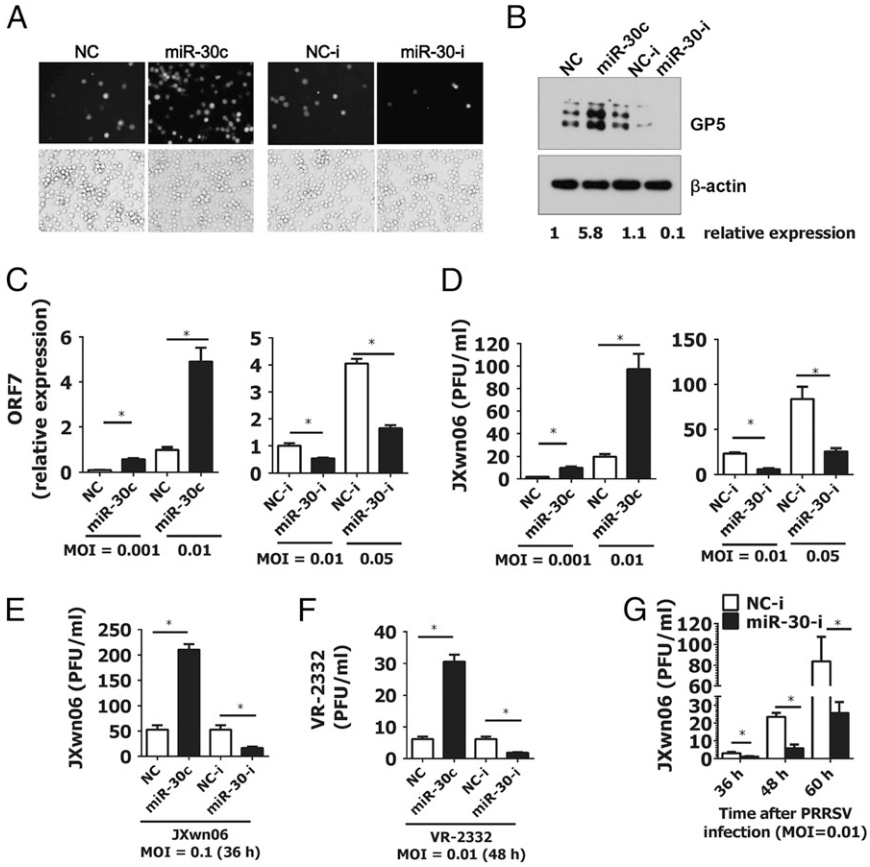

FIGURE 2. miR-30c enhances PRRSV replication. (**A**–**D**) PAMs were transfected with miR-30c mimics, NCs, specific inhibitor of miR-30c (miR-30-i), or negative control inhibitor (NC-i) at a final concentration of 60 nM for 24 h, followed by infection with HP-PRRSV strain JXwn06 for 48 h at an MOI of 0.001, 0.01, or 0.05. Cells were then fixed for immunofluorescent staining of PRRSV N protein (original magnification ×20) (A) or harvested for PRRSV protein GP5 detection using Western blot analysis (B) or qRT-PCR analysis of ORF7 expression (normalized to GADPH) (C). (D) Simultaneously, culture supernatants were collected for quantification of virus yields. PAMs were transfected for 24 h as in (A) and then infected with JXwn06 (MOI = 0.1) (**E**) or VR-2332 (MOI = 0.01) (**F**). Culture supernatants were collected at 36 h (E) or 48 h (F) for quantification of virus yields. (**G**) PAMs were transfected with miR-30 inhibitor or NC inhibitor at a final concentration of 60 nM for 24 h and then infected with JXwn06 for 36, 48, or 60 h at an MOI of 0.01. Culture supernatants were collected to analyze virus yields. Results from one representative experiment with similar results obtained in three independent experiments (A and B) or from three independent experiments [mean ± SD (C–G)] are shown. $*p < 0.05$.

immunofluorescence assay and Western blot analysis using Abs against PRRSV N and GP5 protein, respectively. PRRSV RNA levels were analyzed using qRT-PCR with specific primers detecting ORF7. The growth dynamics of virus are diverse, with viruses having different MOIs. Thus, to draw a clear picture of the effect of miR-30c on PRRSV infection, we used multiple MOIs. Our results showed that ectopic expression of miR-30c significantly increased, whereas inhibition of miR-30c reduced, PRRSV (JXwn06) infection (Fig. 2A), as well as the abundance of viral protein (Fig. 2B) and RNAs (~5.9- or 5.0-fold enhancement when MOI was 0.001 or 0.01 or ~2-fold decrease at MOIs of 0.01 or 0.05) (Fig. 2C). The amount of infectious virus production increased significantly when miR-30c–transfected PAMs were infected with PRRSV at an MOI of 0.001 or 0.01 for 48 h (~5.6- and 4.9-fold increase, respectively) (Fig. 2D). However, when miR-30c inhibitor was applied, the amount of infectious virus decreased significantly (~4.1- and 3.3-fold reduction at MOIs of 0.01 and 0.05, respectively) (Fig. 2D). These results indicate that miR-30c can enhance PRRSV infection. miR-30c also enhanced JXwn06 replication when a higher MOI of 0.1 was used (~4.0-fold increase) (Fig. 2E). To investigate whether the replication of other PRRSV strains can be enhanced by miR-30c, we analyzed the effect of miR-30c on the prototype VR-2332 in PAMs. Our results showed that miR-30c also facilitated VR-2332 replication (~5.0-fold increase), suggesting that the effect of miR-30c on PRRSV infection was not restricted to the particular strain (Fig. 2F). To clearly show the proviral effect of miR-30c on PRRSV infection, we performed an analysis of viral infection in the presence of the miR-30c inhibitor at an MOI of 0.01 for different times. Our results showed that the miR-30c inhibitor reduced viral production to ~36, 24, and 31% relative to that of the negative control mimic (NC) inhibitor at 36, 48, and 60 h post-infection, respectively (Fig. 2G). Taken together, these results suggest that miR-30c facilitates PRRSV infection.

Next, to investigate the underlying mechanisms for miR-30c to enhance PRRSV infection, we examined whether miR-30c could alter IFN-I production and/or signaling. A series of assays was performed to monitor the activation of NF-κB, IRFs, IFN-α promoter, IFN-β promoter, and ISRE after stimulating with poly(I:C). miR-146a was shown to suppress NF-κB activation (18–20), whereas miR-155 was demonstrated to promote IFN-I signaling (23). Thus, we used these two miRNAs as controls. Our data indicated that miR-30c had no effect on the activation of NF-κB, IRFs, IFN-α, or IFN-β promoter (Fig. 3A–C, Supplemental Fig. 2A), but it significantly repressed (~59% reduction) the activation of ISRE under poly(I:C) stimulation (Fig. 3D). However, miR-30c mutant with mutated seed sequences (GUAAACA to GUAUGUA) lost its ability to inhibit the activation of ISRE (Fig. 3D). These results suggest that miR-30c impairs IFN-I signaling instead of its production. To confirm these results, we treated miR-30c–transfected CRL-2843 cells with IFN-α or IFN-β to directly initiate IFN-I signaling. Our results showed that miR-30c downregulated the activation of ISRE stimulated by IFN-I to ~44%, whereas the miR-30c mutant had no effect (Fig. 3E, Supplemental Fig. 2B). Moreover, the repression of IFN-I signaling by miR-30c occurred in a dose-dependent manner (Supplemental Fig. 2C). Other miR-30 family members showed similar inhibitory effects (Supplemental Fig. 2D). Blocking the expression of miR-30c using a specific inhibitor enhanced the induction of ISRE activity ~2-fold (Fig. 3F). These results verified the role of miR-30c as a negative regulator of IFN-I signaling.

IFN-I activates the JAK-STAT signaling pathway to induce hundreds of ISGs to inhibit viral infection. Our results showed that the induction of MX1 and ISG15 (ISGs) was suppressed in

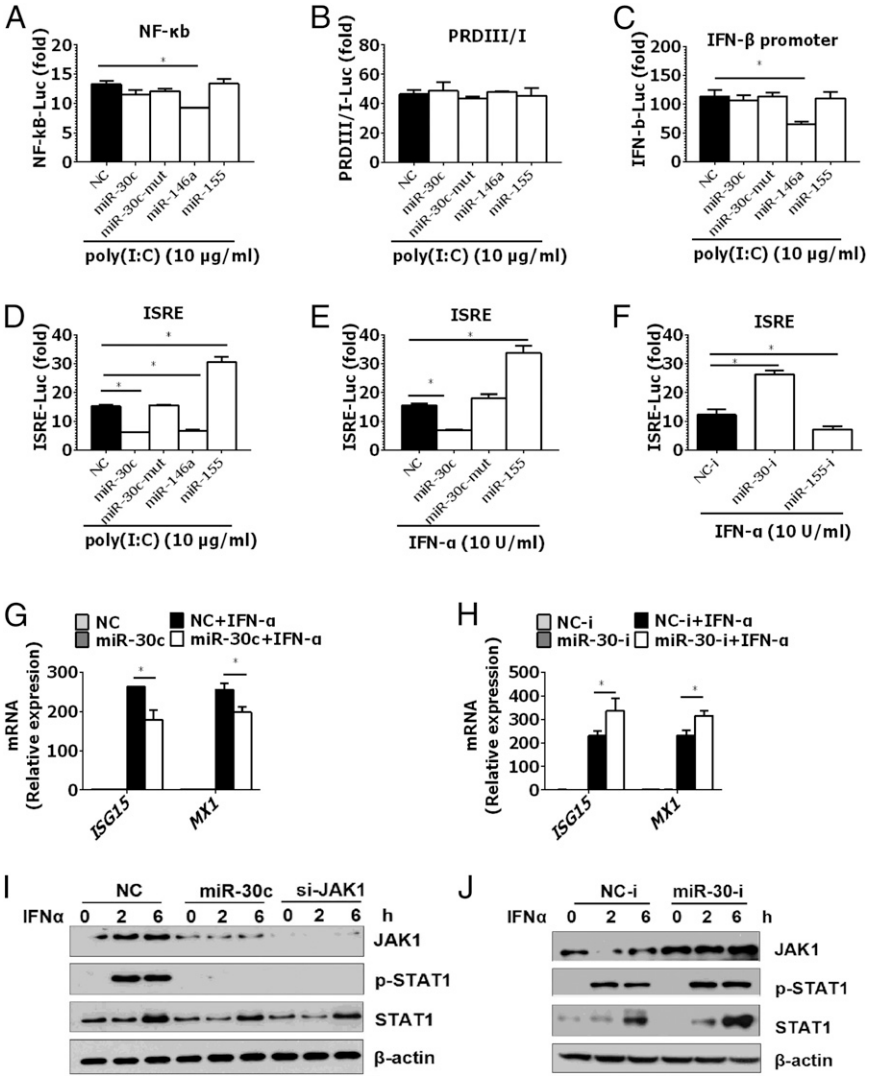

FIGURE 3. miR-30c inhibits IFN-I signaling. IRFs (**A**), NF-κB (**B**), ISRE reporter vectors (**D**–**F**), or IFN-β promoter (**C**) vectors along with pRL-TK were cotransfected with 60 nM of the indicated miRNA mimics (A–E) or inhibitors (F) into CRL-2843 cells for 36 h, followed by incubation with poly(I:C) (10 μg/ml) (A–D) or porcine IFN-α (10 U/ml) (E and F) for 12 h. Then cells were harvested for luciferase assay. CRL-2843 cells were transfected with the indicated NC mimics, miR-30c mimics, si-JAK1 (**G** and **I**), miR-30c inhibitor, or NC inhibitor (**H** and **J**) (60 nM) for 36 h and then stimulated with IFN-α (10 U/ml). Cells were collected 12 h later for qRT-PCR analysis of MX1 and ISG15 expression (normalized to GADPH) (G and H) or harvested for Western blot analysis of JAK1, p-STAT1, or STAT1 at the indicated times (I and J). Results were obtained from three independent experiments [mean ± SD (A–H)] or from one representative experiment with similar results in three independent experiments (I and J). $*p < 0.05$.

miR-30c–transfected CRL-2843 cells stimulated by IFN-α, whereas blocking the endogenous miR-30c expression increased the induction of MX1 and ISG15 (Fig. 3G, 3H, Supplemental Fig. 2E, 2F). STAT1 can be induced by IFN-I and plays a vital role in the JAK-STAT signaling cascade as a transcription factor through tyrosine phosphorylation–dependent activation. To further elaborate the inhibition effect of miR-30c, the activation of STAT1 was analyzed in CRL-2843 cells overexpressed with miR-30c. Our results showed that the levels of STAT1 and phospho-STAT1 induced by IFN-α were decreased in the presence of miR-30c, similar to the results of the knockdown of JAK1 by JAK-1 siRNA (si-JAK1) (Fig. 3I). Opposite effects were observed in cells treated with miR-30c inhibitor (Fig. 3J). Thus, we conclude that miR-30c inhibits IFN-I signaling instead of its production.

miR-30c inhibits IFN-I signaling primarily through targeting JAK1

To investigate which major target of miR-30c is involved in modulating IFN-I signaling, a computational analysis was performed using TargetScan prediction program (http://www.targetscan.org). Analysis showed that miR-30c could target JAK1 through a site in the 3′UTR region conserved in mammals (Fig. 4A). To verify whether JAK1 is a direct target of miR-30c, we cloned the predicted target site in JAK1 into a firefly luciferase reporter vector; meanwhile, a mutant vector was constructed to eliminate the possible recognition by replacing five seed nucleotides (UGUUUAC to UAGGGUC). In the presence of miR-30c, the luciferase activity of JAK1 3′UTR was reduced to ~60% relative to NCs, whereas blockage of endogenous miR-30c resulted in a ~2.5-fold increase in luciferase activity. However, all of the effects produced by miR-30c were eliminated in the vector bearing the mutant target site (Fig. 4B). These results confirmed that the miR-30c target site is harbored in the 3′UTR of JAK1. To further validate JAK1 as a target of miR-30c, its expression was examined in CRL-2843 cells treated with miR-30c mimics or inhibitor. As expected, the mRNA levels of JAK1 were decreased when miR-30c was overexpressed but were increased when the miR-30c inhibitor was applied (Fig. 4C). Moreover, miR-30c mimics significantly suppressed the level of JAK1 protein in a dose-dependent manner (Fig. 4D). Taken together, these results indicate that JAK1 is a target of miR-30c.

To assess the role of JAK1 in miR-30c–mediated inhibition of IFN-I signaling, we constructed two JAK1 expression vectors, with or without the 3′UTR of JAK1. Overexpression of miR-30c inhibited ISRE activation induced by JAK1 bearing the 3′UTR (~50% reduction), whereas it had no significant effect on ISRE activity activated by JAK1 without the 3′UTR (Fig. 4E). In addition, a significant difference was observed in the ISRE activation by JAK1 with or without the 3′UTR (Fig. 4E), implicating that endogenous miR-30c might play a role. As expected, the activation

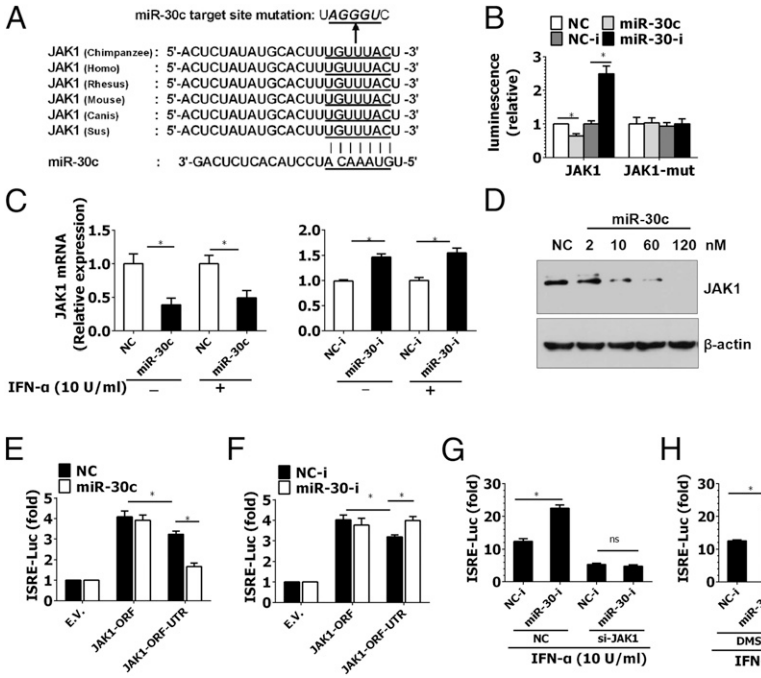

FIGURE 4. miR-30c inhibits IFN-I signaling by targeting JAK1. (**A**–**D**) JAK1 is targeted by miR-30c. (A) Schematic diagram of the predicted target sites of miR-30c in JAK1 3′UTRs of six representative mammals. The predicted target sites of miR-30c are underlined and mutated as indicated. (B) Marc-145 cells were cotransfected with JAK1 or JAK1 mutant luciferase reporter vector (100 ng), pRL-TK, and miR-30c mimics or NC, miR-30c inhibitor or NC inhibitor for 30 h and then harvested for luciferase assay. (C and D) CRL-2843 cells were transfected with miR-30c mimics or NC mimics, miR-30c inhibitor or NC inhibitor [60 nM, except for the dose-dependent assay in (D)] for 36 h and then mock treated or stimulated with IFN-α (10 U/ml) for 12 h. Then, cells were collected for qRT-PCR analysis (C) or Western blot analysis (D) of JAK1 expression. miR-30c suppresses IFN-I signaling via targeting JAK1. ISRE reporter vector, pRL-TK, empty vector (E.V.), or JAK1 expression vectors (0.5 μg) and miRNA mimics (**E**) or inhibitors (**F**) were cotransfected into CRL-2843 cells for 48 h and harvested for luciferase assay analysis. (**G**) CRL-2843 cells were cotransfected with ISRE reporter vector, pRL-TK, si-JAK1, or NC and miR-30c inhibitor or NC inhibitor (60 nM), as indicated for 48 h, followed by stimulation with IFN-α (10 U/ml) for 12 h, and luciferase activities were analyzed. (**H**) CRL-2843 cells were cotransfected with ISRE reporter vector, pRL-TK, for 35 h. Subsequently, cells were treated with DMSO or JAK1 inhibitor (0.5 μM) for 1 h, followed by stimulation with IFN-α (10 U/ml) for 12 h to determine luciferase activities. Results were obtained from three independent experiments [mean ± SD (B, C, and E–H)], or one representative experiment of three independent experiments is shown (D). $*p < 0.05$. ns, not significant.

of ISRE by JAK1 with the 3′UTR was increased from ∼3-fold to 4-fold when miR-30c inhibitor was applied (Fig. 4F). These data suggest that miR-30c exerts its effect specifically by targeting the putative site in the 3′UTR of JAK1.

To confirm this observation, we used si-JAK1 to repress JAK1 expression in the presence of miR-30c inhibitor, followed by IFN-α stimulation. Knockdown of JAK1 efficiently inhibited ISRE activity induced by IFN-α (to ∼40%) and abolished the effect of miR-30c inhibitor on IFN-α–induced ISRE activity (Fig. 4G). A similar effect to si-JAK1 was observed when the JAK1 inhibitor ruxolitinib was applied (Fig. 4H). These results suggest that modulation of IFN-I signaling by miR-30c is primarily achieved through targeting JAK1.

JAK1 is also involved in the transduction of the type II IFN (IFN-γ) signaling pathway (41). To investigate whether miR-30c affects the activation of IFN-γ signaling, we analyzed the activation of IFN-γ signaling using reporter assays and RT-PCR. Our results showed that miR-30c significantly repressed (∼40% reduction) activation of the GAS promoter under IFN-γ stimulation, whereas miR-30c inhibitor enhanced GAS promoter activity to ∼1.5-fold (Supplemental Fig. 3A). In addition, similar results were observed when the induction of IRF1, an IFN-γ–stimulated gene, was analyzed (Supplemental Fig. 3B). These results suggest that miR-30c inhibits IFN-γ signaling. In PRRSV-infected PAMs, IFN-γ was not activated, and IFN-γ stimulated genes were not induced (data not shown). However, we assumed that miR-30c induced by PRRSV might affect the activation of IFN-γ signaling in activated T cells.

miR-30c facilitates PRRSV infection via repressing IFN-I signaling by targeting JAK1

Next, to clarify whether the effect of miR-30c on PRRSV infection is dependent on the impairment of IFN-I signaling, we used a STAT1 activation inhibitor (fludarabine) to repress IFN-I signaling in PAMs transfected with miR-30c inhibitor and analyzed its effect on PRRSV replication. Our results showed that inhibition of PRRSV infection by miR-30c inhibitor was reduced in fludarabine-treated cells (Fig. 5A), suggesting that miR-30c regulates PRRSV infection by targeting the IFN-I signaling pathway. To elaborate the role of JAK1 in the miR-30c–mediated facilitation of PRRSV replication, we performed a knockdown experiment using si-JAK1 in PAMs expressing miR-30c inhibitor to examine its effect on viral replication. Knockdown of JAK1 rescued the viral infection inhibited by the miR-30c inhibitor (Fig. 5B, 5C). Taken together, these results confirm that miR-30c facilitates PRRSV infection primarily through repressing IFN-I signaling by targeting JAK1.

Because miR-30c is upregulated by PRRSV, and overexpression of miR-30c impairs JAK1 expression, we speculated that JAK1 expression might be downregulated during PRRSV infection. To test this hypothesis, we examined the expression kinetics of JAK1 during PRRSV infection. As expected, JAK1 expression was significantly decreased at 24 h postinfection, and an ∼80% reduction was observed at 48 h postinfection (Fig. 5D). However, when miR-30c inhibitor was applied, recovery of JAK1 expression was observed at the mRNA and protein levels upon JXwn06 in-

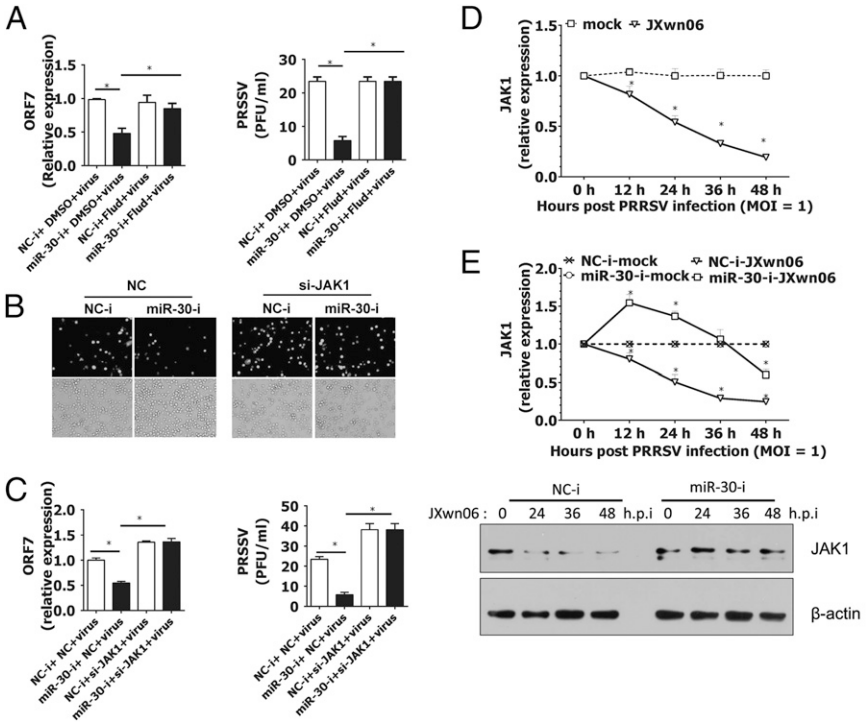

FIGURE 5. PRRSV upregulates miR-30c to enhance its replication by targeting JAK1. (**A**) PAMs were transfected as in Fig. 2. Twenty-four hours later, cells were treated with DMSO or fludarabine (0.5 μM) for 1 h and then infected with JXwn06 (MOI = 0.01) for 48 h. Cells were harvested for qRT-PCR analysis of ORF7, or culture supernatants were collected for quantification of virus yields. (**B** and **C**) PAMs were cotransfected with si-JAK1 or NC, and miR-30c inhibitor or NC inhibitor (60 nM) as indicated. Twenty-four hours later, cells were infected with JXwn06 for 48 h (MOI = 0.01) and detected as in Fig. 2A, 2C, and 2D. (B) Original magnification ×20. (**D**) JAK1 expression is decreased during PRRSV infection. qRT-PCR analysis of JAK1 was performed in PAMs infected with JXwn06 at an MOI of 1 for the indicated times. (**E**) JAK1 expression is rescued by miR-30c inhibitor. qRT-PCR analysis and Western blot analysis of JAK1 expression were performed in PAMs transfected with miR-30c inhibitor or NC inhibitor, followed by infection with JXwn06 (MOI = 1) for the indicated times. Results from three independent experiments [mean ± SD (A and C–E)] and from one representative of three independent experiments with similar results (B and E) are shown. $*p < 0.05$.

fection (Fig. 5E), suggesting that PRRSV infection downregulates JAK1 by upregulating miR-30c.

miR-30c correlates with PRRSV infection in vivo

PAMs are the main target cells for PRRSV, whereas peritoneal macrophages are resistant to PRRSV infection. Thus, we analyzed the endogenous expression of miR-30c in these cells. Interestingly, miR-30c was constitutively expressed at a lower level in peritoneal macrophages than in PAMs (~5.2-fold lower) (Fig. 6A), suggesting that miR-30c might influence PRRSV tropism in vivo.

To investigate whether HP-PRRSV has the ability to induce miR-30c in vivo, six pigs were infected intranasally with HP-PRRSV JXwn06 (1.5 ml × $10^{5.2}$ $TCID_{50}$/ml for each piglet); lung samples were collected at 7 d postinfection and analyzed for miR-30c expression and viral loads. As shown in Fig. 6B, pigs challenged with JXwn06 displayed significantly elevated levels of miR-30c (~6.6-fold) in lungs at 7 d postinfection compared with control pigs ($n = 4$). Other members of the miR-30 family were not altered by PRRSV infection (Fig. 6C, 6D). miR-21, which is not reported to be regulated by PRRSV, was also analyzed, and our results showed that miR-21 production was not altered by JXwn06 (Fig. 6E). To determine whether there is a relationship between miR-30c expression and viral load in HP-PRRSV–infected pigs, we also analyzed the viral loads in the lungs of JXwn06-infected pigs. Interestingly, a significant positive correlation ($R = +0.8763$, $p = 0.0063$) was observed between miR-30c expression and PRRSV viral loads (Fig. 6F). Similar results were attained using PAMs lavaged from the lungs of infected pigs (Supplemental Fig. 4). Collectively, these data indicate that HP-PRRSV infection results in an increase in miR-30c expression in vivo, which is positively correlated with the viral loads in lungs and PAMs of PRRSV-infected pigs.

Taken together, we demonstrated that PRRSV infection significantly induces miR-30c expression in an NF-κB–dependent manner, which targets JAK1 to impair IFN-I signaling and enhance PRRSV replication (Fig. 7).

Discussion

miRNAs have emerged as important and versatile modulators of immune responses and participate actively in the regulation of host–pathogen interactions. In the current study, we found that miR-30c enhanced PRRSV replication. Our results showed that miR-30c was significantly upregulated by PRRSV infection dependent on the NF-κB pathway. Subsequently, we demonstrated that miR-30c impaired the IFN-I signaling pathway by targeting JAK1, resulting in the enhancement of PRRSV infection. Moreover, our results showed that PRRSV infection significantly increased the production of miR-30c in vivo and in vitro. Importantly, there was a positive correlation between miR-30c expression and viral loads in the lungs and PAMs of PRRSV-infected pigs.

Multiple strategies are used by viruses to inhibit the IFN-I system to ensure their successful propagation and spread (9). PRRSV infection induces poor IFN-I responses, and several PRRSV proteins, including Nsp1, Nsp2, Nsp11, N protein (29, 30), and Nsp4 (36, 42), were shown to antagonize IFNs. Nsp1 was identified as the most potent antagonist and is able to inhibit the production and signaling of IFN-I. A previous study demonstrated that Nsp1

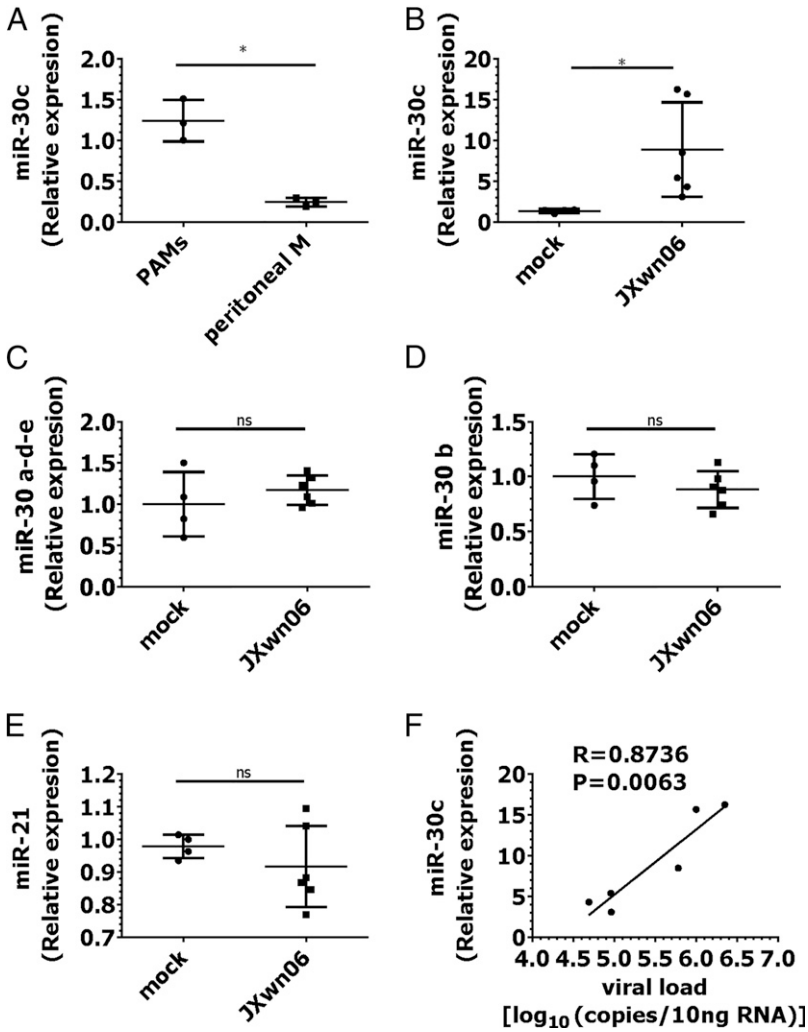

FIGURE 6. miR-30c correlates well with PRRSV infection in vivo. (**A**) qRT-PCR analysis of miR-30c in PAMs and peritoneal macrophages. (**B**–**E**) HP-PRRSV infection enhances miR-30c expression in vivo. qRT-PCR analysis of miR-30c (B), miR-30a-d-e (C), miR-30b (D), and miR-21 (E) in lungs of pigs infected or not with PRRSV JXwn06 for 7 d (mock group, $n = 4$; JXwn06 infection group, $n = 6$). Data in (A)–(E) are mean ± SEM. (**F**) Positive correlation of miR-30c expression and viral loads in lungs of HP-PRRSV–infected pigs. qRT-PCR analysis of miR-30c and ORF7 in lungs of pigs infected with JXwn06 for 7 d ($n = 6$), a linear regression analysis was then performed. *$p < 0.05$. ns, not significant.

inhibits IFN-I signaling via inducing the degradation of karyopherin-α1 (also known as importin-α5), a nuclear transport protein involved in the import of ISG factor 3 to the nucleus (43). Different PRRSV strains exert distinct effects on the IFN-I signaling pathway. For example, VR-2332 and VR-2385 infections decrease KPNA1 expression, whereas the MLV strain of PRRSV does not. However, MLV suppresses the activation of ISG15 and ISG56 induced by IFNs in MARC-145 cells, implying that other mechanisms might contribute to the impairment of IFN-I signaling during PRRSV infection (43). Cellular miRNAs were shown to be used by viruses to evade IFN-I–mediated antiviral responses in host cells (18, 21, 44, 45). However, whether miRNAs participate in PRRSV-mediated evasion of IFN-I–mediated antiviral responses remains elusive. In this article, we described a new strategy used by PRRSV to evade IFN-I–mediated antiviral response by using a host IFN-I signaling suppressor: miR-30c. However, it is difficult to verify whether miR-30c is more or less important in viral pathogenesis than other reported mechanisms. Nevertheless, it is reasonable to speculate that PRRSV benefits itself when PRRSV-induced miR-30c cooperates with other proteins, including Nsp1, Nsp11, Nsp4, N proteins, to interfere with IFN-I–mediated antiviral responses.

Our results showed that miR-30c suppressed IFN-I signaling and significantly promoted viral infection by directly targeting JAK1, a critical signaling component in IFN-I signaling. The importance of JAK1 is emphasized by the fact that some viruses, including human CMV (46) and HSV (47), can reduce the constitutive levels of JAK1 to facilitate their infections. During PRRSV infection, we demonstrated that JAK1 expression was significantly decreased (Fig. 5F). In addition, the impaired expression of JAK1 could be rescued by miR-30c inhibitor (Fig. 5G), confirming that PRRSV-induced miR-30c is responsible for the decreased JAK1.

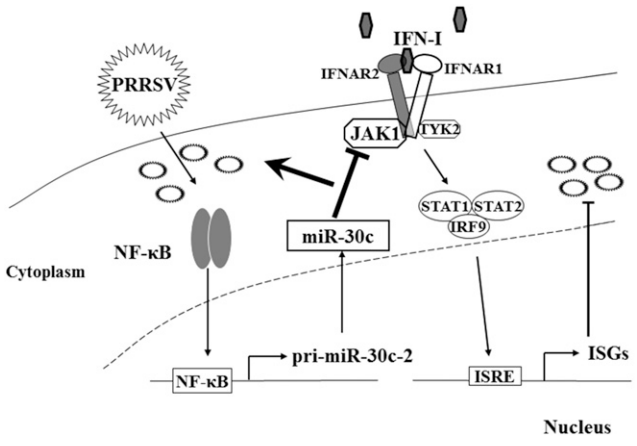

FIGURE 7. PRRSV upregulates miR-30c to enhance viral replication by evading IFN-I–initiated innate immunity. During PRRSV infection, miR-30c is upregulated dependent on the NF-κB pathway. miR-30c interferes with IFN-I signaling by repressing JAK1 expression, leading to viral escape from the host innate-immunity response.

IFN-I is a universal antiviral cytokine. Because the target site of miR-30c in the 3′UTR of JAK1 is conserved in mammals (Fig. 4A), we speculate that miR-30c might negatively modulate IFN-I signaling and exert a broad effect on viral infection. As expected, we found that miR-30c also significantly enhanced the infections of a DNA virus (HSV, HSV-1) and a negative-strand RNA virus (influenza virus WSN) (data not shown). However, we observed no significant changes in the expression of miR-30c during HSV-1 and influenza virus infections (data no shown). Thus, we assume that engaging miR-30c might be a unique strategy used by PRRSV to impair IFN-I responses. Indeed, miR-30c expression corresponds well with viral loads in the lungs of HP-PRRSV–infected pigs, implicating that it plays an important role in PRRSV pathogenesis.

PRRSV has a tropism for cells of the monocyte–macrophage lineage, such as PAMs, but not for peritoneal macrophages. In a previous study, we revealed that miR-181, an effective antiviral miRNA, is expressed at a much higher level in peritoneal macrophages than in PAMs, and the total level of miRNAs that potentially target PRRSV in peritoneal macrophages is also higher than that in PAMs (48, 49). Interestingly, miR-30c, a proviral miRNA, is expressed at lower levels in peritoneal macrophages than in PAMs (Fig. 6A). These data implicate that miRNAs play an important role in determining PRRSV tropism for cells.

In summary, we present a strategy used by PRRSV to escape innate immunity by engaging miRNA, which may help us to further understand PRRSV pathogenesis. In addition, our findings underscore the importance of miR-30c in the regulation of IFN-I signaling and PRRSV infection, as well as broaden our knowledge about the role of miRNAs in host–virus interactions.

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Disclosures

The authors have no financial conflicts of interest.

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