HHS Public Access

Author manuscript

Int J Pharm. Author manuscript; available in PMC 2023 January 25.

Published in final edited form as:

Int J Pharm. 2022 January 25; 612: 121325. doi:10.1016/j.ijpharm.2021.121325.

A new self-attenuated therapeutic influenza vaccine that uses host cell-restricted attenuation by artificial microRNAs

Ke Wen[a,1], **Haiyan Wang**[a,b,1], **Yanping Chen**[a], **Huixiao Yang**[a,b], **Zhichao Zheng**[a,b], **Yongyong Yan**[a,b], **Realivazquez Adilene**[a,c], **Mingtao Zeng**[a,c,*]

[a]Center of Emphasis in Infectious Diseases, Department of Molecular and Translational Medicine, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center El Paso, El Paso, Texas 79905, USA

[b]Affiliated Stomatology Hospital of Guangzhou Medical University, Guangzhou Key Laboratory of Basic and Applied Research of Oral Regenerative Medicine, Guangzhou, Guangdong 510182, China

[c]Graduate School of Biomedical Sciences, Texas Tech University Health Sciences Center El Paso, El Paso, Texas 79905, USA

Abstract

New strategies are urgently needed for developing vaccines and/or anti-viral drugs against influenza viruses, because antigenic shift and drift inevitably occurs in circulating strains each year, and new strains resistant to anti-viral drugs have recently emerged. In our study, we designed and incorporated artificial microRNAs (amiRNAs) into the NA segment of rescued influenza viruses to separately target two host genes, Cdc2-like kinase 1 (*CLK1*) and SON DNA binding protein (*SON*), which were found to play an essential role in virus replication. Mouse epithelial fibroblast (MEF) or human lung carcinoma A549 cells infected with engineered influenza PR8 viruses expressing amiR-30CLK1 (PR8-amiR-30CLK1) or amiR-93SON (PR8-amiR-93SON) had reduced expression of host proteins CLK1 and SON, respectively. All engineered influenza viruses functioned as attenuated vaccines, induced significantly higher antibody responses, and provided greater protective efficacy. In addition, they were found to be safe, based on the mouse weight changes and clinical signs observed. In contrast to the engineered viruses targeting SON, mice treated with engineered viruses targeting CLK1 recovered from weight loss and survived

*Corresponding author: Mingtao Zeng, Ph.D., Tel: +1 915-215-4244; Fax: +1 915-783-1271; mt.zeng@ttuhsc.edu.

[1]Co-first authors.

CRediT authorship contribution statement

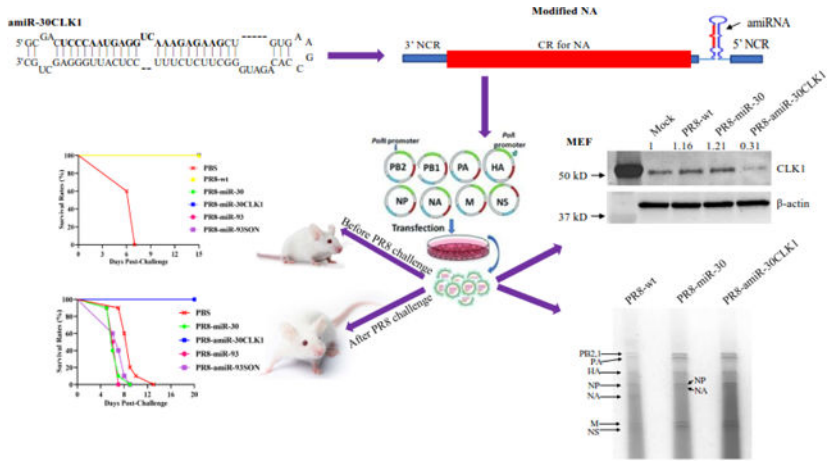
Ke Wen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original and revised draft. **Haiyan Wang:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Yanping Chen:** Data curation, Investigation, Methodology. **Huixiao Yang:** Data curation, Investigation. **Zhichao Zheng:** Data curation, Investigation. **Yongyong Yan:** Data curation, Investigation. **Realivazquez Adilenea:** Data curation, Investigation. **Mingtao Zeng:** Conceptualization, Supervision, Formal analysis, Investigation, Writing – review & editing.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

lethal infection by 6 h after lethal-dose PR8 infection, suggesting that our PR8-amiR-30CLK1 self-attenuated influenza virus (SAIV) could be used as a new therapeutic influenza vaccine.

Graphical Abstract



Keywords

artificial microRNA; Cdc2-like kinase 1; intranasal delivery; live attenuated influenza vaccine; SON DNA-binding protein; therapeutic vaccine

1. Introduction

Influenza is a leading cause of morbidity and mortality around the world, with seasonal viruses affecting up to 15% of the human population, causing severe illness in 3–5 million people and fatalities of ~500,000 individuals each year [1]. Influenza A viruses (IAVs) are the most virulent human pathogens among the four influenza types and are negative-sense, single-stranded, segmented RNA viruses. Currently, vaccines and antivirals are used in prophylactic and therapeutic strategies, respectively, to treat influenza viruses. There are two major classes of vaccines used for prevention of IAV infection: inactivated influenza vaccines (IIVs) and live attenuated influenza vaccines (LAIVs), each with potential shortcomings. For IIVs, boosters are required for enhancing efficacy and protection in both humans and animals [2]. A major shortcoming of LAIVs is the potential for reversion to virulence through point mutations, recombination, or reassortment [3–6]. In order to improve protection, influenza vaccines must be reformulated annually to match the circulating strains, although there are always strains unaccounted for. For antivirals, several drugs are available, including the M2 ion channel inhibitors, amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir [7, 8]. Drug-resistant viruses [9, 10] have suddenly emerged since the introduction of antiviral drugs, and the use of antiviral drugs may also have effects on population vulnerability due to lack of seroconversion [11]. Given that an influenza pandemic has been widely predicted as a result of these trends, there is an urgent need to develop new vaccines and antiviral drugs through novel strategies that provide higher protection and efficacy.

Recent research progress in molecular biology technologies, such as RNA interference (RNAi) and CRISPR/Cas9 knockout screening [12–15], has significantly accelerated research and development into new influenza vaccines and antiviral drugs. For example, many host genes that are critical for influenza replication and involved in almost every stage of influenza replication have been identified [16–20]. In our current study, two candidate host genes, Cdc2-like kinase 1 (CLK1) and SON DNA binding protein (SON), were chosen, since both were found to play an essential role in replication of influenza viruses [18]. CLK1 was demonstrated to be responsible for alternative splicing of the M2 gene of influenza viruses during influenza replication [18, 19, 21], and SON was found to be involved in influenza virus infection [18, 19] by regulating the trafficking of influenza virions to late endosomes. MicroRNAs (miRNAs), utilizing one type of RNAi, are conserved in eukaryotes to post-transcriptionally suppress target gene expression by mRNA cleavage or translational inhibition [22] and are mediated via the interaction between the miRNA seed site (nt 2–8 on the guide miRNA strand) and the 3’ UTR of the target gene [23–25]. Although miRNAs are evolutionarily conserved, a small number are species specific, such as miRNA-93, which is not present in avian cells [26]. With the advances of influenza reverse genetics and miRNA molecular research, influenza viruses have become feasible as vectors to deliver miRNAs in vivo [27]. In our current study, artificial miRNAs (amiRNAs) were designed to target two host genes (CLK1 and SON) by replacing the guide miRNA strands in the miRNA-3 and miRNA-93 backbones and inserting them into the NA segment of influenza virus. Viruses containing amiRNAs were rescued using an eight-plasmid-based reverse genetics system for influenza viruses. The self-attenuated influenza viruses (SAIVs) were expected to express functional mammalian species-specific amiRNAs that inhibit host factors critical for influenza replication and to be used as attenuated vaccines and anti-viral drugs.

2. Material and Methods

2.1. Eggs and cell culture

Embryonated chicken eggs were purchased from Charles River Laboratories (Willimantic, CT) and then incubated at 37.5 °C for up to 9 days for use in the propagation of engineered influenza viruses. MDCK cells (ATCC, cat. no. CCL-34) were cultured in Eagle’s minimum essential medium (EMEM; ATCC, cat. no. 30–2003) supplemented with 10% FBS (Gibco, NY). 293T cells (ATCC, cat. no. CRL-11268), MEF cells (ATCC, cat. no. CRL-2214), and A549 cells (ATCC, cat. no. CCL-185) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, NY) supplemented with 10% FBS, 1% penicillin, and 1 μg/ml streptomycin (Gibco, NY). Cells were cultured at 37°C in 5% CO_2.

2.2. Artificial microRNA design and expression

Mouse CLK1 (NM_001042634.2) and SON (NM_178880) were demonstrated to play an essential role in influenza virus replication [18] and were targeted by our designed artificial microRNAs (amiRNAs) through an online amiRNA designer (http://rnaidesigner.invitrogen.com/rnaiexpress/). Human miR-93 and miR-30 cassettes (Fig. 1A, D) were used as the backbones for the amiRNAs in targeting CLK1 (amiR-30CLK1, Fig. 1B) and SON (amiR-93SON, Fig. 1E), respectively. The original miR-93 and miR-30 were included as negative controls to exclude their functions in virus replication. All miRNAs

and amiRNAs were engineered to encode cleavage sites for two endonucleases (Hpa I and Xho I), one at each end; synthesized by GenScript (Piscataway, NJ); and cloned into the microRNA-expressing plasmid pll3.7 to yield pll3.7-miR-30, pll3.7-amiR-30CLK1, pll3.7-miR-93, and pll3.7-amiR-93SON. For transfection, 1×10^5 293T cells per well were seeded into 6-well plates. The next day, the cells were transfected with 1 μg of pll3.7, pll3.7-miR-30, pll3.7-amiR-30CLK1, pll3.7-miR-93, pll3.7-amiR-93SON, or no plasmid using the transfection reagent TransIT-LT1 (Mirus). Forty-eight hours after transfection, total RNA was extracted with TRIzol® reagent (Thermofisher, cat. no. 15596026) for further processing.

2.3. Virus design, rescue, and titration

The eight-plasmid-based system [28] was used to rescue engineered influenza viruses based on the strain A/Puerto Rico/8-KV20/1934 (H1N1) (PR8). Two endonuclease cleavage sites (HpaI and XhoI) were introduced into pHW186-NA [28] between the NA coding sequence and the 5' NCR for cloning miRNAs or amiRNAs. Rescued engineered viruses were denoted as wild-type PR8 (PR8-wt), PR8 with miR-30 (PR8-miR-30), PR8 with amiR-30CLK1 (PR8-amiR-30CLK1), PR8 with miR-93 (PR8-miR-93), or PR8 with amiR-93SON (PR8-amiR-93SON). Each type of rescued virus was propagated in embryonated chicken eggs and titrated as plaque-forming units (PFU) in MDCK cells using the plaque assay.

2.4. Mammalian cell infection

Cells were seeded in 6-well plates 1 day prior to infection. For the infection, cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS). Cells in one well were detached and quantified and the virus was diluted in serum-free culture medium based on the cell number. The cells in other wells were then infected with virus at an MOI of 0.1. One hour after incubation, the viruses were removed, and the cells were maintained in serum-free culture medium supplemented with TPCK-treated trypsin (Sigma-Aldrich). Forty-eight h after infection, the cells were harvested for extracting cellular RNA and proteins with the TRIzol® reagent (Thermofisher, cat. no. 15596026). RNA was then reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo $(dT)_{18}$ primer.

2.5. Extraction of viral RNA and amplification of specific gene segments

Viral RNA was extracted using the QIAamp Viral RNA kit (Qiagen, Valencia, CA) and then reverse-transcribed to cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with the U12 primer. One microgram of extracted viral RNA was separated on a 4% polyacrylamide gel with urea in TBE buffer for silver staining. The cDNA for viruses or cells was amplified using the Ready PCR Mix (Amresco, Solon, OH) with specific primers as listed in Table 1. Densitometry was performed using Image J analysis software (National Institutes of Health, USA).

2.6. Western blotting

The concentration of protein in the samples was determined using Bradford's method (Kruger, 1994). The samples were loaded on sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked and incubated with the primary antibody, polyclonal rabbit anti-CLK1 (ARP52021_P050) at 4°C overnight. After washing, the membranes were incubated with alkaline phosphatase (AP)-linked goat anti-rabbit IgG (Cell Signaling) at RT for 1 h. The membranes were then washed and developed with chemiluminescent AP substrate before imaging. Densitometry was performed using Image J analysis software (National Institutes of Health, USA).

2.7. Virulence test *in vivo*

BALB/c mice (6–8 weeks old) were purchased from the Jackson Laboratory and randomly divided into groups, with five male mice in each group. For determination of the half mouse-lethal dose (MLD_{50}), PR8 viruses were serially diluted in DPBS, and 50 μl were intranasally inoculated into mice anesthetized by injection with ketamine and xylazine. The MLD_{50} was calculated according to the method of Reed and Muench [29]. After infection, the mice were monitored daily for clinical symptoms, weight loss, and death. Weight loss and survival rates of the remaining mice were observed daily for 21 days. Mice undergoing a weight loss in excess of 30% were euthanized and considered to be at the end of survival. All animal experiments were approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center El Paso.

2.8. *In vivo* study for examining the prophylactic effects of engineered viruses

BALB/c mice (6–8 weeks old) were purchased from the Jackson Laboratory and randomly divided into groups. Each group contained 5 female and 5 male mice, which were intranasally inoculated with 3×10^5 PFU of engineered viruses diluted in 50 μl PBS. Mice were inoculated with a sub-lethal dose (25 PFU) of wild-type PR8 as the positive control and with PBS as the negative control. Blood was collected on days 0, 7, 14, and 21 post inoculation. On day 21 post inoculation, the mice were challenged with $50\times MLD_{50}$ viruses and monitored daily for clinical symptoms, weight loss, and death for 21 days. Mice in excess of 30% weight loss were euthanized in consideration of animal welfare. All animal experiments were approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center El Paso.

2.9. Antibody responses induced by engineered viruses

Antibody titers in sera were determined by ELISA. Four AP-conjugated goat anti-mouse antibodies were used to detect IgA (Bethyl, cat. no. A90–103AP), IgG (cat. no. A90–131AP), IgG1 (cat. no. A90–105AP) and IgG2a (cat. no. A90–107AP). Briefly, 96-well plates were coated with 1 μg/ml PR8 virus hemagglutinin (HA) diluted in 100 μl/well of 50 mM sodium bicarbonate buffer (pH 9.6) overnight at 4°C and then blocked with 300 μl/well of PBS containing 1% BSA for 30 min at RT. The 50-fold-diluted sera in 100 μl/well PBS containing 1% BSA were then added to plates and incubated overnight at 4°C. Commercial mouse sera (Bethyl, cat. no. rs10101) were used as the reference to draw a standard curve for quantifying antibody concentration in sera. After washing, diluted AP-conjugated antibodies

were added to each well (100 μl/well) and incubated for 1 h at RT. The plates were then washed and developed for 20 min with diethanolamine substrate (100 μl/well; KPL, cat. no. 508000). The reaction was then stopped with EDTA stop solution (100 μl/well; KPL), the plates read at 405 nm using the PowerWaveXS2 (Biotek), and the antibody concentrations calculated.

2.10. *In vivo* study for examining the therapeutic effects of engineered viruses

Randomly selected mice (5 female and 5 male in each group) were intranasally infected with 20× MLD_{50} PR8. Six hours later, they were intranasally administered with 3×10^5 PFU of engineered viruses. Mice were then monitored daily for clinical symptoms, weight loss, and death for 21 days. Mice in excess of 30% weight loss were euthanized and considered to be at the end of survival. All animal experiments were approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center El Paso.

2.11. Statistical analysis

Comparisons of antibody concentrations between groups were performed by using nonparametric one-way ANOVA with the Tukey multiple comparison test. The bars for antibody concentrations represent the mean ± standard error of the mean (SEM). Body weight changes are expressed as the percentage of baseline values. Similarly, results are expressed as the mean ± SEM. The t-test was used to compare body weight changes. The log-rank test was performed to establish significant differences in survival curves. The analyses were performed using GraphPad Prism version 7.0 for Windows (GraphPad Software). P values < 0.05 were considered to indicate a significant difference.

3. Results

3.1. Downregulation of CLK1 and SON transcription by transfecting 293T cells with pll3.7-amiRNA plasmids

The sequences and structures of human miR-30 and miR-93 are shown in Fig. 1A and Fig. 1D, respectively. The mature sequences of miR-30 and miR-93 were replaced with sequences targeting CLK1 and SON transcription (Fig. 1B, E in bold), and the resulting miRNAs were denoted as artificial miR-30CLK1 (amiR-30CLK1) and artificial miR-93SON (amiR-93SON). All miRNAs and amiRNAs were synthesized and cloned into the microRNA-expressing plasmid pll3.7 to yield pll3.7-miR-30, pll3.7-amiR-30CLK1, pll3.7-miR-93, and pll3.7-amiR-93SON. The production of functional miRNAs and amiRNAs was verified in several mammalian cell types (Madin-Darby canine kidney [MDCK], mouse epithelial fibroblast [MEF], A549, and 293T cells) in our previous study [30]. In the current study involving the transfection of 293T cells with miRNAs or amiRNAs, the CLK1 and SON transcripts were reduced by 45% and 31%, respectively, at 48 h post transfection compared with no plasmid transfection (Fig. 1C, F). However, the CLK1 and SON transcripts were reduced by less than 10% for transfection with pll3.7, pll3.7-miR-30, and pll3.7-miR-93 compared with no plasmid transfection (Fig. 1C, F).

3.2. The feasibility of incorporating amiRNAs into the NA segment of rescued influenza viruses

As shown in Fig. 2A, miRNAs or amiRNAs were inserted between the NA coding sequence and the terminal noncoding region (NCR) at the 5' end. Influenza viruses with or without engineered NA segments were rescued through the eight-plasmid-based system [28] and propagated in embryonated chicken eggs. The rescued influenza viruses were denoted as wild-type PR8 (PR8-wt), PR8 with miR-30 (PR8-miR-30), PR8 with amiR-30CLK1 (PR8-amiR-30CLK1), PR8 with miR-93 (PR8-miR-93), and PR8 with amiR-93SON (PR8-amiR-93SON). Viruses were then concentrated by ultracentrifugation, and the viral genomes were extracted and detected by silver staining. As shown in Fig. 2B, the wild-type NA segment migrated at 1413 bp, while the engineered NA segments migrated at 1536 bp (based on the miR-30 backbone) or 1534 bp (based on the miR-93 backbone), which are close to the migration of the NP segment (1565 bp). The M and NA segments were also amplified by RT-PCR and separated by electrophoresis. As was detected by silver staining, the wild-type and engineered NA segments had different sizes based on the agarose electrophoresis pattern, whereas the M segments in all rescued viruses had similar sizes (Fig. 2C), which demonstrates the feasibility of incorporating miRNAs and amiRNAs into the NA segments of rescued influenza viruses. It is interesting to note that the quantities of amplified NA segments were lower in engineered viruses than in wild-type viruses, probably due to the hindering effects of the secondary structure of the miRNAs on amplification efficiency.

3.3. Downregulation of CLK1 and SON expression by infection with viruses incorporated with amiRNAs in the NA segment

In order to evaluate the effects of viruses engineered to express amiRNAs downregulating CLK1 and SON expression, MEF and A549 cells were infected with wild-type or engineered viruses at a multiplicity of infection of 1 (MOI=1). Total RNA and protein were extracted for examining CLK1 and SON expression. CLK1 transcripts were reduced by 49% and 61% in MEF and A549 cells, respectively, at 48 h post PR8-amiR-30CLK1 infection compared with no infection (Fig. 3A). Similarly, SON transcripts were reduced by 48% and 66% in MEF and A549 cells, respectively, at 48 h post PR8-amiR-93SON infection compared with no infection (Fig. 3B). However, cells infected with PR8-wt, PR8-miR-30, or PR8-miR-93 only slightly altered CLK1 or SON transcription, with lower levels (<30%) than those with PR8-amiR-30CLK1 or PR8-amiR-93SON infection. Western blot analysis also demonstrated that CLK1 expression was reduced by ~69% in MEF cells and ~45 % in A549 cells infected with PR8-amiR-30CLK1 compared with no infection (Fig. 3C). There was only a small difference in CLK1 expression after infection with PR8-wt, PR8-amiR-30, or negative control in both MEF and A549 cells. No commercial SON antibody to detect SON expression was available prior to manuscript preparation.

3.4 High antibody responses induced by all engineered influenza viruses

Mice inoculated with all engineered viruses (3×10^5 PFU for each) and low-dose PR8 (25 PFU) had significantly higher concentrations of IgA, IgG, IgG1, and IgG2a on days 14 and 21 than the PBS control (Fig. 4), similar to the observed trend of prophylactic effects on weight loss and survival rates. Inoculation with low-dose PR8 induced the highest antibody

responses on days 7, 14, and 21 among all groups, suggesting that the engineered NA might reduce virus replication (as observed in the in vitro study in Fig. 2C) and then induce lower antibody responses. Low-lose PR8 also elicited significantly higher antibody responses on days 7 and 14 and significantly higher IgG1 responses on day 21 than other groups (Fig. 4). In addition, low-dose PR8 elicited significantly higher IgA responses on day 21 than did PR8-miR-93 or PR8-miR-93SON (Fig. 4). All engineered viruses induced similar antibody responses, except that PR8-amiR-30CLK1 induced significantly higher IgA responses on day 21 than did PR8-miR-93 or PR8-amiR-93SON, and PR8-miR-93 induced significantly lower IgG1 responses than did the other three groups (Fig. 4).

3.5 High prophylactic efficacy for all engineered influenza viruses used as vaccines

To examine the prophylactic effects of engineered influenza viruses against viral challenge with lethal titers, mice were intranasally inoculated with 3×10^5 PFU of engineered viruses or low titers (25 PFU) of PR8-wt virus 21 days before challenge with 50× MLD_{50} of PR8. Blood was collected on days 0, 7, 14, and 21 post inoculation for examination of antibody responses. Mice inoculated with all engineered viruses or low titers of PR8-wt virus were completely protected against lethal challenge with PR8, with no morbidity, as measured by weight loss (Fig. 5A), or mortality observed in these mice (Fig. 5B). By contrast, mice inoculated with PBS exhibited rapid weight loss (Fig. 5A), and all died (Fig. 5B) by day 7 post challenge.

3.6. PR8-amiR-30CLK1: therapeutic effects against PR8 infection

To examine the therapeutic effects of engineered viruses against infection with a lethal dose of PR8, mice were infected with 20× MLD_{50} of PR8 6 h before treatment with engineered viruses. Mice treated with PR8-amiR-30CLK1 lost weight until day 7, lost ~25% of their weight by day 7, and then completely recovered from an infection that would have been lethal with PR8 (Fig. 6A). All mice treated with PR8-amiR-30CLK1 survived (Fig. 6B). By contrast, mice treated with PR8-miR-30, PR8-miR-93, PR8-amiR-93SON, or no treatment exhibited rapid weight loss (Fig. 6A), and all died (Fig. 6B). Antibody responses weren’t measured in this experiment since the immune responses induced by the PR8 infection would compound the following antibody responses by our engineered viruses.

4. Discussion

Although there are seasonal influenza vaccines and two classes of anti-influenza virus drugs available for public use, influenza remains a significant public health problem worldwide, due to the rapid antigenic shift and drift of the virus as well as the emergence of drug resistance [31]. This rapid evolution persistently compromises the effectiveness of vaccines and therapeutics, and a further understanding of the complex host cell pathways coopted by influenza viruses for replication may provide new targets and strategies for vaccination and antiviral therapy. amiRNA-expressing vectors provide unique benefits, as they are less toxic than regular shRNA vectors [32–34]. Owing to the specificity and efficiency of gene silencing, there has been increasing interest in whether amiRNAs targeted to the viral genome can control viral replication in infected cells. So far, the amiRNA-mediated antiviral approach has been found useful in treating many viruses, including adenoviruses, rabies

virus, dengue virus, chikungunya, and porcine reproductive and respiratory virus. In all cases, it has been shown to be an efficient inhibitor of virus replication, with minimal or no cytotoxicity [35–38].

In our study, we constructed influenza viruses by incorporating miRNAs or amiRNAs within the NA segment. We found that all engineered viruses could function as attenuated vaccines and provided greater protective efficacies and greater antibody responses. Two engineered viruses incorporating amiRNAs, PR8-amiR-30CLK1 and PR8-amiR-93SON, were demonstrated to inhibit CLK1 and SON expression, respectively, both critical for virus replication, and thereby reduce reproduction of the virus. Interestingly, two viruses engineered to express miRNAs, PR8-miR-30 and PR8-miR-93, were also found to function as attenuated vaccines with a high safety profile, probably because the insertion of miRNAs in the genome interfered with virus replication to attenuate their virulence. For our engineered viruses, there may be a critical concern about their long-term genetic stability after multiple rounds of production in chicken eggs. To enhance genetic stability, other gene segments, such as NS or M, could also be chosen for insertion of miRNAs, since the NS and M segments need to be spliced for virus replication [30, 39].

CLK1 plays a role in the alternative splicing of M2 and SON, which regulate the trafficking of virions to the late endosome [18, 19, 21], and both are involved in influenza virus infection. The engineered viruses targeting them were previously hypothesized to inhibit virus infection and proposed as anti-viral drugs. This proposal received mixed support from our finding that, while an engineered virus targeting CLK1 could be used as a new influenza therapeutic, mice treated with an engineered virus targeting SON could not survive lethal-dose influenza infection. The main reason for this difference in outcome is probably that the roles of CLK1 and SON in influenza pathogenesis are different, and these roles might be differentially complemented by other host proteins. More research is needed about their effects and that of other host genes that are exclusively involved in influenza replication. For example, host genes important for influenza infection and replication that have been identified by RNAi-based studies include those for virus entry [18–20, 40], fusion of the endosomal and viral membrane [17–19, 40], transport of the viral components to the nucleus [18, 19], as well as late events, including export of the vRNP complex and RNA into the cytoplasm [16–20].

In our study, we used human miR-30 and miR-93 as backbones for designing artificial miRNAs. There is no homology within the miR-30 and miR-93 sequences between human and chicken but high homology between human and mice, suggesting that engineered influenza viruses with miRNAs or amiRNAs could be propagated in chicken eggs at reasonable titers and attenuated in mammalian cells. Instead of a single miRNA, two different miRNAs were investigated in our current study to potentially incorporate two or more miRNAs in the future.

5. Conclusions

Two host factors, CLK1 and SON DNA binding protein (SON), were found to play essential roles in replication of influenza viruses and were chosen to be targeted by artificial

microRNAs (amiRNAs), which were incorporated into the NA segment of rescued influenza viruses. Mouse epithelial fibroblast (MEF) and human lung carcinoma A549 cells infected with those engineered PR8 viruses containing amiR-30CLK1 (PR8-amiR-30CLK1) or amiR-93SON (PR8-amiR-93SON) had reduced expression of host proteins CLK1 and SON, respectively. All engineered influenza viruses, including PR8-miR-30, PR8-amiR-30CLK1, PR8-miR-93 and PR8-amiR-93SON could function as attenuated vaccines by inducing significantly higher antibody responses and providing greater protective efficacy. In contrast to PR8-amiR-93SON, mice treated with PR8-amiR-30CLK1 recovered from weight loss and survived lethal infection by 6 h after lethal-dose PR8 infection, suggesting that the engineered influenza virus PR8-amiR-30CLK1 could be used as a new therapeutic influenza vaccine.

Acknowledgements

This work was supported by a grant (AI133207) from the National Institute of Allergy and Infectious Diseases and an internal fund from the Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center El Paso (to M.Z). The funders had no role in study design, data collection and analysis, the decision to publish, or preparation of the manuscript.

References

[1]. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De Leon FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA, Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010, Lancet, 380 (2012) 2095–2128. [PubMed: 23245604]

[2]. Rudolf M, Poppel M, Frohlich A, Breithaupt A, Teifke J, Blohm U, Mettenleiter T, Beer M, Harder T, Longitudinal 2 years field study of conventional vaccination against highly pathogenic avian influenza H5N1 in layer hens, Vaccine, 28 (2010) 6832–6840. [PubMed: 20727963]

[3]. Basha S, Hazenfeld S, Brady RC, Subbramanian RA, Comparison of antibody and T-cell responses elicited by licensed inactivated- and live-attenuated influenza vaccines against H3N2 hemagglutinin, Hum Immunol, 72 (2011) 463–469. [PubMed: 21414368]

[4]. He XS, Holmes TH, Zhang C, Mahmood K, Kemble GW, Lewis DB, Dekker CL, Greenberg HB, Arvin AM, Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines, J Virol, 80 (2006) 11756–11766. [PubMed: 16971435]

[5]. Hoft DF, Babusis E, Worku S, Spencer CT, Lottenbach K, Truscott SM, Abate G, Sakala IG, Edwards KM, Creech CB, Gerber MA, Bernstein DI, Newman F, Graham I, Anderson EL, Belshe RB, Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children, J Infect Dis, 204 (2011) 845–853. [PubMed: 21846636]

[6]. Rahn J, Hoffmann D, Harder TC, Beer M, Vaccines against influenza A viruses in poultry and swine: Status and future developments, Vaccine, 33 (2015) 2414–2424. [PubMed: 25835575]

[7]. Hsieh HP, Hsu JT, Strategies of development of antiviral agents directed against influenza virus replication, Curr Pharm Des, 13 (2007) 3531–3542. [PubMed: 18220789]

[8]. Jefferson T, Demicheli V, Di Pietrantonj C, Rivetti D, Amantadine and rimantadine for influenza A in adults, Cochrane Database Syst Rev, (2006) CD001169.

[9]. Lackenby A, Thompson CI, Democratis J, The potential impact of neuraminidase inhibitor resistant influenza, Curr Opin Infect Dis, 21 (2008) 626–638. [PubMed: 18978531]

[10]. Moorthy NS, Poongavanam V, Pratheepa V, Viral M2 ion channel protein: a promising target for anti-influenza drug discovery, Mini Rev Med Chem, 14 (2014) 819–830. [PubMed: 25342196]

[11]. Conly J, Johnston B, Ode to oseltamivir and amantadine?, Can J Infect Dis Med Microbiol, 17 (2006) 11–14. [PubMed: 18418477]

[12]. Arévalo MT, Navarro A, Arico CD, Li J, Alkhatib O, Chen S, Diaz-Arevalo D, Zeng M, Targeted Silencing of Anthrax Toxin Receptors Protects against Anthrax Toxins, The Journal of biological chemistry, 289 (2014) 15730–15738.

[13]. Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A, Efstathiou S, Atkins JF, Firth AE, Taubenberger JK, Digard P, An overlapping protein-coding region in influenza A virus segment 3 modulates the host response, Science (New York, N.Y.), 337 (2012) 199–204.

[14]. Perreira JM, Meraner P, Brass AL, Functional Genomic Strategies for Elucidating Human-Virus Interactions: Will CRISPR Knockout RNAi and Haploid Cells?, Adv Virus Res, 94 (2016) 1–51. [PubMed: 26997589]

[15]. Savidis G, McDougall WM, Meraner P, Perreira JM, Portmann JM, Trincucci G, John SP, Aker AM, Renzette N, Robbins DR, Guo Z, Green S, Kowalik TF, Brass AL, Identification of Zika Virus and Dengue Virus Dependency Factors using Functional Genomics, Cell Rep, 16 (2016) 232–246. [PubMed: 27342126]

[16]. Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ, The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus, Cell, 139 (2009) 1243–1254. [PubMed: 20064371]

[17]. Hao L, Sakurai A, Watanabe T, Sorensen E, Nidom CA, Newton MA, Ahlquist P, Kawaoka Y, Drosophila RNAi screen identifies host genes important for influenza virus replication, Nature, 454 (2008) 890–893. [PubMed: 18615016]

[18]. Karlas A, Machuy N, Shin Y, Pleissner KP, Artarini A, Heuer D, Becker D, Khalil H, Ogilvie LA, Hess S, Maurer AP, Muller E, Wolff T, Rudel T, Meyer TF, Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication, Nature, 463 (2010) 818–822. [PubMed: 20081832]

[19]. Konig R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, Alamares JG, Tscherne DM, Ortigoza MB, Liang Y, Gao Q, Andrews SE, Bandyopadhyay S, De Jesus P, Tu BP, Pache L, Shih C, Orth A, Bonamy G, Miraglia L, Ideker T, Garcia-Sastre A, Young JA, Palese P, Shaw ML, Chanda SK, Human host factors required for influenza virus replication, Nature, 463 (2010) 813–817. [PubMed: 20027183]

[20]. Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, Gupta PB, Hao T, Silver SJ, Root DE, Hill DE, Regev A, Hacohen N, A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection, Cell, 139 (2009) 1255–1267. [PubMed: 20064372]

[21]. Zu M, Li C, Fang JS, Lian WW, Liu AL, Zheng LS, Du GH, Drug Discovery of Host CLK1 Inhibitors for Influenza Treatment, Molecules, 20 (2015) 19735–19747. [PubMed: 26540031]

[22]. Hannon GJ, RNA interference, Nature, 418 (2002) 244–251. [PubMed: 12110901]

[23]. Filipowicz W, Bhattacharyya SN, Sonenberg N, Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?, Nat Rev Genet, 9 (2008) 102–114. [PubMed: 18197166]

[24]. Friedman RC, Farh KK, Burge CB, Bartel DP, Most mammalian mRNAs are conserved targets of microRNAs, Genome Res, 19 (2009) 92–105. [PubMed: 18955434]

[25]. Ambros V, The functions of animal microRNAs, Nature, 431 (2004) 350–355. [PubMed: 15372042]

[26]. Perez JT, Pham AM, Lorini MH, Chua MA, Steel J, tenOever BR, MicroRNA-mediated species-specific attenuation of influenza A virus, Nat Biotechnol, 27 (2009) 572–576. [PubMed: 19483680]

[27]. Schmid S, Zony LC, tenOever BR, A versatile RNA vector for delivery of coding and noncoding RNAs, J Virol, 88 (2014) 2333–2336. [PubMed: 24307584]

[28]. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG, A DNA transfection system for generation of influenza A virus from eight plasmids, Proc Natl Acad Sci U S A, 97 (2000) 6108–6113. [PubMed: 10801978]

[29]. Reed HMLJ, a simple method of estimating fifty percent endpoints, American Journal of Epidemiology, 27 (1938) 493–497.

[30]. Li J, Arevalo MT, Diaz-Arevalo D, Chen Y, Choi JG, Zeng M, Generation of a safe and effective live viral vaccine by virus self-attenuation using species-specific artificial microRNA, J Control Release, 207 (2015) 70–76. [PubMed: 25858415]

[31]. Ison MG, Antivirals and resistance: influenza virus, Curr Opin Virol, 1 (2011) 563–573. [PubMed: 22440914]

[32]. Boden D, Pusch O, Lee F, Tucker L, Ramratnam B, Human immunodeficiency virus type 1 escape from RNA interference, J Virol, 77 (2003) 11531–11535.

[33]. Boden D, Pusch O, Silbermann R, Lee F, Tucker L, Ramratnam B, Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins, Nucleic Acids Res, 32 (2004) 1154–1158. [PubMed: 14966264]

[34]. Boudreau RL, Martins I, Davidson BL, Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo, Mol Ther, 17 (2009) 169–175. [PubMed: 19002161]

[35]. Bhomia M, Sharma A, Gayen M, Gupta P, Maheshwari RK, Artificial microRNAs can effectively inhibit replication of Venezuelan equine encephalitis virus, Antiviral Res, 100 (2013) 429–434. [PubMed: 23988697]

[36]. Israsena N, Supavonwong P, Ratanasetyuth N, Khawplod P, Hemachudha T, Inhibition of rabies virus replication by multiple artificial microRNAs, Antiviral Res, 84 (2009) 76–83. [PubMed: 19646485]

[37]. Saha A, Bhagyawant SS, Parida M, Dash PK, Vector-delivered artificial miRNA effectively inhibited replication of Chikungunya virus, Antiviral Res, 134 (2016) 42–49. [PubMed: 27565991]

[38]. Xie PW, Xie Y, Zhang XJ, Huang H, He LN, Wang XJ, Wang SQ, Inhibition of Dengue virus 2 replication by artificial micrornas targeting the conserved regions, Nucleic Acid Ther, 23 (2013) 244–252. [PubMed: 23651254]

[39]. Dubois J, Terrier O, Rosa-Calatrava M, Influenza viruses and mRNA splicing: doing more with less, mBio, 5 (2014) e00070–00014.

[40]. Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, Lieberman J, Elledge SJ, Identification of host proteins required for HIV infection through a functional genomic screen, Science, 319 (2008) 921–926. [PubMed: 18187620]

[41]. Wen K, Li G, Bui T, Liu F, Li Y, Kocher J, Lin L, Yang X, Yuan L, High dose and low dose Lactobacillus acidophilus exerted differential immune modulating effects on T cell immune

responses induced by an oral human rotavirus vaccine in gnotobiotic pigs, Vaccine, 30 (2012) 1198–1207. [PubMed: 22178726]

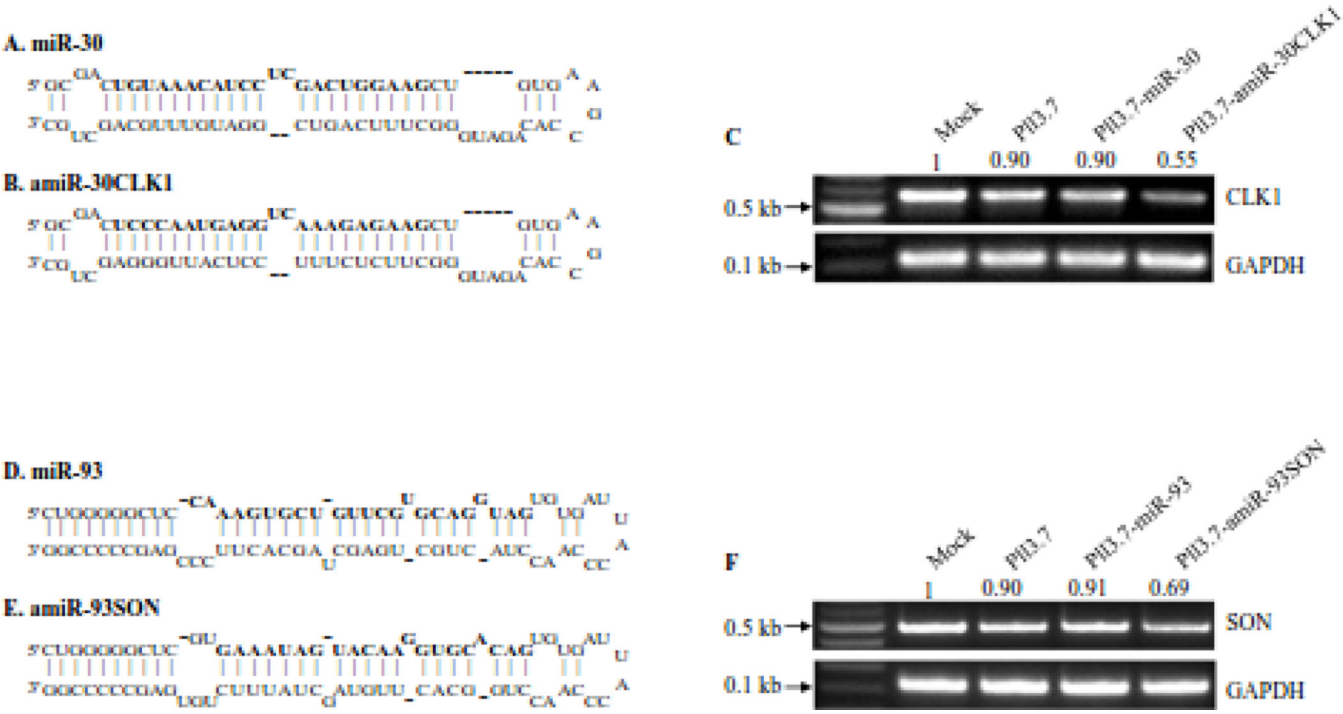


Figure 1. Design of artificial miRNAs (amiRs) based on human miR-30 and miR-93 backbones and detection of CLK1 and SON transcripts.

(A) Sequence and secondary structure of human miR-30. (B) Sequence and secondary structure of amiR-30CLK1. (C) 293T cells were transfected with pll3.7, pll3.7-miR-30, or pll3.7-amiR-30CLK1. Forty-eight hours later, total RNA was extracted and reverse-transcribed, the CLK1 cDNA amplified by PCR, and the products separated by electrophoresis on an agarose gel, with GAPDH used as the control. (D) Sequence and secondary structure of human miR-93. (E) Sequence and secondary structure of amiR-93SON. (F) 293T cells were transfected with pll3.7, pll3.7-miR-93, or pll3.7-amiR-93SON. Forty-eight hours later, total RNA was extracted and reverse-transcribed, the SON cDNA amplified by PCR, and the products separated by electrophoresis on an agarose gel, with GAPDH used as control.

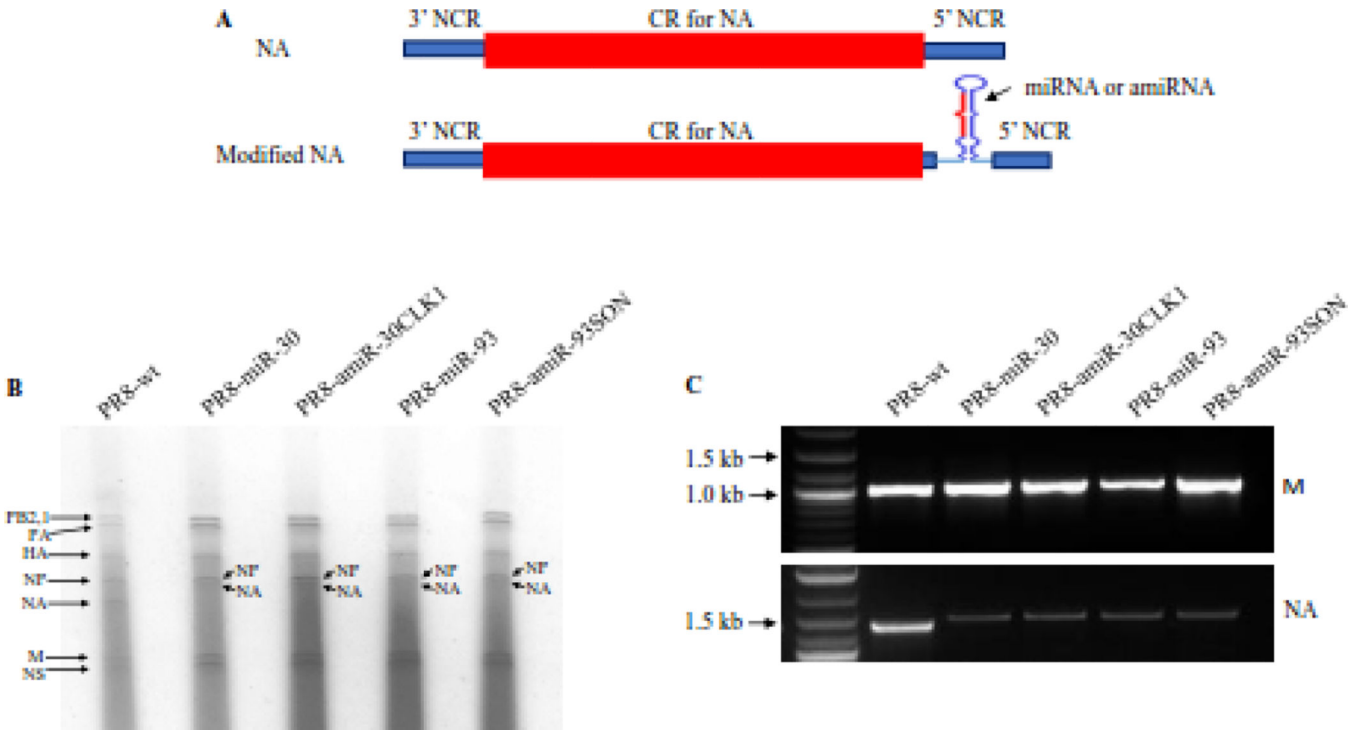


Figure 2. Engineering of the NA segment and verification of rescued influenza viruses.

(A) Diagrams of the original and engineered NA segments. Blue bars represent 3' and 5' noncoding regions (NCRs), and red bars represent the NA coding sequence. (Top) Organization of the original NA segment. (Bottom) Organization of the engineered NA segment with the miRNA- or amiRNA-expressing cassette. (B) Viral RNAs were isolated from PR8-wt, PR8-miR-30, PR8-amiR-30CLK1, PR8-miR-93, or PR8-amiR-93SON, and 1 μg of RNA was separated on a 4% acrylamide gel in TBE with urea for silver staining to detect influenza gene segments. (C) Viral RNAs were reverse-transcribed and then amplified and separated by electrophoresis on an agarose gel.

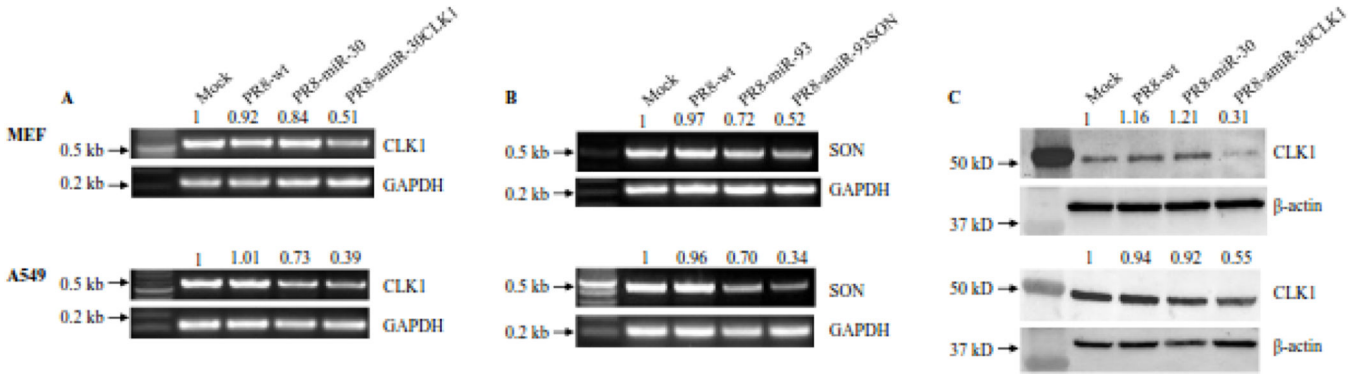


Figure 3. CLK1 and SON expression in MEF or A549 cells infected with wild-type or engineered PR8 viruses.

MEF (top) or A549 cells (bottom) were infected with wild-type or engineered PR8 viruses. Forty-eight hours later, the cells were collected, total RNA extracted and reverse-transcribed, and the proteins extracted to examine CLK1 expression. (A) CLK1 segments were amplified by PCR and separated on an agarose gel. (B) SON segments were amplified by PCR and separated on an agarose gel. (C) CLK1 proteins were detected by western blotting.

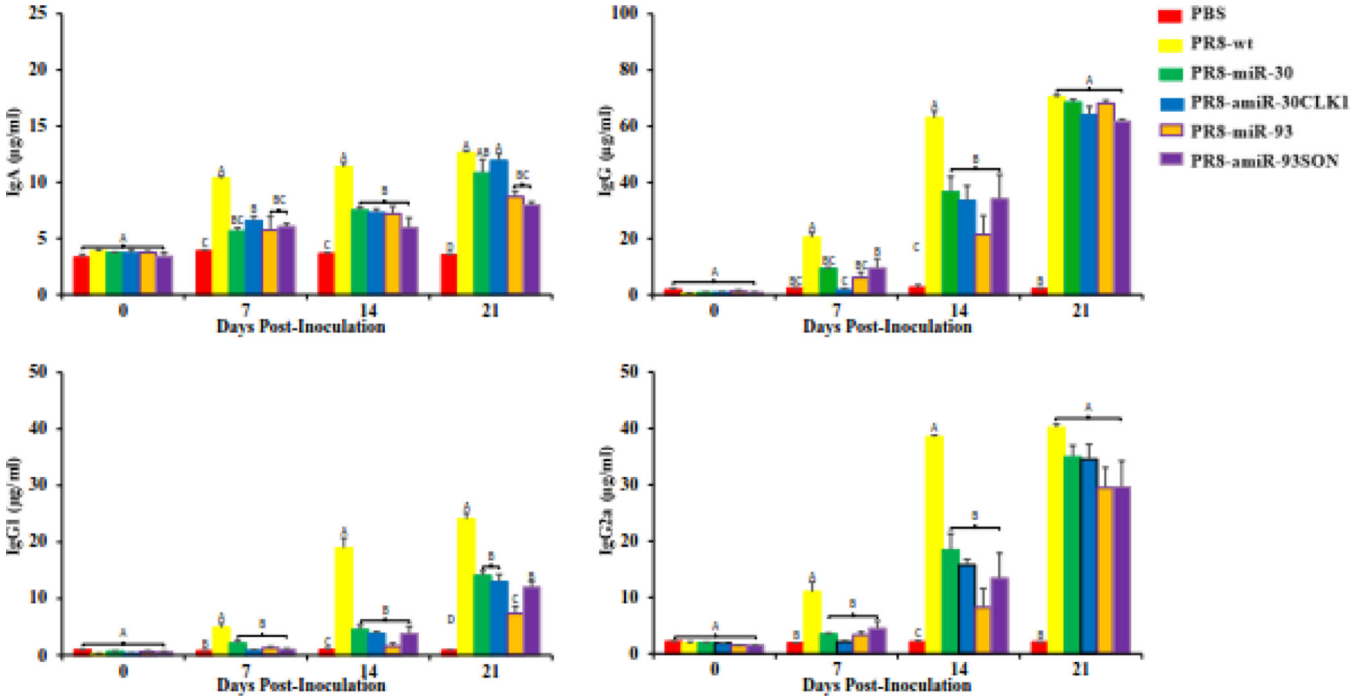


Figure 4. Antibody responses induced by wild-type or engineered PR8 viruses in mouse blood.

BALB/c mice (6–8 weeks old) in each group (n=10) were intranasally inoculated with 3×10^5 PFU engineered viruses, 25 PFU wild-type PR8 (as positive control), or PBS (as negative control). Blood was collected on days 0, 7, 14, and 21 post inoculation. Concentrations of IgA, IgG, IgG1, and IgG2a antibodies were measured by ELISA. Comparisons among groups were performed by using a nonparametric one-way ANOVA with the Tukey multiple comparison test. The bars represent the mean ± standard error of the mean (SEM). P values <0.05 were considered to indicate a significant difference. As described, letter combinations above the bars indicate significant differences between groups, whereas shared letters indicate no significant difference [41].

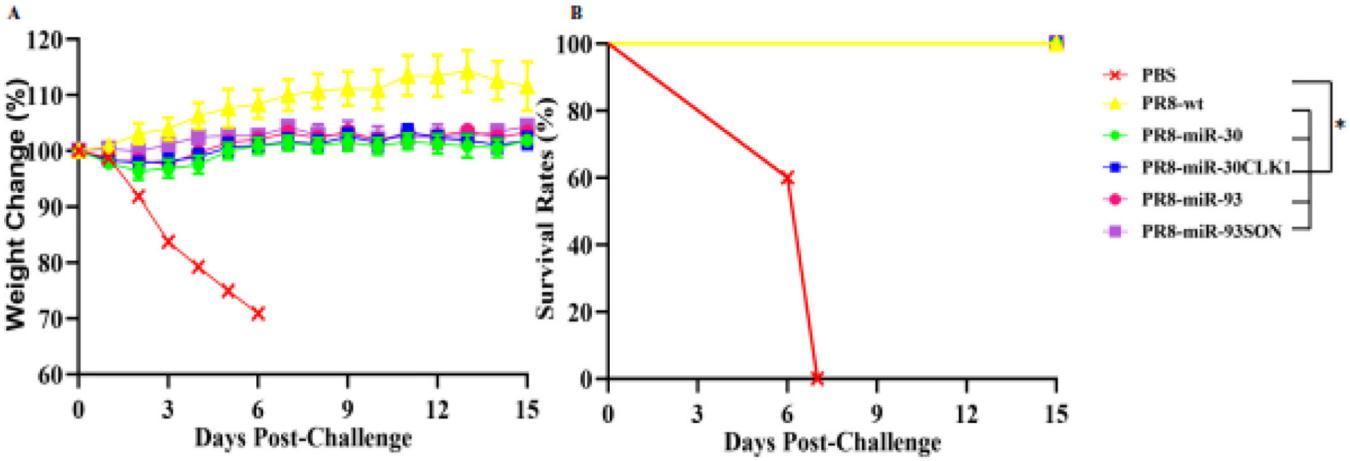


Figure 5. Protection induced by engineered or low-titer (25 PFU) wild-type PR8 viruses against wild-type PR8 challenge in mice.

See Fig. 4 legend for group and inoculation description. On day 21 post inoculation, mice were challenged with 50×MLD_{50} and were then monitored daily for clinical symptoms, weight loss, and death for 21 days. Mice undergoing a weight loss in excess of 30% were euthanized for reasons of animal welfare. Body weight changes (A) were expressed as a percentage of baseline values. The black lines indicate mean values, and error bars represent the SEM. The t-test was used to compare body weight changes. The log-rank test was performed to establish significant differences between survival curves (B). P values < 0.05 were considered to indicate a significant difference. *significant difference in survival rates between groups.

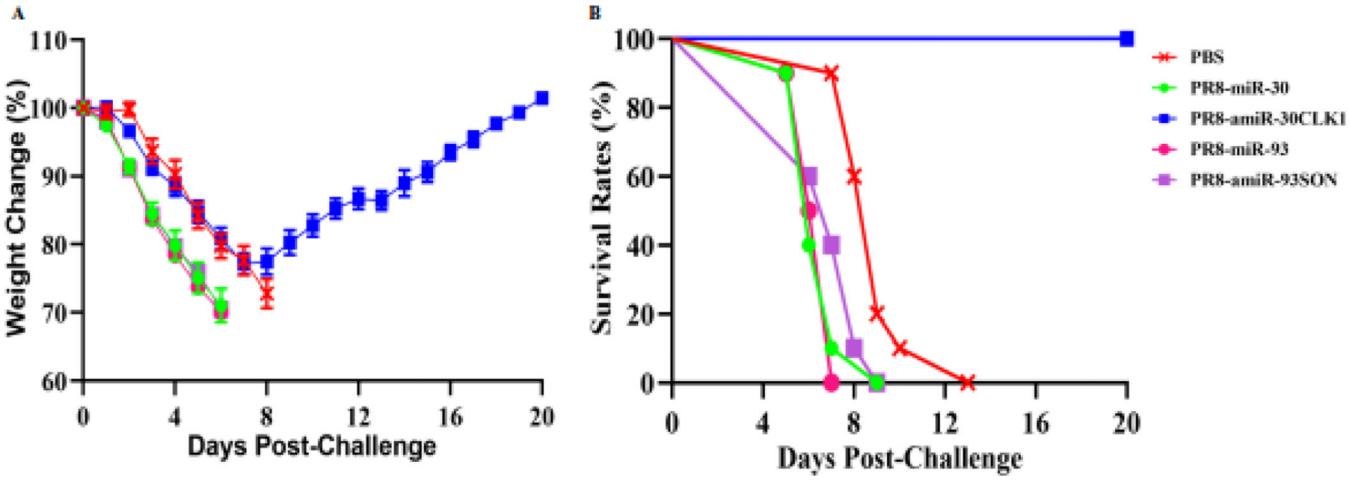


Figure 6. Therapeutic effects of engineered PR8 viruses.

BALB/c mice (6–8 weeks old) in each group (n=10) were infected with 20 × MLD_{50} (10^4 PFU) wild-type PR8. Six hours later, mice were intranasally dosed with 3×10^5 PFU of engineered PR8 viruses. Mouse weight changes (A) and survival rates (B) were recorded for 21 days. See Fig. 5 legend for data description and statistical analysis.

Table 1.

Primers for amplifying gene segments.

Gene segments	Species	Sequences	Sizes (bp)
M	PR8	Forward: 5'-TATTCGTCTCAGGGAGCAAAAGCAGGTAG-3' Reverse: 5'-ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT-3'	1027
NA	PR8	Forward: 5'-TATTGGTCTCAGGGAGCAAAAGCAGGAGT-3' Reverse: 5'-ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT-3'	1413
CLK1	Homo sapiens	Forward: 5'-ATAGCAGTGCCCAGGAGAAC-3' Reverse: 5'-TGATGCTCAAACCATTCCAA-3'	598
CLK1	Mus musculus	Forward: 5'-AGTGGTTTGAGCATCGAGGT-3' Reverse: 5'-TCTGCCAGCAGAACTGTGTT-3'	596
SON	Homo sapiens	Forward: 5'-TCCTGTGCCAGTTGTTTCTG-3' Reverse: 5'-GTCTCACTGGTGGGCAAAAT-3'	501
SON	Mus musculus	Forward: 5'-GTGGGTTCCAGTCGAGAAAA-3' Reverse: 5'-GCAGAGAAGTTCCCAGAACG-3'	504
GAPDH	Homo sapiens	Forward: 5'-GCACCGTCAAGGCTGAGAAC-3' Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'	129
GAPDH	Mus musculus	Forward: 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' Reverse: 5'-TAGTGGGGTCTCGCTCCTGGAAGATG-3'	240