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Effective inhibition of mRNA accumulation and protein expression of H5N1 avian influenza virus NS1 gene in vitro by small interfering RNAs

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Abstract Avian influenza has emerged as a devastating disease and may cross species barrier and adapt to a new host, causing enormous economic loss and great public health threats, and non-structural protein 1 (NS1) is a multifunctional non-structural protein of avian influenza virus (AIV) that counters cellular antiviral activities and is a virulence factor. RNA interference (RNAi) provides a powerful promising approach to inhibit viral infection specifically. To explore the possibility of using RNAi as a strategy against AIV infection, after the fusion protein expression plasmids pNS1-enhanced green fluorescent protein (EGFP), which contain the EGFP reporter gene and AIV NS1 as silencing target, were constructed and NS1-EGFP fusion protein expressing HEK293 cell lines were established, four small interfering RNAs (siRNAs) targeting NS1 gene were designed, synthesized, and used to transfect the stable cell lines. Flow cytometry, real-time quantitative polymerase chain reaction, and Western blot were performed to assess the expression level of NS1. The results suggested that sequence-dependent specific siRNAs effectively inhibited mRNA accumulation and protein expression of AIV NS1 in vitro. These findings provide useful information for the development of RNAi-based prophylaxis and therapy for AIV infection.

Abbreviations

AIV	Avian influenza viruses
HA	Hemagglutinin
NS1	Non-structural protein 1
NEP	Nuclear export protein
RNAi	RNA interference
dsRNA	Double-stranded RNA
IFN	Interferon
CEFs	Chicken embryo fibroblasts
EGFP	Enhanced green fluorescent protein
siRNAs	Small interfering RNAs
shRNA	Short hairpin RNAs
miRNA	microRNAs
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
FCM	Flow cytometry
RT-PCR	Reverse transcription-polymerase chain reaction
M-MLV	Moloney murine leukemia virus
PBS	Phosphate balanced solution

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RPLP0	Acidic ribosomal protein P0
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IgG	Immunoglobulin G
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horseradish peroxidase
mAB	monoclonal Ab
ECM	Enhanced chemiluminescent method
CCD	Charge-coupled device
PA	RNA transcriptase
M2	Matrix
NP	Nucleocapsid protein
MDCK	Madin–Darby canine kidney
CPSF30	Cellular cleavage and polyadenylation specificity factor

Introduction

Avian influenza has emerged as a devastating disease and may cross species barrier and adapt to a new host, causing enormous economic loss and great public health threats. In 1997, H5N1 avian influenza virus (AIV) infected 18 hospitalized patients in the Hong Kong Special Administrative Region, China; six of the patients died (Bender et al. 1999). Analysis of the sequences of all eight RNA segments of the influenza A/Goose/Guangdong/1/96 (H5N1) virus, isolated from a sick goose during an outbreak in Guangdong, China, in 1996, revealed that the hemagglutinin (HA) gene of the virus was genetically similar to those of the H5N1 viruses isolated in Hong Kong in 1997, which suggested that the H5N1 viruses isolated from the Hong Kong outbreaks derived their HA genes from a virus similar to the A/Goose/Guangdong/1/96 virus or shared a progenitor with this goose pathogen (Xu et al. 1999). Since 2003, it has been found in many countries in Asia, Europe, and Africa. These wide distributions and host range, and the ability to transmit interspecies indicate it has pandemic potential.

As the non-structural protein of AIV, non-structural protein 1 (NS1) is a nuclear, dimeric protein that is highly expressed in infected cells and has double-stranded RNA (dsRNA)-binding activity (Hatada and Fukuda 1992; Nemeroff et al. 1995). It is a multifunctional protein that counters cellular antiviral activities and is a virulence factor (Li et al. 2006; Min and Krug 2006). Majority of these functions are connected with overcoming of host antiviral innate immunity mainly via the inhibition of type I interferon (IFN) response (Ehrhardt et al. 2010; Panshin et al. 2010). NS1 gene is critical for the pathogenicity of AIV in chickens, and the amino acid residue Ala149 correlates with the ability of these viruses to antagonize IFN induction in chicken embryo fibroblasts (CEFs) (Long et al. 2008).

As a process of post-transcriptional gene silencing mediated by short dsRNA, RNA interference (RNAi) causes degradation of target mRNAs and has been utilized as a therapeutic strategy by knockdown key target of pathogen or receptor (Hannon 2002).

Small interfering RNAs (siRNAs) specific for matrix (M2), nucleocapsid protein (NP), and RNA transcriptase (PA) genes of H5N1 AIV could effectively inhibit the expression of the corresponding viral protein and potently inhibit AIV production (Ge et al. 2003; Zhou et al. 2007; Sui et al. 2009; Zhang et al. 2009). However, few studies have been performed to explore the possibility of using RNAi against NS1 as a strategy for inhibiting AIV H5N1 replication.

In this study, to explore the possibility of using RNAi targeting NS1 as a strategy against AIV infection, four siRNAs targeting AIV NS1 gene were designed and chemically synthesized, and the cell lines which stably express pNS1-enhanced green fluorescent protein (EGFP) were established and used to evaluate the siRNAs inhibition effects against NS1. Our results indicated that specific siRNAs capable of reducing mRNA accumulation and protein expression of NS1 were identified, which might be of importance as a promising approach for development of anti-AIV therapeutics and knockdown analysis of AIV NS1.

Materials and methods

Virus and gene

Influenza A virus (A/environment/Qinghai/1/2008(H5N1) was isolated from bird feces in Qinghai, People's Republic of China. The accession number of NS1 nucleotide sequences in GeneBank is FJ455824.

Cell line

HEK293 cell line (human embryonic kidney cell line) was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and routinely grown at 37 °C, in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, USA) containing 10 % heat-inactivated fetal bovine serum (FBS; Gibco BRL, USA) supplemented with penicillin (100 U/mL; Gibco BRL, USA) and streptomycin (100 U/mL; Gibco BRL,USA).

Expression constructs

In order to establish NS1-EGFP fusion protein expressing stable cell lines, AIV-NS1 cDNA fragment obtained from plasmid pcDNA3.1-NS1 digested with Xho1 and EcoR1 was cloned into eukaryotic expression vector pEGFP-N1

(Clontech, Japan) digested with Xho1 and EcoR1 to obtain the recombinant fusion protein expressing plasmid, pNS1-EGFP. All constructs were confirmed by DNA sequencing, and EndoFree™ Plasmid Maxi Kit (QIAGEN, Germany) was used to extract the recombinant plasmid for further transfection.

Design and synthesis of siRNAs

Four siRNAs, NS1-siRNA1, NS1-siRNA2, NS1-siRNA3, and NS1-siRNA4, which were targeted NS1, were designed according to the basic principles of siRNA design and synthesized by Shanghai GenePharma Co. (Shanghai, China; Table 1). Scramble sequence was synthesized and used as negative control, and effective EGFP-siRNA was also synthesized and used as positive control.

Expression plasmids transfection and establishment of NS1-EGFP expressing stable cell lines

The plasmid DNA was induced into HEK293 cell as described previously (Liu et al. 2011). Lipofectamine™ 2000 was used to carry pNS1-EGFP and pEGFP-N1 into HEK293 cell. After transfection, limiting dilution cloning in 96-well plates was performed as described of Watson (1979). Cells which stably incorporated the expression plasmid pNS1-EGFP and pEGFP-N1 were selected by survival in the presence of G418 (500 μg/mL; Gibco BRL, USA). Fluorescent microscopy (Olympus X71, Japan) was used to examine the expression of EGFP, and Western blot was performed to confirm the expression of NS1-EGFP fusion protein. Non-transfected HEK293 cells and HEK293 cells transfected with pEGFP-N1 were used as control.

siRNA transfection

According to the protocol of PepMute™ siRNA Transfection Reagent (SignaGen Laboratories, USA), the following procedures were followed to induce siRNAs into NS1-EGFP expressing stable cells and EGFP expressing stable cells, respectively, briefly: at 24 h before transfection, seeded 5×10^5 cells per well (6-well plate). For each well, add 1.0 mL of complete medium with serum and antibiotics freshly 30 min before transfection. Dilute siRNA into 100 μL of working solution of PepMute™ Transfection Buffer (siRNA final concentration is 20 nmol/L). Add 2.0 μL PepMute™ reagent, mix by pipetting up and down, and incubate for 15 min at reverse transcription (RT). Add the transfection mix to the cells dropwise, and gently rock the plate back and forth.

Fluorescence observation and FCM analysis

Expression of NS1-EGFP and EGFP in the stable cells transfected with siRNAs was examined by fluorescent microscopy (Olympus X71, Japan) at 24, 48, and 72 h after transfection. At 48 h after transfection, the cells were digested with 0.25 % trypsin, washed with phosphate balanced solution (PBS), and resuspended in PBS for flow cytometry (FCM) assay using a FACS Calibur flow cytometer (Becton Dickinson, USA) with filters (emission, 507 nm; excitation, 488 nm) for EGFP expression. Samples (10^6 cells each) were counted and analyzed with CellQuest software (Becton Dickinson, USA). Non-transfected HEK293 cells were used as control.

Real-time quantitative RT-PCR

Real-time quantitative RT-polymerase chain reaction (PCR) was performed as described previously (Liu et al. 2011). Briefly, at 48 h post-transfection, high-quality total RNA from transfected stable cells were isolated with PureLink™ RNA Mini Kit (Invitrogen, USA). The absorption of light at 260 and 280 nm (A260/280) and electrophoresis followed by staining with ethidium bromide were used to determine the concentration and integrity of purified RNA, respectively. Moloney murine leukemia virus (M-MLV) first-strand kit (Invitrogen, USA) was used for cDNA synthesis with 10 min incubation at 25 °C, followed 60 min at 37 °C and then at 70 °C for 15 min. One hundred nanograms of total RNA from the NS1-EGFP expressing stable cell lines transfected with siRNAs, and NS1-EGFP expressing stable cell line untransfected was used to determine expression levels of NS1 mRNA with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA) and a ABI7500 (Carlsbad, USA). Comparative CT method was used to compare the NS1 mRNA levels of the stable cells transfected with different siRNAs and mock.

Table 1 Lists of siRNAs sequence

Start site	Sense (5′–3′)	Anti-sense (5′–3′)
EGFP-siRNA (274)	GGCUACGUCCAGGAGCGCACC	UGCGCUCCUGGACGUAGCCUU
NS1-siRNA1 (299)	GGGAUUGGUUAAUGCUCAUTT	AUGAGCAUUAACCAAUCCCTT
NS1-siRNA2 (424)	GAGGCUCUAAUACUACUUATT	UAAGUAGUAUUAGAGCCUCTT
NS1-siRNA3 (461)	GAGCAAUAGUGGGCGAAAUTT	AUUUCGCCCACUAUUGCUCTT
NS1-siRNA4 (548)	GAGGAUUUGAAUGGAAUGATT	UCAUUCCAUUCAAAUCCUCTT

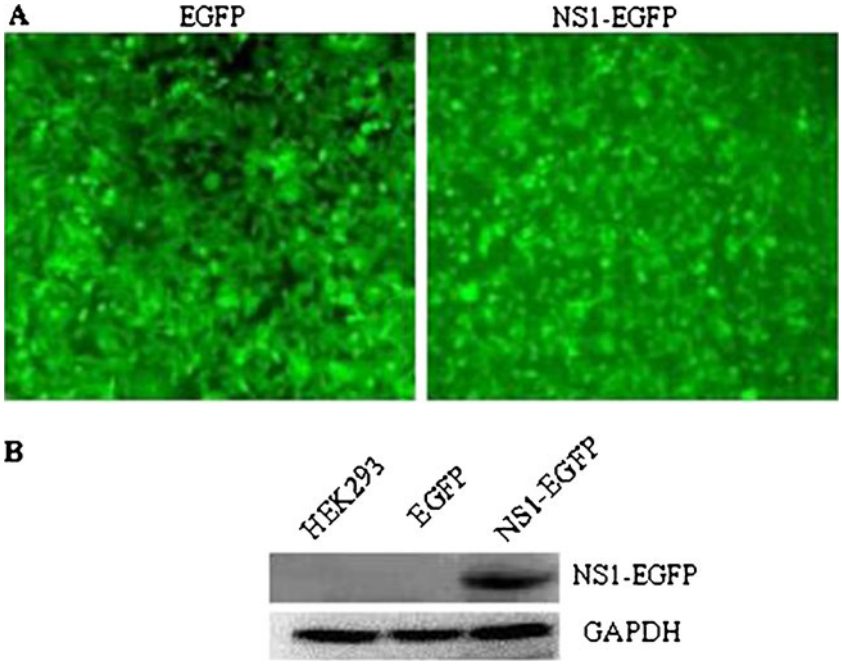


Fig. 1 Expression of NS1-EGFP fusion protein in NS1-EGFP expressing stable cells. **a** Fluorescent pictures of HEK293 cells expressing EGFP alone or NS1-EGFP fusion protein. **b** Western blot analysis of HEK293 cells expressing EGFP alone or NS1-EGFP fusion protein with mouse monoclonal antibodies against NS1

To control variability of RNA input, all reactions were normalized to the Ct value of acidic ribosomal protein P0 (RPLP0). Each assay represents three independent experiments, and all samples were performed in triplicate. The results were expressed as mean±SD. The sequence of the primers for detection of NS1 (FJ455824) and human RPLP0 (NM001002) were as follows:

NS1: forward (110–131), 5′-GCCGAGATCAGAAGTCCCTAAG-3′, reverse (181–202), 5′-TCCGCTCCACTATATGCTTTCC-3′;
RPLP0: forward, 5′-CGCTGCTGAACATGCTCAA-3′, reverse, 5′-ATTGTCGAACACCTGCTGGAT-3′.

Western blot

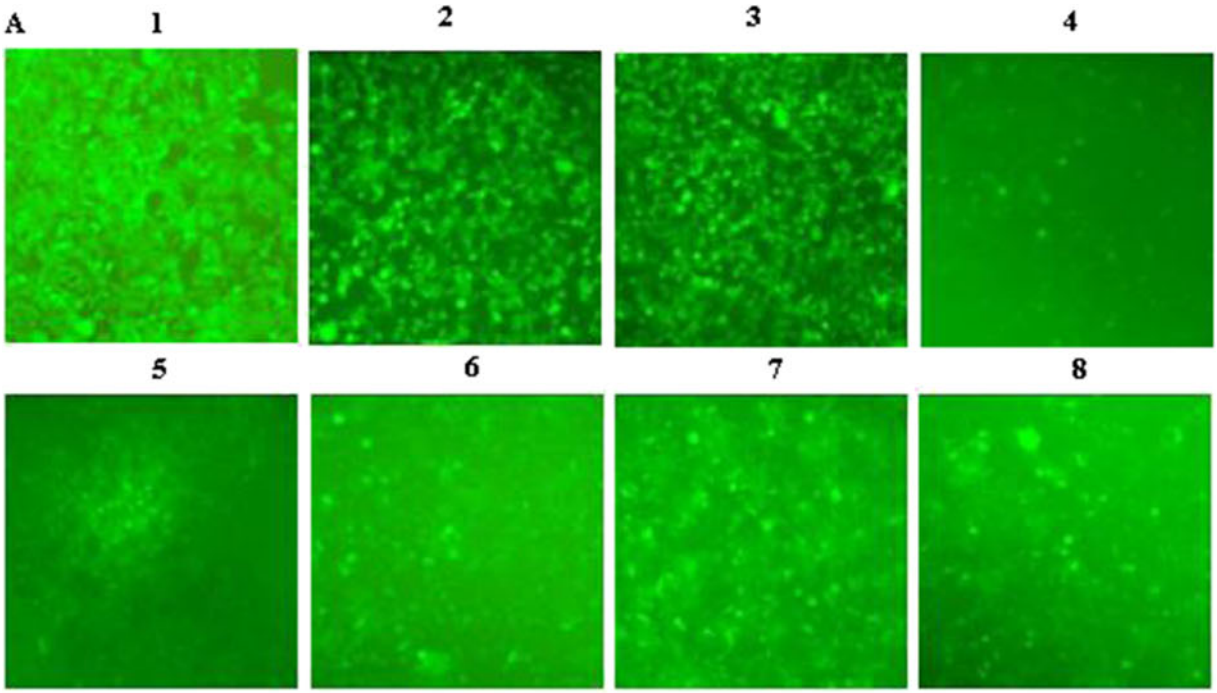
Western blot was performed as described previously (Liu et al. 2011). The primary antibodies were mouse monoclonal IgG1 against NS1 (1:200; Novus Biologicals, USA), rabbit polyclonal anti-EGFP (Novus Biologicals, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal Ab (mAB; Cell Signaling Technology, USA), respectively. The secondary antibodies were horseradish peroxidase (HRP) labeled goat anti-mouse IgG1 (Abcam, USA) and HRP labeled goat anti-rabbit IgG (1:5,000; Abcam, USA). After incubation with the secondary antibodies for 2 h, the membranes were developed with enhanced chemiluminescent method (ECM) kit (BOSTER, China) for 30–300 s to visualize, and the proteins were detected by charge-coupled device (CCD) cameras. BandScan 5.0 software was used to quantify the Western blots for protein expression.

Results

Identification of HEK293 cells which stably express NS1-EGFP and EGFP

After G418 selection, cells which stably incorporated pNS1-EGFP were established, and cells which stably incorporated pEGFP-N1 were also obtained and used as control. The expression of EGFP protein and NS1-EGFP fusion protein in NS1-EGFP and EGFP expressing stable cell lines was examined with fluorescence microscope (Fig. 1a). With mouse monoclonal antibodies against NS1 (Novus Biologicals, USA), the analysis results of Western blot with cell extracts from NS1-EGFP expressing stable cell lines indicated that there was a specific band at the expected molar mass for NS1-EGFP fusion protein, but no NS1 expressing in EGFP expressing stable cell lines (Fig. 1b).

Fig. 2 The percentage of fluorescent cell population and mean fluorescent intensity of NS1-EGFP expressing stable cell lines transfected with siRNAs. **a** Fluorescent pictures of stable cells transfected with siRNAs. *1*, EGFP expressing stable cell lines; *2*, NS1-EGFP expressing stable cell lines; *3*, NS1-EGFP expressing stable cell lines transfected with scramble-siRNA; *4*, NS1-EGFP expressing stable cell lines transfected with EGFP-siRNA; *5*, NS1-EGFP expressing stable cell lines transfected with NS1-siRNA1; *6*, NS1-EGFP expressing stable cell lines transfected with NS1-siRNA2; *7*, NS1-EGFP expressing stable cell lines transfected with NS1-siRNA3; *8*, NS1-EGFP expressing stable cell lines transfected with NS1-siRNA4. **b** FCM analysis of the percentage of fluorescent cell population and mean fluorescent intensity of NS1-EGFP expressing stable cell lines transfected with siRNAs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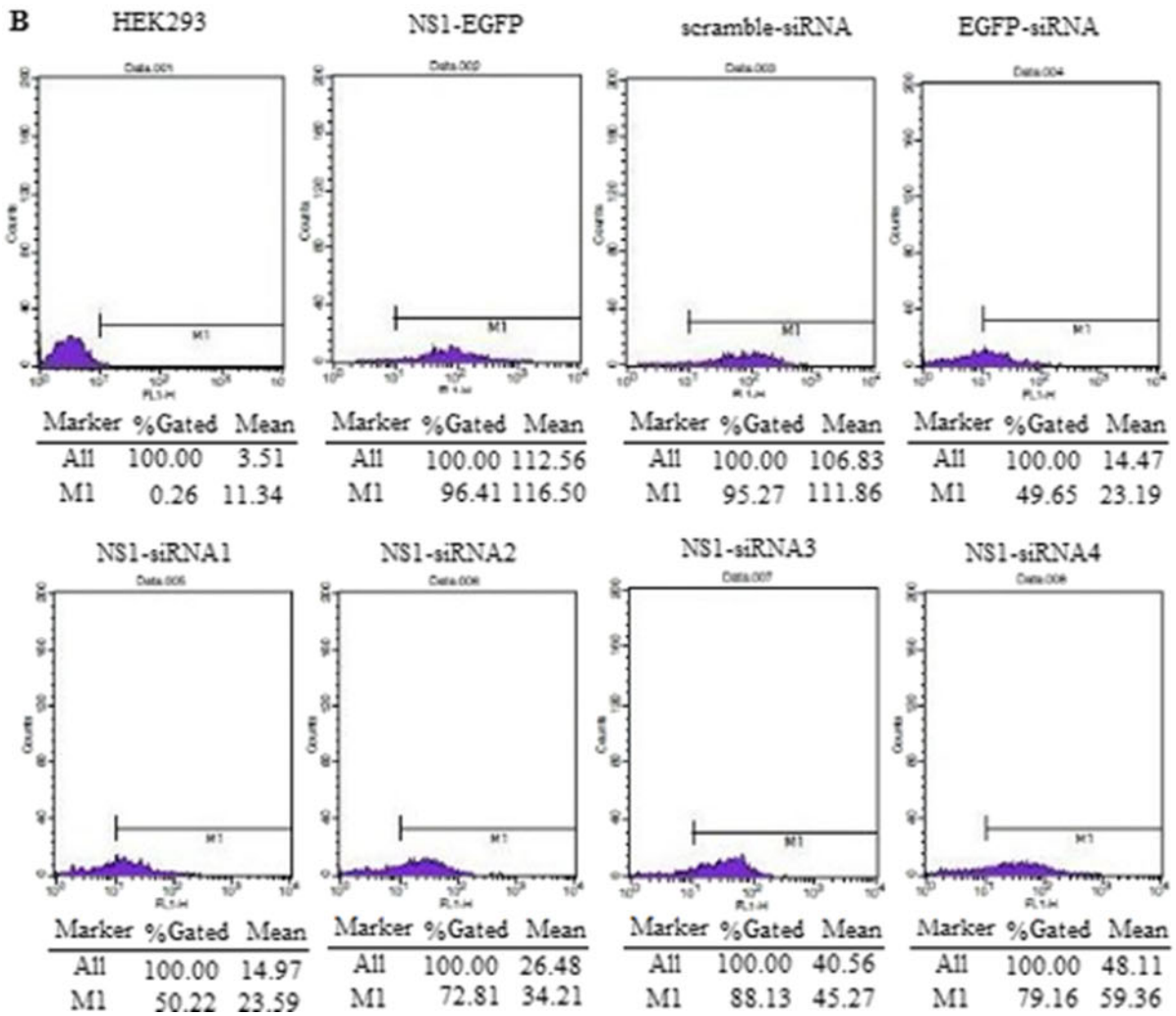
B
HEK293
NS1-EGFP
scramble-siRNA
EGFP-siRNA
Marker %Gated Mean
All 100.00 3.51
M1 0.26 11.34
Marker %Gated Mean
All 100.00 112.56
M1 96.41 116.50
Marker %Gated Mean
All 100.00 106.83
M1 95.27 111.86
Marker %Gated Mean
All 100.00 14.47
M1 49.65 23.19
NS1-siRNA1
NS1-siRNA2
NS1-siRNA3
NS1-siRNA4
Marker %Gated Mean
All 100.00 14.97
M1 50.22 23.59
Marker %Gated Mean
All 100.00 26.48
M1 72.81 34.21
Marker %Gated Mean
All 100.00 40.56
M1 88.13 45.27
Marker %Gated Mean
All 100.00 48.11
M1 79.16 59.36

NS1-EGFP knockdown by siRNAs

The percentage of fluorescent cell population and mean fluorescent intensity reflects the expression level of NS1-EGFP fusion protein, which could be used for the assessment of the inhibition effects of siRNAs on NS1. To analyze whether siRNAs could effectively inhibit NS1-EGFP expression in NS1-EGFP expressing stable cell lines, the fluorescence of the HEK293 stable cell lines was examined under fluorescence microscope at 24, 48, and 72 h post-transfection. Based on the different fluorescence intensity, NS1-siRNA1, NS1-siRNA2, NS1-siRNA3, and NS1-siRNA4 were screened, and microscopic examination revealed that at 48 h post-transfection, the weakest fluorescence was observed in the HEK293 cells transfected with EGFP-siRNA or NS1-siRNA1. Compared with the cells transfected with scramble siRNA, which were used as negative control, the distinct reduction of the percentage of fluorescent cell population and fluorescent intensity of the cells treated with NS1-siRNA1, NS1-siRNA2, NS1-siRNA3, and NS1-siRNA4 was observed, and the inhibition effects of NS1-siRNA1 were the strongest (Fig. 2a).

To further confirm the fluorescence observation results, FCM assay was performed to assess the percentage of fluorescent cell population and mean fluorescent intensity of stable cells transfected with siRNAs, and the data revealed that the fluorescent cell percentage and mean fluorescent intensity of stable cell transfected with NS1-siRNA1, NS1-siRNA2, NS1-siRNA3, and NS1-siRNA4 were 50.22, 23.59; 72.81, 34.21; 88.13, 45.27; and 79.16, 59.36, respectively, and compared with the reduction fold of positive control (1.94, 5.02) and that of negative control (1.02, 1.04). The reduction folds of the percentage of fluorescent cell population were 1.92, 1.32, 1.09, and 1.22, respectively, and the reduction folds of the mean fluorescent intensity were 4.94, 3.41, 2.57, and 1.96, respectively, among which, the silencing effects of NS1-siRNA1 were the strongest (Fig. 2b). These data suggested that specific siRNAs targeting NS1 could silence NS1-EGFP fusion protein expression effectively.

Inhibition of NS1 mRNA accumulation by siRNAs

Real-time quantitative RT-PCR was performed to quantify NS1 transcription level based on the use of SYBR Green in stable cells transfected with siRNAs. Figure 3 depicted the results obtained by qRT-PCR: compared with the inhibition efficiencies of positive control and that of negative control, the inhibition effects of NS1-siRNA1 on NS1 mRNA accumulation were the strongest among four siRNAs, and that of NS1-siRNA3 were the weakest.

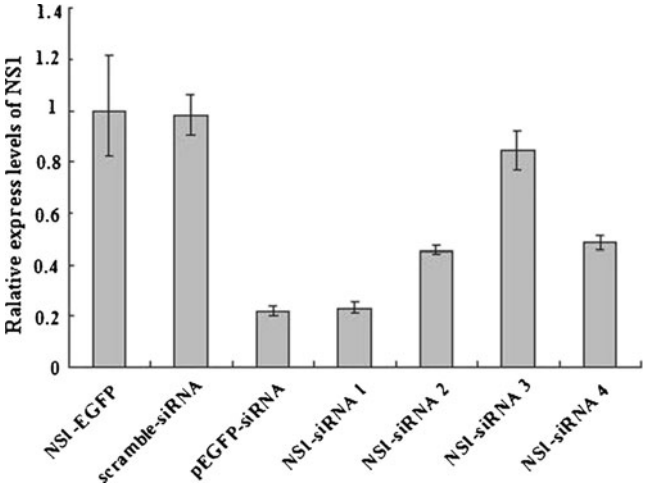

Fig. 3 Relative expression levels of NS1 mRNA in NS1-EGFP expressing stable cells transfected with siRNAs

Inhibition of NS1 protein expression by siRNAs

Western blot was performed on equal amounts of protein harvested from mock or siRNAs transfected stable cell lines at 48 h after transfection using mouse monoclonal antibodies against NS1. GAPDH was used as loading control. The results indicated that NS1 protein expression was reduced in NS1-

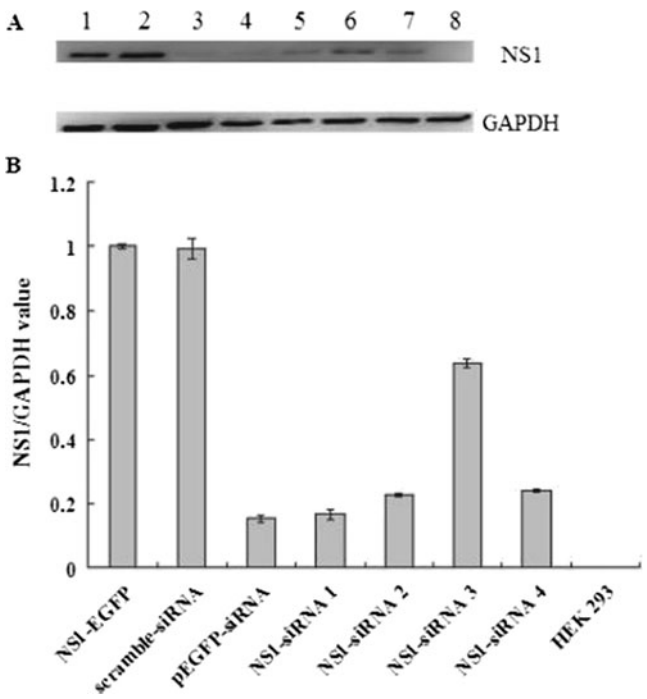

Fig. 4 Inhibition effects of NS1 specific siRNAs on expression of NS1-EGFP fusion protein with Western blot using mouse monoclonal antibodies against NS1. **a** Inhibition effects of NS1 specific siRNAs on expression of NS1-EGFP fusion protein (*1*, without siRNA; *2*, scramble-siRNA; *3*, EGFP-siRNA; *4*, NS1-siRNA1; *5*, NS1-siRNA2; *6*, NS1-siRNA3; *7*, NS1-siRNA4; *8*, HEK293). **b** The quantification with BandScan 5.0 (n=3)

EGFP expressing stable cells transfected with NS1-siRNA1, NS1-siRNA2, NS1-siRNA3, and NS1-siRNA4, and the inhibition effects of NS1-siRNA1 was the strongest, which were more effective than NS1-siRNA2, NS1-siRNA3, and NS1-siRNA4, and which were keeping with the results of FCM analysis and qRT-PCR assay. In addition, all these siRNAs had no effect on the expression of GAPDH (Fig. 4a, b).

Discussion

RNAi is a cellular process regulating gene expression and participating in innate defense in many organisms, and is being developed as a therapeutic strategy for viral diseases (Cullen 2002). siRNAs specific for NP or PA of AIV genome can potently inhibit AIV production in both cell lines and embryonated chicken eggs (Ge et al. 2003). siRNAs specific for M2 and NP genes of H5N1 AIV could effectively inhibit expression of the corresponding viral protein, and these siRNAs could specifically inhibit AIV replication in Madin–Darby canine kidney (MDCK) cells, and significantly reduced lung virus titers in the infected mice and partially protected the mice from lethal AIV challenge (Zhou et al. 2007). siRNA targeting AIV M2 gene potently inhibited viral replication, and not only effective for H1N1 virus but also for highly pathogenic AIV H5N1 (Sui et al. 2009). siRNAs targeting PA could efficiently inhibit AIV H5N1 replication (Zhang et al. 2009).

The NS1 protein of AIV is comprised of two domains: an N-terminal RNA-binding domain (amino acids 1 to 73) and a C-terminal effector domain (amino acid 74 to the C terminus), which binds several host proteins (Golebiewski et al. 2011; Liu et al. 2010). Since NS gene of influenza encodes NS1 and nuclear export protein (NEP) by different mechanisms, our results suggested that NS1-siRNA1 (the start site is 299), which had the strongest silencing effects, is in C-terminal effector domain and has no effects on NEP protein.

The NS1 protein plays a major role in countering the innate immune response to AIV infection, largely by preventing IFN response (Spesock et al. 2011). The knockdown assay of NS1 with siRNAs might become a promising way to activate IFN response.

Fluorescent observation of EGFP and NS1-EGFP expressing stable cell indicated that the fluorescent intensities of EGFP expressing stable cell were stronger than that of NS1-EGFP expressing stable cell significantly; the reason might be the expression level of NS1-EGFP fusion protein was lower than that of EGFP alone.

Based on the different fluorescence intensity at 24, 48, and 72 h after transfection, NS1-siRNA1, NS1-siRNA2, NS1-siRNA3, and NS1-siRNA4 were screened. Microscopic observation of the green fluorescence indicated that the inhibition effects of siRNAs could be detected at 24 h after siRNA transfection, but at 48 h post-transfection, the weakest fluorescence was observed in the HEK293 cells transfected with NS1-siRNA1. In addition, there were so many cells that it was very difficult to obtain a clear picture at 72 h post-transfection, and it was also impossible to observe the inhibition effects of siRNAs. So, it is at 48 h post-transfection that the samples were collected for the inhibitor effect analysis of siRNAs.

Off-target effects, efficient delivery, and stability are major obstacles of in vivo examination of siRNAs. To overcome these disadvantages, recently, various improved siRNAs have been developed, and nanoparticle-based siRNA delivery systems are being tested (Bryan and Steven 2009; Lu et al. 2011).

In conclusion, the results in the study support a role of AIV NS1 specific siRNAs inhibiting AIV NS1 mRNA accumulation and protein expression in vitro, which might play an important role as a molecular tool in anti-viral infection. However, further study concerning NS1-siRNA1's mechanistic role in the process of inhibiting AIV NS1 expression and in vivo assays are both required and warranted.

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