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Original Article

The microRNA-let-7b-mediated attenuated strain of influenza A (H1N1) virus in a mouse model

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Abstract

Introduction: Evaluating the attenuation of influenza viruses in animal studies is important in developing safe and effective vaccines. This study aimed to demonstrate that the microRNA (miRNA)-let-7b-mediated attenuated influenza viruses (miRT-H1N1) are sufficiently attenuated and safe in mice.

Methodology: The pathogenicity of the miRT-H1N1virus was investigated in a mouse model, evaluated with median lethal dose (LD_{50}). The replicative dynamics of the miRT-H1N1, wild type (wt)-H1N1, and scramble (scbl)-H1N1 viruses in the lungs of infected mice were compared. The degrees of lesions and the expression levels of IL-6, TNF-α, and IFN-β in the lungs of mice infected with different viruses were also analyzed.

Results: In miRT-H1N1 virus-infected mice, 100% of mice survived, and a lower pathogenicity was characterized with non-significant weight loss when compared to mice infected with the control wt virus. The miRT-H1N1 virus was not fatal for mice, even at the highest dose administered. The viral load in the lungs of miRT-H1N1-infected mice was significantly lower than that of the wild-type virus-infected mice. Fewer pulmonary lesions and lower levels of selected pro-inflammatory cytokines in the lungs of the mice infected with the miRT-H1N1 virus were also observed. The virulence of the miRT-H1N1 virus reduced significantly, suggesting that the miRT-H1N1 virus was safe for mice.

Conclusions: Our study demonstrated that the miRNA-mediated gene silencing is an alternative approach to attenuating the pathogenicity of wt influenza viruses that have potential in the development of influenza vaccines.

Key words: microRNA-let-7b; influenza virus; vaccine; mice

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Introduction

In the spring of 2009, the H1N1 influenza A virus, which emerged from Mexico and the United States and then spread all around the world in several months, caused acute respiratory diseases in human beings. This novel H1N1 influenza A virus had high transmissibility among humans, which posed a serious concern in public health. The virus was characterized as having originated as swine-derived influenza virus, because a large part of its genome was from swine influenza virus isolated from pigs [1,2].

Vaccination is considered valuable in lowering the chance of being infected and in reducing disease severity in humans to fight against influenza. Current live attenuated influenza vaccines (LAIV) have limitations in protecting humans against viruses and are normally designed to help target the virus whose antigen matches the vaccine, not the latest viruses. There has been an urgent need for new approaches to develop influenza vaccines to prevent influenza infections, especially to combat a future highly pathogenic influenza pandemic [3]. The mechanism of miRNA-mediated viral gene silencing may serve as a novel attenuation strategy, in which a target sequence is inserted into viral RNA to make a recombinant and attenuated virus [4]. At present, this attenuation strategy has already been applied to lentiviruses, picornaviruses, and rhabdoviruses [4,5].

When a massive number of people are to be inoculated with a vaccine, the safety of the vaccine is

always a concern. Common side effects for viral vaccine immunization include fever, vomiting, and muscle aches at the injection site. In worst-case scenarios, more severe reactions may occur (*e.g.*, anaphylaxis). Acute hypersensitivity is a well-known adverse reaction to vaccines [6]. There should be more concern about the safety of LAIVs when compared with inactivated trivalent influenza vaccines. LAIV can cause some unique adverse reactions (*e.g.*, asthma) [7,8]. Therefore, sufficient tests in animal models are required to estimate and evaluate the safety of an attenuated vaccine in order to develop an influenza vaccine safe for human use [9].

In this study, we performed animal experiments that have been widely applied to the research in live attenuated influenza vaccines. The procedure of this method has been described in section 210.11, volume 21, of the Code of Federal Regulations [3]. According to the protocol, mice are inoculated with a large dose of the candidate vaccine; this is followed by close observation of their weight loss and manifestation of clinical symptoms for 14 days. The purpose of pre-clinical safety tests in mice is to demonstrate that the candidate vaccine is attenuated. Previously, we used the A/Nanjing/108/2009 H1N1 as the backbone strain. Using a reverse genetics approach, we inserted a targeting sequence of miRNA-let-7b into a site in the viral PB1 gene through point mutagenesis. We succeeded in constructing the miRNA-let-7b-mediated attenuated H1N1 influenza A virus. Also, we demonstrated its decreased replication capacity in a cell culture. This study, which is a continuation of the previous project, aimed to test the pathogenicity changes of the miRNA-let-7b-mediated attenuated H1N1 influenza A virus. Our data demonstrated that the recombinant virus is attenuated and safe in a mouse model.

Methodology

Cells

Madin-Darby canine kidney (MDCK) (Invitrogen Corp, Carlsbad, USA) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, USA) with 10% fetal calf serum (HyClone, Logan, USA). Cells were maintained at 37°C in 5% CO_2.

Application of virus

In this study, A/Nanjing/108/2009 (H1N1) viruses (wt-H1N1 virus) were isolated in China in 2009 [10,11]. Reassortant virus was generated previously by incorporating miRNA-let-7b response elements (MREs) into the open-reading frame of the viral PB1 gene, termed miRT-H1N1 virus. The codon sequence was changed according to the instructions of influenza A virus amino acids so as to construct the DNA sequence of scbl-H1N1 virus, ensuring the right amino acid sequence and avoiding the recognition and degradation of miRNA-let-7b [5]. Cells were harvested 48 hours after transfection and then injected into the allantoic fluid of 10-day-old embryonated chicken eggs. Live virus was isolated 48 hours after infection and quantified by hemagglutination assay using chicken red blood cells and was titered in 24- and 96-well culture plates containing MDCK cells. All operations with influenza viruses were conducted in biosafety level 3 (BSL3) containment laboratories for Disease Control and Prevention.

Animals

Four- to six-week-old female BALB/c mice, purchased from Jinling Hospital (Nanjing University School of Medicine, Nanjing, China), weighing 18 to 20 grams, were used in this study and housed under standard temperature and light and dark cycles. All experiments adhered to the Nanjing University's Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of Nanjing University.

Pathogenicity studies

Mice were anesthetized with chloral hydrate. The virus titer was tenfold serially diluted and 50 μL of the diluted virus was used to inoculate each mouse. For each dose, ten mice were inoculated as a group. In the negative control group, each mouse was inoculated with 50 μL of phosphate-buffered saline (PBS). Survival rates and body weight loss were examined daily for 14 days post-infection (dpi). The mice were euthanized if they lost > 25% of their original body mass. The percentage of body weight change was calculated by dividing the daily weight by the initial (on day 0) weight of each mouse. The mean body weight loss of each mouse was calculated by obtaining the average value of each mouse body weight change from the first day to the fourteenth day. The 50% lethal dose (LD_{50}) values were calculated by the Reed-Muench method. The research protocol for the use of mice in this study was approved by Nanjing University's Regulations for Animal Care and Use.

Histopathological analysis

Twenty mice per group were anesthetized and then intranasally inoculated with 50 μΛ of 10^5 tissue culture

infective dose $(TCID)_{50}$ of viruses. Mice inoculated with PBS alone were used as negative controls. At 3, 5, 8, 10, and 14 dpi, four mice were sacrificed, and the left lobe of the lung was harvested and fixed in 10% phosphate-buffered formalin for pathological examination. The formalin-fixed lungs were processed for paraffin embedding. The paraffin-embedded tissues were cut into 5-μm-thick sections, and then stained by using hematoxylin and eosin (H&E). The left lobe of the lung was used for performing lung histopathologic studies, while the right lobe was grounded for titration of infectious viral titers, total RNA extraction, and measurement of inflammatory cytokines. The histopathologic score (HPS) was determined by a trained pathologist under a blind controlled condition.

Viral titration

Mouse tissue homogenates were prepared and titrated for viral titers in MDCK cells. Lung homogenates were prepared in 1 mL of PBS containing antibiotics. Tissue homogenates clarified by low-speed centrifugation were titrated in 24- and 96-well tissue culture plates containing MDCK cell monolayers, as described elsewhere [12]; viral titers were calculated and expressed as $\log_{10}TCID_{50}$/g of tissue [13].

Viral RNA of the M gene assay

Tissue homogenates from 3, 5, 8, 10 and 14 dpi were analyzed by a real-time polymerase chain reaction (PCR) according to the manufacturer's protocol. Total RNA was isolated from the right lobe of the lung using the RNeasy Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's protocol. Real-time PCR was performed using a 7300 real-time PCR system (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. RNA purity determination, cDNA synthesis, and real-time PCR were performed as described previously [14]. The β-actin gene was used as an internal control. Fold change of each gene expression level was calculated using the ΔΔCT calculation. Primer sequences are listed as follows. For □-actin, m-β-actin forward: 5'-TGCTGTCCCTGTATGCCTCT-3'; reverse: 5'-TTTGATGTCACGCACGATTT-3'. For viral M gene, m-M forward: 5'-ATGGATGTCAATCCGACTC-3'; reverse: 5'-TTTGCTGCAATGACGAGAGG-3'.

Quantification of cytokines and chemokines

To carry out the inflammatory cytokine analysis, the right lobes of lungs from 3, 5, 8, 10, and 14 dpi were analyzed by real-time PCR according to the manufacturer's protocol. Tissue homogenates were analyzed for mRNA transcript levels of mouse IL-6, TNF-α, and IFN-β. The primer sequences are listed as follows. For IL-6, forward: 5'-ACAACCACGGCCTTCCCTAC-3'; reverse: 5'-TCTCATTTCCACGATTTCCCAG-3'. For IFN-β, forward: 5'-AGCACTGGGTGGAATGAGAC-3'; reverse: 5'-TCTCCCAC GTCAATCTTTCC -3'. For TNF-α forward: 5'-CGAGTGACAAGCCTGTAGCCC-3'; reverse: 5'-GTCTTTGAGATCCATGCCGTTG-3'.

Statistical analyses

All data were expressed as mean ± SD. Differences in body weight loss in mice were analyzed using analysis of variance (ANOVA). Student's *t*-test was used when comparing the values of two different groups, and values of $p < 0.05$ were considered significant. The statistics tests were performed in SPSS software (SPSS, Chicago, USA).

Results

Replication of the recombinant viruses in chicken embryos

The 10-day-old embryonated eggs were inoculated with 200 μL miRT-H1N1, scbl-H1N1, and wt-H1N1virus. At 48 hours post-inoculation, allantoic fluids were collected and virus titers were tested. The virus titers were all above $10^{8.1}$ $TCID_{50}$/mL, suggesting that the virus replicated vigorously and was not attenuated in embryonated eggs (Table 1).

Decreased pathogenicity of the miRT-H1N1 virus in mice

Viral pathogenicity of the mutant viruses in a BALB/c mouse model was tested. The data demonstrated that the miRT-H1N1 virus showed less

Table 1. Virus titer in in embryonated chicken eggs at 48 hours post-infection (hpi).

Virus	hpi	Virus titer ($\log_{10}$ $TICD_{50}$/mL [mean ± SD]) in: Chicken eggs
wt-H1N1	48	7.9 ± 0.2
miRT-H1N1	48	8.1 ± 0.5
scbl-H1N1	48	8.0 ± 0.2

Viral titers from miRT-H1N1, wt-H1N1, and scbl-H1N1 viruses infections of 10-day-old embryonated chicken eggs. Allantoic fluid was titered in 96-well culture plates containing MDCK cells expressed as $\log_{10}$ $TCID_{50}$/mL. Data are the means of four independent experiments.

pathogenicity when compared with the wt-H1N1 viruses. Five mice in each group were inoculated intranasally with the mutant and wt virus at the dose range of 10^7~10^3 $TCID_{50}$ per mouse, and their morbidity and mortality were observed for 14 days pi. Mice inoculated with 10^7 $TCID_{50}$ of the miRT-H1N1 virus lost less than 11% of their initial body weight (9.89% ± 4.34 %) (Figure 1A), and all of them survived (Figure 2A). In comparison, mice inoculated with the wt-H1N1 viruses lost more body weight (20.26% ± 6.76%) ($p < 0.05$) (Figure 1 A). Although the mice infected with 10^5 $TCID_{50}$ of the wt-H1N1 virus still maintained an 80% survival rate (Figure 2 B), they showed more weight loss (14.6% ± 6.13 %) than the mice infected with miRT-H1N1 (4.97% ± 1.15%) (Figure 1C). In contrast, the scbl-H1N1 group showed a body weight loss and survival rate that were similar to those of the wt-H1N1 group ($p > 0.05$) (Figures 1 and 2). The wt-H1N1 viruses were fatal to mice (the LD_{50} value was $10^{5.3}$ $TCID_{50}$), but the miRT-H1N1 virus was not severely pathogenic to mice. Even being administrated the highest dose of the miRT-H1N1, none of the mice died

Figure 1. Body weight changes in infected mice. Mice were intranasally inoculated with 50 µL 10^7(**A**), 10^6(**B**), 10^5(**C**), 10^4(**D**), or 10^3(**E**) $TCID_{50}$ of miRT-H1N1, wt-H1N1, scbl-H1N1 virus or phosphate-buffered saline (PBS). Body weights were monitored daily. Data are reported as percent changes in body weight of mice. A total 15 of infected mice were euthanized on days 5 to 8, because they lost more than 25% of their body weight.

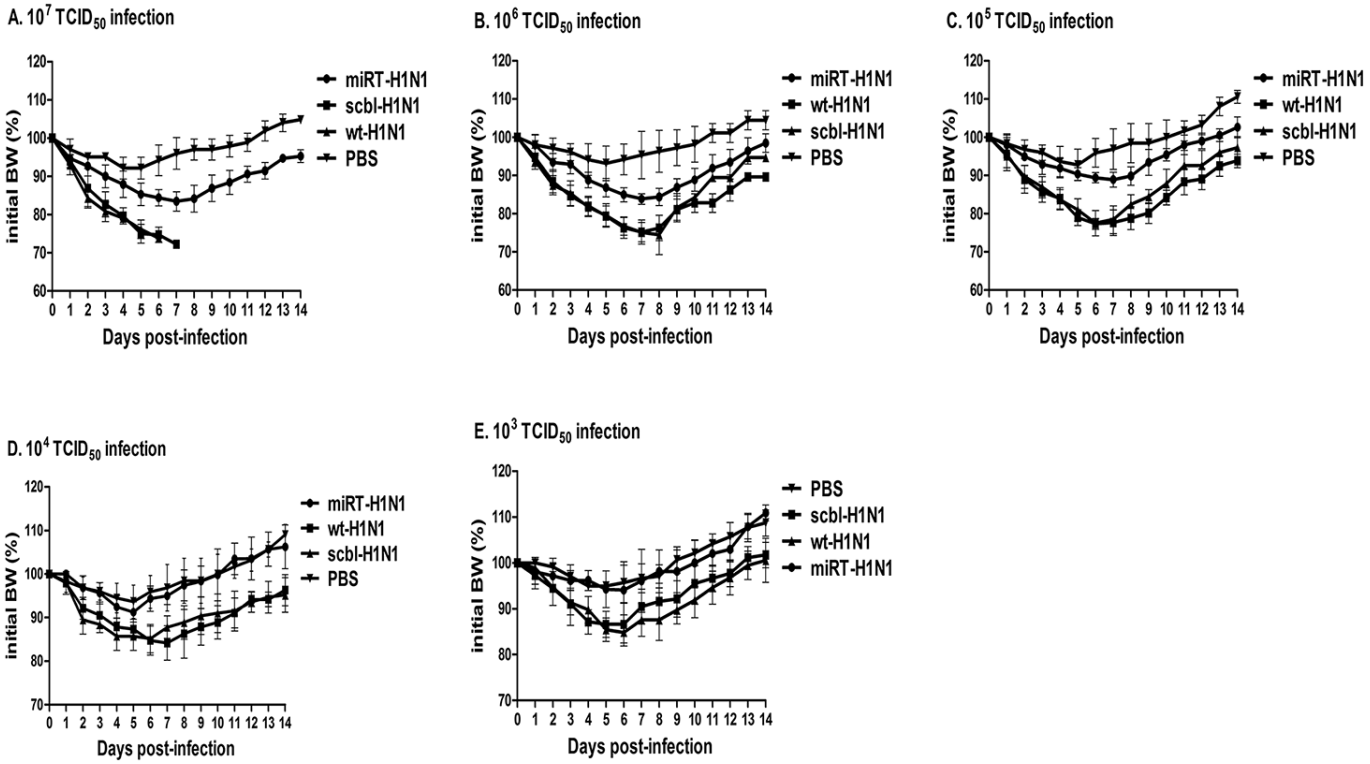


Figure 2. Survival rates of mice inoculated with tenfold dilutions of viruses. (**A**) Survival rates of mice inoculated with 10^7TCID_{50} wt-H1N1, scbl-H1N1 and miRT-H1N1 viruses. (**B**) Survival rates of mice inoculated with 10^5TCID_{50} wt-H1N1, scbl-H1N1, and miRT-H1N1 viruses.

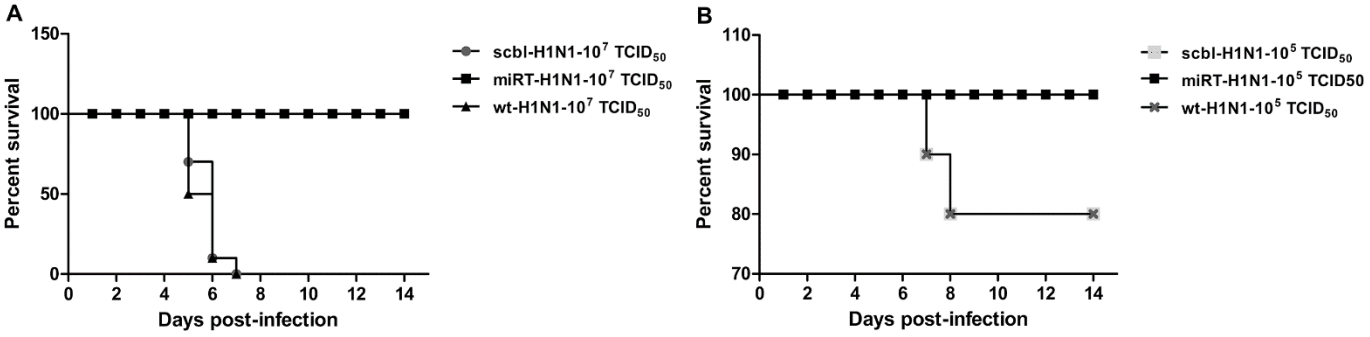
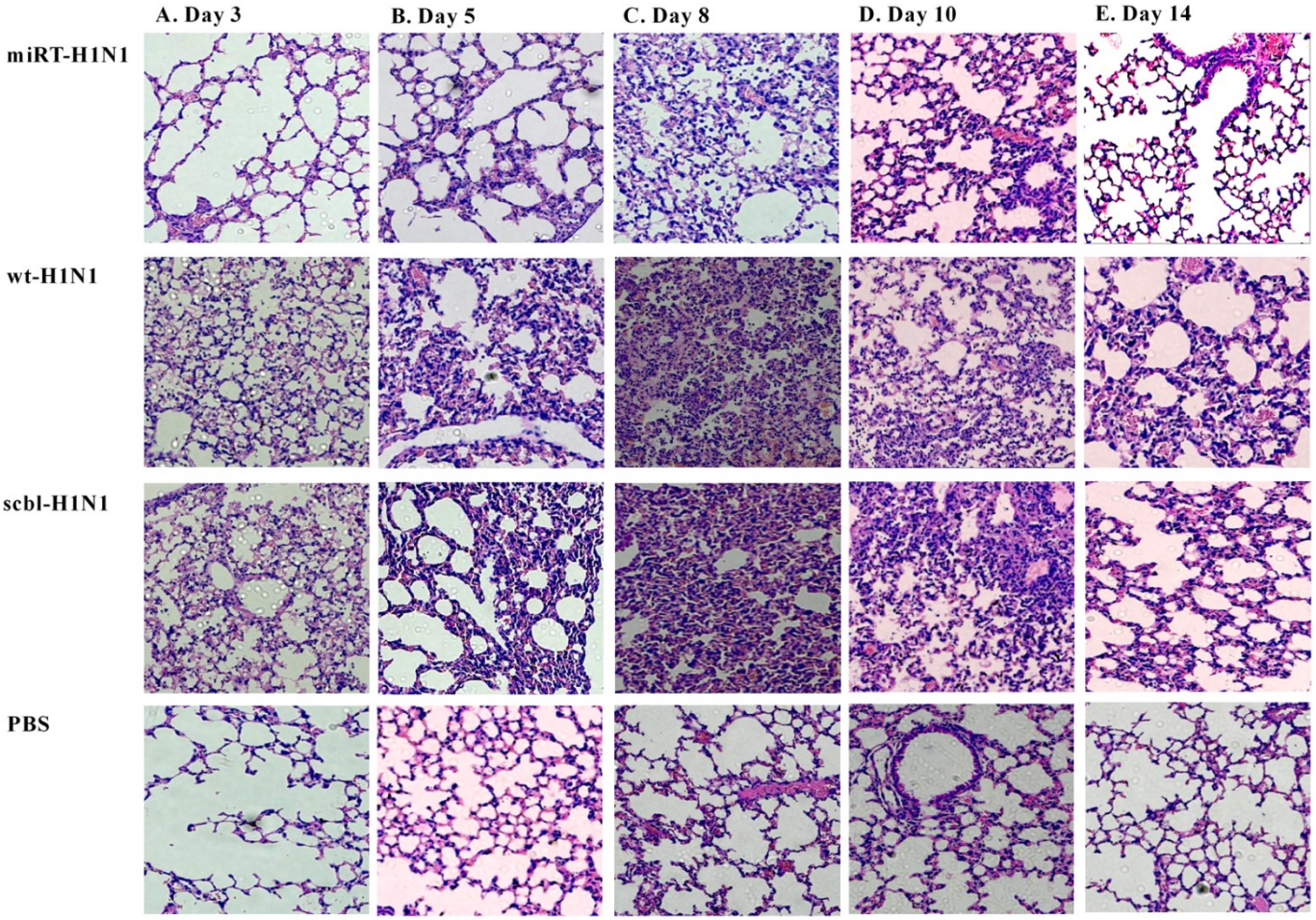
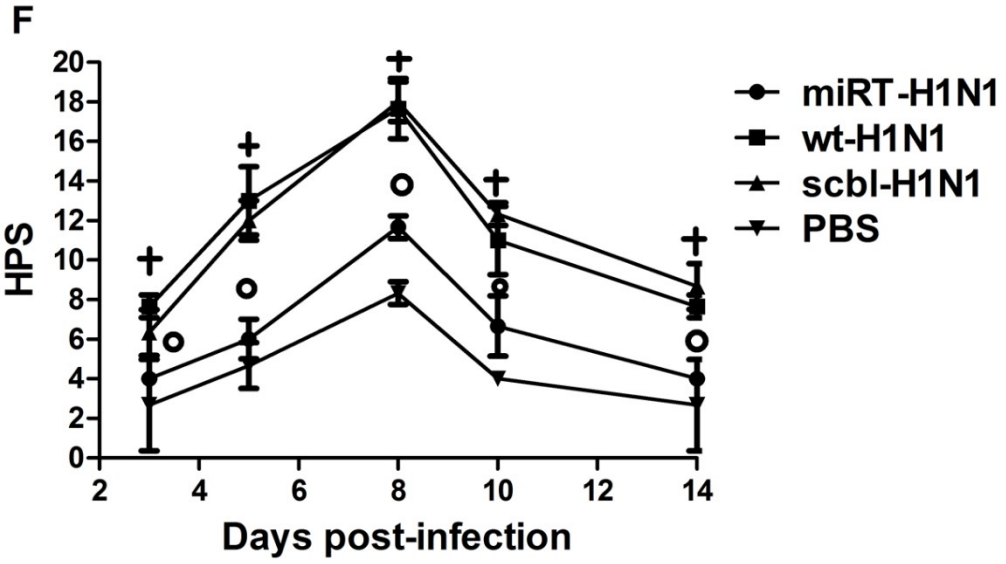


Figure 3. Pathological changes in lung tissues of mice infected with wt-H1N1, miRT-H1N1, scbl-H1N1 viruses and phosphate-buffered saline (PBS). The pathological changes in lung tissues of mice inoculated with miRT-H1N1, wt-H1N1, scbl-H1N1viruses and PBS at (**A**) 3 days post-infection (dpi), (**B**) 5 dpi, (**C**) 8 dpi, (**D**) 10 dpi, and (**E**) 14 dpi. (**F**) Histopathologic score of lung sections at 3 dpi, 5 dpi, 8 dpi, 10 dpi, and 14 dpi. Histopathologic score (HPS) is given as mean ± standard deviation. Comparison between mice that received the wt-H1N1 viruses and those that received the scbl-H1N1 virus ($^{+}p > 0.05$). Comparison between mice that received the wt-H1N1 and miRT-H1N1 viruses ($^{O}p < 0.05$).





(LD_{50} >10^7 $TCID_{50}$). The LD_{50} value of the virus in the scbl group was similar to that of the wild type (the LD_{50} value was $10^{5.38}$ $TCID_{50}$). In sum, the results demonstrated that the miRT-H1N1 virus showed lower pathogenicity and was attenuated significantly in mice.

Milder histopathologic changes in the lungs of the mice infected with the miRT-H1N1 virus

To examine the pathogenicity of the miRT-H1N1 virus in inducing pneumonia and pulmonary histopathology in mice, challenge experiments with the various viruses were performed as described previously. Three days after inoculation, the mice were necropsied, and histopathologic changed in the lungs were examined. The changes in the miRT-H1N1 group were similar to those in the PBS-infected mice (Figure 3A), with a similar histopathologic score (HPS) ($p > 0.05$) (Figure 3F). Five days after infection, significantly severe pulmonary pathologic changes and a higher HPS were shown in the lungs of the wt-H1N1 group, compared with those of the miRT-H1N1 group. More neutrophils were infiltrated and accumulated in the lungs of wt-H1N-infected mice (Figure 3B). Eight days after infection, the differences of the lesions in the lungs were the most significant. Microscopically, more severe lung lesions characterized by multifocal to widespread necrotizing bronchiolitis and more severe interstitial pneumonia were found in the wt-H1N1 group. In contrast, significantly fewer lung lesions and lower HPSs were exhibited in the lungs of the miRT-H1N1 group (Figure 3C, 3F). There was no significant difference in the pathologic degrees of the mice administrated scbl-H1N1 viruses and those of the mice administrated wt-H1N1 viruses (Figure 3A–3E). The HPSs were similar to those of the wt-H1N1 group ($p > 0.05$) (Figure 3F). In sum, the miRT-H1N1 virus was much less pathogenic in the mouse model.

Inhibited replication in mutant virus-infected mice

Viral titers were determined in the lungs of mice to compare the viral replicative dynamics in mice. The mean titers at 3 and 5 dpi in the lungs of mice inoculated with the wt-H1N1 virus were $10^{5.37}TCID_{50}$ /g and $10^{3.71}TCID_{50}$ /g. In contrast, the mean titers in mice inoculated with miRT-H1N1 virus were $10^{2.58}TCID_{50}$ /g and $10^{1.49}TCID_{50}$ /g ($p < 0.05$) (Figure 4). In the lungs of mice, all of these viruses were undetectable at later time points (8 and 10 dpi). The mean virus titers at 3 and 5 dpi in the lungs of mice inoculated with scbl-H1N1 viruses were $10^{5.49}TCID_{50}$ /g and $10^{3.49}TCID_{50}$ /g, similar to those of the wt-H1N1 group ($p > 0.05$) (Figure 4). Therefore, the miRT-H1N1 was restricted in replication when compared with the wt-H1N1virus.

To further characterize the viral replication, the viral matrix (M) gene copy numbers were measured in the lungs of mice by real-time PCR analysis (Figure 5). The amount of the M gene copy detected in the lungs infected with wt-H1N1 viruses was significantly higher than that detected in the lungs infected with miRT-H1N1 viruses. Remarkably, three days after infection, the difference in M gene copy numbers between the wt-

Figure 4. Virus titers in the lung of mice infected with wt-H1N1, miRT-H1N1, and scbl-H1N1 viruses. Mice were intranasally inoculated with 10^5 $TCID_{50}$ (each in 50 μL) of the three viruses. Lung were collected from mice (n = 4) on the indicated days post-infection (dpi), and titers were determined in MDCK cells (*$p < 0.05$). The dashed horizontal line indicates the lower limit of detection.

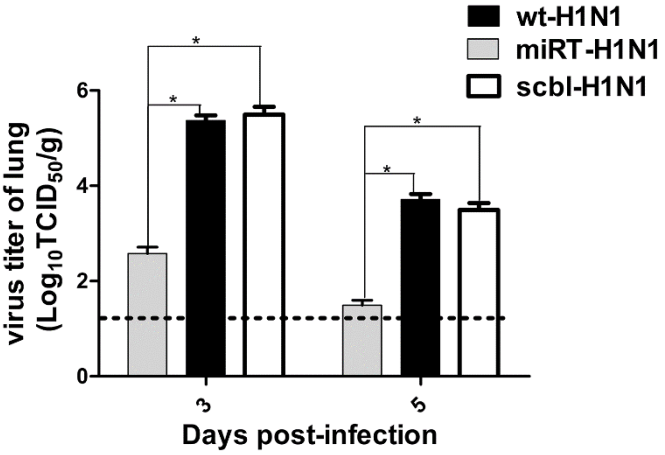
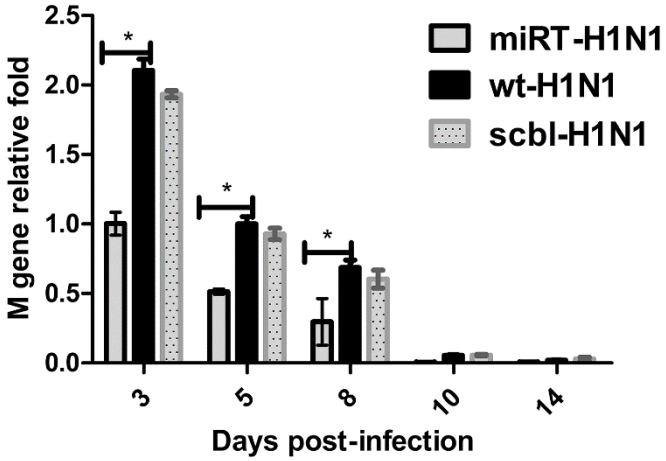


Figure 5. The RNA replication levels of viral nucleic acid M gene in lung tissues of mice infected with viruses. In each virus group, there were 4 mice. Their lungs were harvested at 3 days post-infection (dpi), 5 dpi, 8 dpi, 10 dpi, and 14 dpi. The viral RNA replication was then calculated through real-time polymerase chain reaction (PCR). Each value represents the mean of each cohort, and the error bars indicate SD (*$p < 0.05$).



H1N1 group and the miRT-H1N1 group was the most obvious ($p < 0.05$). Although the virus M gene maintained at low levels at other time points after infection, the differences in M gene copies between the attenuated strain group and the wt-H1N1 group was significant ($p < 0.05$), which was consistent with the detected difference in viral titer levels. In addition, the scbl-H1N1 virus showed viral M gene copies similar to that of the wt-H1N1 virus ($p > 0.05$).

Decreased pro-inflammatory responses in the lungs of mice infected with the mutant virus

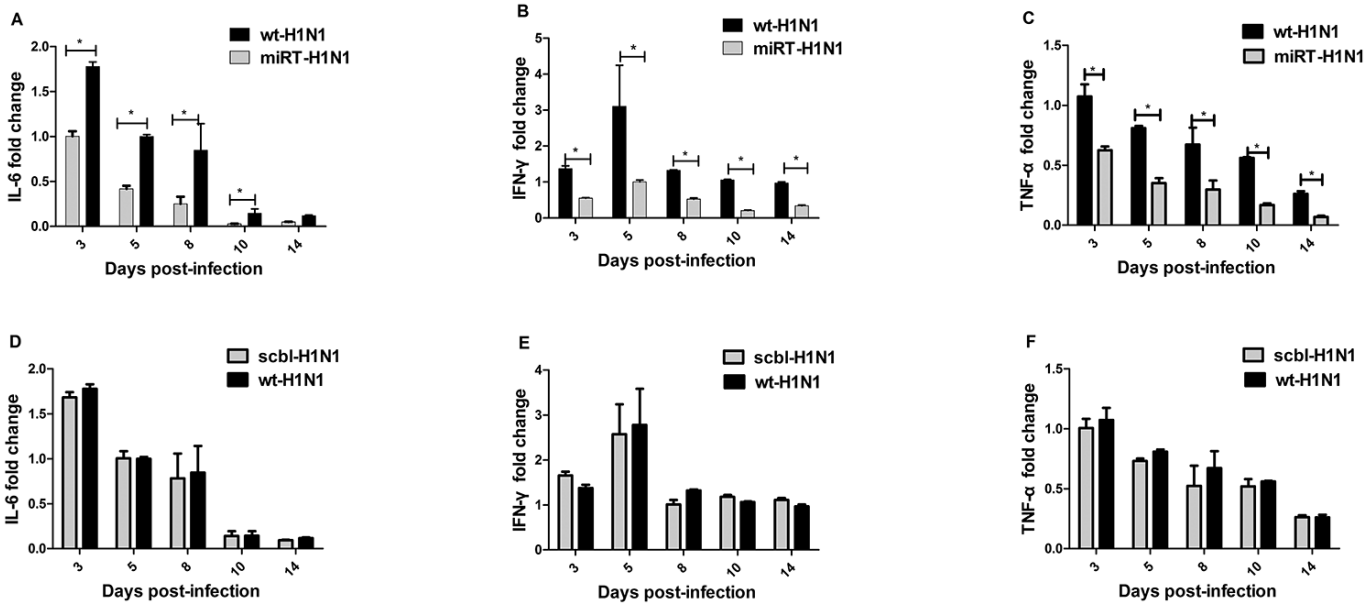
Various inflammatory mediators, such as chemokines and cytokines, which are induced by the viruses, contribute to pathogenicity of the lungs in influenza infection. Levels of IL-6, TNF-α, and IFN-γ in the lungs of mice infected with various viruses were measured as described previously. A significant decline of pro-inflammatory cytokines, including IL-6, TNF-α, and IFN-γ, was found in the lungs of mice inoculated with miRT-H1N1 viruses ($p < 0.05$) (Figures 6A, 6B, 6C). No obvious differences in cytokine induction between the sclb-H1N1 group and the wt-H1N1 group were observed ($p > 0.05$) (Figures 6D, 6E, 6F). This result was consistent with the histopathology in the lungs of the mice infected with the mutant and control viruses. In sum, the results indicated a decreased pro-inflammatory cytokine response induced in miRT-H1N1 virus-inoculated mice.

Discussion

Vaccine is the most effective approach to protect humans against influenza infection [15]. However, because of the restrictions, LAVIs can only offer suboptimal protection [1]. miRNA-mediated gene silencing may better solve this problem [16]. In general, vaccines are safe and effective, but very rare adverse reactions may occur. Public concerns about the safety of vaccines have grown increasingly in recent years. The animal model used in pre-clinical studies is the first step in developing safe vaccines. In order to reduce adverse events occurring in later clinical applications, more efforts should be made to verify viral attenuation in animals. It plays an important role in the process of developing vaccines. This study aimed to demonstrate that the miRNA-let-7b-mediated attenuated virus is safe for mice.

It is important to evaluate the virulence of attenuated vaccines in preliminary studies. The technology is not novel, but this kind of safety test has been widely used in all attenuated live influenza vaccines. The cold-adapted influenza A/AA/6/60 (H2N2) (AA ca) virus has been widely used as the backbone of the live attenuated trivalent seasonal influenza vaccine. The replication of the AA ca virus was restricted in nasal turbinates of mice, unlike the wild-type virus. The mean virus titers were $10^{4.6}$ and $10^{6.2}$ $TCID_{50}$/g in nasal turbinates of mice infected with

Figure 6. The pro-inflammatory factor and inflammatory factor in lung tissues of mice infected with wt-H1N1, miRT-H1N1, scbl-H1N1 viruses and phosphate-buffered saline. Their lungs were harvested at 3 days post-infection (dpi), 5 dpi, 8 dpi, 10 dpi, 14 dpi, and cytokines were detected by real-time polymerase chain reaction (PCR). The difference in IL-6 (**A**), INF-γ (**B**), and TNF-α (**C**) between the miRT-H1N1 and the wt-H1N1 groups. The difference in IL-6 (**D**), INF-γ (**E**), and TNF-α (**F**) between the sclb-H1N1 and the wt-H1N1 groups. Each value represents the mean of each cohort, and the error bars indicate SD (*$p < 0.05$).



the AA ca and the AA wt, respectively, two days after infection [17]. The replication kinetics of A/California/72009 (CA/7 ca), whose internal protein gene segments were derived from the AA ca, were also determined in mice. The viral titer of the CA/7 ca was almost $10^{2.0}$ $TCID_{50}$/g [18] two days after infection. M (NSL-88R) mutant is a live attenuated H1N1 M1 mutant and was generated by reverse genetics. The attenuation of the M (NLS-88R) mutant in mice was confirmed by significant reductions of viral replications in the lungs when compared with the wild-type virus. Being inoculated with M (NLS-88R) mutant at the dose range of 10^4~10^6 plaque-forming units (pfu)/mouse resulted in no morbidity or mortality, with $MLD_{50} > 10^6$ pfu/ mouse. Two days after infection, the viral titer of the M (NLS-88R) mutant was almost 100 times lower than that of the wild-type strain [7]. In 2009, a report described the effectiveness of harnessing the endogenous miRNA silencing mechanism to achieve species-specific attenuation of the influenza A virus. Mice infected with 10^3 pfu of the MRE-based vaccines showed a less than 10% weight loss and a 100% survival rate [19]. In general, if the vaccine does not produce any clinical signs of disease, characterized by a less than 10% weight loss and a 100% survival rate, and the viral load in the lungs of mice infected with attenuated viruses is at least 100 times lower than that of mice infected with the wild type, the candidate vaccine is attenuated in mice.

We had previously generated a novel attenuated vaccine by inserting the miRNA-let-7b tag into the viral PB1 gene. The attenuation of miRT-H1N1 viruses has been confirmed by significantly reduced viral replication in cultured cells [5]. In this study, we further assessed the attenuation of miRT-H1N1 viruses in a mouse model. The common parameters here included the viral titers, survival rate, and body weight loss, which were used to evaluate viral pathogenicity in mice. In addition, lung lesions, pathology scores, viral RNA replication, and inflammatory mediators were also monitored to ensure the safety of mice. In our research, the lungs were the best choice when measuring the viral titers and pathology. Although miRNA let-7 RNA is widely expressed in human tissues [2], it is highly expressed in the lungs [20]. Influenza virus replication generally starts in the respiratory tract. Whether or not it will be restricted in the respiratory tract depends on the virulence of the viral strains and host immunity [21]. Pulmonary tissue lesions caused by influenza are the main harmful results of the disease. Lung injury may be the most effective parameter in assessing the prevention and cure of the disease [22]. Our data showed that the mice inoculated with the miRT-H1N1 virus showed minimal body weight loss and a 100% survival rate even when administrated the highest dose. miRT-H1N1 viral titers in the lungs of infected mice were 100 times lower than the wt-H1N1 viral titers. These data demonstrated that the miRT-H1N1 virus was attenuated in mice. In addition, the mice administrated miRT-H1N1 viruses showed significantly milder pathologic changes and lower HPSs than the mice in the wt-H1N1 group. In accordance with the results of the virus titration assay, significantly lower copy numbers of the viral M gene were detected in mice infected with miRT-H1N1 virus. The levels of IL-6, TNF-α, and IFN-γ also decreased significantly in the lungs of the mice inoculated with the miRT-H1N1 virus. These data provide additional evidence to ensure the safety of the attenuated viruses. In contrast, all of the parameters examined in mice infected with scbl-H1N1 viruses were similar to those in mice infected with wt-H1N1 viruses. Therefore, our results demonstrated that the MREs, which were inserted into PB1 for miRNA let-7 recognition and targeting, can inhibit virus production in mice profoundly, and that the compromised viral replication is caused by miRNA.

Further issues raised in this research will be studied in our future studies. Reversion of the LAIVs, especially to a virulent phenotype, is a particularly serious concern. Previous studies have claimed an advantage for multiple MREs to be incorporated within the viral genomes to prevent viral escape [23,24]. We may consider a similar strategy for our further development of effective and safe influenza vaccines.

Conclusions

Our study demonstrated that the miRNA-let-7b meditated influenza virus is attenuated in mice and the approach of microRNA targeting for attenuation can be considered to be a rational method for increasing vaccine safety.

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Authors' contributions

Shi Y, Xing Z ,Sun WK designed research; Tan MM, Xia D, Ding Y and Liu ZC performed research; Xing Z, Sun WK, Feng CL, Su X, Shen XY and Tan MM analyzed data and wrote the paper.

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