Small Interfering RNA Targeting M2 Gene Induces Effective and Long Term Inhibition of Influenza A Virus Replication

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Abstract

RNA interference (RNAi) provides a powerful new means to inhibit viral infection specifically. However, the selection of siRNA-resistant viruses is a major concern in the use of RNAi as antiviral therapeutics. In this study, we conducted a lentiviral vector with a H1-short hairpin RNA (shRNA) expression cassette to deliver small interfering RNAs (siRNAs) into mammalian cells. Using this vector that also expresses enhanced green fluorescence protein (EGFP) as surrogate marker, stable siRNA-expressing cell lines were successfully established and the inhibition efficiencies of rationally designed siRNAs targeting to conserved regions of influenza A virus genome were assessed. The results showed that a siRNA targeting influenza M2 gene (siM2) potently inhibited viral replication. The siM2 was not only effective for H1N1 virus but also for highly pathogenic avian influenza virus H5N1. In addition to its M2 inhibition, the siM2 also inhibited NP mRNA accumulation and protein expression. A long term inhibition effect of the siM2 was demonstrated and the emergence of siRNA-resistant mutants in influenza quasispecies was not observed. Taken together, our study suggested that M2 gene might be an optimal RNAi target for antiviral therapy. These findings provide useful information for the development of RNAi-based prophylaxis and therapy for human influenza virus infection.

Introduction

Influenza A virus (IAV) remains a scourge on human health [1,2,3]. Its antigen drifts and shifts are an ever-changing challenge for available vaccines [4,5]. The appearance of drug resistance is the main hurdle for the development of antiviral drugs [6,7,8,9]. Given the limitations of current anti-influenza A virus strategies, the need for novel strategies for prevention and treatment of IAV is evident [10]. In this regard, RNA interfering (RNAi) technology holds great promise to inhibit the replication of IAV, including H5N1 virus.

RNAi is a form of posttranscriptional gene silencing mediated by short double-stranded RNA, known as small interfering RNA (siRNA) [11,12]. In this process, the cellular complex Dicer cleaves a double-stranded RNA (dsRNA) molecule to yield double-stranded duplexes 21–25 nucleotides in length. These siRNAs then guide the RNAi induced silencing complex (RISC) to cleave target mRNAs that share sequence identity with the siRNA [13,14,15]. Since it was first demonstrated that adding exogenous, synthetic siRNA molecules to mammalian cells can induce RNAi, there have been rapidly expanding efforts to develop RNAi therapies that induce the degradation of target messenger RNA (mRNA) involved in genetically inherited diseases or acquired disorders [16,17,18,19,20,21,22].

IAV is an enveloped, negative-stranded RNA virus. The unique property of single-stranded RNA virus itself makes RNAi an attractive approach for development of anti-avian influenza therapeutics. The single-stranded viral genome, consisting of 8 segments contained at least 10 open reading frames (ORFs), serves as template for both viral genome replication and subgenomic RNA synthesis. It has been reported that siRNAs respectively targeting to the viral genes of polymerase 1 (PB1), polymerase 2 (PB2), polymerase A (PA), nucleocapsid protein (NP), non-structure proteins (NS1 and NS2), matrix proteins (M1 and M2), especially those specific for NP, PA and PB1, can potently inhibit replication of influenza A viruses [16,23,24,25,26]. However, it has been reported that HIV and HCV may develop siRNA-resistant mutations quickly [17,27,28], and therefore abrogated the further RNAi treatment. Thus, the evaluation of long term inhibition efficiency of designed siRNAs and screening of the emergence of siRNA resistance mutants are also an important research target.

In the present study, we identified an effective siRNA targeting M2 gene (siM2), a highly conserved gene in IAV, as compared to a reported effective siRNA targeting NP gene (siNP). We further established cell lines which stably expressing the shRNAs by transducing lentiviral-shRNA vectors to Madin-Darby cannie kidney (MDCK) cells. Using these two cell lines, we evaluated long term antiviral effects of these siRNAs against IAV subtypes H1N1 and H5N1 and further screened the potential siRNA-resistant viral mutations. Our results showed that rationally designed siM2 conferred long term effective inhibition for IAV replication. It was
further demonstrated that no siRNA-resistant viral mutation appeared in siM2 targeting sequence even after the virus was cultured in the shRNA expressing stable cell line for 40 passages.

Results

Screening Effective siRNAs Targeting M2 Gene

Two siRNAs targeting the M2 gene were rationally designed by siRNA target designer (the sequences of siRNAs are shown in the supporting information Table S1) and their effect in inhibiting the virus replication was assessed in MDCK cells. Two siRNAs targeting the NP gene were included in the experiments as controls. The results showed the siRNA M-950 exhibited a good inhibition effect with dose dependent manner, while another siRNA M-126 just slightly inhibited virus replication even at a concentration of 100 nM (Fig. 1A). Fig. 1B showed that the siRNA NP-1496 could inhibit influenza virus replication, while siRNA NP-336 had no inhibition effect, which is consistent with the previous report [25].

The siM2 Exhibited Higher Inhibitory Effect of H1N1 Virus than siNP in Stable Cell Lines

Based on the above results, the lentiviruses expressing the shRNAs M2-950 or NP-1496 were constructed and transduced into MDCK cells to establish two stable cell lines, shM2-MDCK and shNP-MDCK. MDCK cells and the MDCK cells transduced by blank lentivirus (Mock MDCK) were used as controls. The cell lines were infected with H1N1 virus at a moi of 0.005 and culture supernatants were harvested at indicated time-points to determine the virus titer by plaque assay. As shown in Fig. 2, virus replication kinetics of Mock MDCK is similar with that of MDCK, indicating that lentivirus integration didn't influence virus replication. Virus titers in shNP- and shM2-MDCK cell cultures were 2 to 10 folds lower than the controls MDCK and Mock MDCK cultures, suggesting that virus replication had been suppressed by the expressed shRNAs in both shM2-MDCK and shNP-MDCK cells. Notably, siM2 exhibited a better inhibition effect, showing about 2-fold lower viral titer than siNP, although the expression levels of siM2 and siNP were similar (ΔCt siM2 = 6.68, siNP = 6.95).

The siM2 Abolished not only M2 mRNA but also siNP mRNA Accumulation in the Stable Cell Lines

We also measured the accumulation of mRNA for NP and M2 gene in infected MDCK, Mock MDCK, shM2-MDCK and shNP-MDCK cells. The mRNAs were extracted from the cells harvested at 1, 2, 4 and 24 hrs post-infection and tested by real-time RT-PCR. The mRNA expression level is normalized by copy

Figure 1. Effects of chemically synthesized siRNAs on influenza virus production. (A) siRNA M-950 is effective for influenza virus inhibition. (B) siRNA NP-1496 can inhibit influenza virus replication at indicated siRNA concentration. An unrelated siRNA targeting GFP was used as negative control. MDCK cells were transfected by chemically synthesized siRNAs, and infected with H1N1 virus at a moi of 0.005 in 8 hrs after transfection. Viral culture supernatants were collected at 48 hrs post infection. The viral load was detected by Q RT-PCR and expressed by relative viral RNA copies. The data were from three replicates of the experiments and presented as the mean value+SD. doi:10.1371/journal.pone.0005671.g001

Figure 2. Inhibition of influenza H1N1 virus production on stable cell lines. Stable cell lines were infected by H1N1 virus at a moi of 0.005. Virus titer was measured at the indicated time points after infection. Data were from three replicates of the experiments. doi:10.1371/journal.pone.0005671.g002
number of β-Actin. The M2 mRNA level in shM2-MDCK cells harvested at 4 hrs and 24 hrs post-infection was significantly lower than those in MDCK, Mock MDCK and shNP-MDCK cells (Fig. 3A). Similarly, the NP mRNA level in shNP-MDCK cells collected at 4 hrs and 24 hrs after the viral infection was significantly suppressed as compared to those in MDCK and Mock MDCK cells (Fig. 3B). Interestingly, siM2 could also inhibit the accumulation of NP mRNA (Fig. 3B), suggesting that the siM2 might have a broad inhibitory effect.

NP Protein Expression was Suppressed in Virus Infected shM2-MDCK Cells

To further confirm whether the suppression of NP mRNA in shM2-MDCK cells indeed affect NP protein expression, the NP protein level was tested by an indirect immunofluorescence assay. As shown in Fig. 4, EGFP fluorescence, an indicator of shRNA expression, was detected in Mock MDCK, shNP-MDCK and shM2-MDCK but not in MDCK cells, while NP protein was detected in MDCK and Mock MDCK cells but not in shNP-MDCK and shM2-MDCK cells. The results were consistent with above viral mRNA results, indicating that siM2 indeed suppressed the NP protein expression.

siM2 Resistant Virus Mutant was not Observed Even after 40 Passages

To test if siM2 siRNA-resistant virus mutant would quickly appeared when cultured in shM2-MDCK cells, H5N1 virus was continually cultured in shM2-MDCK cells for 40 passages. Every 10 passages, the culture supernatant was collected and tested by plaque reduction assay. No obvious larger size of plaque was found. Ten plaques with relative larger size were picked to further identify potential mutation in the siRNA targeting region by sequencing. The results showed that no mutation appeared in the siM2 targeting region even after 40 passages of the cultures (Fig. 6).

Discussion

The principal finding of this study is that rationally designed siRNA targeting influenza M2 gene (M-950) conferred effective long term inhibition against influenza A virus replication. Such high suppressive effect is not only against H1N1 influenza A virus but also against a highly pathogenic H5N1 subtype. In the previous related studies, Ge and his co-workers [25] screened
Figure 4. Viral protein levels in stable shRNA-expressing cell lines infected by H1N1 virus. The indicated cell lines were infected with H1N1 virus for 6 hrs and stained with anti-NP antibody. Green represents signal from EGFP in the lentiviral vectors, while red (texas red) represents staining with anti-NP antibody. Images were taken by using a fluorescence microscope under a 400 magnification. All the pictures were captured under the same exposure time and gain.

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Figure 5. Inhibition of influenza H5N1 virus production on stable shRNA expressing cell lines. (A) Inhibitory effects of siM2 and siNP were detected by plaque reduction assay after the stable cell lines were infected with 0.05 moi of H5N1 virus for 72 hours. (B) The stable cell lines were infected with indicated doses of H5N1 virus. Virus titers were measured at the indicated time points after infection. The data were presented as the mean values of two experiments.

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siRNAs targeting to 6 conserved genes of influenza A virus and showed that NP-1496 was the best since it can confer a more than 200-folds inhibition of H1N1 virus. Li et al [29] and Tomkines et al [23] further confirmed that NP-1496 provided high anti-H5N1 effect. We therefore included NP-1496 as a positive control in this study. Our results showed that siRNA M-950 exhibited similar (Fig. 1) or even slight higher (Fig. 2) inhibitory effect against IAV replication as compared to that of NP-1496. A recent report by Zhou et al [30] also showed that several siRNAs targeting NP and M genes exhibited effective inhibition against influenza A virus replication in cultured MDCK cells and in animal models. However, sequences of their reported siRNAs targeting M2 gene are completely different from the siRNA M-950. Furthermore, chemically synthesized siRNAs or plasmid based shRNAs were always delivered by transfection in previous related studies, whereas we used a lentivirus system to deliver selected shRNAs. Although the integration property of lentivirus has abrogated it to be used in human, it is helpful for our study purpose to successfully establish stable cell lines persistently expressing siRNAs.

In this study we found that siM2 not only decreased the level of M2 mRNA but also the level of NP mRNA, suggesting that siM2 has a broad inhibition manner in the process of influenza virus replication. Ge et al have reported a similar broad inhibition of siRNAs [25]. In their study, NP-1496 and PA-2087 provided a broad inhibition to H1N1 influenza virus, which not only abolished the accumulations of specific NP or PA mRNAs but also inhibited the accumulations of mRNAs for M, NS1, PB1, PB2 and PA or NP genes. A possible explanation is that some double stranded siRNAs may result in IFN responses or activate a RNA degradation pathway, e.g. Phosphorylated protein Kinase R (PKR) [9,31,32]. However, the mechanisms of this broad inhibition of some siRNAs are still not very clear yet. From the standpoint of viral target choice in RNAi based antiviral therapy, NP protein is required for elongation and antiternimation of nascent cRNA and vRNA transcripts [33,34]. Without newly synthesized NP, further viral transcription and replication are blocked. While, M2 plays a critical role in the assembly of infectious virus particles. Thus, the potent antiviral effect of siM2 may be attributed to its broad inhibitory effect.

Depending on the stringency of siRNA-target base pairing, siRNA treatment may cause selection of siRNA-resistant viruses, and this has been shown with HIV and HCV [17,27,28], and therefore abrogated the further medication or treatments. Using lentiviral delivery system, we established stable cell lines persistently expressing shRNA, which provided a more convenient experimental approach to study long term inhibition effect of siRNAs and screen for siRNA resistant virus mutants in quasispecies in vitro. Our results showed that H5N1 virus cultured in shM2-MDCK were equally susceptible to siM2 as the original virus even after 40 passages. Moreover, sequencing of siM2 targeted region in 10 such independent plaque purified virus isolates revealed sequence identical to the parental one. The current data have shown no insertion, deletion and nucleotide substitution in the siRNA target sequence, therefore demonstrated siM2 possessed good long term inhibition effect for influenza virus replication without the problem of siRNA resistant mutants.

Taken together, all the findings about effective RNAi target, lentiviral vector delivery and the establishment of stable shRNA expressing cell lines in our study provide rational information for the development of siRNAs as prophylaxis and therapy for influenza virus infection in humans.

Materials and Methods

Cell lines and viruses

MDCK and Human embryonic kidney 293T cells were respectively maintained in MEM and DMEM (Invitrogene, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U penicillin G/mL and 100 ug streptomycin/mL). Influenza virus strains A/New Caledonia/20/1999 (H1N1) and A/Hong Kong/486/97 (H5N1) used in these experiments were prepared in MDCK cells and virus titters were determined by TCID50. All experiments with H5N1 virus were performed in BSL-3 laboratory.

Preparation and transfection of siRNAs

The siRNAs targeting M or NP gene of influenza A virus were designed by siRNA target designer version 1.51 from Promega (http://www.promega.com/siRNADesigner/program/). The duplexes of designed and previously reported siRNAs were synthesized by Invitrogen [USA] (the sequences were shown in the supporting information Table S1). The siRNAs were reverse transfected to MDCK cells using Lipofectamine™ RNAiMAX (Invitrogen, USA) as described in company’s instruction. After
incubated the cells for 16–18 hrs, the cells were infected with the viruses and followed by detection of viral replication. 24 hours after infection, RNA were extracted from the cells and followed by real time RT-PCR to detect the relative quantities of replicated viral RNA.

**Construction of lentiviral vectors**

The H1-promoter-driven shRNA cassettes were constructed by annealing two primers containing the 19-nt sense and reverse complementary targeting sequences with a 9-nucleotide loop - TTCAAGAGA and flanking MluI and CiaI cloning sites (the sequences of shRNA were shown in the supporting information Table S1), and then cloned into the 3′-end of the H1 promoter in the LVTHM plasmid [35,36]. The sequences of the insertions were confirmed by DNA sequencing.

**Generation of recombinant lentivirus**

Lentiviral vectors with shRNA expression cassette were produced by calcium phosphate-mediated, three-plasmid transfection of 293T cells [37]. Briefly, 293T cells (2.5×10⁶ cells in a 75T flask) were transfected with 20 μg LVTHM or LVTHM-shRNA, 15 μg psAX2 and 6 μg pMD.2G and cultured in DMEM supplemented with 10% FBS and antibiotics. Virus supernatants were collected on day 3 post-transfection, filtered through a 0.45 μm pore-size filter, ultracentrifuged at 40,000 g for 1 hr, the infected medium was removed and resuspended in PBS. Virus stocks were titered by infecting Hela cells with virus dilutions in DMEM and 8 μg polybrene (hexadimethrine bromide, Sigma) ml⁻¹ and analyzed for EGFP expression with a flow cytometer (BD Bioscience Immunocytometry Systems, USA). Data was processed with Cellquest software. Titters of the virus stocks were routinely 10⁻¹⁰ transduction units (TU) ml⁻¹.

**Establishment of shRNA expressing stable cell lines**

Lentiviral stocks were used to transduce MDCK cells. After 3 days of transduction, the medium containing the lentivirus was replaced with complete culture medium. The transduced MDCK was sub-cultured every 3–4 days for 4 weeks to get stable viral genome integration. Sorting of live GFP positive cells was performed using a FACStar instrument (Becton Dickinson, USA). Plating 10⁶ cells in a 25 cm² flask and the stable shRNA expressing cell lines were named. After 1 hr incubation, the virus was removed and the cultures were overlaid with 1% semi solid agar-MEM. Three days after infection, plaques were visualized by staining of crystal violet.

**Real-time RT-PCR**

Real-time RT-PCR was carried out as described previously [39]. Briefly, H1N1 or H5N1 virus infected MDCK, Mock MDCK, shNP-MDCK and shM2-MDCK were harvested at 1, 2, 4 and 24 hr after infection. Total RNA was extracted from the infected cell samples using RNeasy RNA isolation Kit (Qiagen, Germany) and reverse transcribed using Superscript II Reverse Transcriptase and Oligo dT primer (Invitrogen, USA), according to the manufacturer’s protocol. Viral mRNA copies were measured by SYBR green M×3000 Real-Time PCR System (Stratagen, USA), using primers NP-Forward: 5′-GAC CAG GAG TGG AGG AAA CA-3′, NP-Reverse: 5′-CGG CCA TAA TGG TCA CTC TT-3′; M2-Forward: 5′-CGT CGC TTC TTT AAA TAC GGT TTG-3′, M2-Reverse: 5′-GTA CC AAT CAT CAG CAT CAC CAT TC-3′ β-Actin-Forward: 5′-CC TGC ACC ACT GGC ATC GTG AT-3′, β-Actin-Reverse: 5′-GTA TTG TCC GAC TAC AGG TCT TG-3′. The reactions were performed at 95°C 10 min, 40 cycles of 95°C 1 min, 60°C 1 min, 72°C 1 min, followed by melting curve analysis according to instrument documentation (Stratagene M×3000). All reactions were done in triplicates and the results were normalized by β-actin.

**Indirect Immunofluorescence Assay**

Indirect immunofluorescence assay was performed as described previously [40,41] with some modification. MDCK, Mock MDCK, shNP-MDCK and shM2-MDCK cells grew on micro cover glasses (Thomas, USA) were infected with 1 moi of H1N1 virus for 6 hrs. After washed with PBS, the cells were fixed in 4% paraformaldehyde for 15 mins at RT and then permeabilized in 0.1% Triton X-100 for 3 mins at RT. After washed with PBS again, the cells were incubated with 1:50 diluted mouse anti-NP antibody (Abcam, UK) for 30 mins in dark at RT. The cells were washed three times in PBS with 1% FCS and incubated with 1:500 diluted Texas red-conjugated anti-mouse IgG (Abcam, UK) for 30 mins in the dark at RT. The cells were washed and mounted. Slides were viewed under an Olympus fluorescence microscope (Olympus, Germany).

**Screening siRNA resistant mutants on shM2-MDCK**

The screening of potential siRNA resistant mutants were performed in our established stable shRNA-expressing cell lines according to previously described protocols [42] with some modification. Briefly, the shM2-MDCK cells in a T25 cm² flask were infected with H5N1 virus for 6 hrs. After washed with PBS, the cells were fixed in 4% paraformaldehyde for 15 mins at RT and then permeabilized in 0.1% Triton X-100 for 3 mins at RT. After washed with PBS again, the cells were incubated with 1:50 diluted mouse anti-NP antibody (Abcam, UK) for 30 mins in dark at RT. The cells were washed three times in PBS with 1% FCS and incubated with 1:500 diluted Texas red-conjugated anti-mouse IgG (Abcam, UK) for 30 mins in the dark at RT. The cells were washed and mounted. Slides were viewed under an Olympus fluorescence microscope (Olympus, Germany).
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