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Human H1 promoter expressed short hairpin RNAs (shRNAs) suppress avian influenza virus replication in chicken CH-SAH and canine MDCK cells

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ABSTRACT

Influenza A virus represents a significant health and economic threat to both humans and animals worldwide. The polymerase III human H1 promoter was used to express nine short hairpin RNAs (shRNAs) targeting nucleocapsid protein (NP) or the acidic component of polymerase complex (PA) genes of avian influenza virus. Tested shRNAs led to a decrease of up to 400 fold in infective titre and up to 80% in the level of viral NP and PA mRNAs in chicken CH-SAH cells ($p < 0.05$). In mammalian MDCK cells a decrease of up to 10^6-fold in infective titre, and up to 90% in the level of viral mRNAs ($p < 0.05$) was detected. While RNAi silencing played a major role in viral inhibition, transfection of any plasmid induced an antiviral interferon response in MDCK cells. In CH-SAH cells, no induction of IFN response was observed by constructs, and the IFN response was suppressed by influenza virus infection. Simultaneous induction of RNAi and IFN responses in CH-SAH cells resulted in 18,500-fold decrease in infective titre ($p < 0.05$). We also identified novel efficient and conserved RNAi target site in the NP gene which can be used in antiviral cocktails of shRNAs to block the escape of influenza virus.

1. Introduction

Influenza is one of the most widespread zoonotic infectious diseases yet repeated attempts to control it in humans and animals have failed. In humans, influenza A virus produces seasonal epidemics with up to 20% of population being infected resulting in 250,000–500,000 deaths worldwide each year (Peiris et al., 2007; Maines et al., 2008). Rarely, the appearance of new influenza strains (1918 H1N1, 1957 H2N2 and 1968 H3N2) might result in a worldwide pandemic with millions of deaths. Influenza also affects human companion and livestock animals, and recently avian and swine influenza viruses resulted in zoonotic human infections.

Avian influenza (fowl plague) in poultry can range from asymptomatic infection with low pathogenic strains to systemic disease with 100% mortality with highly pathogenic H5 and H7 strains (Swayne and Halvorson, 2008). The unprecedented pandemic of highly pathogenic H5N1 strain in 2003–2009 spread quickly through Asia, Europe and Africa and decimated the worldwide poultry industry. H5N1 virus also showed an alarming ability to directly infect humans (Peiris et al., 2007; Maines et al., 2008). As of July 1, 2009, World Health Organization (WHO) reported 436 laboratory-confirmed cases of human infections with about a 60% fatality rate (WHO, 2009a). Recent genomic studies indicate that the pandemic virus of 1918, responsible for 50 million deaths worldwide, similarly arose from avian influenza viruses adapting to humans (Taubenberger et al., 2005).

Swine influenza (H1N1 and H3N2) viruses regularly cause outbreaks of respiratory disease among pigs and are common throughout pig populations worldwide (Thacker and Janke, 2008). In the North-Central USA 51% of pigs have antibody evidence of infection with H1N1 (CDC, 2009). In addition, swine has long considered to be an ideal mixing vessel for adaptation of avian influenza virus to a mammalian host. Recently, WHO declared a world wide public health emergency due to human epidemics caused by a novel swine-origin influenza A (H1N1) virus produced by triple reassortment of sequences from swine, avian and human influenza viruses of North American, European and Asian lineages (Garten et al., 2009; Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, in press). On June 11, 2009, first time in 41 years WHO officially announced level six human pandemic. As of July 1, 2009, World Health Organization (WHO) reported 77,201 laboratory-confirmed cases of human infections in 120 countries with 332 deaths (WHO, 2009b). There is a real concern that virus can mutate

Abbreviations: IFN, interferon; NP, nucleocapsid protein; PA, acidic component of polymerase complex; Pol, polymerase; RNAi, RNA interference; siRNA, short interfering RNA; shRNA, short hairpin RNA.

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or reassort with H5N1 avian strain creating highly pathogenic and readily transmissible novel influenza strain.

Similarly, recent evidence of influenza adaptation to a human primary companion animals, such as dogs and cats, have great potential public health impact. H5N1 avian influenza virus demonstrated ability to cause systemic disease in domestic cats, which previously were considered resistant to influenza (Kuiken et al., 2004). Likewise, less well-known recent adaptation of equine influenza A (H3N8) virus to dogs created new intimately associated with humans source of zoonotic infection (Crawford et al., 2005).

These examples again demonstrate continuous evolution of animal influenza virus and its ability to adapt to a new host. Widespread presence of animal influenza virus might lead to new zoonotic infections, and creation of new pandemic influenza A strains through gene reassortment with human influenza virus. Influenza represents a health and economic threat to both humans and animals worldwide. The development of novel anti-influenza approaches against influenza virus in human and in companion and livestock animals is urgently needed.

Several promising alternatives to antiviral drugs and vaccine development, including antisense, ribozyme, and more recently RNA interference (RNAi), have been used to inhibit viral reproduction by directly targeting viral RNAs. RNAi is a recently discovered natural antiviral mechanism present in a variety of eukaryotic species, and might also be the most promising new approach for targeting viruses (Haasnoot et al., 2007; Ding and Voinnet, 2007; Carthew and Sontheimer, 2009). Up to a 30,000-fold inhibition of human influenza virus was achieved using synthetic siRNA in MDCK cells (Ge et al., 2003). While siRNA application could be advantageous for treating an ongoing influenza infection, the siRNA effectors are quickly degraded *in vivo* providing only transient protection. The expression of short hairpin RNA (shRNA) from vectors offers a possibility for achieving extended viral inhibition without the need of repeated introduction. Polymerase III type 3 RNase P RNA H1 (H1) and small nuclear RNA U6 (U6) promoters are often used for RNAi silencing. These promoters naturally direct the synthesis of small, highly abundant noncoding RNA, do not contain intragenic control regions, and have well defined termination sequences of 4–5 thymidines. While both promoters are highly efficient in shRNA silencing of cellular mRNAs, viruses often use a number of approaches to suppress host cellular and immune response, which might affect the efficiency of RNA silencing. Nonstructural (NS1) protein of influenza virus is known to act as an inhibitor of the IFN response, impairs the post-transcriptional processing and nuclear export of cellular transcripts, and also inhibits RNAi silencing in *Drosophila* and plant cells (Hale et al., 2008).

The goal of this project was to test applicability of human H1 promoter to produce anti-influenza shRNA for long term protection against influenza virus in mammalian and avian cells. In addition a novel conservative and efficient RNAi target site was developed to be used in antiviral cocktails of shRNAs to decrease the possibility of influenza virus escape from RNAi silencing.

2. Materials and methods

2.1. Cell cultures

In the preliminary study of avian cell cultures (chicken embryo fibroblasts primary cell culture, Quail Japanese fibrosarcoma QT-35 and chicken hepatoma CH-SAH), the tested strains of avian influenza virus replicated to the highest titre in chicken hepatoma CH-SAH cells which were used for further work. CH-SAH cell line was established from hepatocellular carcinoma induced in a chicken by diethylnitrosamine and was supplied by Solvay company (Mendota Heights, MN) (Alexander et al., 1998). Madin-Darby canine kidney (MDCK) cells was chosen as the model mammalian cell line commonly used for *in vitro* studies of influenza viruses and for testing anti-influenza drugs. The cells were grown in Dulbecco's modified Eagle medium/F-12 (D-MEM/F-12) (Sigma) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM HEPES, 200 U/mL penicillin and 80 μg/mL streptomycin at 37 °C in the presence of 5% CO_2.

2.2. Virus propagation and virus titration

The following avian influenza virus avirulent strains were propagated in embryonated chicken eggs and tested in MDCK and CH-SAH cells: A/turkey/Ontario/6118/68 (H8N4), A/turkey/Massachusetts/3740/65 (H6N2), A/duck/Czech/56 (H4N6), A/Quail/Italy/1117/65 (H10N8). Virus stock was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs and incubated at 37 °C for 48 h. The allantoic fluids were harvested and stored at −80 °C. Virus titre was measured by hemagglutination or 50% tissue culture infectious dose ($TCID_{50}$) assay. A/Quail/Italy/1117/65 strain was chosen for the study as it replicated to high titres in both cell lines with strong cytopathic effect (CPE) at 48 h post-infection and was suitable for TCID50 assay.

2.2.1. Hemagglutination (HA) assay

The allantoic fluids from all eggs were harvested and titrated by the hemagglutination (HA) test using 0.5% chicken red blood cells (CRBC) (Webster et al., 2002). The HA assay was carried out essentially as described. Briefly, 50 μl volumes of the allantoic fluids or culture supernatants were serially diluted 2-fold in PBS in U shaped 96-well plates. Subsequently, 50 μl of a 0.5% suspension of chicken erythrocytes were added per well. The plates were incubated for 1 h at 4 °C and the HA patterns were read visually. Wells containing an adherent layer of erythrocytes were scored as positive.

2.2.2. Assay and determination of 50% tissue culture infectious doses ($TCID_{50}$)

$TCID_{50}$ indicates the dilution of virus that will produce a cytopathic effect in 50% of the infected cultures (Webster et al., 2002). Culture supernatants were assayed using a 10-fold dilution for virus cytopathic effect in MDCK. For virus propagation in cell cultures the growth medium was removed and the cell monolayer in a 24-well plate was inoculated with 100 μl/well of virus suspension. Plates were incubated for 1 h on a rocking platform at room temperature. After 1 h the inoculum was removed, and the cell monolayer was washed with phosphate buffered saline (PBS), and D-MEM/F12 medium containing 2 μg/mL TPCK treated trypsin was added to cells. Cells were analyzed for CPE at 48 h post-infection (p.i.). To quantify the titre of progeny virus in supernatants, 50 μl of 10% BSA was added to the cell supernatants as a stabilizer, and the supernatants were harvested and kept at −70 °C for $TCID_{50}$ assay. Virus titration was done in MDCK cells that were plated in 96-well plates at 3×10^4 cells/well density 24 h prior to the assay to achieve 75–90% confluency. Ten-fold serial dilutions from 1:10 to $1:10^8$ were made for each sample, and from each dilution 25 μl/well of virus suspension was added to the cells in quadruplicates. Cells were stained with 0.1% crystal violet at 48 h p.i. and wells with CPE were scored as positive for virus growth. $TCID_{50}$/mL was calculated by the Reed and Muench mathematical technique as described by Villegas (Villegas, 1998).

2.3. Construction of short hairpin RNA (shRNA) expressing constructs

Two complementary oligonucleotides (Suppl. Table 2) were synthesized for each construct (Sigma). The oligonucleotides were

diluted to a concentration of 1 μg/μl in nuclease-free water and annealed in 50 μl annealing mixture containing 2 μl of siRNA sense template, 2 μl of antisense template and 46 μl of 1X DNA annealing solution. The mixture was heated to 90 °C for 3 min then cooled to 37 °C and incubated for 1 h. Five μl of the annealed siRNA template was diluted with 45 μl of nuclease-free water for a final concentration of 8 ng/μl. The inserts were cloned into BamH I and Hind III sites of the pSilencer 3.1-H1 neo vector according to the protocols recommended by the manufacturer (Ambion). A 100 μl aliquot of DH5α competent cells was transformed with 5 μl of five times diluted plus-insert ligation product by heat-shock transformation. Plasmid DNA was isolated from three selected clones from each plate using QiAprep Spin Miniprep Kit (Qiagen). The regions of the plasmids containing the inserts were sequenced to verify the presence of the expected sequences using M13 forward primer. shRNA constructs used in the experiments were purified from bacterial cultures with the Qiagen Plasmid Midi Kit.

2.4. Transfection of shRNA constructs into the cells

Cells were transfected with shRNA plasmids using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instruction. Briefly: cells were seeded a day prior to transfection in 24-well tissue culture plates, so by the time of the transfection the cell monolayer would be ~95% confluent. For each well 0.8 μg of plasmid was diluted in 50 μl of Opti-MEM. Lipofectamine 2000 (Invitrogen) was diluted in 50 μl of Opti-MEM in a separate tube and incubated for 5 min at room temperature. A ratio of 1 μg of DNA: 3 μl Lipofectamine was used for transfection of CH-SAH cells, and 1 μg of DNA: 5 μl Lipofectamine 2000 was used for transfection of MDCK cells. The plasmid DNA and Lipofectamine were mixed and incubated for 30 min at room temperature. The growth medium from the cells was replaced with fresh medium without antibiotic and FBS, and the plasmid-Lipofectamine complexes were then added to each well of cells. Four hours later the transfection medium was replaced with fresh growth medium containing antibiotics and FBS.

For anti-influenza activity shRNA constructs were transfected into the cell cultures and 18 h later the cells were infected with A/Quail/Italy/1117/65 (H10N8) virus at a multiplicity of infection (moi) of 0.001. Forty-eight hours post-infection cell culture supernatant was harvested, and $TCID_{50}$ assay was applied to determine infectious viral titre as described previously. Transfection efficiency was monitored by transfecting the same number of cells with the same amount of pEGFP-N1 plasmid (BD Biosciences). GFP expressing cells were viewed under a fluorescent microscope (Leica), and the total number of cells in bright field and cells demonstrating GFP fluorescence under the UV light was determined. Pictures were taken using the OpenLab 4 program (Improvision). Expression of EGFP in cells was also quantified by FASCan flow cytometer (Becton Dickinson). Cells were trypsinized, washed in PBS and resuspended in 500 μl of PBS containing 2% FBS. Data from 10,000 cells were collected. Data analysis was performed using CellQuest software supplied by the manufacturer.

2.5. Real-Time RT-PCR

Primers for the Real-Time RT-PCR for each gene of interest (influenza virus NP, PA; chicken IFN-β, OASL, ACTB; canine OAS1, ACTB) were designed using PerlPrimer software across intron/exon boundaries (Suppl. Table 1) (Marshall, 2007). All primers were tested for specific amplification by agarose gel electrophoresis and sequencing of PCR products (Laboratory Services Division, University of Guelph, Guelph, ON). Primer sets that resulted in 90–110% amplification efficiency were chosen for quantification.

To prevent re-infection the transfected cells were harvested at 3 h p.i. for RNA extraction. Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instruction. The total RNA concentration was quantified by Eppendorf Biophotometer Spectrophotometer (Germany). The quality of the RNA was evaluated using 18S and 28S ribosomal RNAs (rRNA) with 2100 Bioanalyzer (Agilent). The electroforegramms showed that there was no degradation of RNA at the tested time. Complementary DNA (cDNA) synthesis was performed in a TGradient thermocycler (Biometra, UK). Equal amounts of RNAs were used for cDNA synthesis using oligo (dT)12–18 primer (Invitrogen) and SuperScript II RNase H reverse transcriptase (Invitrogen) in a total volume of 20 μl. The concentrations of all cDNAs were determined using the Picogreen dsDNA Quantitation Kit (Molecular Probes).

Real-Time RT-PCR was performed in an ABI Prism 7000 (Applied Biosystems) and analyzed with the ABI Prism 7000 SDS Software. Reaction mixtures were prepared with Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). Equal amounts of cDNA were used for Real-Time RT-PCR. The PCR mixture was incubated at 95 °C for 2 min and 45 cycles were performed at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s. The specificity of amplification during Real-Time PCR was monitored by the dissociation curve, running some of the products in an agarose gel and sequencing. The relative amounts of RNA transcripts of the genes of interest in treated and control cells were determined by Relative quantification with standard curve method. All PCR reactions were run in duplicate. The Ct values that varied by >0.5 unit (cycle) between duplicates were discarded. The duplicate Ct values were averaged, and the amounts of transcripts present for each gene in each sample were normalized to the ACTB gene (housekeeping gene).

2.6. Statistical analysis

Data were analyzed by ANOVA using the GLM procedure of SAS software as a completely randomized block design. The modified Levene (Brown and Forsythe) test for homogeneity of variances (HOV) showed no evidence of any differences in the group variances. The Tukey test was used to perform pair wise comparisons among the group means. Kolmogorov–Smirnov test was used for statistical analysis of flow cytometry data. All experiments were repeated in triplicate. The level of significance chosen to determine whether the data obtained from treated and control samples were significantly different was $p < 0.05$.

3. Results

3.1. Design of novel shRNAs

A significant problem with designing anti-influenza RNAi is the enormous diversity of avian influenza virus strains. As even a single mutation in the target site can abolish RNAi inhibition, it is important to identify sites that would be highly conserved across different strains especially in pathogenic H5, H7 and H9 avian influenza subtypes. The NP gene is one of the most highly conserved genes of influenza virus and is thus the prime target for RNAi (Heiny et al., 2007; Obenauer et al., 2006). The complete nucleotide sequences of NP gene were uploaded from the Influenza Sequence Database (http://www.biohealthbase.org/) (Squires et al., 2008) and aligned by multiple sequence alignment program ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Thompson et al., 1994). In addition, the NP sequences of avian influenza viruses aligned using MUSCLE were kindly provided by Dr. John C. Obenauer (St. Jude Children's Research Hospital, Memphis, USA) (Obenauer et al., 2006).

Fifty siRNAs were designed against the NP gene using several siRNA design programs (http://www.dharmacon.com, http://www.biopredsi.org, http://www.mwg-biotech.com). In addition, the secondary structure of the antisense strand of the siRNA was determined using Vienna RNA Package (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and RNA Structure 4.3 (http://128.151.176.70/RNAstructure.html). Antisense sequences producing weak secondary structure were selected as suggested (Patzel et al., 2005). Also all siRNA sequences were screened for high homology to chicken and canine sequences to decrease the possibility of off-target silencing. The anti-EGFP siRNA (Cao et al., 2004), anti-influenza NP-1496 (NP a/b) and PA-2087 (PA) siRNAs (Ge et al., 2003) were converted into shRNA for use as positive controls. The previously tested scrambled shRNA sequence was used as a negative control. Preliminary experiments demonstrated that the 4-nt loop (NPb) (Siolas et al., 2005) worked with higher efficiency in both chicken and mammalian cells than the 11-nt miR-26b loop (NPa) (Miyagishi et al., 2004), so all novel shRNAs were designed with a 4-nt loop (CCAA) (Suppl. Table 2). Several G:U wobble mutations were introduced into the sense strand of shRNAs to increase RNAi efficiency, decrease the possibility of interferon induction and self-targeting, and to facilitate sequencing and propagation of the shRNA vectors in *Escherichia coli* (Miyagishi et al., 2004).

3.2. Inhibition of production of influenza virus by shRNAs transcribed by H1 promoter in avian and mammalian cells

The transfection procedure was optimized using pEGFP plasmid to achieve up to 45% transfection rate for CH-SAH cells and up to 25% in MDCK cells (not shown). The presence of functional RNAi machinery was tested in both cell lines by co-transfection of pEGFP and previously tested anti-GFP shRNA constructs (Stewart et al., 2008). The quantification by flow cytometry showed that GFP fluorescence was decreased by 23% in chicken CH-SAH cells ($p < 0.05$) and by 84% in mammalian MDCK cells when compared to the scrambled shRNA control ($p < 0.05$) (Fig. 1). This experiment suggested the presence of functional RNAi machinery in both cell cultures, although ~4-fold higher efficiency of GFP silencing was detected with MDCK cells. It also demonstrated that the human H1 promoter can be used to express shRNA in both canine MDCK and avian CH-SAH cell cultures.

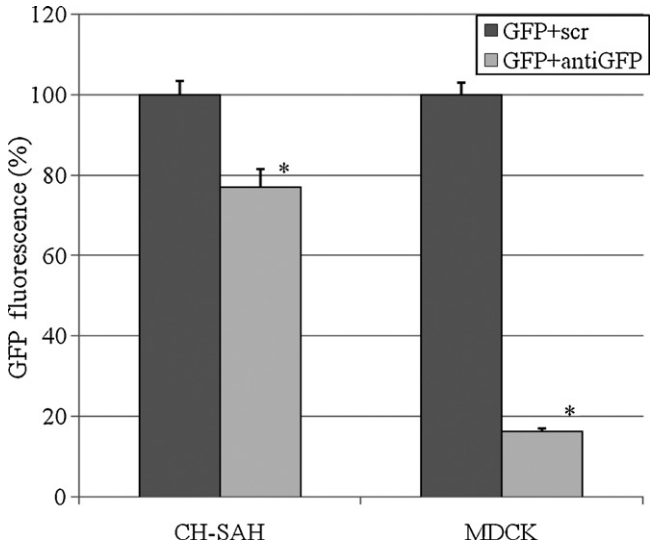

Fig. 1. Fluorescence levels of GFP in MDCK and CH-SAH cells co-transfected with anti-GFP shRNA. The error bars represent standard deviations ($N = 3$ biological replicates). Total fluorescence was found by multiplying the number of GFP expressing cells by mean fluorescence. The total fluorescence of cells co-transfected with pEGFP and scrambled shRNA plasmids is considered 100%. GFP + scr, cells co-transfected with pEGFP and scrambled shRNA plasmids; GFP + anti-GFP, cells co-transfected with pEGFP and anti-GFP shRNA plasmids. *Statistically significant difference with GFP + scr control ($p < 0.05$).

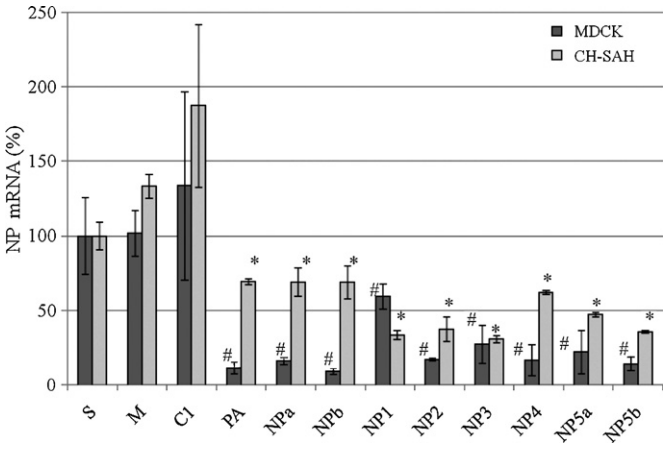

Fig. 2. Relative expression of the NP gene determined by Real-Time RT-PCR in CH-SAH cells transfected with shRNA expressing constructs against NP or PA gene of avian influenza virus 3 h p.i. The error bars represent standard deviations ($N = 3$ biological replicates). Relative amounts of mRNA transcripts of the genes of interest in treated and control cells were determined by Relative quantification with standard curve method and normalized to the ACTB housekeeping gene. The values for scrambled control were considered 100%. Constructs are listed in Suppl. Table 2. S, cells transfected with scrambled shRNA construct; M, mock-transfected cells; C1, cells not transfected but infected; PA, cells transfected with shRNA constructs against PA gene; NP, cells transfected with shRNA constructs against NP gene. #MDCK, statistically significant difference with scrambled control ($p < 0.05$). *CH-SAH, statistically significant difference with scrambled (S) control ($p < 0.05$).

3.2.1. Real-Time RT-PCR

A key feature of the classical RNAi is that inhibition of gene expression happens by destruction of the targeted mRNA. To evaluate viral mRNA degradation, the cells were harvested 3 h p.i., before new virions are released starting at ~4 h, and used for Real-Time RT-PCR. In order to correctly quantify the amounts of mRNAs, the normalization was done to ensure that recorded changes were not caused by technical variability or global transcript degradation due to viral infection or cell death. To decrease technical variability equal amounts of RNA were used for reverse transcription, and equal amounts of cDNA were used for Real-Time RT-PCR reaction. The quantification of viral mRNA was normalized to the expression of housekeeping genes. Two housekeeping genes, GAPDH and ACTB, previously used for viral studies were evaluated using BestKeeper software (Li et al., 2005b; Satterly et al., 2007; Pfaffl et al., 2004). The ACTB was chosen as the least affected by experimental treatments in both cell lines.

Three negative controls were included in each experiment: non-transfected infected cells (C1), mock-transfected cells (M), and the cells that were transfected with scrambled shRNA construct (S). There were no significant differences between these three negative controls, which indicates that the transfection of construct or expression of scrambled shRNA did not affect level of viral NP mRNA (Fig. 2). Real-Time RT-PCR analysis of cells transfected with anti-influenza virus shRNA constructs showed a 30–70% decrease of NP mRNA levels in CH-SAH ($p < 0.05$) and a 40–90% decrease in MDCK cells ($p < 0.05$) compared to scrambled control. These results confirmed that RNAi was caused mainly by degradation of viral mRNAs and not by microRNA-like translational repression. In cells transfected with anti-PA shRNA constructs, not only a decrease in PA mRNA (not shown) but also a significant reduction in NP mRNA was detected indicating degradation of other viral mRNAs. Similarly, a

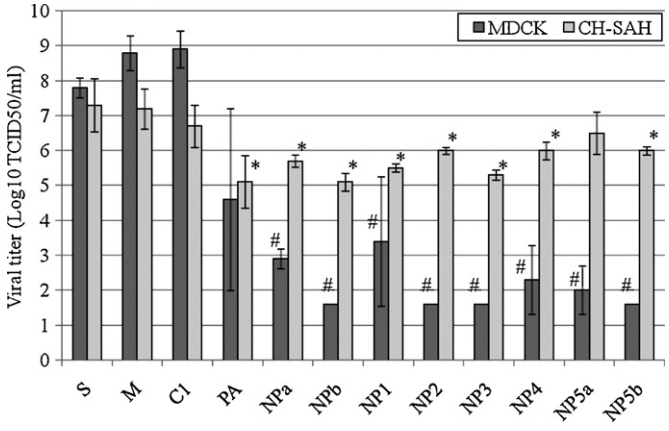

Fig. 3. Infective titres ($TCID_{50}$) of avian influenza virus in MDCK and CH-SAH cells transfected with shRNA expressing constructs against PA or NP gene. The error bars represent standard deviations ($N = 3$ biological replicates). Constructs are listed in Suppl. Table 2. #MDCK, statistically significant difference with scrambled (S) control ($p < 0.05$). *CH-SAH, statistically significant difference with scrambled (S) control ($p < 0.05$).

significant decrease in PA mRNA was observed in cells transfected with anti-NP shRNA construct indicating general destabilization of viral mRNA (not shown).

3.2.2. Viral infectious titre ($TCID_{50}$)

While significant decrease in viral NP mRNA was demonstrated in previous experiment, it was essential to demonstrate that introduction of anti-influenza shRNAs will lead to decrease in viral infectious titre ($TCID_{50}$). In CH-SAH cells there were no significant differences in virus titre between three negative controls, which indicates that the transfection of construct or expression of scrambled shRNA did not affect viral replication (Fig. 3). In contrast, the transfection of anti-influenza shRNA constructs led to up to 4-fold decrease in HA titre (not shown) and 6–160-fold decreased $TCID_{50}$ titre compared to the scrambled control ($p < 0.05$). In MDCK cells transfection with scrambled control led to a 10-fold reduction in virus titre ($p = 0.6$) indicating possible non-RNAi antiviral effect. The inhibitory effect for the anti-influenza shRNA constructs was much higher in MDCK cells than in avian CH-SAH cells, with up to 64-fold decrease in HA titre (data not shown) and 1.6×10^2–1.6×10^6-fold decrease in viral infectious titre compared to the scrambled control ($p < 0.05$). We cannot claim a titre lower than 40 $TCID_{50}$/mL as it is the detection limit for $TCID_{50}$ assay (Log 10(40 $TCID_{50}$/mL) = 1.6) (Flint et al., 2009). Still with the most efficient constructs (NPb, NP2, NP3, NP5b) no infectious viral particles were detected in cell supernatant, and the cell monolayer was intact at 48 h p.i., while in negative controls (C1, M, S) the entire cell monolayer was destroyed and detached from the surface (Suppl. Fig. 1).

3.3. Interferon response (IFN) in infected CH-SAH and MDCK cells transfected with a shRNA expressing plasmids

It was important to verify that inhibition of viral replication was due to RNAi silencing and not due to induction of the antiviral interferon response (IFN) by shRNA constructs. The induction of IFN was analyzed by monitoring mRNA levels of IFN response genes (ISG) 2′,5′-oligoadenylate synthetase (OAS1/OASL) and interferon-beta (IFN-β) commonly used to monitor IFN response to RNAi (Bridge et al., 2003; Stewart et al., 2008). Real-Time RT-PCR for chicken OASL was developed to monitor its response in challenged samples.

Not transfected and not infected cells (C2) served as an additional negative control to evaluate the level of OAS under normal

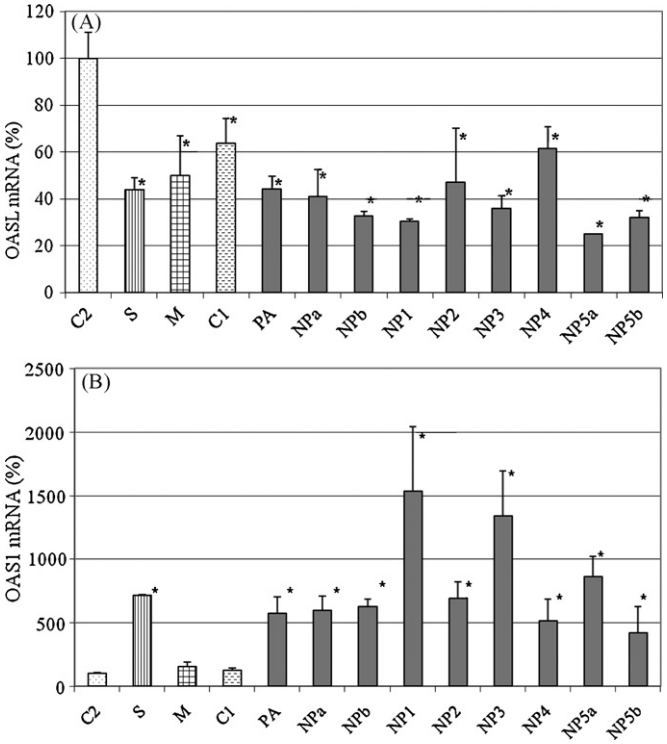

Fig. 4. (A) CH-SAH and (B) MDCK. Relative expression of IFN inducible OASL/OAS1 gene as determined by Real-Time RT-PCR in cells transfected with shRNA constructs (23 h post-transfection) and infected with avian influenza virus (3 h p.i.). The error bars represent standard deviations ($N = 3$ biological replicates). Relative amounts of mRNA transcripts of the genes of interest in treated and control cells were determined by Relative quantification with standard curve method and normalized to the ACTB housekeeping gene. The values for scrambled control were considered 100%. Constructs are listed in Suppl. Table 2. C2, cells not transfected and not infected. *Statistically significant difference with C2 control ($p < 0.05$).

conditions. Surprisingly, in CH-SAH instead of induction, a 38–75% decrease ($p < 0.05$) in OASL mRNA level was observed in all infected cells compared with non-infected C2 control (Fig. 4A). A similar decrease in expression was observed for chicken IFN-β gene in all infected cells (not shown). In experiments with MDCK cells the results were dramatically different. OAS1 expression was not significantly affected by infection alone (Fig. 4B). In contrast, a 4–15-fold increase ($p < 0.05$) of OAS1 expression was observed in all MDCK cells transfected with a shRNA expressing plasmid.

3.4. Induction of interferon response in non-infected CH-SAH and MDCK cells transfected with a shRNA expressing plasmids

IFN response induction was also evaluated in the absence of viral infection. Poly(I:C), a synthetic dsRNA that mimics a viral-infected state in cells was used as a positive control for induction of IFN response by dsRNA (Colby, 1971). The pBluescript plasmid was used as a control for induction of IFN response by unmethylated CpG DNA motifs in this plasmid. The results showed that in the absence of viral infection there were no significant differences in the expression of OASL gene in CH-SAH cells (Fig. 5A). Similar results were observed for chicken IFN-β gene (not shown). These suggest that previously observed reduction of OASL and IFN-β mRNAs were caused by avian influenza virus. Transfection of plasmids did not lead to induction of IFN response, which suggests that CH-SAH cells did not respond to unmethylated CpG DNA motifs. Transfection with poly(I:C) did resulted in up to a 250-fold induction of OASL expression ($p < 0.05$) indicating the presence of

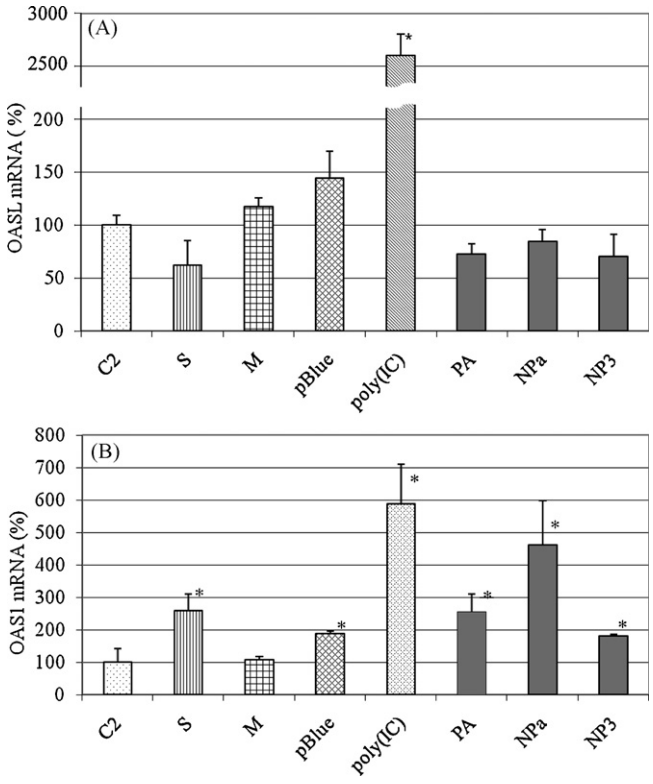

Fig. 5. (A) CH-SAH and (B) MDCK. Relative expression of interferon inducible OASL/OAS1 gene determined by Real-Time RT-PCR in non-infected cells transfected with shRNA constructs. The error bars represent standard deviations ($N = 3$ biological replicates). Relative amounts of mRNA transcripts of the genes of interest in treated and control cells were determined by Relative quantification with standard curve method and normalized to the ACTB housekeeping gene. The values for scrambled control were considered 100%. Constructs are listed in Suppl. Table 2. pBlue, cells transfected with pBluescript plasmid; Poly (IC), cells transfected with 0.25 μg of poly(I:C) as a positive control for induction of IFN response by dsRNA.*Statistically significant difference with C2 control ($p < 0.05$).

a functional response to dsRNA. In MDCK cells, the expression of OAS1 was upregulated by up to 5 fold ($p < 0.05$) by transfection with any plasmid (pBluescript, pSilencer) and up to 6-fold by transfection with poly(I:C) into the cells ($p < 0.05$) (Fig. 5B). The results indicated the presence of a functional IFN response to both dsRNA and unmethylated CpG DNA motifs in MDCK.

3.5. Effect of combined RNAi and IFN response on viral replication ($TCID_{50}$) in CH-SAH

The results of the present study identified significant differences in the inhibition of influenza virus replication in two cell cultures using the same shRNA constructs. It was hypothesized that induction of both IFN response and RNA interference in MDCK cells led to an additive inhibition of influenza virus production. To test this, CH-SAH were transfected with shRNA construct, or IFN inducing agent poly(I:C) or both poly (I:C) and shRNA construct. The induction of only IFN response with poly(I:C) in control (C1) decreased influenza titre by 920 fold ($p < 0.05$) (Fig. 6). Similarly, induction of only RNAi in CH-SAH transfected with shRNA constructs but not treated with poly(I:C) decreased infective titre by 50–370 fold compared to scrambled control ($p < 0.05$). Induction of both IFN and RNAi responses resulted in 1600–18,500-fold decrease in infective titre ($p < 0.05$).

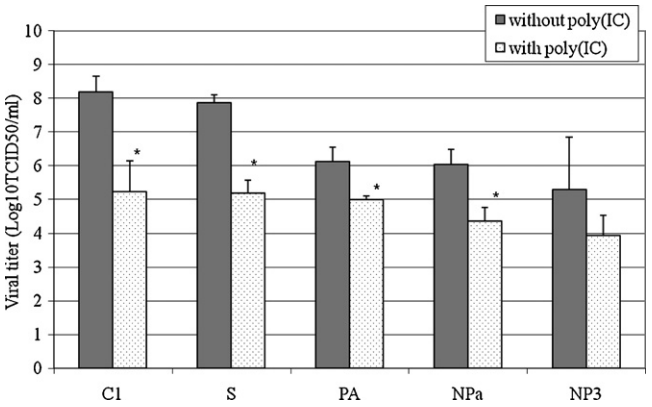

Fig. 6. Effect of induction of IFN response on infective viral titres ($TCID_{50}$) in CH-SAH cells transfected with shRNA expressing constructs. IFN response was induced by co-transfection with 0.25 μg poly(I:C). The error bars represent standard deviations ($N = 3$ biological replicates). Constructs are listed in Suppl. Table 2. *Statistically significant difference with corresponding sample not treated with poly (I:C) ($p < 0.05$).

4. Discussion

4.1. Inhibition of replication of influenza virus with anti-influenza shRNAs expressed by Pol III human H1 promoter

In this work it was demonstrated that anti-influenza shRNAs expressed by Pol III human H1 promoter induce highly efficient inhibition of influenza virus replication in both avian CH-SAH and canine MDCK cells. In CH-SAH cells transfection of shRNA constructs targeting NP or PA mRNA of influenza virus resulted in significant decreases of up to 80% in the levels of influenza NP mRNA and up to 370 fold in virus infective titre. MDCK cells transfected with the same shRNA constructs showed even higher efficiency, with significant decreases up to 90% in the level of viral mRNA, and up to 10^6-fold in influenza virus infective titre. Decreased levels of NP mRNA demonstrated that the inhibition of influenza virus likely occurs through classical RNAi mediated degradation of targeted mRNA and not through translational inhibition. No viral particles were detected in MDCK cells transfected with NPb, NP2, NP3, and NP5b constructs, which might indicate a possibility of achieving sterilizing immunity to influenza virus using RNAi. While RNAi inhibition was lower in chicken cells, previous studies demonstrated that vaccines causing as little as 5- to 10-fold reduction in lung virus titre protected mice against lethal influenza virus challenge (Liang et al., 1994; Epstein et al., 2002).

It was important to verify that the observed inhibitions of virus replication were caused by RNAi and did not arise from unintended side effects of RNAi. RNAi mediated off-target silencing of unrelated genes could be triggered by a small region of homology in the sense or antisense strand of an RNAi molecule and in some cases was shown to produce a novel phenotype (Jackson et al., 2003; Scacheri et al., 2004). While all shRNA sequences were screened for high homology to chicken and canine sequences, it is known that target recognition by RNAi machinery involves Watson–Crick base pairing of nucleotides 2–8 in the seed region of the guide strand (Carthew and Sontheimer, 2009), potentially affecting large numbers of host mRNAs. However, it seems unlikely that the same cellular gene important for influenza virus replication would be affected by off-target silencing with different constructs targeting sites in both NP and PA mRNAs in both chicken and canine cells.

Recently, we and others reported that high expression from shRNA expression vectors might lead to high levels of unprocessed RNAi substrates saturating cellular export and RNAi machinery,

thus interfering with processing/export of endogenous miRNAs *in vitro* and *in vivo* (Grimm et al., 2006; Stewart et al., 2008; Castanotto et al., 2007). It was demonstrated that some viral and cellular miRNAs are essential for replication of a number of viruses. For example, liver-specific miR-122 miRNA is required for hepatitis C virus (HCV) replication (Jopling et al., 2005), while all examined herpesviruses produce viral miRNAs required for the latent and lytic stage of the viral life cycle (Cullen, 2009). In this case non-specific interference with miRNA processing or export might have affected replication of the influenza virus. However, a large variability in influenza inhibition was observed between different shRNA constructs expressed using the same H1 promoter. In addition, transfection of scrambled shRNA had no effect on viral replication in CH-SAH cells. It did lead to some reduction in viral titre in MDCK cells, but this effect was likely caused by induction of an IFN response in MDCK cells.

Induction of a cellular IFN response is another common side effect of RNAi in animal cells (Hornung et al., 2005; Judge et al., 2005). Induction of IFN in some cases resulted in misinterpretation of the therapeutic effects of RNAi molecules (Kleinman et al., 2008). IFN induction is less common by intracellularly expressed shRNA, as they resemble endogenous miRNA. Nevertheless, IFN response is a well-known cellular defence against viral infection and its induction might have resulted in observed inhibition of influenza replication (Garcia-Sastre and Biron, 2006; Pichlmair and Reis e Sousa, 2007). However no induction of classical IFN responsive OASL or IFN-β genes was observed in infected or non-infected CH-SAH cells. Similarly, transfection of scrambled control had no significant effect on $TCID_{50}$ titre. These results indicate that RNAi and not a nonspecific IFN response was responsible for the observed inhibition of avian influenza virus in chicken CH-SAH cells. In contrast, in MDCK cells a significant 4–15-fold induction of OAS1 expression was observed in all cells transfected with shRNA constructs. However, induction of IFN response by scrambled shRNA resulted in only a non-significant 10-fold reduction in viral titre, while several anti-influenza shRNA constructs led up to a 10^6-fold decrease in titre. Also, no correlation was observed between the level of IFN induction and efficiency of influenza virus inhibition. For example, the NP1 construct, which induced the highest OAS1 response, was one of the least efficient in influenza inhibition. This indicated that sequence specific RNAi and not nonspecific IFN induction played the major role in inhibiting viral replication in MDCK cells.

Our results indicate that shRNAs expressed by the H1 promoter are highly efficient at targeting influenza virus and achieved a high level of suppression. Several groups have used different RNAi approaches to demonstrate inhibition of human and avian influenza virus including highly pathogenic H5, H7, and H9 subtypes. However, a high level of viral titre reduction, essential to prevent possible escape, was not achieved with all the constructs. In the first application of RNAi up to 30,000-fold inhibition of human influenza virus was achieved with synthetic siRNA targeting NP and PA mRNA in MDCK cells (Ge et al., 2003). The human U6 promoter was used to express shRNA targeting M1 mRNA, which led to an 80% reduction in influenza virus titre in MDCK cells (Hui et al., 2004). Another group also used human U6 promoter targeting NP mRNA and achieved up to 40,000-fold inhibition of the H5N1 strain of influenza virus with NP shRNA constructs (Li et al., 2005a). Several groups also tested Pol I and Pol II promoters but usually with less success. M2 targeted shRNA expressed from the very robust ribosomal RNA polymerase I promoter led only to a 10-fold decrease in infectious titre (McCown et al., 2003). Pol II promoters can provide cell or tissue specific expression of shRNA which would limit possible side effects of ubiquitous induction anti-influenza RNAi. However, the cytomegalovirus promoter (CMV) has been proven ineffective in silencing of some cellular mRNAs and HIV virus (Xia et al., 2002; Boden et al., 2003). Similarly, M2 or NP-specific shRNAs expressed by the CMV promoter led only up to 43-fold reduction in influenza titre in MDCK cells (Zhou et al., 2007). A possible problem with using Pol II promoters against influenza virus is that NS1 protein is known to impair the post-transcriptional processing and nuclear export of cellular Pol II transcripts, which likely reduced the efficiency of Pol II expressed RNAi molecules (Hale et al., 2008). NS1 does not seem to prevent nuclear export of RNAs lacking poly(A) sequences, which might explain why much higher efficiency can be achieved with Pol III promoters. Overall Pol III type 3 H1 and U6 promoters seem to be preferable for anti-influenza RNAi as they allow high ubiquitous expression of shRNA and are not affected by influenza NS1 protein.

4.2. Differences between avian and mammalian cell cultures in RNAi efficiency

When comparing silencing efficiency of the same constructs, up to 10^4-fold higher reduction of virus titre was observed in mammalian MDCK cells compared to avian CH-SAH cells. Similarly, the same anti-GFP shRNA construct was four times more efficient in MDCK cells than in CH-SAH cells despite an almost 2-fold lower rate of plasmid transfection. There could be several reasons for these differences in RNAi efficiency.

First, it is possible that establishment of the CH-SAH cells resulted in the loss/downregulation of RNAi proteins. For example, loss of IFN response is often observed in established cell cultures such as HeLa. It has been also reported that different tissues express Dicer at different levels leading to variation in RNAi silencing even in the same species (Sago et al., 2004; Peng et al., 2006). Also as the majority of RNAi design rules were developed using mammalian systems, it is possible that shRNA design might not be optimal for avian cells leading to lower RNAi efficiency.

It is also suggested from our results that induction of IFN response in MDCK cells and its suppression in avian cells by influenza infection was partially responsible for differences in influenza virus inhibition. Transfection of shRNA constructs into MDCK cells resulted in a 4–15-fold induction of OAS1 gene, and led to non-significant 10-fold decrease in the infectious titre with scrambled shRNA. In contrast transfection with shRNA constructs induced expression of OASL gene in CH-SAH cells. When an IFN response was induced in CH-SAH cells, it did lead to an additional 14–46-fold decrease in infective titre indicating possibility of additive inhibition. Avian influenza infection also led to a 2-fold decrease in OASL and IFN-β expression in all infected CH-SAH cells, while no similar effect was seen with MDCK cells. This decrease is probably related to NS1 protein of avian influenza virus, which is a known inhibitor of IFN responses (Hale et al., 2008). It has been reported that the sequestration of dsRNA by the influenza A virus NS1 protein in infected cells prevents dsRNA mediated activation of PKR, induction of the NF-κB pathway, IFN-β synthesis and OAS1/RNase L activation. NS1 protein also impairs the post-transcriptional processing and nuclear export of cellular pre-mRNAs in order to counteract the host antiviral response. This might explain the decrease in the expression level of processed OASL and IFN-β mRNAs as detected by Real-Time RT-PCR. The absence of similar effect in MDCK cells is likely due to infection with avian influenza virus not adapted to mammalian cells. It has been suggested that the ability of the NS1 protein to inhibit the IFN response is host-specific (Geiss et al., 2002), and that NS1 proteins from different strains varied in their subcellular localization and in their ability to inhibit the IFN-β pathway (Hale et al., 2008).

Similarly, it might be suggested that the avian specific RNAi suppression ability of NS1 protein of avian influenza virus might be responsible for the observed differences in RNAi efficiency between

avian and mammalian cells. Due to the importance of an antiviral RNAi response it is not surprising that multiple RNAi suppressors have been identified in plant, insect and animal viruses (de Vries and Berkhout, 2008). Moreover, mammalian RNAi pathway participates in inhibition of influenza virus (Matskevich and Moelling, 2007). The NS1 protein can act as an RNAi suppressor in plants and *Drosophila* cells, although reports with mammalian cells are still controversial (Hale et al., 2008; de Vries et al., 2009). Recently it was reported that NS1 proteins from different strains of influenza virus varied in their ability to suppress shRNA-mediated silencing of a luciferase reporter gene and functionally complement the HIV Tat RNAi suppressor (de Vries et al., 2009).

Finally, the differences in silencing efficiencies between two cell cultures were likely caused by differences in the relative strengths of the Pol III human H1 promoter in avian and mammalian cells. For example human H1 promoter is almost two times more efficient in mammalian CHO cells than in chicken CEF and DT-40 cells (Yuan et al., 2006). In the future, RNAi constructs with chicken Pol III promoters might be required to achieve higher efficiency in avian cells. Still it will be important to optimize the shRNA expression to achieve the desired virus inhibition with minimal cellular toxicity. It has been demonstrated that high level of anti-hepatitis shRNA expression achieved with the U6 promoter caused toxic effects in the liver and lethality in mice (Grimm et al., 2006).

4.3. Developing novel RNAi target site for influenza

The major problem with designing RNAi constructs for influenza virus is that there are few conservative sites, which also allow highly efficient RNAi silencing. As a result the majority of articles testing anti-influenza RNAi utilize the NP-1496 target site (Ge et al., 2003). However, it has been estimated that at least four RNAi targets will be required to reliably suppress viral replication of fast mutating influenza virus (Chen et al., 2008). It has been also demonstrated that prevention of HIV-1 escape in cell culture can be reliably achieved by simultaneous expression of four shRNAs (ter Brake et al., 2008). In this research we identified a highly efficient and conserved RNAi target site (NP2, NP3 and NP5b) in the NP gene of influenza virus. Our analysis indicate that for the most efficient anti-influenza shRNA constructs the target site is completely conserved in 95% for NP (a/b), 85% for NP2 and NP3, and 96% for NP5b, among 202 sequences of H5N1 present in the Influenza virus database. Such sequence conservation suggests that mutations in this region are likely to be deleterious for viral reproductive ability, and even if the virus escapes through mutations, its replication would likely be severely crippled. Pairing of nucleotides 2–8 in the seed region of the guide strand is often sufficient to ensure mRNA degradation or translational repression (Carthew and Sontheimer, 2009), so it is likely that even broader strain coverage can be achieved with tested constructs. For example, it has been demonstrated that mutations in position 1–2 and 18–19 of target site did not result in HIV escape from RNAi (von Eije et al., 2008).

5. Conclusions

In this paper, we demonstrated that anti-influenza shRNAs expressed with Pol III human H1 promoter can achieve a significant reduction of influenza virus infective titre in chicken and mammalian cells. In CH-SAH cells, no induction of IFN response was observed after transfection of shRNA constructs, and the IFN response was suppressed after virus infection. Induction of an IFN response in CH-SAH cells additionally decreased the viral titre by up to 50 fold, but still did not achieve the same level of inhibition as in MDCK cells. We also identified a novel highly efficient and conserved RNAi target site in the NP gene of influenza virus which can be used in antiviral cocktails of shRNAs to prevent influenza virus escape from RNAi silencing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2009.08.009.

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