FULL PAPER

Virology

Antiviral effects against influenza A virus infection by a short hairpin RNA targeting the non-coding terminal region of the viral nucleoprotein gene

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ABSTRACT. RNA interference (RNAi) can inhibit *Influenza A virus* (IAV) infection in a gene-specific manner. In this study, we constructed a transgene expressing a short hairpin RNA (shRNA) that targets the noncoding region of the IAV RNA gene encoding nucleoprotein (NP). To investigate the antiviral effects of the shRNA, we generated two transgenic mouse lines with this transgene. Unfortunately, there was no apparent difference in IAV resistance between transgenic and non-transgenic littermates. To further investigate the antiviral effects of the shRNA, we prepared mouse embryonic fibroblasts (MEFs) from transgenic and non-transgenic mice. In experimental infections using these MEFs, virus production of mouse-adapted IAV strain A/Puerto Rico/8/1934 (PR8) in the transgenic MEFs was suppressed by means of the down-regulation of the viral RNA gene transcription in the early stages of infection in comparison with non-transgenic MEFs. These results indicated that expression of the shRNA was able to confer antiviral properties against IAVs to MEFs, although the effects were limited. Our findings suggest that the shRNA targeting the noncoding region of the viral RNA (vRNA) of NP might be a supporting tool in developing influenza-resistant poultry.

KEY WORDS: antiviral effect, influenza A virus, RNA interference, short hairpin RNA, transgenic mouse

Avian influenza viruses belonging to the genus *Influenzavirus A* of the *Orthomyxoviridae* family are economically important pathogens in poultry worldwide. Influenza A viruses (IAVs) are negative-sense, single-stranded, segmented RNA viruses and are divided into subtypes based on the antigenic specificities of surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) [15]. Almost all possible combinations of IAV subtypes have been isolated from avian species. The optimal method of disease prevention is the immediate culling of affected poultry. An antibody-based vaccination against HA and NA might temporarily reduce the risk of disease spread in the vicinity of infected poultry farms. However, annually updated vaccines would be required because of frequent minor (antigenic drift) and major (antigenic shift) changes in glycoproteins. A possible strategy to control avian influenza in poultry flocks could be the use of genetic modification to confer infection resistance [7].

RNA interference (RNAi) is an attractive technology that specifically suppresses gene expression. RNAi induced by short hairpin RNA (shRNA) has been effective in inhibiting IAV infection in a gene-specific manner [1]. Several studies have demonstrated that shRNAs specific for influenza mRNA can effectively inhibit replication of different virus subtypes both *in vitro* and in mice [12, 14, 21]. In these reports, the shRNA targets were located in coding regions of the viral RNA (vRNA) genes.

The terminal noncoding region (NCR) of vRNA of IAV is critical for viral polymerase binding, cap snatching, transcription initiation, packaging, and replication [2, 8, 11]. The NCR sequences at the 3′ and 5′ termini of each vRNA are highly conserved among all IAVs [11]. Thus, the terminal regions of vRNAs may be a potential RNAi target for antiviral effects against broad

subtypes of IAV, as evidenced by a synthetic small interfering RNA (siRNA) specific for the NCR of the PB1 gene that partially inhibited virus production in Madin-Darby canine kidney (MDCK) cells [5].

The nucleoprotein (NP) associates with PB2, PB1, and PA to form a viral ribonucleoprotein complex that is responsible for viral replication and transcription [10]. The NP is highly conserved among IAVs and has been suggested as an antiviral target. We have previously demonstrated an anti-NP monoclonal antibody that conferred remarkable protective effects against heterosubtypic influenza virus infections in transgenic mice [3]. In this study, we investigate the antiviral potential of an shRNA targeting the NP vRNA as another tool for gene modification. We generated transgenic mice expressing a shRNA targeting the 5′ terminus of the NP vRNA and conducted experimental infections to evaluate IAV infection resistance.

MATERIALS AND METHODS

Virus and cells

IAV H1N1 strain A/Puerto Rico/8/1934 (PR8) was a kind gift from Dr. Takeshi Ichinohe at The University of Tokyo, Japan. The virus was propagated in 10-day-old embryonated chicken eggs at 37°C for 2 days, and the allantoic fluid was stored −80°C until use. MDCK cells and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum at 37°C in 5% CO_2.

Generation of transgenic mice

The shRNA target sequence was located in the 5′ terminus of the vRNA (antisense RNA) segment containing the NP gene; 5′-UAGAAACAAGGGUAUUUUUCU-3′ (nucleotides 1,543–1,563). The oligonucleotides and their complements were synthesized by GenScript Japan Inc. (Tokyo, Japan) and cloned into an altered pMSCV vector with the insertion of a β-actin promoter. The transgene fragment was isolated from the constructed plasmid by *Stu*I (Takara Bio Inc., Kusatsu, Japan) digestion. The DNA fragment containing the β-actin promoter and the short hairpin sequence was microinjected into nuclei of C57BL/6 mouse eggs to generate transgenic mice. Genotyping of transgenic mice as previously described [3], was performed by PCR using the primer set 5′-AGGGGGACACCAGCAAGGAG-3′ and 5′-CATTCAGGCTGCGCAACTGT-3′. The ethics committees of Kyushu University, Japan, approved all animal experimentation protocols.

Experimental infection in mice

Experimental infection in mice was carried out in the Biosafety Level 2 facility at the Center of Biomedical Research, Research Center for Human Disease Modeling, Graduate School of Medical Sciences, Kyushu University, Japan. The allantoic fluid containing strain PR8 was diluted in phosphate-buffered saline to prepare the inoculum. Mice aged 6–8 weeks were anesthetized by intraperitoneal injection [9] and then intranasally inoculated with 40 μl of the diluted allantoic fluid. Survival and body weights of the infected mice were recorded for 14 days.

Quantitative analysis of siRNA in MEFs

MEFs were prepared according to standard procedures, and total RNA was extracted from $1 × 10^6$ cells using a miRNeasy Mini Kit (Qiagen, Hilden, Germany). The siRNA expression level in the transgenic MEFs was quantified via cDNA synthesis and real-time PCR (RT-PCR) using a miRNA 1st-Strand cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and miRNA QRT-PCR Detection Kit (Agilent Technologies), respectively. A siRNA-specific forward primer (5′-AGAAAAATACCCTTGTTC-3′) and the universal reverse primer supplied with the kit were used for RT-PCR.

Experimental infection in MEFs

For the investigation of one-step viral growth in transgenic MEFs, the cells were seeded in 35 mm diameter dishes ($1 × 10^6$ cells per dish). Strain PR8 was adsorbed onto the cells at a multiplicity of infection (MOI) of 0.01 at 37°C for 1 hr. Then, the cells were washed to remove any non-adherent virus and cultured in 3 ml DMEM without trypsin. The supernatant (500 μl) was harvested from the infected cells at 8 and 24 hr post-infection, and a plaque formation assay was performed using MDCK cells cultured into DMEM containing 0.8% SeaPlaque™ Agarose (Lonza, Basel, Switzerland) and 2 μg/ml of TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO, U.S.A.).

Semi-quantitative analysis of the viral gene expression

With the RNeasy Plus Mini-Kit (Qiagen), total cellular RNA was extracted from MEFs after 8 and 24 hr post-infection with strain PR8 as described above. To synthesize complementary DNAs derived from mRNA genes of the viral NP and mouse GAPDH, reverse transcription was conducted using PrimeScript reverse transcriptase (Takara). Then, real-time PCR reactions with the synthesized cDNA and SsoAdvanced Universal SYBR Green Supermix (Bio-rad, Hercules, CA, U.S.A.) were performed on CFX Connect™ Real-Time PCR Detection System (Bio-rad). The cycle conditions were according to manufacturer's instructions and the comparative threshold cycle (Ct) method was used to compare the level of gene expression. The expression level of the viral mRNA was normalized against that of mouse GAPDH mRNA. The primers used for reverse transcription and real-time PCR are described elsewhere [4, 20].
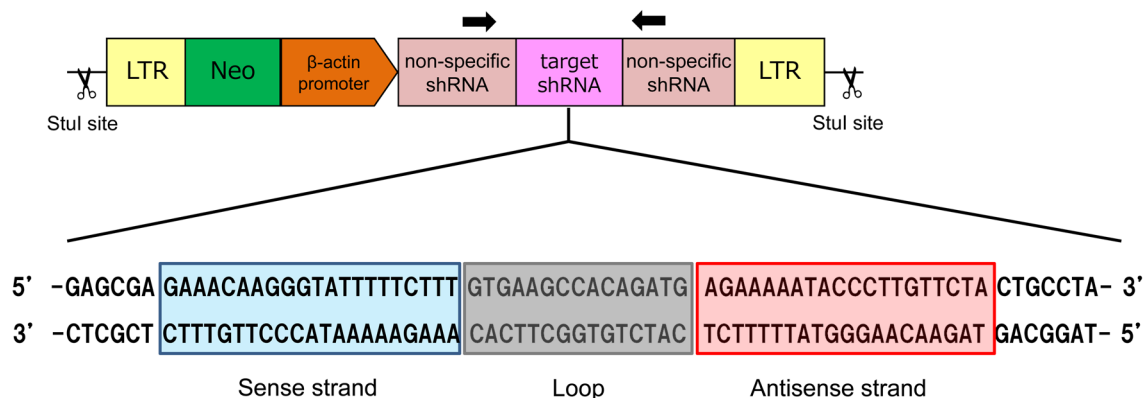
Fig. 1. Schematic representation of the shRNA expression vector. This vector includes a β-actin promoter driving shRNA expression. The arrows denote the PCR primers used to identify transgene integration in the transgenic mice.

Table 1. Expression levels of siRNA targeting the vRNA of NP gene in MEFs

Cell lines		Genotyping [a]	Expression level of siRNA (10^5 copy/cell)
Line 6522	A	TG	11.0
	B	TG	4.9
	C	TG	4.7
	D	TG	2.4
	E	Non-TG	Below the detection limit [b]
Line 6837	F	TG	1.2
	G	TG	1.3
	H	TG	2.2
	I	Non-TG	Below the detection limit
	J	Non-TG	Below the detection limit

a) MEFs were prepared from transgenic (TG) embryos or non-transgenic (Non-TG) embryos. b) The detection limit of siRNA was 8.6×10^4 copy/cell.

RESULTS

We selected a highly conserved sequence (bases 1,543–1,563) of the vRNA of NP to construct a transgene expressing a shRNA targeting the NCR of the NP vRNA. Oligonucleotides forming one target and two nonspecific shRNAs were inserted into an altered pMSCV vector. The expression of the shRNAs was under the control of a β-actin promoter. The schematic structure of the transgene and the shRNA sequences are presented in Fig. 1.

To examine the antiviral potential of the shRNA targeting the NCR of the NP vRNA against IAV infection, we generated transgenic mice with the transgene that was isolated from the constructed pMSCV plasmid by *Stu*I digestion. Two transgenic mouse lines, 6522 and 6837, were established, and no apparent abnormalities were observed. Since the shRNA could not be detected in total RNA isolated from transgenic mouse tail and lung by quantitative RT-PCR (lower than the limit of detection: 8.6×10^4 copies/cell; data not shown), MEFs were prepared from mouse embryos of each transgenic mouse line. Following total RNA extraction from each transgenic MEF to analyze shRNA expression in the mice, the shRNA expression level was measured by quantitative RT-PCR. In the transgenic MEFs of lines 6522 and 6837, $2.4–11.0 \times 10^5$ and $1.2–2.2 \times 10^5$ copies/cell of the shRNA were detected, respectively. The shRNA expression in non-transgenic MEFs was lower than the limit of detection (Table 1).

To investigate IAV infection resistance in the transgenic mice, a mouse-adapted strain PR8 was intranasally inoculated into the mice of lines 6522 and 6837 at a low infective titer (400 plaque forming units/mouse) because the shRNA expression levels were considered limited. There was no significant difference in clinical signs and mean weight loss between transgenic and non-transgenic littermates in both lines (Fig. 2).

Subsequently, we performed a one-step viral growth analysis in MEFs to examine the resistance of transgenic MEFs. Because the MEFs used in the present study were extremely sensitive to trypsin, which is required for the efficient multiple-step growth of low pathogenic influenza viruses in cell culture, the MEFs infected with strain PR8 at an MOI of 0.01 were cultured in the absence of trypsin. The supernatants were collected at 8 and 24 hr post-infection and were subjected to viral titration by plaque formation assay using MDCK cells. As shown in Fig. 3, the virus titers in all transgenic MEFs of lines 6522 (cell lines A–D) and 6837 (cell lines F–H) were significantly lower than those in non-transgenic MEFs (cell lines E, I, and J) at 8 hr post-infection ($P<0.01$),
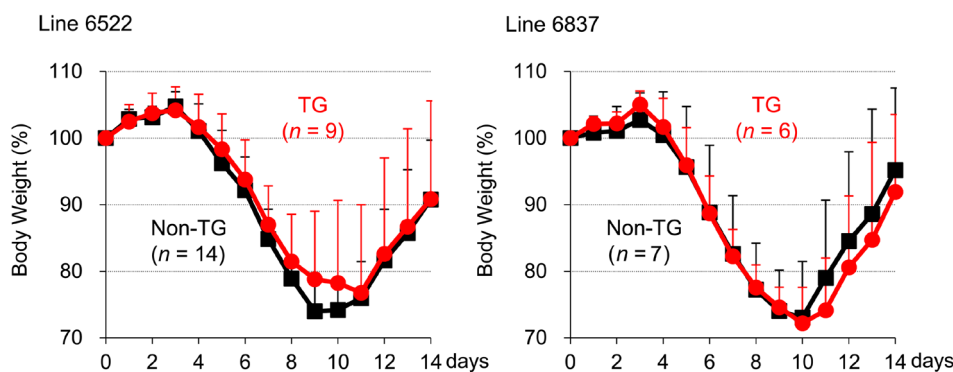
Fig. 2. Experimental infections in transgenic mice expressing shRNA. Transgenic (TG) and non-transgenic (non-TG) mice were challenged intranasally with IAV strain PR8. Mice body weight during the challenge phase (14 days) was calculated relative to their starting body weight. Error bars indicate standard deviation.
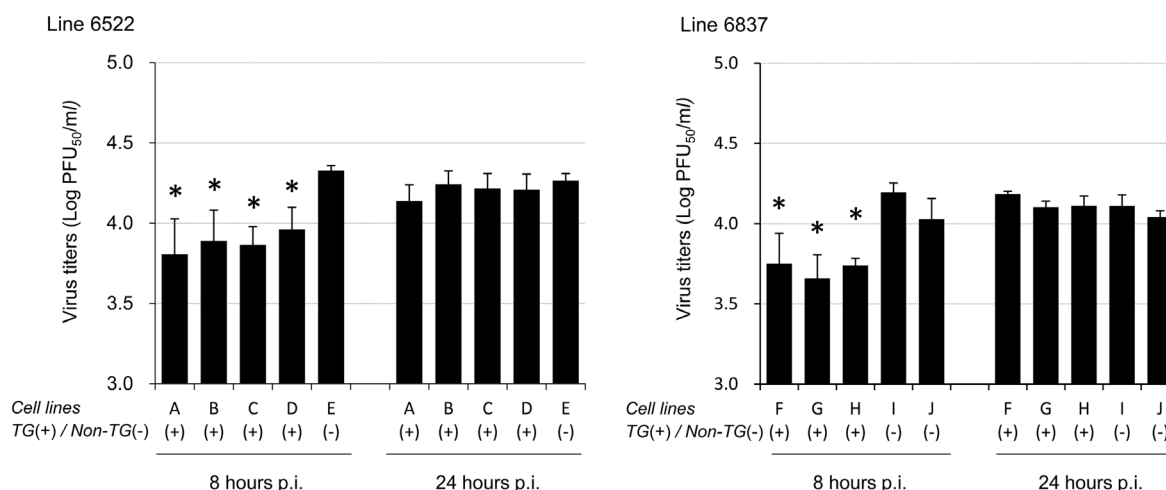

Fig. 3. Viral replication in transgenic mouse embryonic fibroblasts (MEFs) expressing shRNA. MEFs were infected with PR8 at an MOI of 0.01. The culture supernatants were collected at 8 and 24 hr post-inoculation for virus titration in MDCK cells. The mean values of the virus titers were determined in triplicate. Error bars indicate standard deviation. MEF cell lines A–D and F–H were prepared from transgenic mouse embryos of lines 6522 and 6837, respectively. MEF cell lines E, I, and J were prepared from non-transgenic mouse embryos. Statistical analysis was performed using Student's *t*-test, and * indicates $P<0.01$; significant differences in transgenic MEFs versus non-transgenic MEFs (A–D vs. E in cell line 6522, F–H vs. I in cell line 6837).

although the difference was within 1 log unit. However, the viral titers at 24 hr post-inoculation were almost similar with those in non-transgenic MEFs. To confirm the suppression of the NP gene transcription, we examined the accumulation of the NP mRNA in MEFs at 8 and 24 hr post-infection by semi-quantitative real-time PCR analysis (Fig. 4). As compared to non-transgenic MEFs, the accumulation of the NP mRNA in all transgenic MEFs were significantly low at 8 hr post-infection. Although the expression level in cell line F was rather low, other transgenic MEFs were comparable to those in non-transgenic MEFs at 24 hr post-infection. These results indicated that the shRNA targeting the 5′ terminal NCR of the NP vRNA suppressed the virus replication in transgenic MEFs by means of the restricted transcription of the viral RNA gene at the early stage of infection, and at least 1.2×10^5 copies/cell of the shRNA was required for viral suppression.

DISCUSSION

In this study, we demonstrated that a shRNA targeting the 5′ terminal NCR of the NP vRNA conferred an antiviral effect against IAV infections in cultured transgenic MEFs at the early stage of infection (Fig. 3). However, this effect was limited (difference within 1 log unit) and not observed at 24 hr post-infection (Fig. 3). It was concluded that knockdown of the NP vRNA significantly suppressed viral replication in the transgenic animals at the early stage of the viral infection because vRNAs, including the NP vRNA, are released from viral particles immediately after IAV infection. The results of semi-quantitative analysis of the viral gene expression in infected MEFs supports this hypothesis (Fig. 4). It appeared that the limited effect was due to a relatively low shRNA
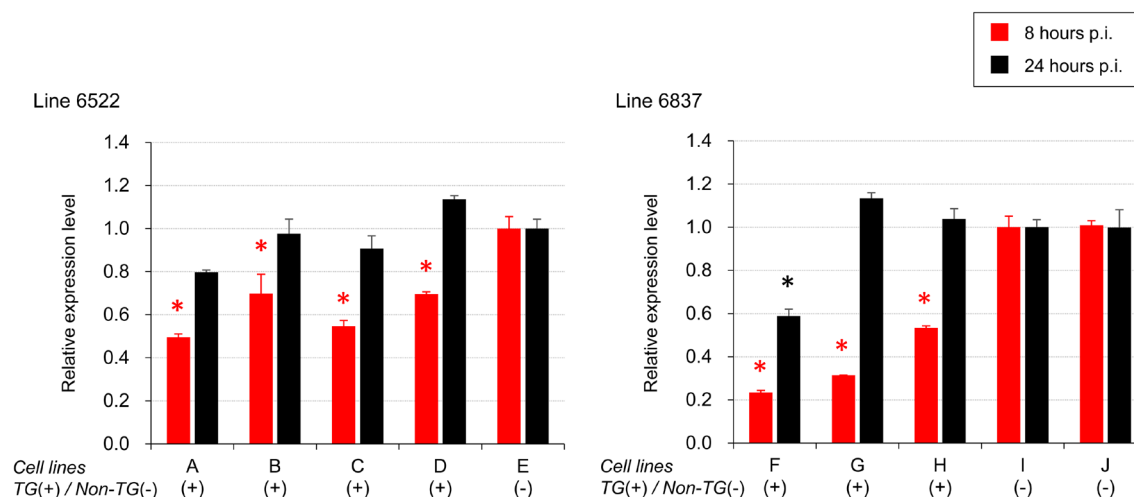
Fig. 4. Expression levels of a viral gene in transgenic MEFs expressing shRNA after infection with strain PR8. Total RNA was extracted from MEFs infected with PR8 at an MOI of 0.01 after 8 and 24 hr post-inoculation. To determine the expression level of mRNA gene of the viral NP, reverse transcription and semi-quantitative real-time PCR were carried out. The results are presented in relative value to the expression level in non-transgenic MEFs (cell lines E and I for transgenic lines 6522 and 6837, respectively), and error bars represent the mean ± SEM of duplicate experiments. MEF cell lines A–D and F–H were prepared from transgenic mouse embryos of lines 6522 and 6837, respectively. MEF cell lines E, I, and J were prepared from non-transgenic mouse embryos. Statistical analysis was performed using Student's t-test, and * indicates $P<0.01$; significant differences in transgenic MEFs versus non-transgenic MEFs (A–D vs. E in cell line 6522, F–H vs. I in cell line 6837).

expression level in the transgenic MEFs. In both tails and lungs of the transgenic mice, the expression levels were lower than the limit of detection (data not shown). Therefore, the shRNA expression level was insufficient to prevent severe weight loss in the infected transgenic mice (Fig. 2).

In this study, we used an RNA polymerase II (Pol II) β-actin promoter (Fig. 1) because shRNA expression controlled by a promoter would be a suitable tool for providing tissue-specific antiviral strategies with relatively few side effects. Several groups have revealed that Pol II is capable of expressing high levels of functional shRNA, and different Pol II promoters, including CMV-IE, ubiquitin-c, and CAG promoters, are available for shRNA synthesis [18, 19, 22–24]. Our results indicate that a much stronger promoter capable of enhancing the expression level of the shRNA is required to exert an enhanced antiviral effect in the transgenic mice.

Integration sites influence transgene expression levels in transgenic mice [6, 17]. Since the transgenic mice in this study were generated by the direct microinjection of a DNA fragment into the pronucleus of fertilized mouse eggs, it is thought that integration occurs into random regions of different chromosomes as a concatemer (several transgene copies). Thus, the mice may exhibit different levels of transgene expression. Therefore, to conduct a detailed analysis of the antiviral potential of the shRNA, knock-in mice in which the DNA fragment is inserted at a determined genomic locus should be prepared.

Recently, we reported that transgenic mice expressing anti-NP human monoclonal antibodies exhibited a marked resistance to lethal infection with heterosubtypic IAVs including different clades of highly pathogenic H5N1 avian influenza viruses [3]. Although the antiviral mechanism observed in transgenic mice remains unclear, the anti-NP antibodies may induce indirect antiviral effects, such as antibody-dependent or complement-dependent cytotoxicity, because transgenic MEFs expressing the anti-NP antibodies did not demonstrate any resistance to IAV infection [3]. In the present study, we demonstrated that virus production at the early stage of IAV infection was suppressed in transgenic MEFs expressing the shRNA targeting the 5′ terminal NCR of the NP vRNA (Fig. 3), indicating that this shRNA exerted direct antiviral effects on transgenic MEFs. The combination approach of direct and indirect antiviral effects may be useful for developing influenza-resistant animals that express the shRNA as a direct agent for the suppression of viral replication and secrete the anti-NP monoclonal antibodies as an indirect agent for the viral elimination.

We previously reported that the suppression of viral replication at the early stage of infection is significantly important for the recovery from severe influenza virus infections in mice [3]. Other researchers have reported that the suppression of viral replication in the early stage of infection might favorably change the severity of influenza in vivo [13, 16]. In this study, we demonstrated that a shRNA targeting the 5′ NCR of the viral NP gene was able to suppress the virus replication by means of the down-regulation of the viral RNA gene transcription at the early stage of infection in transgenic MEFs. However, the antiviral potential of the shRNA alone was insufficient for developing influenza-resistant animals. Therefore, the shRNA may contribute to the development of influenza-resistant animals as a supporting tool.

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