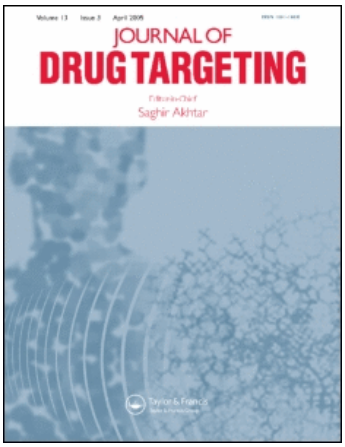

Journal of Drug Targeting
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640314

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Congsheng Cheng [ab]; Lihong Yao [b]; Aijun Chen [b]; Runqing Jia [b]; Lifang Huan [b]; Jianqiang Guo [b]; Hong Bo [a]; Yuelong Shu [a]; Zhiqing Zhang [b]
[a] State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, P.R. China [b] State Key Laboratory for Molecular Virology and Genetic Engineering, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, P.R. China

Online Publication Date: 01 February 2009

To cite this Article Cheng, Congsheng, Yao, Lihong, Chen, Aijun, Jia, Runqing, Huan, Lifang, Guo, Jianqiang, Bo, Hong, Shu, Yuelong and Zhang, Zhiqing(2009)'Inhibitory effect of small interfering RNA specific for a novel candidate target in PB1 gene of influenza A virus',Journal of Drug Targeting,17:2,133 — 139

To link to this Article: DOI: 10.1080/10611860802473048

URL: http://dx.doi.org/10.1080/10611860802473048

Journal of Drug Targeting, February 2009; 17(2): 133–139

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RESEARCH ARTICLE

Inhibitory effect of small interfering RNA specific for a novel candidate target in PB1 gene of influenza A virus

CONGSHENG CHENG[1,2], LIHONG YAO[2], AIJUN CHEN[2], RUNQING JIA[2], LIFANG HUAN[2], JIANQIANG GUO[2], HONG BO[1], YUELONG SHU[1], & ZHIQING ZHANG[2]

[1]*State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100 Yingxin Jie, Xuanwu District, 100052 Beijing, P.R. China, and* [2]*State Key Laboratory for Molecular Virology and Genetic Engineering, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100 Yingxin Jie, Xuanwu District, 100052 Beijing, P.R. China*

(*Received 28 May 2008; accepted 11 September 2008*)

Abstract
Influenza, mainly caused by influenza virus, is becoming one of the major concerns in the world. Limitation in vaccines necessitates the urgent development of new therapeutic options against this virus. In the present study, we designed small interfering RNA (siRNA) targeting overlapping gene of PB1 and PB1-F2 gene of the influenza A virus and investigated its effect against influenza A virus infection. A reduction in virus-associated cell apoptosis was observed in A549 cells treated with this siRNA. Furthermore, its antiviral effect was confirmed by different methods. Also, a marked decrease of virus titer in chicken embryos treated with the siRNA was observed. The findings of this work highlight the potential of this shared region to be an additional therapeutic target for the treatment of influenza virus infection.

Keywords: *Influenza A virus, small interfering RNA, PB1, PB1-F2*

Introduction

Influenza A virus is known to be the major pathogen of influenza, one of the most common and important respiratory diseases affecting all ages (Taubenberger and Morens 2007). With the prospect of another pandemic influenza fresh in our consciousness, there are more serious concerns about the measures available to control an avian or human pandemic of influenza A. Although vaccines provide protection against influenza virus, the vaccine strains have to be changed almost every year due to the antigen shift and drift in virus surface protein (Fedson 2003). The advantage of anti-influenza drugs in influenza control is their independence with the antigenic changes in the virus, which means that they can be used against emerging strains for which no vaccines are available. Therefore, the spread of virus in the body can be slowed down and enough time may be acquired for host cells initiating immune system. For these reasons, antivirals constitute an important element in the global campaign against seasonal flu and potential pandemic. The main problem with the present antivirals is the emergence of resistance through point mutations in viral genes (De Clercq 2006). New drugs are urgently needed for the preparedness of seasonal and pandemic flu.

RNAi, a powerful functional genomics tool, seems to be a promising way to explore new antiviral agents. It is a universal phenomenon of post-transcriptional gene silencing which is initiated by double-stranded RNA and leads to specific degradation of homologous

Correspondence: Y. Shu, State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100 Yingxin Jie, Xuanwu District, 100052 Beijing, P.R. China. Tel/Fax: 86 10 63580764. E-mail: yshu@cnic.org.cn; Z. Zhang, State Key Laboratory for Molecular Virology and Genetic Engineering, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100 Yingxin Jie, Xuanwu District, 100052 Beijing, P.R. China. Tel: 86 10 63519655. Fax: 86 10 63580764, 86 10 63532053. E-mail: zhang_zq@hotmail.com

ISSN 1061-186X print/ISSN 1029-2330 online
DOI: 10.1080/10611860802473048

RNAs. This process involves the generation of 21-nucleotide small interfering RNAs (siRNAs), which in association with RISC are used as guides to target specific RNA substrate. Such RNAs can lead to the degradation of targeted sequences when introduced directly into mammalian cells (Elbashir et al. 2001). In recent years, RNAi has been called on to silence deviant or disease-causing genes, such as viral genes or oncogenes. Recently, siRNAs have been used to treat respiratory syncytial virus infection and progressed into phase II clinical trial (De Clercq 2006). All these pave the way for siRNA-based anti-influenza drugs for clinical applications.

PB1 is the product of the second segment of influenza A virus genome and functions as the "classic polymerase" responsible for polymerization and endonuclease. Recently, a novel protein encoded by an alternate reading frame in the PB1 gene segment of influenza A virus has been discovered and shown to enhance viral virulence in a mouse model (Chen et al. 2001; Zamarin et al. 2006). In this study, we designed siRNA targeting highly conserved sequence shared by PB1 and PB1-F2 within the type A influenza virus genomes, and tried to find out whether this siRNA could inhibit virus replication both *in vitro* and *in vivo*.

Materials and methods

Cells and virus

Madin Darby Canine Kidney (MDCK) cell lines and A549 cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Influenza A/CNIC/246/2000 (H1N1) virus was isolated by the Chinese National Influenza Center, passaged, and adapted in A549 and MDCK cells prior to use.

Design and synthesis of siRNA

siRNAs were rationally designed according to new strategies (Birmingham et al. 2007). The candidate siRNAs scored five or more were selected and subjected to a BLAST search against GenBank to ensure that they are unique to influenza virus genome sequences only. Based on the panels of siRNA transfection, we select one conserved site in H5N1 and H1N1 influenza virus genome: PB1-259 (5′-CCAAGTGGCTATGCC-CAAA-3′) for further study. As a control, siRNA with the sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used. All siRNAs were synthesized by Guangzhou RiboBio Co., Ltd (Guangzhou, China), and stored at −70°C before use.

Cell transfection

siRNA transfection was performed according to the instructions in the HiPerFect Transfection Reagent Handbook. The optimized conditions were: 2×10^5 cells, 30 μl HiPerFect Transfection reagent (Qiagen, Valencia, CA, USA), and 1.25 μl (50 nM) siRNA in each well of a 6-well plate. The efficiency of cell transfection was monitored by co-transfection of 1.25 μg pEGFP-N1 plasmid and 50 nM siRNA targeting Green Fluorescent Protein (GFP) gene and observed under fluorescent microscopy 12 h after transfection.

Viral infection and titration

Twenty-four hours after siRNA transfection, the cells were infected with influenza A virus as described previously (Turpin et al. 2005). Briefly, A549 cells in a 6-well plate (Falcon, Becton Dickinson, San Jose, CA, USA) were challenged with A/CNIC/246/2000 virus at a multiplicity of infection of 0.1 plaque-forming units (PFU)/cell. After absorption at 37°C for 1 h, the cells were washed three times with phosphate-buffered saline (PBS) to remove unabsorbed viruses, and then cultured in serum-free Dulbecco's Modified Eagle Medium (MEM) supplemented with 1.0 μg/ml TPCK trypsin (Sigma, St Louis, MO, USA) at 37°C. After 48-h incubation, the cells were frozen and thawed for three cycles, and clarified by low-speed centrifugation (500*g* for 10 min). The supernatants were collected for the determination of virus yield by hemagglutination assay (Gubareva et al. 1998). Triplicate cultures were used so as to perform statistical analysis.

Quantification of viral genomic RNA by quantitative real time RT-PCR

Antiviral activities were also assessed by quantitative real-time RT-PCR (Sequence Detection System 7700; Applied Biosystems, Foster City, CA, USA). Forty-eight hours post-infection, viral RNA extraction and RT-PCR were performed as described (Cheng et al. 2008). Experiments were performed with 500 ng total RNA in a reaction volume of 50 μl, using the *TaqMan* One-Step RT-PCR Master Mix Reagent (Applied Biosystems). Primer and probe sequences specific for the PB1 gene include 5′-AGTACTCAGAAAGAGG-AAGATGGAC-3′ (forward primer), 5′-AATACAC-AATCTGTTTGGGCATAGC-3′ (reverse primer), and 5′-(FAM) ACCGAAACTGGAGCACCGCAA-CTCA-3′ (probe). The total RNA amount was corrected by performing a parallel real-time RT-PCR experiment targeting the human β-actin gene as an internal control. The primer and probe sequences for β-actin include 5′-GCGCGGCTACAGCTTCA-3′ (forward primer), 5′-TCTCGTTAATGTCACGCA-CGAT-3′ (reverse primer), and 5′-FAM-CACCAC-GGCCGAGCGGGA-3′ (probe). And β-actin RNA was used to generate a standard.

Cell apoptosis assay

siRNA transfection and virus infection were performed as above. To evaluate the inhibition effect of siRNA on PB1-F2, cell apoptosis assay was performed. Briefly, 15-h post-infection, A549 cells (2×10^5) were double stained with Annexin V-FITC and propidium iodide (PI). The cells were washed with PBS and resuspended in 200 μl Annexin V-binding buffer [10 mM Hepes/-NaOH (pH 7.4), 150 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM KCl] supplemented with 4 μg/ml Annexin V-FITC (Nanjing KeyGen Biotechnology Co., Ltd, Nanjing, China). After 10-min incubation in the dark, the cells were washed once before the addition of 1 μg PI/ml of cell suspension and incubated for 10 min in the dark. Single staining using Annexin V-FITC or PI alone was performed as controls (Vermes et al. 1995). Flow cytometry analysis was performed using a FACSstar® plus flow cytometer (Becton Dickinson) equipped with Lysis II software.

Western blot

To determine whether this siRNA could down–regulate PB1 protein expression, virus from cell supernatants 72-h post-infection was concentrated. Total protein was then quantified using the Bradford assay (Bio-Rad, Philadelphia, PA, USA). Equal amounts of protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were then transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk containing 0.1% Tween 20 for 1 h. The blots were probed with primary goat antibody against PB1 protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse antibody against β-actin (Santa Cruz) for 1 h, followed by incubation with different horseradish peroxidase-conjugated secondary antibodies. Finally, PB1 and β-actin expression were detected by TMB reagents (Promega, Madison, WI, USA).

Antiviral activity of the siRNA in chicken embryos

Inhibitory effects of PB1 siRNA against influenza A virus was determined in eggs as described (Ge et al. 2003). For each egg, 30 μl Oligofectamine (Invitrogen) was diluted with 30 μl Opti-MEM (GIBCO, Gaithersburg, MD, USA). 2.5 nmol (10 μl) siRNA was mixed with 30 μl Opti-MEM and added to diluted Oligofectamine, and the mixture was incubated at room temperature for 30 min. The mixture was then combined with 100 μl influenza virus strain A/CNIC/246/2000 (5000 PFU/ml) and immediately injected into the allantoic cavity of a 10-day embryonated chicken egg. The eggs were incubated at 35°C for 17 h and the allantoic fluid was harvested for plaque reduction assay.

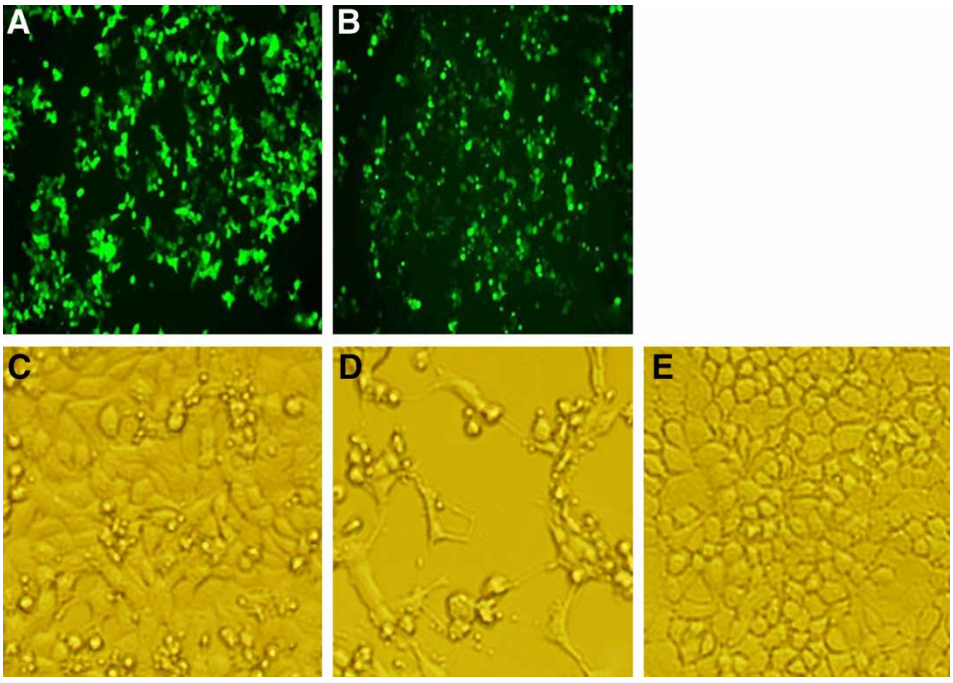

Figure 1. Microscopic observation of transfection efficacy and CPE of virus-infected cells. A549 cells were (A) transfected with pEGFP-N1 plasmid alone or (B) co-transfected with pEGFP-N1 and siRNA specific to GFP gene. Quenching of fluorescence was detected (B) 12-h post-infection, which demonstrated effective transfection condition. (C) Then, A549 cells were transfected with siRNA of PB1 under the same conditions above and were infected with A/CNIC/246/2000 (H1N1) virus. (A) The supernatants were inoculated to MDCK cells. As controls, the cells transfected with control siRNA and challenged with virus showed CPE (D), while the cells without virus infection showed no CPE (E).

Results

siRNA targeting overlapping region attenuate influenza A virus infection in vitro

Since PB1 siRNA could reduce virus-associated cell apoptosis, the studies were carried out to determine whether it has potential as an antiviral agent. We first examined the transfection efficiency by siRNA targeting GFP gene in A549 cells. At 12-h post-transfection, the expression level of GFP was down-regulated (Figure 1(A),(B)). Then A549 cells were transfected with siRNA targeting overlapping gene followed by infection with influenza virus strain A/CNIC/2000/246. Twenty-four hours later, the supernatants were collected and inoculated on MDCK cells. Cytopathic effect (CPE) was evaluated as the indication of virus replication. A significant reduction in CPE (~75%) was observed in PB1 siRNA-treated group, while control siRNA showed no reduction in CPE (Figure 1(C)–(E)).

Since siRNA functions by degrading RNAs that share sequence homology, we next examined the effect of siRNA on the abundance of viral genomic RNA in virus-infected cells by using quantitative real-time RT-PCR. The relative viral genome copy numbers revealed that the viral genomic RNA was able to achieve a >60% reduction. By contrast, control siRNA showed no inhibitory effect on viral genomic RNA. This result demonstrated the PB1 siRNA target specifically viral genomic RNA and abolishes virus replication in cultured cells (Figure 2(A),(B)).

We further evaluated the effect of PB1-silencing siRNA on virus replication by HA assay. We collected the supernatants from siRNA-treated influenza A virus infected A549 cells 72-h post-infection, and determined the virus titer by hemagglutination assay.

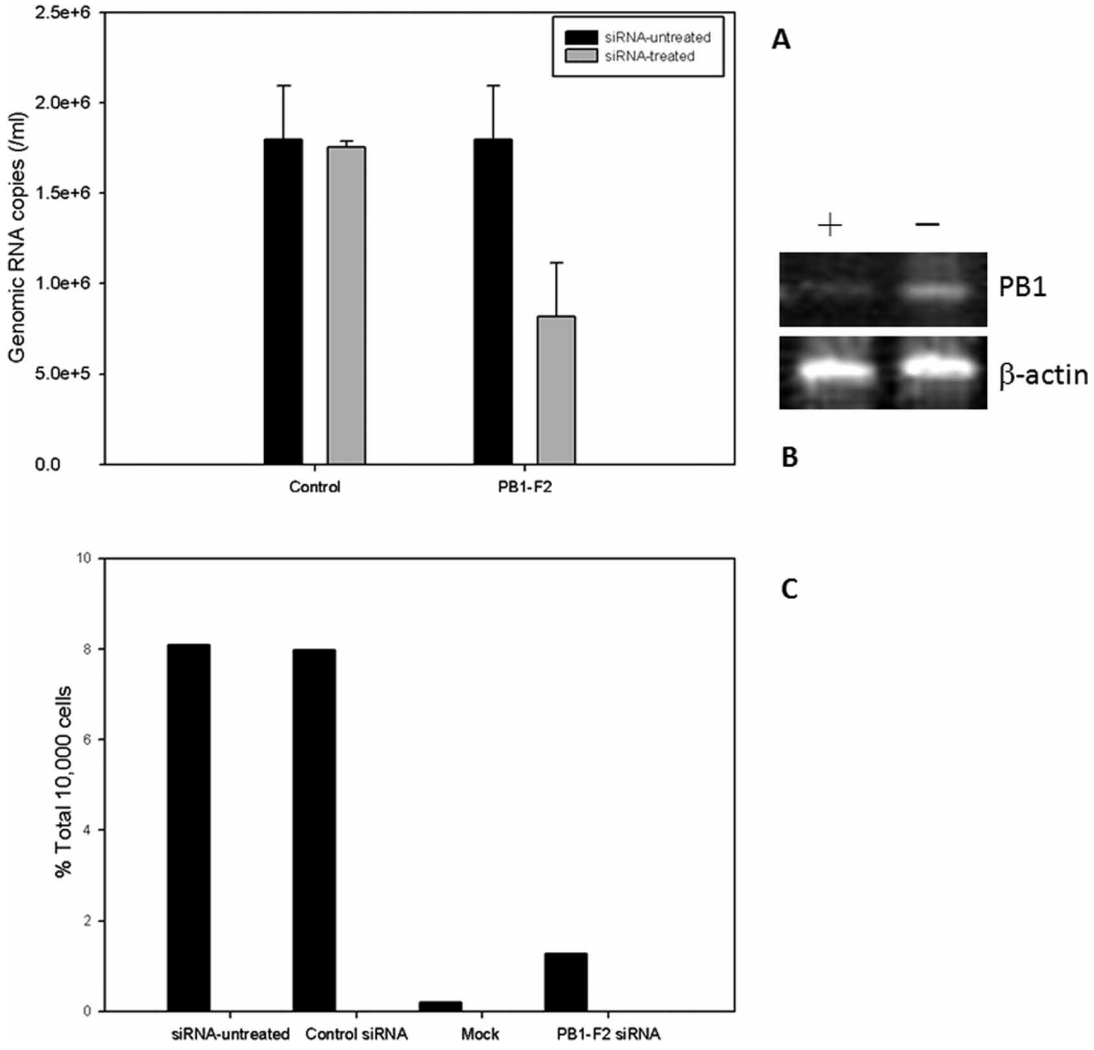

Figure 2. Inhibition of virus replication *in vitro* by siRNA targeting the overlapping gene. A549 cells were transfected with PB1 siRNA or control siRNA and challenged with H1N1 virus. Forty-eight hours later, virus loads were investigated by quantitative real-time RT-PCR with β-actin as an internal control. (A) A decrease in RNA copies is indicative of inhibitory effect of siRNA. (B) The RT-PCR products were visualized in agarose gel electrophoresis. (C) The virus titer was determined 72-h post-infection by hemagglutination assay and a dramatic reduction in virus yield was observed after treatment of PB1 siRNA.

As a control, untreated influenza virus-infected cells were also included. The results indicated that the cells transfected with the control siRNA did not exhibit any reduction in virus titer compared with untreated virus-infected A549 cells. However, a reduction of 84% in virus titer was observed in the cells treated with PB1 siRNA (Figure 2(C)).

siRNA targeting overlapping region of PB1 and PB1-F2 can inhibit virus-induced cell apoptosis and reduce target protein production

As the targeted sequence is shared by PB1-F2 and PB1, and the former has a number of unique features, including apoptotic or proapoptotic properties, we herein investigated its putative role in virus-induced apoptosis in epithelial cell lines by RNAi method. A549 cells were transfected with PB1 siRNA followed by challenging with H1N1 influenza A virus. To eliminate background apoptosis aroused by siRNA or transfection reagents, a series of control were set up. As shown in Figure 3, untreated or control siRNA-treated cells showed no obvious apoptosis. However, apoptosis in PB1 siRNA-treated cells decreased by $\sim$20% after virus challenge, while control siRNA-treated cells demonstrate dramatic apoptosis.

As the chosen siRNA targeting both PB1-F2 and PB1 effectively reduced virus-associated apoptosis, the next question was whether these siRNAs would be able to specifically reduce PB1 proteins that are produced during infection. Equal amounts of siRNA-treated and control siRNA-treated samples were analyzed by the western blot. Using β-actin as an internal control, it can be observed that the expression level of PB1 protein could be diminished by the specific siRNA (Figure 4).

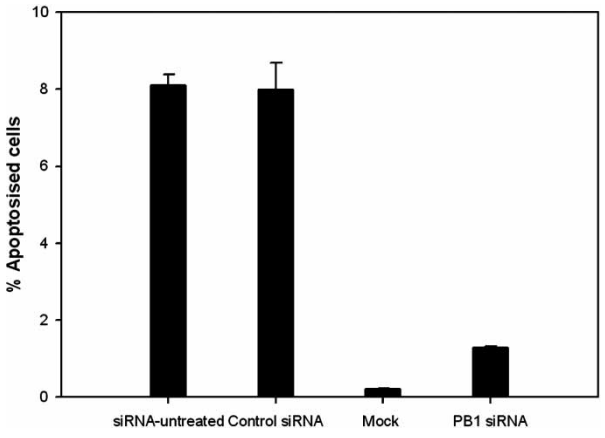

Figure 3. Reduction of virus-associated cell apoptosis by PB1 siRNA. A549 cells were transfected with PB1 siRNA or control siRNA and challenged with H1N1 virus. The cells were digested and collected from 6-well plates for apoptosis analysis using Annexin V-FITC dual staining kit 15-h post-infection. As controls, the cells transfected with control siRNA (mock group) and infected with virus (no siRNA-treated) were also included.

siRNA abolish virus infection in embryonated chicken eggs

To expand the *in vitro* findings, the antiviral effect of the PB1 siRNA was evaluated in chicken embryos. Each of 10-day-old chicken embryos was treated with 2.5 nmol siRNA and followed by challenging with 100 μl of 5000 PFU/ml influenza virus A. Seventeen hours post-infection, the allantoic fluid was collected for plaque reduction assay on MDCK cells. The PB1 siRNA alone, as well as negative (distilled H_2O) and positive controls (influenza virus only), was included in the injections. As shown in Figure 5, injection of control siRNA did not interfere with virus replication. However, a significant reduction in virus titer was observed in chicken embryos treated with PB1-siRNA ($P < 0.05$). These results were in agreement with the *in vitro* experiments (Figure 5).

Discussion

RNAi is a process of sequence-specific, post-transcriptional gene silencing that is initiated by double-stranded RNA. RNAi appears to be ideal for inhibiting RNA virus, especially influenza virus infection for the reason that their mRNAs, both vRNA and cRNA, could be potential targets for siRNA-mediated degradation (Bennink and Palmore 2004). Second, the influenza virus genome consists of eight segmented RNAs, encoding a total of 11 proteins. Each protein is either an integral component of the viral structure or plays critical role during the virus life cycle. Interfering with the production of any one of them is likely to have severe consequences on viral replication and production (Ge et al. 2004).

To our knowledge, little study is involved in using the overlapping region of PB1 and PB1-F2 as a target to abolish virus infection, although many studies have confirmed siRNA-mediated gene silencing in influenza

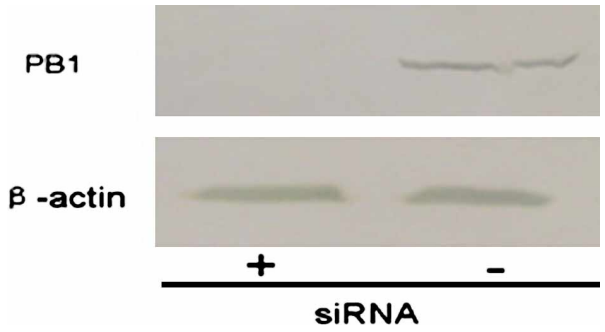

Figure 4. Western blot analysis of viral PB1 protein. Samples from siRNA-treated and control siRNA-transfected groups were run on 10% SDS–PAGE, and then transferred to nitrocellulose membranes for blotting. Cellular β-actin was used as an internal control.

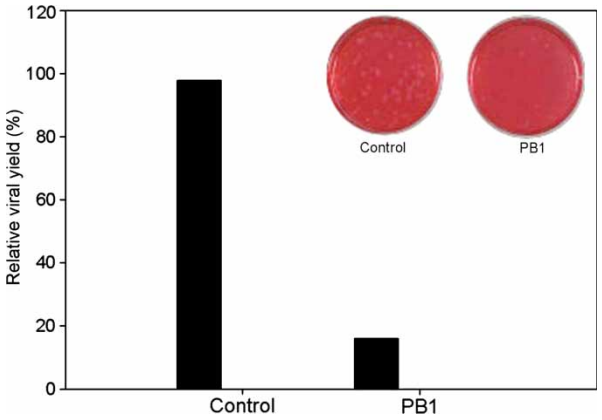

Figure 5. *In vivo* antiviral activity of PB1 siRNA. Embryonated chicken eggs were injected with Oligofectamine containing 30 nmol PB1 siRNA or control siRNA. Seventeen hours later, viruses were titrated on MDCK cells with plaque reduction assay. Virus plaques were counted (right upper) and viral yield was calculated as the ratio of PB1-F2 siRNA against non-specific control.

virus. The aim of this study was to determine whether this shared region can be targeted to inhibit influenza A virus replication and to explore the possibility of this region as new drug target.

We first facilitated screening of active siRNAs by reporter plasmid-containing GFP gene. Low expression of GFP protein by siRNA was achieved in the preliminary study. Owing to these results, we have identified some effective siRNAs against H5N1 and H1N1 influenza A virus (data not shown) and presented here against PB1 for further study. PB1 is known to be part of influenza virus RNA polymerase and plays an important role in both transcription and replication of the RNA genome. PB1-F2 was found to be translated from an alternative (+1) ORF in the polymerase PB1 gene (nt 120–381). Previous studies demonstrated that the exposure of cells to a synthetic version of PB1-F2-induced apoptosis and resulted in variably sized pores in planar lipid membrane (Chen et al. 2001; Chanturiya et al. 2004). In this study, the influence of siRNA on virus-induced apoptosis was investigated and the siRNA was shown to inhibit apoptosis in A549 cells. The result partly confirmed previous reports on the role of PB1-F2 in virus-induced cell apoptosis. Recent studies have also confirmed the role of PB1-F2 in pathogenesis (Coleman 2007; McAuley et al. 2007). We hypothesized that the encoded gene could be explored as novel drug targets to clear virus infection.

For this reason, we picked up this siRNA for further study and sought to determine whether the siRNA is shown to be most efficient in inhibiting the virus in cell culture. Virus CPE indicated that this siRNA could inhibit virus production. In order to verify whether virus production from siRNA-treated cells is reduced accordingly, viral particles were titrated in the supernatants from the cells transfected with siRNA and infected with influenza virus, both by real-time quantitative RT-PCR, and western blot, and by the determination of virus hemagglutinin titer. Because RNA was detected 24-h post-infection, we took this RNA as viral RNA from new virion and obvious decrease was observed compared with control siRNA-transfected group. This inhibitory effect was confirmed at the protein level as shown in western blot. Abolishment of the virus infection was also confirmed by hemagglutinin assay, which indicated that this siRNA could specifically inhibit virus replication *in vitro*.

We then addressed the question of antiviral activity of the siRNA in chicken embryos. Treatment with the siRNA resulted in a substantial reduction in virus titer as determined by plaque reduction, suggesting that influenza virus infection can be blocked by siRNA targeting the gene overlap. Recent study has shown that PB1-F2 served as an indirect regulator of the influenza virus polymerase activity via its interaction with PB1 (Mazur et al. 2008). Lack of PB1-F2 during infection resulted in an altered localization of PB1 and decreased viral polymerase activity and mutant viruses devoid of a functional PB1-F2 reading frame exhibited a small plaque phenotype. As a result, the dramatic reduction in cell apoptosis and viral titer after this siRNA transfection is probably due to a mixed effect of reducing the production of both PB1-F2 and PB1 proteins. Thus, this region can be used as a novel candidate target for anti-influenza drug development. Further study should be performed using an expression plasmid-containing PB1-F2 but not the entire virus to study the specific cellular changes upon reduction of PB1-F2 by siRNA treatment.

Recently, it was reported that expression of the influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia (McAuley et al. 2007). If an antiviral antagonist of the PB1-F2 is available, the targeted destruction of professional antigen-presenting cells could be inhibited. Therefore, the ability to clear virus and more importantly to fend off opportunistic bacterial infections would be maintained (Coleman 2007). If the inhibitory effect was contributed mainly by PB1-F2, our investigation here may aid the development of new drugs against influenza virus.

Acknowledgements

This work was supported by the National Key Technology R&D Program of China (2006BAD 06A15) and National Natural Science Foundation of China (Key project 30599433).

Declaration of interest: The authors report no conflicts of interest.

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