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Effective small interfering RNAs targeting matrix and nucleocapsid protein gene inhibit influenza A virus replication in cells and mice

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Abstract

RNA interference (RNAi) is a powerful tool to silence gene expression. Small interfering RNA (siRNA)-induced RNA degradation has been recently used as an antivirus agent to inhibit specific virus replication. Here, we showed that several siRNAs specific for conserved regions of influenza virus matrix (M2) and nucleocapsid protein (NP) genes could effectively inhibit expression of the corresponding viral protein. We also evaluated the antiviral potential of these siRNAs targeting M2 and NP of H5N1 avian influenza virus (AIV), which are essential to viral replication. We investigated the inhibitory effect of M2-specific siRNAs and NP-specific siRNAs on influenza A virus (H5N1, H1N1 and H9N2) replication in Madin-Darby canine kidney (MDCK) cells and BALB/c mice. The results showed that treatment with these siRNAs could specifically inhibit influenza A virus replication in MDCK cells (0.51–1.63 $TCID_{50}$ reduction in virus titers), and delivery of pS-M48 and pS-NP1383 significantly reduced lung virus titers in the infected mice (16–50-fold reduction in lung virus titers) and partially protected the mice from lethal influenza virus challenge (a survival rate of 4/8 for H1N1 virus-infected mice and 2/8 for H5N1 virus infected mice). Moreover, the treatment of pS-M48 and pS-NP1383 could suppress replication of different subtypes of influenza A viruses, including a H5N1 highly pathogenic avian isolate strain. The results provided a basis for further development of siRNA for prophylaxis and therapy of influenza virus infection in humans and animals.

Keywords: RNA interference; Influenza virus; NP; M2; Cells; Mice

1. Introduction

RNA interference (RNAi) is a process by which double-stranded RNA directs sequence-specific degradation of homologous messenger RNA. Small interfering RNA (siRNA), short 21–26 nt, is a powerful tool for sequence-specific, post-transcriptional gene silencing. Many studies had shown that siRNA could significantly suppress gene expression when delivered into mammalian cells in vitro (McManus and Sharp, 2002; Dykxhoorn et al., 2003). Subsequently, a number of studies demonstrated that siRNA inhibited viral gene expression and replication of RNA viruses in vitro (Gitlin and Andino, 2003; Haasnoot et al., 2003), including HIV (Jacque et al., 2002), polio virus (Gitlin et al., 2002), hepatitis C virus (Kapadia et al., 2003), West Nile virus (McCown et al., 2003), and influenza virus (Ge et al., 2003).

Influenza A virus contains eight RNA segments of negative polarity in its genome. To design siRNAs that remain effective despite antigenic drifts and shifts, we have focused on regions of the viral genome that are conserved among different subtypes and strains of virus from human, chicken, duck, equine, and swine. Essential to viral replication, NP and M2 proteins provide ideal targets for RNAi. Moreover, NP and M2 genes are highly conserved across subtypes of influenza A virus; therefore, siRNAs against these genes should inhibit most influenza viruses. Thus, we chose the two genes as the target sequence and designed a total of 5 siRNAs specific for NP or M2 genes.

We reported here NP- and M2-specific siRNA specifically inhibited accumulation of NP and M2 mRNA, respectively, and could potently inhibit influenza virus production in vitro and in vivo. Inhibition by the most potent siRNAs was a result of sequence-specific interference with viral mRNA accumulation

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as well as the inhibition of viral RNA transcription across several subtypes of influenza A virus.

2. Materials and methods

2.1. Viruses, cells and animals

Influenza A virus strains used in this study were A/chicken/Hubei/327/2004 (H5N1), A/Duck/Hubei/W1/2004 (H9N2), and A/Hubei/2003 (H1N1) (a mouse-adapted strain passaged in the BALB/c mice). All experiments with H5N1, H9N2, H1N1 viruses were conducted under BSL-3+ containment. HeLa cells and MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Cultures were incubated at 37 °C with 5% CO_2. Five to six weeks old female BALB/c mice were purchased from Experimental Animal Center, Institute of Medicine, Hubei Province.

2.2. Construction of plasmids

The coding regions of NP and M2 gene were, respectively, amplified from the entire length of NP and M gene segments from A/chicken/Hubei/327/2004 (H5N1) virus described previously (Zhou et al., 2006). Full-length and truncated M2, sM2, M2 without residues 26th–55th cDNA were produced by PCR as described previously (Frace et al., 1999). NP and sM2, the PCR products, were respectively cloned between BamHI and HindIII sites of the vector pCD-EGFP, fused to the N-terminal of the enhanced green fluorescent protein (EGFP) under the control of immediate early promoter of human cytomegalovirus (CMV). The eukaryotic expression vectors pNP-EGFP and psM2-EGFP were used as a reporting system for monitoring function of the siRNAs.

2.3. Target sequence selection and vector construction

As the AAGN18UU sequence (N, any nucleotide) has been found to be preferred for siRNA-mediated gene silencing under the control of the Pol U6 promoter (Elbashir et al., 2002), we searched for this sequence in the ORF of NP and sM2 gene. Five target sequences (M-48, M-754, M-949, NP-749 and NP-1383) in the coding region of NP and sM2 gene were selected according to the web-based criteria (www.ambion.com). And these selected sequences were submitted to a BLAST search against human genome sequence to ensure that human genome was not targeted. To construct hairpin siRNA expression cassette, the following DNA oligonucleotides were synthesized: M-48, M-754, M-949, NP-749 and NP-1383 (Fig. 1). The 21 nt target sequences served as a basis for the design of the two complementary 55-mer siRNA template oligonucleotides that were synthesized, annealed, and inserted into BamHI and HindIII sites of the siRNA expression vector pSilencer4.1-CMV neo (Ambion). The recombinant plasmids were designated as pS-M48, pS-M754, pS-M949, pS-NP749 and pS-NP1383. The pSilencer 4.1-CMV neo negative control (Ambion) was a negative control plasmid (pS-Negative), encoding a hairpin siRNA whose sequence was not found in mice, human and influenza virus genome databases.

Fig. 1. Schematic diagrams of annealed siRNA template insert. Five siRNA target sequences (M-48, M-754, M-949, NP-749 and NP-1383) were selected and served as a basis for the design siRNAs template insert. Five pairs of two complementary 55-mer siRNA template oligonucleotides base on the target sequences were synthesized, annealed and inserted into the BamHI and HindIII sites of pSilencer4.1-CMV neo vector. About 9-nt spacer was introduced in the sequences. The transcript was predicted to fold back on itself to form hairpin siRNAs with a nine-base pair stem-loop structure.

2.4. Transfection of the siRNA expression cassette into HeLa cells

HeLa cells were plated onto six-well plates and cultured at 37 °C and 5% CO_2 overnight. When the cell layer reached 70–80% confluence, 3 μg of pS-M48, 3 μg of pS-M754 and 3 μg of pS-M949 were cotransfected, respectively, with 3 μg of psM2-EGFP, and meanwhile, 3 μg of pS-NP749 or 3 μg of pS-NP1383 were also cotransfected, respectively, with 3 μg of pNP-EGFP, both using Lipofectamine™ 2000 (Invitrogen) according to the Manufacturer's recommendations. And 3 μg of pNP-EGFP and 3 μg of psM2-EGFP were cotransfected, respectively, with 3 μg of pS-Negative as a negative control. Non-transfected HeLa cells were also used as a control.

2.5. Analysis of EGFP expression in HeLa cells and flow cytometry assay

At 24 h posttransfection, EGFP expressions were examined with a fluorescence microscope (Olympus, Japan). At 48 h posttransfection, the transfected cells and the control cells were washed gently in phosphate-buffered saline (PBS), trypsinized and resuspended in PBS. EGFP positive cells and EGFP expression signal were evaluated by the FACSCalibur Flow Cytometry System, assisted by Cell Engineering Center of Tongji Medical College.

2.6. Western blot analysis

At 48 h posttransfection, the transfected cells and the control cells were harvested and mixed with an equal volume

of 2× sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris–HCl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), then boiled for 10 min, separated on 5% stacking/12% separating SDS-polyacrylamide gels, and electroblotted to PVDF membranes (Millipore, Bedford, MA). The blots were blocked with 1% bovine serum albumin (BSA) in TBS-T buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20), and incubated for 30 min at room temperature with anti-NP monoclonal antibodies or rabbit anti-sM2 antiserum which was generated by immunization of Japan Big Ear White rabbits using purified recombinant sM2 protein (data not shown). After being washed three times in TBS-T, the blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG or goat anti-rabbit IgG (Southern Biotech). 3,3-Diaminobenzidine tetrahydrochloride (DAB) was used as the substrate for membrane development. A duplicate of the blots were analyzed in western blotting using the monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ambion) to verify the sample loading.

2.7. Reverse transcription (RT)-PCR

Specific silencing of target genes were also confirmed by RT-PCR and sequencing. Total RNAs from the transfected cells or the control cells were isolated by phenol–chloroform extraction and ethanol precipitation. To eliminate traces of DNA, samples were incubated with RNase-free DNase 1 (Fermentas) for 30 min at 37 °C, and then the DNase was inactivated by incubating at 65 °C for 10 min. To detect NP, M2, EGFP and GAPDH mRNA expression in HeLa cells, 2 μg of RNA extracts was used as the template for RT-PCR amplification using a slightly modified protocol of a commercial Kit (RT-PCR Kit with avian myeloblastosis virus [AMV]; version 2.1; Takara). For retrotranscription of NP and sM2 mRNA, the primers described above were employed. Primers for retrotranscription of EGFP mRNA were 5′-CTTGGATCCGTGAGCAAGGGCGAGGAG-3′ (sense), 5′-TCTGGATCCGCTTTACTTGCACAGCTC-3′ (antisense) and primers for retrotranscription of GAPDH mRNA were 5′-CCTTCATTGACCTCCACTAC-3′ (sense), 5′-GTTGTCATACTTCTCATGGTTC-3′ (antisense). RT-PCR products were further cloned into pMD-18T for sequencing.

2.8. Influenza virus infection and assay of virus titers in MDCK cells

To test whether the expressed siRNAs inhibited influenza virus production, we first assessed the growing capacity of influenza A virus in MDCK cells expressing siRNAs. MDCK cells were plated onto six-well plates and cultured at 37 °C and 5% CO_2 overnight. When the cell layer reached 70–80% confluence, 3 μg of pS-M48, 3 μg of pS-M754, 3 μg of pS-M949, 3 μg of pS-NP749 and 3 μg of pS-NP1383, respectively, were cotransfected with 3 μg of pS-Negative as described above. Non-transfected MDCK cells were also used as a control. At 18 h posttransfection, infection was done with subtype H1N1, H5N1 and H9N2 virus at a multiplicity of infection (moi) of about 0.01, respectively. Briefly, after removing the culture medium, H1N1, H5N1 and H9N2 virus (300 μl) in infection medium (2.5 μg/ml trypsin), respectively, were added to each well. After incubation for 1 h at room temperature, 3 ml of infection medium was added to each well, and the cells were cultured at 37 °C under 5% CO_2. Forty eight hours postinfection, supernatants were harvested from the infected cultures and virus titer ($TCID_{50}$) was determined three times on MDCK cells.

2.9. PEI-mediated DNA transfection, influenza virus infection and assay of lung virus titers in mice

The siRNA expression plasmids were mixed with PEI (Sigma) at a nitrogen/phosphorus weight ratio (N/P ratio) of 10 at room temperature for 20 min. For i.v. administration, 200 μl of the mixture containing 100 μg of DNA was injected into 6-week-old female BALB/c mice through the vena caudalis. At 15 h later, 50 μl of PBS containing the indicated doses (10^6 $TCID_{50}$) of viruses, H1N1, H5N1 and H9N2, respectively, were instilled into anesthetized mice through nostrils. In this experiment, mice were anaesthetized with ketamine/xylazine as described in the study by Tompkins et al. (2004). At 24 h postinfection, the lungs were harvested. The virus titers, determined as described previously (Ge et al., 2004), were shown as $\log_{10}$ tissue culture infectious dose ($TCID_{50}$). Student's *t*-test was performed as indicated.

2.10. Viral challenge assay in mice

Groups of 6-week-old female BALB/c mice (four groups of eight each) were injected with the PEI-mediated pS-Negative plasmids or siRNA expression plasmids (pS-M48 + pS-NP1383). At 18 h later, mice were infected H1N1 (10^6 $TCID_{50}$) or H5N1 (100 LD_{50}) virus, and monitored for body weight and mortality until all animals had succumbed to the infection or were recovering.

3. Results

3.1. siRNAs synthesized in vivo specifically silence the NP and sM2 gene of AIV in HeLa cells

Compared with the negative control (pNP-EGFP and psM2-EGFP were respectively cotransfected with pS-Negative), cotransfection of sM2 specific siRNA expression plasmids with psM2-EGFP caused significant reduction in EFGP signal so did the cotransfection of NP specific siRNA expression plasmids with pNP-EGFP (Fig. 2A). In contrast, cotransfection of pCD-EGFP with the siRNA expression plasmids caused no significant reduction of EGFP expression compared with the control (data not shown).

The inhibitory effects of the siRNAs on expression of EGFP were quantitatively validated by flow cytometry assay at 48 h posttransfection. The extent of EGFP down regulation was quantitated by assessing the mean fluorescence of the positive cells (M2-values) and the rate of EGFP positive cells (Fig. 2B). Compared with the pS-Negative control, the M2-values of the cells transfected with sM2-specific expression plasmids pS-M48,

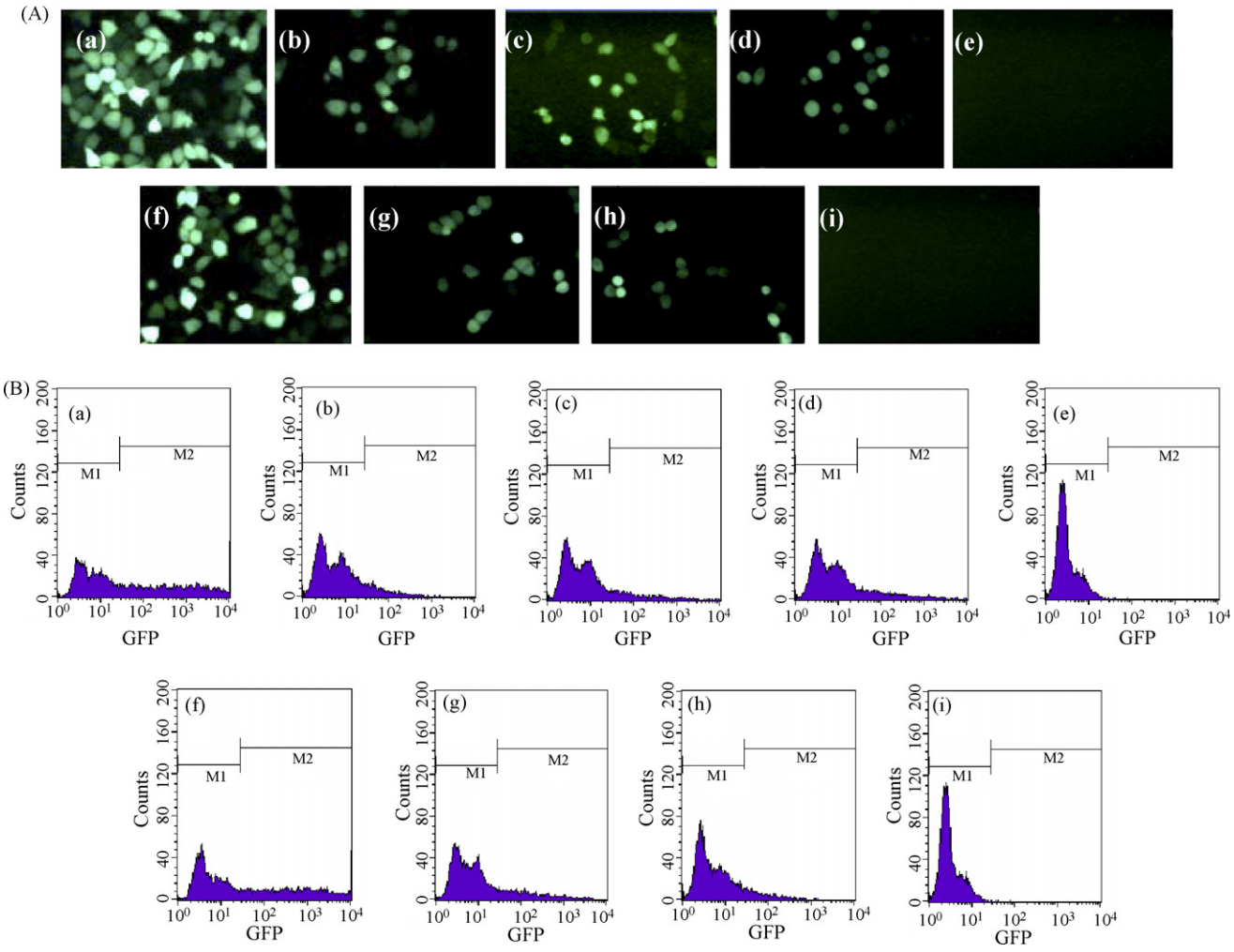

Fig. 2. Transient expression of siRNAs conferred the sequence-specific inhibition of expression of influenza A virus NP and sM2 in HeLa cells. (A) Fluorescence detection of cotransfection of psM2-EGFP and pNP-EGFP, respectively, with their corresponding siRNA expression plasmids 24 h posttransfection. (B) Flow cytometry analysis of cotransfection of psM2-EGFP and pNP-EGFP, respectively, with their corresponding siRNA expression plasmids 24 h posttransfection. EGFP expression level in cells cotransfected with (a) psM2-EGFP and pS-Negative; (b) psM2-EGFP and pS-M48; (c) psM2-EGFP and pS-M754; (d) psM2-EGFP and pS-M949; (e) cell control; (f) pNP-EGFP and pS-Negative. g) pNP-EGFP and pS-NP749. (h) pNP-EGFP and pS-NP1383. (i) cell control.

pS-M754 and pS-M949, were reduced by 97.8%, 94.6% and 92.2%, respectively; and the EGFP positive cells were reduced by 79.8%, 74.3% and 67.6%, respectively. The M2-values of the cells transfected with NP-specific expression plasmids pS-NP749 and pS-NP1383 were reduced by 92.3% and 97.7%, respectively; and the EGFP positive cells were reduced by 72.9% and 78.2%, respectively, compared with the pS-Negative control.

The similar inhibitory effects of the siRNAs were also confirmed by Western blot and RT-PCR analysis. Western blot revealed the expression of NP-EGFP and sM2-EGFP were effectively inhibited by their specific siRNA expression plasmids, not by pS-Negative. No significant difference in the expression of GAPDH was observed among different groups (Fig. 3A). The results of RT-PCR suggested that the inhibitory effects occurred at the transcriptional or post-transcriptional level. The results of Western blot (Fig. 3B) suggested that the inhibitory effects occurred at the translational level.

All the above results indicated that transient expression of siRNAs conferred sequence-specific inhibition of the expression of NP and sM2 gene of AIV in HeLa cells.

3.2. Transient expression of siRNAs confers specific inhibition of influenza virus production in MDCK cells

As shown in Table 1, in blank control cells (no siRNA) and pS-Negative transfected cells, virus titers ($TCID_{50}$) were obviously higher than that of NP- or M2-specific siRNAs expression plasmids transfected cells. The result showed the five siRNAs could inhibit replication of influenza A virus subtypes (H5N, H9N, H1N1) and caused a 2–43-fold reduction in virus titers in MDCK. Among these five siRNAs, pS-M48 (21–38-fold reduction in virus titers) and pS-NP1383 (28–43-fold reduction in virus titers) showed a higher inhibition activity. But no significant inhibition was observed in pS-Negative transfected

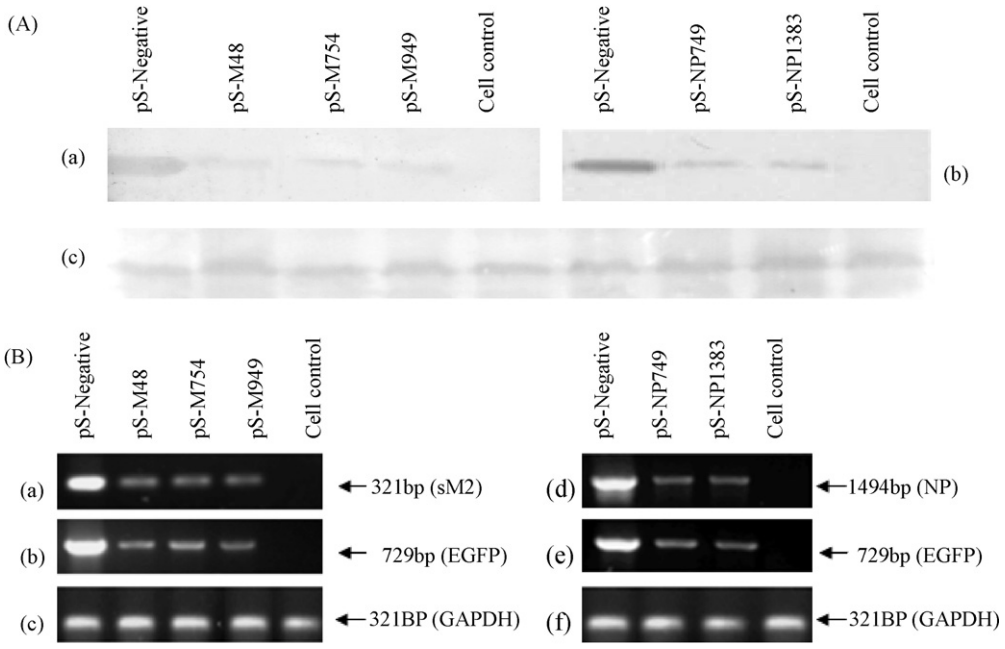

Fig. 3. (A) Western blot analysis showed that these specific siRNAs plasmids inhibited sM2 or NP protein expression. (a) The expression of sM2 protein in siRNAs transfected cells and controls. (b) The expression of NP protein in siRNAs transfected cells and controls. (c) The expression of GAPDH protein in siRNAs transfected cells and controls. (B) The mRNA level of NP, sM2, EGFP and GAPDH were evaluated by RT-PCR 48 h posttransfection. (a and b) The mRNA level of sM2 and EGFP in transfected cells and controls. (d and e) The mRNA level of NP and EGFP in transfected cells and controls. (c and f) The mRNA level of GAPDH in transfected cells and controls.

cells, indicating that non-specific siRNA did not interfere with influenza virus production.

3.3. Treatment with siRNA-expressing plasmids rapidly induced an antiviral response in BALB/c mice

To assess whether RNAi could inhibit influenza virus replication in vivo, we used an established murine model of influenza virus infection. siRNA expression plasmids pS-M48, pS-NP1383 and pS-M48 + pS-NP1383 were combined with polyethylenimine (PEI) and injected into BALB/c mice. For i.v. administration, 200 μl of the mixture containing 100 μg of DNA (pS-Negative, pS-M48 or pS-NP1383) was injected into 6-week-old female BALB/c mice by vena caudalis (50 μg of each with pS-M48 and pS-NP1383 combined). At 18 h later the mice were administered intranasally under anesthesia with H5N1, H9N2, or H1N1 viruses (Tables 2 and 3). The mice were sacrificed 24 h postinfection and lung homogenates were assayed for virus titers.

Virus titers (H5N1 AIV) were significantly reduced in lungs of the mice given pS-M48, pS-NP1383 or pS-M48 + pS-NP1383 compared with that of pS-Negative treatment (Table 2). The lung virus titers of pS-M48, pS-NP1383, and pS-M48 + pS-NP1383 treatment had, respectively, 1.21 log $TCID_{50}$ (16-fold), 1.4 log $TCID_{50}$ (25-fold) and 1.7 log $TCID_{50}$ (50-fold) reduction, compared with that of pS-Negative treatment. The lung virus titers of pS-M48 + pS-NP1383 treatment had 1.57 log $TCID_{50}$ (37-fold) and 1.61 log $TCID_{50}$ (40-fold) reduction, respectively in H1N1-

Table 1
Influenza virus specific siRNAs treatments inhibit H1N1, H5N1 and H9N2 virus replication in MDCK cells

Groups	Challenge virus					
	H1N1		H5N1		H9N2	
	Log $TCID_{50}$[a]	Fold reduction[b]	Log $TCID_{50}$[a]	Fold reduction[b]	Log $TCID_{50}$[a]	Fold reduction[b]
Cell control	6.87	–	8.33	–	6.54	–
pS-Negtive	6.90	–	8.33	–	6.55	–
pS-M48	5.57	21	6.88	28	4.97	38
pS-M754	6.30	4	7.88	3	5.97	4
pS-M949	6.39	2	7.57	6	5.63	8
pS-NP749	5.98	8	7.23	13	5.80	6
pS-NP1383	5.27	43	6.77	36	5.11	28

–, not applicable.
[a] The virus titer ($TCID_{50}$) was the mean value of the three times determination on MDCK cells.
[b] Compared with the pS-Negative-treated group.

Table 2
Influenza virus specific siRNAs treatments inhibit H5N1 influenza A virus replication in vivo

Treatment[a]	*n*	Mean lung virus titer[b] (log $TCID_{50}$)	*P*-Value[c] (*t*-test)	Fold reduction[d]
pS-Negtive	4	8.24 ± 0.13	–	–
pS-M48	4	7.03 ± 0.09	<0.001	16
pS-NP1383	4	6.84 ± 0.17	<0.001	25
pS-M48 + S-NP1383	4	6.54 ± 0.28	<0.001	50

–, not applicable.
[a] BALB/c mice were treated as indicated and challenged with 1×10^6 $TCID_{50}$ of H5N1. Two days later, animals were sacrificed and lungs were collected for virus titers.
[b] Expressed as $\log_{10}TCID_{50}$/ml ± S.E.M.
[c] One-way ANOVA statistical analysis on log-transformed data, followed with comparison with control (pS-Negative) by Dunnett's method.
[d] Compared with the pS-Negative-treated group.

or H9N2-infected mice, compared with that of pS-Negative treatment (Table 3). Compared with pS-Negative control group, the lung virus titers of the RNA interference groups were significantly reduced ($P < 0.001$). Additionally, Lung virus titers in untreated, PBS plus delivery vehicle-treated, and pS-Negative-treated mice were identical (data not shown), showing that pS-Negative treatment did not affect virus replication.

The results indicated the designed siRNAs targeting with NP and M2 gene were effective in inhibiting replication of different subtypes of influenza A virus in the lungs. When pS-M48 and pS-NP1383 were used together, the inhibitory effect was better.

To test whether the inhibition of virus replication by siRNA treatment was adequate for protection, grouped BALB/c mice were pretreated i.v. with pS-M48 + pS-NP1383, and 18 h later with lethal H1N1 or H5N1 challenge. We did not include H9N2 virus, because it was minimally lethal in mice (Yu et al., unpublished data). The mice treated with pS-M48 + pS-NP1383 and challenged with a lethal dose (10^6 $TCID_{50}$) of H1N1 resulted in 50% (4/8) survival, whereas almost 90% of the pS-Negative treated mice died (Fig. 4a and c). In the case of H5N1 virus, influenza specific siRNA protected two of the eight mice (2/8) challenged with a lethal dose (100 LD_{50}) of H5N1 virus that killed all of the control mice (Fig. 4b and d). Additionally, morbidity as indicated by weight loss was significantly reduced for H1N1 and H5N1. The results suggested that pS-M48 + pS-NP1383 could effectively alleviate the morbidity of the influenza A virus infected mice and could partially protect the mice challenged with lethal dose H1N1 and H5N1 influenza virus.

4. Discussion

RNA interference had been used as an effective antiviral strategy for its specific silencing of viral gene expression in mammalian cells. In this study, five siRNAs targeting NP or M2 gene were designed to inhibit AIV NP or M2 gene expression in HeLa cells. We used a modified CMV promoter, a typical RNA pol II promoter, to drive the transcription of the siRNAs. To provide a reporting system for monitoring the effects of the siRNA, two eukaryotic expression plasmids pNP-EGFP, psM2-EGFP were constructed, in which the NP and sM2 gene were fused to the 5′-end of the EGFP coding sequence, and cotransfected with their specific siRNA expression plasmids. So the inhibitory effects of the NP-specific siRNAs on the NP expression or the M2-specific siRNAs on the sM2 expression could be indirectly evaluated by the expression of EGFP in the transfected cells.

The results showed that the NP-specific siRNAs pS-NP749, pS-NP1383 could effectively down-regulate the expression of NP, and the M2-specific siRNAs pS-M48, pS-M754, and pS-M949 could also effectively down-regulate the expression of M2. These findings were demonstrated through fluorescence microscopic observation, flow cytometry, Western blot and RT-PCR analysis. Additionally, expression of the housekeeping gene GAPDH was also analyzed by Western blot and RT-PCR, and no significant difference in the expression of GAPDH was observed between the siRNAs treatment groups and pS-Negative treatment groups. The results suggested that five siRNAs target-

Table 3
Influenza virus specific siRNA treatments significantly decreased lung virus titers in mice infected with H1 and H9 influenza A virus

2 Challenge virus[a]	Treatment	Mean lung virus titer[b] (log $TCID_{50}$)	*P*-Value[c] (*t*-test)	Fold reduction[d]
H1N1	pS-Negtive	6.53 ± 0.28	–	–
	pS-M48 + pS-NP1383	4.96 ± 0.15	0.00045	37
H9N2	pS-Negtive	6.49 ± 0.41	–	–
	pS-M48 + pS-NP1383	4.88 ± 0.14	0.00183	40

–, not applicable.
[a] BALB/c mice ($n = 4$) were treated as indicated and challenged with 1×10^6 $TCID_{50}$ of H1N1 or 1×10^6 $TCID_{50}$ of H9N2. Two days later, animals were sacrificed and lungs were collected for virus titers.
[b] Expressed as $\log_{10}$ $TCID_{50}$ /ml ± S.E.M.
[c] One-way ANOVA statistical analysis on log-transformed data, followed with comparison using Student's *t*-test.
[d] Compared with the pS-Negative-treated group.

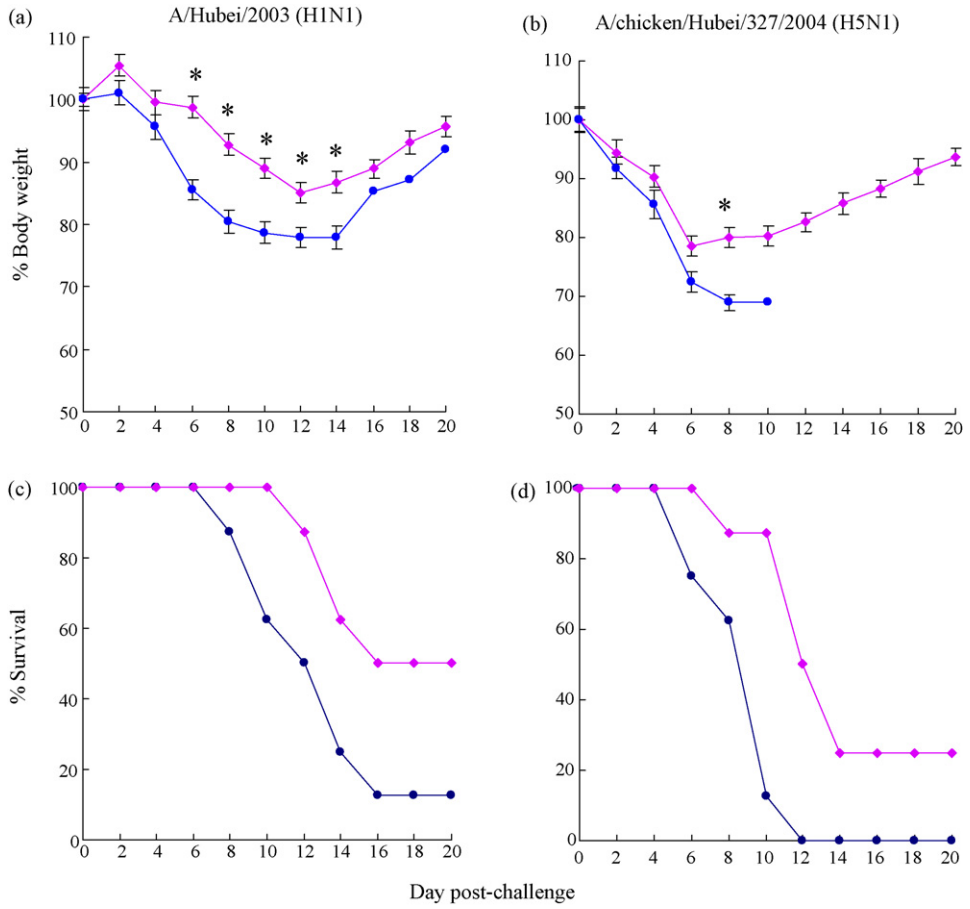

Fig. 4. Influenza virus specific siRNA treatments were broadly cross-reactive and partially protected mice against lethal challenge with pathogenic H5 and H1 influenza A viruses. BALB/c mice (eight per group) were treated with pS-Negative (●) or pS-M48 + pS-NP1383 (♦) and challenged with H1N1 virus (a and c), or H5N1 virus (b and d). The percent of initial body weight (a and b) and survival rate postchallenge (c and d) were shown. Error bars (a and b) depict the standard error of the mean. (*)Groups differ for weight loss, $P < 0.05$ (Student's *t*-test). pS-M48 + pS-NP1383 groups differ from pS-Negative groups for survival.

ing NP or M2 gene did not affect expression of the cellular gene GAPDH.

In order to confirm that the inhibitory effects of the siRNAs were specific to NP or M2 gene, pCD-EGFP was cotransfected with the five siRNAs in HeLa cells. Compared with the pCD-EGFP transfected cells, fluorescence microscopy and flow cytometric assay revealed no obvious difference in EGFP signals in the cotransfected cells (data not shown). This indicated that inhibitory effects of the siRNAs were specific for the NP and M2 genes, but not to EGFP.

To test whether the siRNAs inhibited influenza virus production, we examined their effects in MDCK cells. Our results indicated that specific siRNAs dramatically inhibited viral replication in cultured MDCK cells. We found that NP- or M2-specific siRNA inhibited the accumulation of all viral RNAs in infected cells. Probably, in the presence of NP- or M2-specific siRNA, the newly transcribed NP or M2 mRNA is degraded, resulting in inhibition of NP or M2 protein synthesis. Without newly synthesized NP or M2, further viral transcription and replication are blocked, as is new virion production. The results were consistent with the study by Ge et al. (2003).

In this report we showed that siRNAs could potently inhibit influenza virus production by interfering with the accumulation of not only mRNA, but also viral RNAs. This broad inhibition was virus specific, as it did not significantly affect RNAs transcribed from cellular genes. These findings might have significant implications for the use of siRNA for prophylaxis and therapy of influenza virus infection, and for the mechanisms underlying influenza virus transcription and replication.

Recent reports showed that, within the M gene, one siRNA (targeting nucleotides 18–38 of M cDNA) interfered the most effectively against M2 protein expression and virus replication (McCown et al., 2003). Ge et al. (2003) observed NP (NP-1496) and PA (PA-2087) gene targets were both the most effective in virus inhibition. In our study, effective siRNA target sites with M2 and NP gene differed from the published reports. We found that one M2-specific siRNA (M-48) and one NP-specific siRNA (NP-1383) were potent inhibitor of both protein synthesis and virus replication, whereas other siRNAs caused relatively weaker inhibition of influenza A virus replication. When 100 μg of DNA (50 μg of each with pS-M48 and pS-NP1383 combined) was used, about 37–50-fold reduction in lung virus titers were observed in H1N1, H9N2 or H5N1 virus-infected mice. In the study by Tompkins et al. (2004) using hydrodynamic i.v. delivery combined with i.n. delivery of siRNA could cause about 9–56-fold reduction in lung virus titers. Lung virus titer reductions of

this magnitude had been shown to accompany survival of lethal challenges in vaccine development studies (Epstein et al., 2002).

siRNAs were negatively charged and did not readily cross cell membrane. An effective siRNA-mediated prevention and treatment of influenza virus infection required efficient means to deliver siRNAs into epithelial cells of the respiratory tract. We demonstrated that i.v. injected siRNA-PEI complexes could deliver siRNA to lung epithelial cells and specifically inhibit virus replication in the site of infection.

Ge et al. had shown that siRNA reduced influenza virus production in the lungs when given i.v. to mice either before or after virus infection, and that DNA vectors from which shRNAs could be transcribed also significantly inhibited virus production in the lungs when administered i.v. (Ge et al., 2004). In this research, the effect of siRNA treatment of established infection was not included. It was also demonstrated that siRNA could be effective when given to animals before lethal influenza infection, which were consistent with the study by Tompkins et al. (2004). The data reported here suggest that this intervention would be useful during influenza outbreaks and that siRNA could be given as a preventive medicine in the face of a pandemic.

Influenza NP and M2 proteins are essential to viral replication, providing ideal targets for RNAi. Moreover, NP and M2 genes are highly conserved across subtypes of influenza A virus. Therefore, siRNAs against these genes should inhibit most influenza A viruses. This hypothesis is supported by our results of specific inhibition of replication of H1, H5, and H9 influenza A subtypes in cells and in vivo. Thus, pS-M48 + pS-NP1383 treatment could suppress replication of several subtypes influenza A viruses, including a H5N1 highly pathogenic avian isolate strain.

The siRNA target sequences are identical in A/chicken/Hubei/327/2004 (H5N1), A/Duck/Hubei/W1/2004 (H9N2), and A/Hubei/2003 (H1N1). However, there are naturally occurring influenza variants that have mismatches in the targeted regions. It will be important to test the ability of these siRNAs to inhibit replication of viruses lacking complete identity. Targeting multiple elements within the influenza genome decreases the likelihood of mismatches in all RNAi targets and could also reduce the likelihood of development of siRNA resistant virus escape variants (Tompkins et al., 2004).

Influenza virus infection has the potential to become a much more dangerous disease than it is at present because of easy transmission, antigenic shift and drift of the virus, and the limited efficacy of current vaccines and therapy. Our study showed that these siRNAs significantly reduced lung virus titers in the infected mice. Although the designed siRNAs could only partially protect mice from lethal challenge, especially H5N1 challenge, this study provided a basis for further development of siRNA for prophylaxis and therapy of influenza virus infection in human and animals.

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