MicroRNA-33a disturbs influenza A virus replication by targeting ARCN1 and inhibiting viral ribonucleoprotein activity

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In order to explore the roles of microRNA(s) [miRNA(s)] in the influenza A virus life cycle, we compared the miRNA profiles of 293T and HeLa cell lines, as influenza A virus can replicate efficiently in 293T cells but only poorly in HeLa cells. We analysed differentially expressed miRNAs and identified five, including miR-33a, that could disturb influenza A virus replication significantly. Using TargetScan analysis, we found that ARCN1 could be a potential target of miR-33a. To confirm whether miR-33a could truly target ARCN1, we generated a luciferase reporter for the ARCN1 3′ untranslated region (UTR) and performed a luciferase assay. The data indicated that miR-33a could suppress the luciferase activity of the reporter for the ARCN1 3′ UTR but not a reporter in which the predicted miR-33a targeting sites on ARCN1 3′ UTR were mutated. We performed immunoblotting to confirm that miR-33a could downregulate the protein level of ARCN1. Consistently, the level of ARCN1 protein in HeLa cells was significantly lower than that in 293T cells. We also demonstrated that ectopic expression of ARCN1 could partially rescue the inhibitory effect of miR-33a on virus replication. Furthermore, we demonstrated that miR-33a could impede virus replication at the stage of virus internalization, which was similar to the pattern for knockdown of ARCN1, indicating that miR-33a inhibits influenza virus infection by suppressing ARCN1 expression. In addition, we found that miR-33a could also weaken the viral ribonucleoprotein activity in an ARCN1-independent manner. In conclusion, we found that miR-33a is a novel inhibitory factor for influenza A virus replication.

INTRODUCTION

Influenza A virus is a major pathogen of seasonal flu, which has a tendency to cause pandemic outbreaks such as those that occurred in 1918, 1957, 1968 and 2009. It is an enveloped virus with a genome of eight segmented negative-sense ssRNA. Upon infection, influenza virus enters the cell by receptor-mediated endocytosis. Subsequently, the virus is uncoated and the viral ribonucleoprotein (vRNP) complex is released into the cytoplasm and then transported into the nucleus to initiate virus replication. The progeny viruses are assembled at the plasma membrane, and bud and are released from the infected cell (Noda & Kawaoka, 2012; Pinto et al., 1992; Stauffer et al., 2014). During viral infection, a number of host proteins are involved in multiple aspects of the virus life cycle; for example, cyclinT1/CDK9 functions as an adaptor to mediate the interaction of vRNP and RNA polymerase II, and to promote viral transcription (Zhang et al., 2010); COPI components play important roles in influenza virus entry and viral membrane protein expression and assembly (Sun et al., 2013); cyclophilin A can interact with the influenza A virus matrix protein (M1) and inhibit the translocation of M1 protein into the nucleus (Liu et al., 2009); IFITM3 restricts influenza virus replication and acts as an essential barrier to influenza A virus infection in vivo and in vitro (Brass et al., 2009; Desai et al., 2014; Everitt et al., 2012).

In order to explore further the mechanisms by which viruses complete their life cycle, researchers have taken the approach of genome-wide knockdown screening to identify host proteins that are important for the virus life cycle, to provide clues for further investigations (Brass et al., 2009; Hao et al., 2008; Karlas et al., 2010; König et al., 2010). In addition, microRNAs (miRNAs) have been found to play important roles in the virus life cycle. It has been confirmed that miRNAs can modulate the influenza virus life cycle by directly repressing viral protein expression or controlling the host immune response (Bugele et al., 2013; Song et al., 2010). In return, it has been reported that many viruses have adapted strategies to eliminate miRNAs harmful for their replication.
In order to explore the roles of miRNA(s) involved in the influenza A virus life cycle, we compared the miRNA profiles of 293T and HeLa cell lines, as influenza A virus can replicate efficiently in 293T cells but only poorly in HeLa cells. We found that miR-33a is an inhibitory factor for influenza A virus replication. We demonstrated that miR-33a can target the COPI subunit ARCN1 3' UTR and reduce its expression level, and consequently impair virus internalization. MiR-33a can also weaken the influenza virus vRNP activity through an ARCN1-independent pathway. Our results revealed the mechanism of how miR-33a inhibits influenza virus infection.

RESULTS

Identification of miRNAs suppressing influenza A virus replication

Influenza A virus infection involves many host factors including miRNAs (Li et al., 2010; Loveday et al., 2012). It has been reported that influenza A virus infection causes changes in the profile of host miRNAs. However, the mechanisms by which miRNAs regulate the replication of influenza A virus have not yet been fully elucidated. In order to identify the miRNA(s) involved in the life cycle of influenza A virus, we compared the miRNA profiles of 293T and HeLa cells by searching reported data (Betel et al., 2008; Ruike et al., 2008), as influenza virus H1N1 A/WSN/33 can replicate efficiently in 293T cells but only poorly in HeLa cells (Fig. 1a, b). We selected 12 candidate miRNAs and then detected their expression in HeLa, 293T and A549 cells by quantitative real-time PCR (qRT-PCR). As shown in Fig. 1(c), these 12 miRNAs were expressed at higher levels in HeLa cells and at lower levels in 293T and A549 cells. As A549 cells are derived from human lung tissue and influenza A virus can replicate well in these cells, our functional studies were carried out mostly in A549 cells. To examine the functions of candidate miRNAs in influenza A virus replication, we overexpressed them in 293T and A549 cells and then infected the cells with influenza virus A/WSN/33. The cell lysates were harvested for immunoblotting with antibodies against the viral nucleoprotein (NP) and M1 (two abundant viral proteins of influenza A virus). As shown in Fig. 1(d), the expression of NP and M1 were repressed significantly in five of the cell cultures with overexpressed miRNAs (miR-21, miR-27a, miR-29a, miR-30a and miR-33a) compared with control cells. Meanwhile, the NP and M1 protein levels were upregulated significantly in HeLa cells transfected with inhibitors of miR-21, miR-30a and miR-33a (Fig. 1e), indicating that these miRNAs are potential regulators of influenza A virus replication. Subsequently, the endogenous level of these five miRNAs was detected in A549 cells at three time points after influenza A virus infection. As shown in Fig. 1(f), the expression of miR-33a was repressed most significantly after influenza A virus infection in A549 cells. We then examined whether miR-33a could inhibit other strains of influenza A virus. Similar experiments were performed as described in Fig. 1(f). The data showed that miR-33a could also inhibit the replication of influenza A virus H3N2 and H9N2 (Fig. 1g). Therefore, we further studied the mechanism of miR-33a in regulating influenza A virus replication.

miR-33a inhibits the replication of influenza A virus by targeting ARCN1

To unveil the mechanism by which miR-33a suppresses influenza virus infection, we focused on searching for the potential target of miR-33a. Recently, the importance of host genes for influenza virus infection has been identified through genome-wide knockdown screenings from several groups (Brass et al., 2009; Hao et al., 2008; Karlas et al., 2010; König et al., 2010). Among these genes, we predicted that ARCN1 could be a potential target of miR-33a by carrying out an analysis with TargetScan (http://www.targetscan.org/). To confirm this prediction, we generated an ARCN1 3' untranslated region (UTR) luciferase reporter plasmid and transfected it into 293T cells with or without miR-33a. The cell lysates were subjected to a dual-luciferase assay. As shown in Fig. 2(a), the luciferase activity was significantly reduced in miR-33a-transfected cells compared with that in control cells, suggesting that miR-33a targeted the ARCN1 3' UTR. Consistently, the amount of ARCN1 protein was greatly reduced in miR-33a-transfected cells (Fig. 2b, c). We also examined the amounts of ARCN1 and miR-33a in HeLa, 293T and A549 cells by immunoblotting and quantitative PCR. The data showed that the level of endogenous ARCN1 in HeLa cells was lower than that in 293T and A549 cells while the amount of miR-33a in HeLa cells was significantly higher than in the other two cell lines (Fig. 2d–f), indicating that the levels of ARCN1 and miR-33a are negatively correlated.

To further confirm that miR-33a can target the ARCN1 3' UTR, we transfected miR-33a inhibitor with ARCN1 3' UTR luciferase reporter into HeLa cells. The cell lysates were subjected to a luciferase assay and immunoblotting analysis. The data showed that both luciferase activity and the amount of ARCN1 protein in miR-33a inhibitor-transfected cells were significantly increased (Fig. 2g–i).

There are two potential miR-33a target sequences in the ARCN1 3' UTR (nt 116–122 and nt 399–405). Therefore, we generated ARCN1 3' UTR-mutated luciferase reporters in which the mutations were made in each or both of the predicted target sequences for miR-33a (Fig. 3a) and then transfected WT or mutated luciferase reporters with control or miR-33a mimic into 293T cells. The cell lysates were subjected to a luciferase assay. The data showed that mutation of a single target sequence of the ARCN1 3' UTR could partially reverse the inhibitory effect of miR-33a, and that the luciferase activity of the ARCN1 3'
miR-33a targets ARCN1 and inhibits influenza A vRNP

Fig. 1. Identification of miRNAs inhibiting influenza virus replication. (a) HeLa, 293T and A549 cells were infected by influenza virus A/WSN/33 (H1N1) at an m.o.i. of 1 for 8 h. The cells lysates were harvested and subjected to immunoblotting with antibodies against viral nucleoprotein (NP)/33 (H1N1) at an m.o.i. of 1. After three washes with PBS, the cells were cultured with fresh medium. At various time points post-infection (p.i.), the supernatants M1 against β-actin as an internal control. (b) Influenza virus growth curve in different cell lines. HeLa, 293T and A549 cells were infected with influenza virus A/WSN were collected for determination of viral titre by plaque assay. (c) Relative expression of 12 candidate miRNAs in HeLa cells and 293T or A549 cells. Total RNAs were extracted from HeLa and 293T or A549 cells, and the ratio of the relative expression of
the candidate miRNAs was detected by qRT-PCR. The expression of U6 small nuclear RNA was used as a loading control. (d) Screening for miRNAs that inhibit influenza A virus replication. miRNAs and control miRNA were synthesized and transfected into 293T or A549 cells for 24 h followed by infection with influenza virus A/WSN/33 at an m.o.i. of 0.5 for 16 h. The cell lysates were harvested for immunoblotting with antibodies against the indicated proteins. (e) HeLa cells were transfected with inhibitors of miR-21, miR-27a, miR-29a, miR-30a or miR-33a for 48 h. The cells were then infected with influenza virus A/WSN/33 (H1N1) at an m.o.i. of 1 for 16 h. The cell lysates were harvested for immunoblotting with antibodies against the indicated proteins. The amounts of NP and M1 were quantified and the results shown below the blots represent the means of three independent experiments relative to the control, which was given a value of 1. (f) miR-33a expression was significantly reduced following influenza A virus infection in A549 cells. A549 cells were infected by influenza virus A/WSN/33 at an m.o.i. of 0.1. Cells were collected at 0, 6, 12 and 18 h p.i., and the endogenous level of five miRNAs were detected by qRT-PCR. The results were analysed by Student’s t-test and *P<0.05 was considered statistically significant. Error bars represent SD. •P<0.05, **P<0.01. (g) A549 cells were infected with influenza A virus H9N2 or H3N2 at an m.o.i. of 0.5 for 16 h. The cell lysates were harvested for immunoblotting with antibodies against the indicated proteins.

UTR reporter with mutation of both target sequences was no longer inhibited by miR-33a (Fig. 3b). Taken together, these results indicated that miR-33a repressed endogenous ARCN1 expression by directly targeting the 3' UTR of ARCN1 mRNA.

miR-33a disturbs the replication of influenza A virus by reducing the level of ARCN1

As ARCN1 is a direct target of miR-33a, we wondered whether miR-33a disturbed the replication of influenza A virus by suppressing ARCN1 expression. In order to address this question, we transfected A549 cells with miR-33a mimic or inhibitor and then infected the cells with influenza A virus (H1N1, H9N2 or H3N2). The cell lysates were harvested for immunoblotting analysis, while the supernatants were subjected to a plaque assay to measure the viral titre. As shown in Fig. 4(a), the expression of ARCN1 was repressed by the miR-33a mimic and upregulated by the miR-33a inhibitor. Meanwhile, viral NP and M1 protein levels were significantly decreased in miR-33a mimic-transfected cells and increased in miR-33a inhibitor-transfected cells (Fig. 4a, upper panel). A similar pattern was observed for the viral titre in the supernatants from the cells transfected with miR-33a mimic or miR-33a inhibitor (Fig. 4a, lower panel). Consistently, similar results were obtained in experiments with influenza A virus H9N2 or H3N2 strain (Fig. 4b, c). These results implied that miR-33a may inhibit the replication of influenza A virus by reducing the level of ARCN1. To confirm this further, we examined whether ectopic expression of ARCN1 could reverse the inhibitory effect of miR-33a on influenza A virus replication. We transfected A549 cells with miR-33a alone or with FLAG–ARCN1-expressing plasmid followed by infection of the cells with influenza A virus. The cell lysates were harvested for immunoblotting analysis (Fig. 4d, upper panel) and the viral titres in the supernatants were measured (Fig. 4d, lower panel). The data showed that both the level of NP and M1 proteins and the viral titre in miR-33a and pCMV-FLAG-ARCN1-co-transfected cells were higher than that in cells transfected with miR-33a alone, indicating that expression of FLAG-ARCN1 could partially reverse the inhibitory function of miR-33a on the replication of influenza A virus. These results indicated that miR-33a disturbs the replication of influenza A virus by repressing ARCN1 expression.

miR-33a interferes with the internalization of influenza A virus

In order to determine the step(s) in which miR-33a interferes with influenza A virus, we first tested whether miR-33a affected virus attachment. We transfected A549 cells with miR-33a or small interfering RNA (siRNA) targeting ARCN1, and then infected the cells with Alexa Fluor 488-conjugated A/WSN/33 virus in different doses. The amount of Alexa Fluor 488-conjugated A/WSN/33 bound to the cells was quantified by flow cytometry. The data indicated that there was no significant difference in fluorescence intensity among these groups (Fig. 5a, b), suggesting that miR-33a did not affect the virus bound to the cells. Subsequently, we examined the effect of miR-33a on influenza virus internalization. A549 cells were transfected with siRNA targeting ARCN1 (si-ARCN1), miR-33a or control RNA with or without FLAG–ARCN1 expression vector and infected with Alexa Fluor 488-conjugated A/WSN/33 virus for 30 min. The cells were fixed and labelled with wheat germ agglutinin–Alexa Fluor 350 to delineate the plasma membrane and subjected to confocal microscopy. ARCN1 was detected by anti-ARCN1 antibody with TRITC-labelled secondary antibody. As shown in Fig. 5(c), the miR-33a-treated cells exhibited a significant defect in virus uptake compared with the control cells. In addition, we measured the number of internalized particles and calculated the ratio of internalized particles versus total number of virus particles. The data showed that the ratio of internalized virus in miR-33a- or si-ARCN1-transfected cells was dramatically reduced compared with that in the control cells, while FLAG–ARCN1 could rescue the effect of miR-33a (Fig. 5d). These results indicated that miR-33a can cause a significant defect in virus uptake. It has been
miR-33a targets ARCN1 and inhibits influenza A vRNP

Fig. 2. miR-33a represses ARCN1 expression by targeting its 3' UTR. (a) The full-length ARCN1 3' UTR was cloned into the pmirGLO vector, following the coding region of firefly luciferase as an ARCN1 3' UTR luciferase reporter. 293T cells were transfected with the ARCN1 3' UTR reporter and miR-33a mimics or control miRNA for 48 h. The cell lysates were harvested for a luciferase assay. (b) 293T cells were transfected with a miR-33a mimic (miR-33a) or control miRNA for 48 h. The cell lysates were harvested for immunoblotting analysis with antibodies against the indicated proteins. (c) The relative protein level of ARCN1 was quantified by Quantity One software (Bio-Rad). (d–f) Total RNAs and proteins were prepared from 293T, A549 and HeLa cells. The total RNAs were subjected to qRT-PCR for miR-33a with U6 small nuclear RNA as an internal control (d). The proteins were used for immunoblotting with antibodies against the indicated proteins (e), and the relative expression of ARCN1 was quantified by Quantity One (f). (g) HeLa cells were co-transfected with miR-33a inhibitor or control with ARCN1 3’ UTR luciferase reporter for 48 h. The cell lysates were then harvested for a luciferase assay. (h) HeLa cells were transfected with miR-33a inhibitor or control for 48 h. The cell lysates were then harvested for immunoblotting analysis with antibodies against the indicated proteins. (i) The relative expression of ARCN1 was quantified by Quantity One. The data were analysed by Student's t-test and P<0.05 was considered statistically significant. Error bars represent sd. *P<0.05, **P<0.01.
reported that, as a component of the COPI complex, ARCN1 plays an important role in the internalization of influenza A virus (Sun et al., 2013). Our data suggested that miR-33a could influence the internalization of influenza A virus by reducing the level of ARCN1 protein.

**miR-33a inhibits influenza A virus vRNP activity**

As ARCN1 overexpression cannot fully rescue the inhibitory function of miR-33a on the replication of influenza A virus, we hypothesized that miR-33a might interfere with other crucial steps of the virus life cycle, such as viral RNA synthesis. Therefore, we generated 293T–influenza A virus–Luc cells in which an A/WSN/33 NP promoter luciferase reporter was integrated into the genome of 293T cells. The luciferase gene will be transcribed and expressed in the presence of influenza A virus polymerase. Therefore, the activity of vRNP could be monitored by measuring luciferase activity.

To examine whether miR-33a could disturb the activity of influenza vRNP, we transfected 293T–influenza A virus–Luc cells with miR-33a mimic or control miRNA for 48 h. The cell lysates were harvested for a luciferase assay. As shown in Fig. 6(a), the luciferase activity was significantly reduced in miR-33a mimic-transfected cells, while expression levels of the vRNP components were similar in each group (Fig. 6b). Meanwhile, the inhibitory effect of miR-33a on vRNP activity was not rescued by ARCN1 overexpression (Fig. 6c, d). These results indicated that miR-33a weakens the vRNP activity through an ARCN1-independent pathway.

In conclusion, we showed that miR-33a is an inhibitory factor for influenza A virus replication. We revealed that miR-33a can target the 3' UTR of ARCN1 and reduce its translation.

**Fig. 3.** Identification of target sites of miR-33a on ARCN1. (a) Predicted target sites of miR-33a in the 3' UTR of ARCN1. Perfect matches are indicated by vertical lines. Single or double mutations in 3' UTR of ARCN1 were made in the predicted target region of miR-33a-binding sites and the mutated nucleotides are shown in red. (b) 293T cells were transfected with WT ARCN1 3' UTR or mutated luciferase reporters and miR-33a mimic or control miRNA for 48 h. The cell lysates were harvested for a luciferase assay. The data were analysed by Student's t-test and P<0.05 was considered statistically significant. Error bars represent SD. *P<0.05, **P<0.01.
miR-33a targets ARCN1 and inhibits influenza A vRNP

(a) H1N1

(b) H9N2

(c) H3N2

(d) miR-33a

FLAG–ARCN1

NP

M1

β-Actin

Virus titre

(log10 p.f.u. ml⁻¹)

miR control

miR-33a mimic

Inhibitor control

miR-33a inhibitor

miR control

miR-33a mimic

Inhibitor control

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expression level, and consequently interferes with virus internalization. In addition, we found that miR-33a can weaken the vRNP activity in an ARCN1-independent manner. Our study provides more evidence that miRNAs are involved in regulating the replication of influenza A virus.

**DISCUSSION**

To regulate virus replication, miRNA may target three types of genes: genes involved in the antiviral immune response, host genes required for the virus life cycle or viral genes (Izzard *et al.*, 2014; Ma *et al.*, 2012; Rosenberger *et al.*, 2012; Song *et al.*, 2010; Zhang *et al.*, 2014). We found that miRNA-33a exerts its inhibitory effect by repressing the expression of ARCN1, a host factor that is crucial for influenza virus replication. ARCN1, also known as δ-COP, is one of the nine subunits of the COPI complex. Previous genome-wide siRNA screening studies have indicated that at least four of the COPI subunits are important for influenza A virus replication. Knockdown of δ-COP by siRNA causes a significant reduction in influenza A virus entry (König *et al.*, 2010; Sun *et al.*, 2013). Through dissection of the influenza A virus infection cycle by RNA silencing and pharmacological perturbation, Sun *et al.* (2013) found that long-term ARCN1 deletion leads to indirect effects on influenza A virus entry by impairing cargo uptake and vesicular trafficking to late endosomes. In addition, they also reported that acute treatment with COPI complex inhibitors repressed viral membrane protein expression and assembly, implying a potential role of ARCN1 in later steps of influenza A virus infection. However, the detailed mechanism needs further elucidation.

In the present study, we demonstrate that miR-33a can inhibit influenza A virus internalization by repressing ARCN1. However, the mechanism of how ARCN1 assists the virus in entering the cell is still not well understood.

miRNAs modulate gene expression by binding to complementary segments in the 3' UTR of mRNAs in most cases. Generally, each miRNA can be involved in a very complex network by targeting multiple genes (De Tullio *et al.*, 2014). To date, miR-33a has been confirmed to be involved in modulating sterol homeostasis by targeting ABCA1, ABCB11 and ATP8B1 (Allen *et al.*, 2012; Wang *et al.*, 2014; Wijesekara *et al.*, 2012). As sterol is one of the most important components of the cell membrane and the coat of influenza virus, it is reasonable to postulate that these genes may also be involved in virus replication. There are also reports indicating that miR-33a can regulate cell-cycle progression by repressing Pim-1, CDK6 and CCND1 (Cirera-Salinas *et al.*, 2012; Thomas *et al.*, 2012). It has been found that a disturbed cell cycle occurs during influenza virus infection (He *et al.*, 2010). However, whether miR-33a can influence virus replication by regulating the cell cycle needs to be further elucidated.

The influenza vRNP complex contains four subunits and, following virus entry into host cells, vRNPs are released from the endosomes and then transported into the nucleus, where they function as templates for genome transcription and replication. Subsequently, the vRNPs are transported out of the nucleus into the plasma membrane for assembly into newly forming virions. Therefore, the vRNP subunits trafficking between the nucleus and cytoplasm are also very important for completing the whole virus replication cycle. The vRNP trafficking steps are associated with several importin proteins such as importin-β1 and importin-α1 and -α7, and are also related to the sumoylation system (Eisfeld *et al.*, 2015; Han *et al.*, 2014; Hudjetz & Gabriel, 2012). Our results demonstrated that miR-33a can weaken the vRNP activity, and this was not associated with reducing the expression of vRNP components, suggesting that disturbed vRNP trafficking-related steps may be involved.

In summary, we showed that miR-33a is an inhibitory factor for influenza A virus replication. We demonstrated that miR-33a disturbs the influenza A virus life cycle by repressing ARCN1 expression and weakening vRNP activity. This research provides more evidence to show that host miRNAs are involved in influenza virus infection, which will provide new opportunities for designing anti-influenza virus drugs.

**METHODS**

**Cell lines and viruses.** Madin–Darby canine kidney (MDCK) cells, human embryo kidney 293T cells, human type II alveolar epithelial A549 cells and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FBS (HyClone). Recombinant influenza virus A/WSN/33 was generated by reverse
miR-33a targets ARCN1 and inhibits influenza A vRNP

Fig. 5. miR-33a weakens the internalization of influenza virus. (a) A549 cells were transfected with miR-33a, siRNA targeting ARCN1 (si-ARCN1) or control miRNA, and incubated with different doses of Alexa Fluor 488-labelled A/WSN/33 virus on ice for 45 min. The amount of Alexa Fluor 488-labelled A/WSN/33 bound to the cells was quantified by flow cytometry. The cell lysates were harvested for immunoblotting with the antibodies against the indicated proteins (b). The data were analysed by one-way ANOVA, and \( P < 0.05 \) was considered statistically significant. Error bars represent SD. (c) A549 cells were transfected with si-ARCN1, miR-33a or control RNA with or without FLAG–ARCN1 expression vector. At 48 h after transfection, the cells were infected with Alexa Fluor 488-labelled A/WSN/33 virus on ice for 45 min and then cultured at 37 °C for 30 min. The plasma membrane was labelled using wheat germ agglutinin–Alexa Fluor 350, and the ARCN1 level was detected by anti-ARCN1 antibody with TRITC-labelled secondary antibody. The cells were subjected to confocal microscopy. The cell membrane was visualized as blue, A/WSN/33 virus as green and TRITC–ARCN1 as red, at 30 min p.i. Bars, 10 μm. (d) The internalized virus particles versus the total number of virus particles per cell were counted and values are shown as a dot plot. Data were analysed by Student’s \( t \)-test. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).
genetics as described previously (Neumann et al., 1999). Influenza virus A/Chicken/Liaoning/1/100 (H9N2) was kindly provided by Wenjun Liu, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. Influenza virus A/Jiangxi/262/2005 (H3N2) was kindly provided by Jinghua Yan, Institute of Microbiology, Chinese Academy of Sciences.

Plasmids, antibodies and miRNAs. The full-length 3′ UTR of ARCN1 was cloned into the 3′ UTR luciferase reporter plasmid pmirGLO (Promega). The ARCN1 coding region was cloned into the HindIII and KpnI sites of the pFLAG-CMV2 vector. pREP4-FluA-Luc plasmid was kindly provided by Andrew Pekosz (Johns Hopkins University, MD, USA). Plasmids for expressing PB1, PB2, PA or NP were from the influenza A virus reverse genetics system. The full coding region of ARCN1 was cloned into the pFLAG-CMV2 vector to construct the FLAG–ARCN1 expression vector. The FLAG–ARCN1 expression vector lacked the 3′ UTR and therefore was not subject to miR-33a repression.

Fig. 6. miR-33a inhibits the influenza virus vRNP activity. (a, b) 293T–influenza A virus–Luc cells were transfected with miR-33a mimic or control miRNA for 24 h, followed by transfection with pRL-TK and plasmids expressing influenza A virus PB1, PB2, PA or NP for 48 h. The cell lysates were harvested and subjected to a luciferase assay (a) and immunoblotting analysis (b). (c, d) 293T–influenza A virus–Luc cells cells were transfected with miR-33a mimic or control miRNA for 24 h and then transfected with pRL-TK and plasmids for expressing influenza A virus PB1, PB2, Myc–PA or NP and FLAG–ARCN1 or control vector for 48 h. The cell lysates were harvested and subjected to a luciferase assay (c) and immunoblotting with antibodies against the indicated proteins (d). The data for the luciferase assays were analysed by Student’s t-test, and *P < 0.05 was considered statistically significant. Error bars represent sd. **P < 0.01. The amount of NP, PB1, PB2, Myc–PA, ARCN1 and FLAG–ARCN1 in the immunoblot was quantified and the results represent the means of three independent experiments.
Mouse anti-M1 mAb and rabbit anti-NP polyclonal antibody were kindly provided by Wenjun Liu (Institute of Microbiology, Chinese Academy of Sciences). Mouse anti-β-actin mAb was purchased from Sangon Biotech Co. and rabbit anti-ARCN1 polyclonal antibody was purchased from Abcam. miRNA mimics or inhibitors were purchased from GenePharma Co. The miRNA inhibitors were ssRNA molecules, the sequences of which were the reverse complement of the miRNAs. Sequence-scrambled single- or double-stranded short RNA was used as the negative control for miRNA inhibitors or mimics separately. The miRNA mimic inhibitors were transfected into cells using Lipofectamine 2000 (Invitrogen) at 40 pmol for a 12-well plate and 10 pmol for a 48-well plate. The sequence of the double-stranded miR-33a mimic was: 5′-GUCAUGUAGUGCAUGGCA-3′ and 5′-UUCAGUAACUGAACGUAC-3′; and the sequence of the miR-33a inhibitor was 5′-UGCAAGUGCAACUACAGC-3′. For transfection of A54 cells, the cells were seeded in 12-well plates miR-33a inhibitor was 5 % low-melting-point agarose in DMEM with 2 % FCS. Subsequently, 1 μg of the indicated plasmid(s) mixed with Lipofectamine 2000 was added into 100 μl serum-free DMEM medium for 15 min and then added to the cells for 12 h. The medium was then changed to DMEM with 10 % FCS serum.

**Immunoblotting.** Protein extracts (20 μg) were subjected to electrophoresis with 10 % polyacrylamide gel and blotted onto PVDF membranes (Millipore Corp.). The membrane was incubated with primary antibody for 2 h at room temperature, followed by incubation with HRP-conjugated rabbit anti-mouse or goat anti-rabbit IgG. The signal was detected with chemiluminescence reagent (Engreng Biosystems).

**Plaque assay.** MDCK cells were plated in 35 mm dishes and serial dilutions of viral stock were added for 2 h. Unabsorbed virus was removed by washing with serum-free DMEM. The cells were then overlaid with 1 % low-melting-point agarose in DMEM with 2 μg TPCK-treated trypsin (Sigma) ml⁻¹ and cultured for 3 days. The visible plaques were counted and viral titres were calculated. All data were expressed as the mean of triplicate samples.

**Dual-luciferase assay.** For the dual-luciferase assay, 293T cells were seeded in 48-well plates and transfected with luciferase reporter vectors together with 10 pmol miR-33a mimic or inhibitor using Lipofectamine 2000. At 48 h post-transfection, the cell lysates were harvested for the luciferase assay using a Dual-Luciferase Assay kit (Promega). The results were expressed as relative luciferase activity (firefly luciferase/Renilla luciferase). Each treatment was performed in triplicate in three independent experiments.

**RNA extraction and quantitative PCR.** Total RNA was extracted from the cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. The amount of miR-33a was measured by stem-loop quantitative PCR with U6 small nuclear RNA as the internal control. Each sample was measured in triplicate and the experiment was repeated at least three times.

**Non-specific amine-reactive labelling of virus.** Non-specific amine-reactive labelling was performed as described by Sun et al. (2013). Briefly, 50 μl influenza virus A/WSN/33 (2 × 10⁶ PFU/ml) was mixed gently with 47 μl 0.1 M freshly prepared 0.1 M NaHCO₃ (pH 8.5) and 6 μg Alexa Fluor 488 carboxylic acid, succinimidyl ester (Invitrogen) in the dark for 60 min. An NAP-5 exclusion column (GE Healthcare) was used to separate free dyes from labelled virus using elution buffer [50 mM HEPES (pH 7.4), 145 mM NaCl]. The excess Alexa Fluor 488-labelled virus was aliquotted and stored at −80 °C.

**Virus binding assay.** A549 cells were transfected with miR-33a or control miRNA for 48 h, and incubated with Alexa Fluor 488-labelled influenza virus A/WSN/33 at different m.o.i. on ice for 45 min. Unbound virus was removed by washing with pre-chilled PBS. The cells were trypsinized and collected, and then fixed with 2 % paraformaldehyde followed by flow cytometry analysis.

**Generation of 293T–influenza A virus–Luc Cells.** Cells were transfected with the influenza A virus promoter reporter plasmid pRep4-FluA-Luc for 48 h. The cells were then selected with hygromycin (250 μg ml⁻¹) for 3 weeks and maintained in hygromycin (100 μg ml⁻¹), and were named 293T–influenza A virus–Luc cells.

**Detection of influenza virus polymerase activity.** To measure the influenza virus polymerase activity, 293T–influenza A virus–Luc cells in 24-well plates were transfected with miR-33a mimic or control miRNA for 24 h, and then transfected with pRL-TK and plasmids expressing influenza A virus PB1, PB2, Myc-PA or NP with or without FLAG–ARCN1 expression vector for 48 h. The cell lysates were harvested and subjected to a luciferase assay. The luciferase activity was normalized against Renilla luciferase activity.

**Statistical analysis.** The results of the two independent groups were analysed by Student’s t-test. All data were analysed using SPSS version 16. P < 0.05 was considered statistically significant.

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