RNA interference of influenza A virus replication by microRNA-adapted lentiviral loop short hairpin RNA

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Limitations of the current vaccines and antivirals against influenza A virus (IAV) pandemic underscore the urgent need for developing novel anti-influenza strategies. RNA interference (RNAi) induced by small interfering RNA (siRNA) has become a powerful new means to inhibit viral infection in a gene-specific manner. However, the efficacy of the siRNA delivery platform and the relatively high cost of administration have hindered widespread application of siRNA.

In this study, we developed a microRNA (miRNA)-30-based lentivirus delivery system by embedding a synthetic short hairpin RNA (shRNA) stem into the context of endogenous precursor of miRNA-30 (shRNAmir) to express a silencer of the influenza gene. We showed that the miRNA-based lentivirus vector was able to express and process a single nucleoprotein (NP)-targeting shRNAmir, which could potently inhibit IAV replication. We further showed that miRNA-based lentivirus vector carrying tandemly linked NP and polymerase PB1 shRNAmirs could express and process double shRNAmirs. Despite the relatively low levels of NP and PB1 miRNAs produced in the stably transduced cells, the combination of two miRNAs exerted a great degree of inhibition on influenza infection. Given the advantage of combinatorial RNAi in preventing emergence of mutant virus, miRNA-based lentiviral vectors are valuable tools for antiviral activities. To the best of our knowledge, this is the first study demonstrating that a miRNA-based RNAi strategy can be applied for better control of influenza virus infection.

INTRODUCTION

Influenza A virus (IAV), a segmented RNA virus of the family Orthomyxoviridae, causes annual epidemics and occasional pandemics that have severe consequences for human health and the global economy (Bouvier & Palese, 2008; Tumpey & Belser, 2009). Vaccination is considered the best option for control of influenza infection. However, limitations on vaccine efficacy and the lengthy time required for vaccine design and production against emerging strains have stimulated the development of antivirals. From the therapeutic perspective, antivirals are available to treat influenza virus infection based on matrix M2 or neuraminidase (NA) inhibition. However, the constant emergence of both types of antiviral-resistant influenza strains limits their efficacy in the long term (Saladino et al., 2010). The continued threat of pandemic outbreaks and the limitations of current antiviral strategies underscore the urgent need for developing new influenza therapies.

RNA interference (RNAi) is an evolutionarily conserved and sequence-specific gene silencing mechanism in eukaryotes. Because of the high sequence specificity, RNAi has been widely used to investigate gene functions and to inhibit virus infection. RNAi can be accomplished by introducing synthetic small interfering RNAs (siRNAs), by expressing simple stem–loop short hairpin RNAs (shRNAs) from a DNA vector or by mimicking the naturally occurring microRNAs (miRNAs) with sequences for desired targets. Transfection of synthetic siRNAs into mammalian cells can trigger the RNAi process and subsequently degrade the target mRNA (Hannon, 2002). A wide range of viruses, including human immunodeficiency virus HIV-1, hepatitis C virus, hepatitis B virus, dengue virus and coronavirus, have been successfully targeted by siRNAs (Haasnoot & Berkhout, 2006; Haasnoot et al., 2003). For influenza virus, we and others have shown that siRNAs specific for influenza miRNAs can effectively inhibit replication of different virus subtypes, both in cell culture and in mice (Ge et al., 2003, 2004; Lin et al., 2012a; Tompkins et al., 2004). However, the efficacy of the siRNA delivery platform and relatively high cost of administration restrict the widespread application of siRNA (Dykxoorn & Lieberman, 2006). In addition to transfection of synthetic siRNAs, shRNAs have often been applied to enable a stable and long-term knockdown of target, which can be mediated by a viral vector. In this
system, shRNAs transcribed from the RNA polymerase III (Pol-III) promoter in the nucleus enter the cytoplasm, where the enzyme Dicer processes shRNA into effective siRNAs. Most shRNA expression vectors use Pol-III promoters, including the U6, H1 and tRNA promoters, which can direct high levels of shRNA expression, resulting in highly efficacious silencing (Liu & Berkhourt, 2013; Tiscornia et al., 2003). However, it was found that the exceedingly high level of shRNA expression increases the possibility of off-target silencing and elicits non-specific effects such as IFN response and severe cellular toxicities (Bridge et al., 2003; Grimm et al., 2006; Jackson et al., 2003). An alternative silencer expression strategy has recently been developed by embedding synthetic shRNA stems into the context of an endogenous precursor of the miRNA backbone (shRNAmir) driven by polymerase II (Pol-II) or Pol-III (Chang et al., 2006).

Cellular miRNAs are the natural inducers of RNAi. Primary microRNAs (pri-miRNAs) are transcribed by Pol-II (Cullen, 2004; Lee et al., 2002). The pri-miRNAs are cropped by the nuclear Drosha–DCGR8 complex into miRNA precursors (pre-miRNAs) with a length of ~70 nt. Pre-miRNAs form a transport complex with expotin-5 and translocate to the cytoplasm, where Dicer processes the pre-miRNA and produces a miRNA duplex of ~22 bp. The antisense strand, which contains the mature miRNAs, then programmes RISC (RNA-induced silencing complex) to trigger the procedure of gene silencing (Cullen, 2004; Kim, 2005). A recent advance in gene silencing methodology is to replace the natural stem sequence of pri-miRNA with synthetic target-specific sequence. The mimics of pri-miRNA, shRNAmirs, serve as the natural substrates in miRNA biogenesis pathways and can trigger potent gene knockdown (Chung et al., 2006; Liu et al., 2008). Moreover, shRNAmir-based systems are less prone to cause toxicity by interfering with the endogenous miRNA pathway (Boudreau et al., 2009; McBride et al., 2008). A number of studies have indicated that escaped mutant viruses can easily overcome single-shRNA-mediated inhibition (Gitlin et al., 2005; Wilson & Richardson, 2005). In order to increase the genetic barrier to virus escape, simultaneous expression of multiple RNAi triggers has been developed, known as the combinatorial RNAi (co-RNAi) approach (Grimm & Kay, 2007).

In this study, we chose miRNA-30 (miR-30) as the backbone to generate antiviral shRNAmirs. Among the naturally occurring miRNAs, the miR-30-based system is a well-established and commonly used platform for generating shRNA-based RNAi; its effectiveness and versatility in a broad range of cell types and in animals has already been proven (Fellmann et al., 2013). We investigated whether miR-30-based shRNAmir targeting the influenza nucleoprotein (NP) gene would be a promising antiviral strategy. Furthermore, we tested whether a tandem shRNAmir lentiviral vector that simultaneously expresses two miRNAs targeting the NP and polymerase PB1 genes would enforce the inhibition of influenza virus infection, as the combinatorial RNAi strategy would prevent the possible emergence of resistant virus.

RESULTS

Inhibitory activity of shRNAmir-NP

A number of chemically synthetic siRNAs targeting the conserved regions of the viral genome have been identified and they could provide potent inhibition of IAV infection. Among them, NP1496-siRNA and PB1-2257-siRNAs show very potent inhibition (Ge et al., 2003). To investigate whether shRNAmir embedded into a miR-30 context would have any antiviral activity, we first designed a single shRNAmir based on the sequence of NP1496-siRNA. It is known that miR-30-based vectors generate 22 nt mature miRNAs. Thus, 2 nt (AC) at the 3' end and an additional A at the 5' end were added to the sense strand of NP1496-siRNA, making the previously 19 nt strand 22 nt long. The miR-30-based shRNAs retain a bulge at the base of the shRNA stem that is predicted to exist in the bona fide miR-30 primary transcript. To ensure the presence of the bulge in the shRNA stem, the first base of the sense strand was converted from A to C. The 110 nt shRNAmir-NP hairpin unit was generated by adding flanking sequence derived from the natural miR-30 pri-miRNA (Fig. 1a), and was cloned into a lentiviral vector, pGIPZ, under the control of the human cytomegalovirus promoter (Fig. 1b).

Taking advantage of the high transfection efficiency of 293T cells, we transfected these cells with target-expressing plasmid pcDNA-NP alone, or co-transfected them with pcDNA-NP together with either shRNAmir-NP or non-silencing mammalian gene off-target shRNA, to determine the inhibitory activity of shRNAmir-NP. At 24 h post-transfection (p.t.), a large amount of NP protein was detected in pcDNA-NP-transfected cells and non-silencing shRNAmir-expressing cells (Fig. 1c). In contrast, the transient expression of shRNAmir-NP showed full capability of gene knockdown. Next, we transduced 293T cells with the lentiviral vectors at an m.o.i. of 1. When the stably transduced cells were transfected with pcDNA-NP, a profound reduction (80%) of NP expression was observed in shRNAmir-NP-transduced cells (Fig. 1d).

Since Madin–Darby canine kidney (MDCK) cells are fully susceptible to IAV infection, to investigate the antiviral effect of shRNAmir-NP, stably transduced MDCK cells (m.o.i. 1) were infected with PR8 at an m.o.i. of 0.001. At 24 h post-infection (p.i.), the shRNAmir-NP induced an appreciable RNAi effect, resulting in a significant inhibition of viral NP and non-structural NS1 protein synthesis (Fig. 2a). In line with these results, efficient replication of IAV was observed in non-silencing shRNAmir-expressing cells, comparable with that in non-shRNA-transduced cells. However, the virus titre in shRNAmir-NP-expressing cells was over 1 log lower than that in control samples (Fig. 2b). To determine a dose-dependent inhibition effect of shRNAmir, we transduced cells with lentiviral vector at an m.o.i. of 10. At the higher m.o.i., shRNAmir-NP conferred an ~2 log inhibition of virus replication compared with that in control cells (Fig. 2c). These results demonstrated that
influenza-specific shRNA in miR-30-based lentiviral vector is capable of offering potent gene silencing and efficient inhibition of virus replication in a dose-dependent manner.

**Inhibition efficacy of shRNAmir-NP-PB1 on NP and PB1 gene expression**

We validated that the miR-30-based shRNA delivery system is able to provide potent inhibition of IAV replication. With this promising finding, we further developed a co-RNAi approach by linking shRNAmir-NP tandemly with an additional influenza PB1-gene-specific shRNAmir, aiming to increase the antiviral efficiency and genetic barrier for virus escape. The 19 nt sense strand of PB1-2257 siRNA was extended to 22 nt and the first base at the 5'-end was converted from A to C. To ensure the correct secondary structure of pri-miRNA as the substrate of the Drosha–DGCR8 complex, a set of sequences flanking each shRNA stem was carefully designed and tested. The flanks were selected when the predicted structure of pri-miRNA fulfilled the criteria for the correct Drosha–DGCR8 processing and the folded pri-miRNA demonstrated the greatest thermodynamical stability. Flank 1 was the original sequence upstream of pre-miR30. Flank 2,

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**Fig. 1.** Schematic representation of miR-30-based shRNA vector design and inhibition of NP expression by shRNAmir-NP. 
(a) Sequence of the 106 nt shRNAmir-NP hairpin unit. The folding is based on rna.tbi.univie.ac.at. The appended bases are shown in black boxes. The mature miRNA/guide strand is shown in light grey. The potential cleavage sites for Dicer and Drosha are indicated by black arrowheads. The flanking sequences from natural miR-30 are marked in dark grey. (b) Structure of lentiviral vector used in this study. The miR-30 shRNA unit was cloned into the pGIPZ lentiviral vector, in which shRNAmir expression is driven by an human cytomegalovirus promoter. 5' LTR, 5' long-terminal repeat; RRE, Rev response element; tGFP, turboGFP reporter; IRES, internal ribosomal entry site; PuroR, puromycin resistance; WPRE, woodchuck hepatitis post-transcriptional regulatory element; 3' SIN LTR, 3' self-inactivating long-terminal repeat. (c) Inhibition of NP expression in transiently shRNAmir-NP-expressing 293T cells. Cells were co-transfected with NP expression plasmid (pcDNA-NP) and pGIPZ shRNAmir-NP or non-silencing shRNA lentiviral vector. Twenty-four hours p.t., the expression level of NP was examined by Western blotting. (d) Reduced expression of NP in stably transduced 293T cells (m.o.i. 10). Stably transduced cells were transfected with pcDNA-NP. Twenty-four hours p.t., the inhibition of NP expression was evaluated by Western blotting. The level of β-actin serves as internal loading control. NP protein levels were first normalized to the levels of β-actin in each sample and then compared with that in shRNAmir-null cells and expressed as percentage change.
between the two shRNA stems, was composed of the natural flanking sequence downstream of pre-miR30 and its reversed sequence with modifications. Downstream of shRNAmir-PB1 was appended flank 3, which is the reversed sequence of flank 1 with deletion of the last 5 nt at the 3′ end (Fig. 3a). As a control, a single shRNAmir-PB1 expression vector was constructed in a similar way to shRNAmir-NP.

We first examined whether miRNAs were expressed from each shRNAmir construct by Northern blotting. 293T cells were transfected with each shRNAmir construct and total RNA was isolated at 48 h p.t. Each probe detected signals that corresponded to the specific miRNAs. Single shRNAmir-NP expressed stronger miRNA-NP than did the double shRNAmir construct shRNAmir-NP-PB1. Similar expression levels of miRNA-PB1 derived from either single or double shRNAmir constructs were observed (Fig. 3b). Next, we evaluated the gene silencing effect of shRNAmir-NP-PB1 in transiently transfected cells. 293T cells were co-transfected with each shRNAmir construct together with pcDNA-NP or pEF-PB1-myc. At 24 h p.t., target protein expression levels were evaluated. When compared with the non-silencing sample, the RNAi induced by shRNAmir-NP-PB1 provided complete suppression of NP and an appreciable knockdown of PB1-myc, which were comparable to the individual RNAi effect of shRNAmir-NP or shRNAmir-PB1, respectively (Fig. 3c). To examine the gene knockdown efficacy of the stably expressed shRNAmir-NP-PB1, 293T cells were transduced with each lentiviral vector at an m.o.i. of 10 and were then transfected with pDNA-NP or pEF-PB1-myc. All stably transduced cell lines were viable and no differences in growth

![Fig. 2. Inhibition of IAV infection in shRNAmir-NP-transduced MDCK cells. (a) Stably transduced (m.o.i. 1) cells were infected with PR8 at an m.o.i. of 0.001. At 24 h p.i., cell lysates were harvested and levels of NP and NS1 were assessed by Western blotting. β-Actin level serves as the internal loading control. The percentage protein level changes were calculated as described in the legend of Fig. 1. (b) Virus titre in the supernatant was determined by plaque assay. (c) Stably transduced (m.o.i. 10) cells were infected with PR8 at an m.o.i. of 0.001. Virus titres (shown as mean±SE) were determined at 24 h p.i. **P<0.01.](image-url)
Fig. 3. Schematic representation of tandem shRNAmir-NP-PB1 construction and target silencing with miRNAs. (a) The structure of shRNAmir-NP-PB1. shRNAmir-NP and shRNAmir-PB1 units were linked tandemly with designed intervening sequences. The guide strand of miRNA-NP is shown in light grey. The guide strand of miRNA-PB1 is shown in dark grey. The folding of shRNAmir-NP-PB1 is based on rna.tbi.univie.ac.at. (b) Detection of miRNAs produced from shRNAmir constructs. Total RNA was extracted from transfected 293T cells and used for Northern blotting. SYBR Gold staining of 5S rRNA served as loading control. (c) 293T cells were co-transfected with target protein expression plasmid (pcDNA-NP or pEF-PB1-myc) and an indicated shRNAmir construct. At 24 h p.t., the expression levels of NP and PB1-myc were examined
rates were observed. shRNAmir-NP induced a robust RNAi effect, resulting in over 95% suppression of NP (Fig. 3d). Compared with shRNAmir-NP, less reduction (~60%) of NP was detected in shRNAmir-NP-PB1-expressing cells. For PB1-myc knockdown, the shRNAmir-NP-PB1 provided effective inhibition, with 2.7-fold reduction of PB1-myc expression over that in non-silencing cells. In contrast, there was no apparent suppression of PB1-myc in shRNAmir-PB1-expressing cells.

Expression and processing of antiviral miRNAs in stably transduced cell lines

To understand whether the inhibition efficacy is correlated with the miRNA expression levels, total RNA was extracted from m.o.i. 10-transduced 293T cells. The miRNA corresponding to each shRNAmir was evaluated by Northern blotting. shRNAmir-NP yielded much miRNA-NP, with an intense signal (Fig. 4a). However, there was no miRNA-PB1 detected from shRNAmir-PB1, which could provide an explanation of the inefficient suppression of PB1-myc in shRNAmir-PB1-transduced 293T cells. The miRNA-NP signal was weaker in shRNAmir-NP-PB1-transduced cells than in shRNAmir-NP-transduced cells. miRNA-PB1 was produced from shRNAmir-NP-PB1 at a relatively low level. This suggested that in shRNAmir-NP-PB1-transduced 293T cells the processing of the shRNAmir-NP unit might be more efficient than that of the shRNAmir-PB1 unit. The expression and processing of miRNAs in transduced MDCK cells were also evaluated. As shown in Fig. 4b, the expression of miRNAs from shRNAmir-PB1 cannot be detected in MDCK cells. The expression levels of miRNA-NP and miRNA-PB1 were lower in shRNAmir-NP-PB1-transduced MDCK cells than in transduced 293T cells.

Inhibitory activity of shRNAmir-NP-PB1 on IAV replication

Even though the miRNA levels are low in shRNAmir-NP-PB1 stably transduced MDCK cells, we were still interested in evaluating the ability of shRNAmir-NP-PB1 to inhibit IAV infection. shRNAmir-PB1 was excluded from the following inhibitory activity test as shRNAmir-PB1 failed to express active miRNA in transduced cells. The stably transduced MDCK cell lines (m.o.i. 10) were infected with PR8 at an m.o.i. of 0.001. At 24 h p.i., cell lysates were harvested for Western blotting with antibodies specific for PB1, NP and NS1 proteins; and virus titre in the supernatant was determined by plaque assay. As seen in Fig. 5, non-silencing shRNAmir did not interfere with PR8 replication, as similar levels of viral protein synthesis and virus production were achieved as in the non-transduced cells. In shRNA-NP-transduced cells, a complete suppression of PB1 and significant reductions of NP (95%) and NS1 (86%) were achieved by efficiently silencing the NP gene.

Fig. 4. Assessment of miRNAs produced in stably transduced 293T and MDCK cells. Total RNAs were isolated from transduced 293T cells (a) and MDCK cells (b), respectively, and were hybridized with probes specific for miRNA-NP or miRNA-PB1. SYBR Gold staining of the 5S rRNA serves as a sample loading control.
A decline in viral proteins was also observed in shRNAmir-NP-PB1-transduced cells (Fig. 5a). Consistent with these results, the virus titres from shRNAmir-NP and shRNA-mir-NP-PB1 were dramatically decreased (>2 logs) as compared with the non-silencing or non-transduced cells. shRNAmir-NP-PB1 demonstrated an inhibitory capability against virus production similar to that of shRNAmir-NP (Fig. 5b).

To investigate the antiviral activity of shRNAmirs against a different IAV strain, stably transduced MDCK cells were infected with SIV/SK02 at an m.o.i. of 0.001. The virus titre in the supernatant was determined at 24 and 48 h p.i., respectively. With a lower dose of transduction (m.o.i. 1), efficient virus replication was detected in the non-transduced cells. In contrast, SIV/SK02 replication was potently inhibited by shRNAmir-NP, with an \( \sim 3.5 \) log decrease of virus production at 24 h p.i. (Fig. 6a). At 48 h p.i., both shRNAmir-NP and shRNAmir-NP-PB1 maintained strong suppression by providing \( \sim 3.5 \) log inhibition of virus production over non-transduced cells (Fig. 6d). It was noted that virus replication was impaired in the non-silencing-shRNA-transduced cells in a dose-dependent manner, especially at 24 h p.i.

**DISCUSSION**

Previous studies have shown that properly designed siRNAs are robust inhibitors of IAV replication. Ge et al. (2004) demonstrated that shRNAs expressed from DNA vectors could provide long-lasting antiviral effects *in vivo*. For this approach, siRNAs were processed from simple stem–loop shRNAs transcribed by Pol-III promoters, and subsequently triggered RNAi. However, it has been reported that enhanced expression of shRNA driven by the potent Pol-III promoter would interfere with the regulatory function of endogenous miRNA through competing for critical RNAi components, thus causing cellular toxicity (Castanotto et al., 2007; Grimm et al., 2006). To overcome this limitation, we investigated whether microRNA-adapted
shRNAs would provide any RNAi effect on IAV replication. We first constructed shRNAmir-NP lentiviral vector and showed that the NP gene could be potently silenced by shRNAmir-NP in both transient transfection and stably transduced cells. Further, we demonstrated that shRNA-mir-NP could provide efficient inhibition of influenza infection in tissue culture. Next, we developed a tandem miR-30 shRNA vector expressing shRNAmir-NP and shRNAmir-PB1. In a transient expression manner, shRNAmir-NP-PB1 could successfully express and process NP and PB1 miRNAs, resulting in potent NP and PB1 silencing. The miRNA expression level and silencing effect of shRNAmir-NP-PB1 are similar to those of single shRNAmir, thus verifying the successful design and vector construction of

**Fig. 6.** Inhibitory activity of shRNAmir against SIV/SK02 infection. The stably transduced MDCK cells with different lentiviral vectors at an m.o.i. of 1 (a, b) or 10 (c, d) were infected with SIV/SK02 at an m.o.i. of 0.001. Virus replication (shown as mean±SE) was determined at 24 (a, c) and 48 h.p.i. (b, d) by plaque assay. *P<0.05; **P<0.01; ***P<0.001.
shRNAmirs. In stably transduced cells, shRNAmir-NP-PB1 produced only moderate levels of miRNAs, and thus was not able to enhance knockdown of targets and inhibition of virus replication. These results indicated that the inhibition ability of shRNAmirs is closely correlated with the expression levels of active miRNAs. Cells stably transduced with shRNAmir-NP-PB1 could not produce high levels of miRNAs; it is possible that random lentiviral integration into the genome increases the likelihood of inefficient expression of shRNAmirs. It is commonly acknowledged that integration site has important consequences for both the expression of the transgene and the phenotype of the host cell (DeKelver et al., 2010). Therefore, screening the monoclonal shRNAmir-expressing cells may result in more efficient miRNA expression and targeted knockdown. Alternatively, targeted integration at a defined locus such as the AAVS1 locus (safe harbour site) may also improve miRNA expression (DeKelver et al., 2010; Khan et al., 2011). We also found that expression levels of miRNAs in tandem-shRNAmir-transduced 293T and MDCK cells were different (Fig. 4); this suggested that the efficiency of expression of pri-miRNA and processing of miRNAs may vary in different cell lines.

We noticed that pGIPZ non-silencing shRNAmir, which contains non-homologous sequence to known mammalian genes, had some inhibitory effect on SIV/SDK2 virus replication, but not on PR8 virus. We analysed all eight segments of the SIV/SDK2 genome to see if there are any possible homologies with the non-silencing shRNAmir. We did not find any specific sequence 22 nt in length that was completely targeted by non-silencing shRNAmir. However, we found that in the NS1 gene there are three copies of a 7 nt sequence complementary to the non-silencing shRNAmir. This three regions are in close proximity to each other, which was not found in the PR8 NS1 gene. It has been reported in a number of studies that miRNA can interact with the target mRNA in an imperfect seed-binding manner, albeit less frequently (Chi et al., 2012; Grimson et al., 2007; Martin et al., 2014). This might explain the inhibitory effect of the non-silencing shRNAmir on SIV/SDK2 replication.

In this study, we provided proof of the concept that a miRNA-based RNAi strategy can be applied for better control of influenza virus infection. We showed that the miRNA-based lentivirus vector was able to express and process a single shRNAmir targeting NP, which could potently inhibit IAV replication. We further showed that the miRNA-based lentivirus vector could express and process double shRNAmirs. Despite the relatively low levels of miRNAs derived from shRNAmir-NP-PB1, the combination of two miRNAs exerted a similar degree of inhibition against PR8 infection to shRNAmir-NP, and greater inhibitory activity against SIV/SDK2 infection than shRNAmir-NP did. Given the advantage of combinatorial RNAi in preventing emergence of mutant virus by increasing the genetic barrier for virus escape, it is worth improving the miRNA-based lentivirus to achieve maximal antiviral activities. Some key factors to be considered include the spacer length and the sequence composition between the two miRNAs. It would also be interesting to investigate whether switching the locations of two shRNAs in the double shRNAmir construct would affect the expression of both miRNAs and the associated target knockdown.

**METHODS**

**Cells and viruses.** Madin–Darby canine kidney (MDCK) and human embryonic kidney 293T cells were maintained in minimal essential medium and Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, respectively. Influenza virus A/Puerto Rico/8/34 (PR8) was propagated in 11 day-old embryonated chicken eggs. A/Swine/Saskatchewan/18789/02 (SIV/SDK2) was propagated in MDCK cells.

**miR-30-based shRNA design and plasmid construction.** Two siRNAs, NP-1496 and PB1-2257, have been identified in previous reports as strong inhibitors of influenza virus replication (Ge et al., 2003, 2004). We designed miR-30 shRNAs based on the sequences of these siRNAs, following the procedure described previously (Chang et al., 2013). Briefly, the sequence of the passenger strand of siRNA was extended from 19 to 22 nt by addition of 2 nt to the 3’ end and 1 nt to the 5’ end. The first base of the sense strand was converted from A to C in order to generate the appropriate bulge in the shRNA stem. The flanking sequence derived from the miR-30 pri-miRNA and restriction sites XhoI and EcoRI were added to the shRNA oligonucleotide. The flanking sequences were designed to keep the tandem shRNAmir-NP-PB1 in an appropriate secondary structure, which facilitates proper miRNA processing. Sequences of miR-30 shRNAs were as follows: shRNAmir-NP, 5’-CCTGGAAGGTATAGTTGCTGTTGACAGTGACGCGATCTGTTCCACCATTGAAGAGGAAAGGCAACAGATGTAGTCTCCGAAGAAATAAGATCCTTGCGTCTGCTGCTTATCC-3’; shRNAmir-PB1, 5’-CCTGGAAGGTATAATGTGCTGCTGCTGAGTGACGCGATCTGTTCCACCATTGAAGAGGAAAGGCAACAGATGTATCTTCAATGGTGGAACAGATCCTTGCCTCGAGAAGGTATAG-3’. The sequence of the passenger strand of siRNA was extended from 19 to 22 nt by addition of 2 nt to the 3’ end and 1 nt to the 5’ end. The first base of the sense strand was converted from A to C to generate the appropriate bulge in the shRNA stem. The flanking sequence derived from the miR-30 pri-miRNA and restriction sites XhoI and EcoRI were added to the shRNA oligonucleotide. The flanking sequences were designed to keep the tandem shRNAmir-NP-PB1 in an appropriate secondary structure, which facilitates proper miRNA processing. Sequences of miR-30 shRNAs were as follows: shRNAmir-NP, 5’-CCTGGAAGGTATAGTTGCTGTTGACAGTGACGCGATCTGTTCCACCATTGAAGAGGAAAGGCAACAGATGTATCTTCAATGGTGGAACAGATCCTTGCCTCGAGAAGGTATAG-3’; shRNAmir-PB1, 5’-CCTGGAAGGTATAATGTGCTGCTGCTGAGTGACGCGATCTGTTCCACCATTGAAGAGGAAAGGCAACAGATGTATCTTCAATGGTGGAACAGATCCTTGCCTCGAGAAGGTATAG-3’. The sequence of the passenger strand of siRNA was extended from 19 to 22 nt by addition of 2 nt to the 3’ end and 1 nt to the 5’ end. The first base of the sense strand was converted from A to C to generate the appropriate bulge in the shRNA stem. The flanking sequence derived from the miR-30 pri-miRNA and restriction sites XhoI and EcoRI were added to the shRNA oligonucleotide. The flanking sequences were designed to keep the tandem shRNAmir-NP-PB1 in an appropriate secondary structure, which facilitates proper miRNA processing. Sequences of miR-30 shRNAs were as follows: shRNAmir-NP, 5’-CCTGGAAGGTATAGTTGCTGTTGACAGTGACGCGATCTGTTCCACCATTGAAGAGGAAAGGCAACAGATGTATCTTCAATGGTGGAACAGATCCTTGCCTCGAGAAGGTATAG-3’; shRNAmir-PB1, 5’-CCTGGAAGGTATAATGTGCTGCTGCTGAGTGACGCGATCTGTTCCACCATTGAAGAGGAAAGGCAACAGATGTATCTTCAATGGTGGAACAGATCCTTGCCTCGAGAAGGTATAG-3’.

**Plasmid pcDNA-NP was generated in our laboratory as described previously** (Lin et al., 2012b). Plasmid pEF-PB1-myc encoding PB1 with myc tag fused at its C terminus was generated by inserting the full-length PB1 sequence of PR8 into the XhoI and NotI sites of pEF/myc/cto (Life Technologies).

**Transfection experiments.** One day before transfection, 1 × 10⁶ 293T cells were grown in complete culture medium to 70–80% confluence. Cells were transfected with the indicated plasmids using the TransIT-LT1 reagent (Mirus). At 24 h p.t., cell lysates were harvested for Western blotting. To prepare the samples for Northern blotting, 7.5 × 10⁵ 293T cells were cultured 1 day before transfection. Cells were transfected with 5 μg of each pGIPZ shRNAmir plasmid. Two days after transfection, total RNAs were extracted and subjected to Northern blotting.
Western blotting. Cell lysates were collected from the various experiments in RIPA buffer (0.05 M Tris/HCl, pH 8.0, 0.15 M NaCl, 0.1 % SDS, 1 % NP-40 and 1 % deoxycholic acid). Western blot analysis was performed by a standard protocol and imaged using the LiCor Bioscience Odyssey infrared imaging system as described previously (Li et al., 2008). Antibodies used in this study were as follows. Rabbit polyclonal NS1 and NP antibodies were generated in our laboratory (Shin et al., 2007a, b). Mouse anti-myc tag and mouse anti-actin were purchased from Cell Signaling, and goat anti-PB1 from Santa Cruz. Secondary antibodies IRdye 800-conjugated donkey anti-mouse IgG, IRdye 800-conjugated donkey anti-goat IgG and IRdye 680-conjugated goat anti-rabbit IgG were purchased from LI-COR Bioscience.

Lentiviral vector production and transduction. Ten micrograms of each pGIPZ shRNAmir plasmid and 5 µg of each packaging plasmid pMD2.G and psPAX2 (Addgene) were co-transfected into 293T cells using the calcium phosphate precipitation method. At 24 h p.t., the transfection efficiency was evaluated by the presence of expressed TurboGFP and the medium was replaced with DMEM containing 5 % FBS and 50 µg ml⁻¹ gentamicin. The culture supernatant was collected 48 h after the medium change. Non-adherent cells and debris were removed by centrifugation. The supernatants were passed through a 0.45 µm pore filter, and concentrated by ultracentrifugation at 23 000 r.p.m. for 2 h at 4 °C. The titre of lentiviral particles was determined using the procedure for the GIPZ Lentiviral vector system (GE Healthcare). 293T or MDCK cells were seeded in a six-well plate. On the next day, cells were transduced with lentiviral vector at the indicated m.o.i. in 1 ml culture medium without FBS and with 8 µg Polybrene ml⁻¹. At 6 h p.t., an additional 1 ml complete medium was added to the cells. Three days after transduction, puromycin (InvivoGen) selection was applied to obtain the stably transduced cell clones. The transduced cells were infected with IAV at an m.o.i. of 0.001. The supernatants and cell lysates were harvested at designated time points for the various assays.

miRNA detection by Northern blotting. Total RNAs were extracted from either transfected 293T cells, or stably transduced 293T or MDCK cells with TRIZol (Invitrogen). Forty micrograms of total RNA was electrophoresed in a 15 % denaturing polyacrylamide gel. To check equal loading, the upper part of the gel above 80 bases was cut off and stained with 1 × SYBR Gold Nucleic Acid Gel Stain (Life Technologies) for 20 min. The rRNA (5S rRNA) bands were visualized under UV light. RNA on the lower part of the gel was electrotranferred to an Amersham Hybond-N membrane (GE Healthcare) in a semi-dry transfer apparatus (Bio-Rad) at 1 mA cm⁻² for 1.5 h at 4 °C. RNA was cross-linked to the membrane using UV light at a wavelength of 254 nm and a dose of 1200 µl cm⁻² × 100. Two biotinylated oligonucleotides complementary to the antisense sequences of mature miRNAs were used as the probes: 5'-GGATCTTATTTC-TTCGGGAG-3' (miRNA-NP) and 5'-GATCTTGTCACCATTGA-3' (miRNA-PB1). Hybridizations were performed with 10 pmol biotin-labelled probes in 6 ml ULTRAhyb Ultra-sensitive Hybridization buffer (Ambion) overnight at 42 °C. After hybridization, the blots were washed twice with 2 × SSC/0.1 % SDS, followed by two washes with 0.1 × SSC/0.1 % SDS at 42 °C. The biotin signals were detected by the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) as per the manufacturer’s instruction.

Statistical analysis. The statistical significance of differences was calculated using GraphPad Prism 6 (GraphPad Software) with one-way ANOVA followed by Tukey post-test to obtain the P value. Data are shown as mean ± SE of three technical replicates from one representative experiment. Significant differences between treatments and controls are shown as: *P<0.05; **P<0.01; ***P<0.001.

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REFERENCES


