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Down-regulation of viral replication by lentiviral-mediated expression of short-hairpin RNAs against vesicular stomatitis virus ribonuclear complex genes

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ABSTRACT

Vesicular stomatitis virus (VSV) causes great economic impact to livestock industry and is a prototype for studying non-segmented negative-stranded RNA (NSNR) viruses. In this study, we evaluated the antiviral potential of unique short-hairpin RNA (shRNA) targeting genes that form the ribonuclear protein (RNP) complex of VSV serotype Indiana (VSIV). We used lentiviral vectors to construct cell lines that stably expressed one of seven shRNAs targeting the RNP genes of VSIV, namely nucleocapsid (N), phosphoprotein (P), or polymerase (L). We reported two N-shRNA sequences targeting the 5′ or 3′ end of N that significantly reduced N, P, and L viral transcripts ($p < 0.001$), reduced viral protein expression, and reduced the viral particles shed in Vero cells ($p < 0.01$). When we analyzed the sequence diversity in the target region of this N-shRNA from two field isolates, we detected a single base substitution outside the seed region. We also reported five other shRNA sequences targeting components of the viral RNA that significantly reduce N, P, and L viral transcripts ($p < 0.001$) but failed to efficiently impair viral replication. The differences in the efficiency of the shRNAs tested were not due to mismatches within the target region in the genome of VSIV. Although partial silencing of viral transcripts by single shRNAs impaired but did not block VSIV replication, the combination of the shRNAs identified here into a multiple shRNA vector may result in inhibition of viral replication. These data contribute to ongoing development of RNAi-based technologies to combat viral diseases.

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1. Introduction

Vesicular stomatitis virus (VSV) belongs to the *Rhabdoviridae* family, order Mononegavirales (Wertz et al., 1998; Lyles and Rupprecht, 2007). The viral infection is characterized by vesicles that produce to ulcerative lesions in mouth, teats, and/or coronary bands of cattle, horses, and pigs (Martinez et al., 2003). The disease represents great economic impact (Letchworth et al., 1999; Rodriguez, 2002; Howerth et al., 2006) particularly in endemic countries. Clinical manifestations of VSV infection are indistinguishable from those of foot and mouth disease (FMD) (Martinez et al., 2003; Rodriguez, 2002) but unlike FMDV, outbreaks of VSV are periodically reported in United States and other countries in Central and South America. Therefore, efforts to rapidly control VSV outbreaks are relevant to both limit the impact of the viral infection on the livestock industry due to quarantines as wells as the impact on animal health.

VSV is also considered a model for studying non-segmented negative-stranded RNA (NSNR) viruses (Stillman et al., 1995), a group of many significant pathogens of humans, animals, plants, and insects (Wertz et al., 1998; Lyles and Rupprecht, 2007). VSV encodes five genes, in the following sequential order from a single polymerase entry site: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and polymerase (L) (Lyles and Rupprecht, 2007; Flanagan et al., 2001; Whelan and Wertz, 2002; Clarke et al., 2007). The location of the genes in the genome (Fig. 1A) constitutes a conserved strategy for transcriptional attenuation and gene regulation, thus relative molar ratios of the N, P, and L are crucial for optimal RNA replication (Wertz et al., 1998; Flanagan et al., 2001). The ribonuclear complex (RNP), which functions as the transcription and replication unit, is composed by the L, P, and N proteins (Rubio et al., 1980). N protein is the most conserved and abundant viral protein expressed in infected cells (Rodriguez et al., 2002). It wraps the negative-strand genome RNA along its full-length to protect it from nuclease-mediated degradation (Li and Pattnaik, 1999). The phosphoprotein mediates the binding of L protein to the N protein–RNA complex and it functions as an essential transcription factor for the viral polymerase (Li and Pattnaik, 1999; Bitko and Barik, 2001). The large (L) protein is the major subunit of the multifunctional RNA dependent-RNA polymerase (RdRP) which performs the genome replication and mRNA processing (Fu, 2005; Rahmeh et al., 2010).

RNA interference (RNAi) is a versatile tool to induce sequence-specific post-transcriptional silencing of gene expression (Fire

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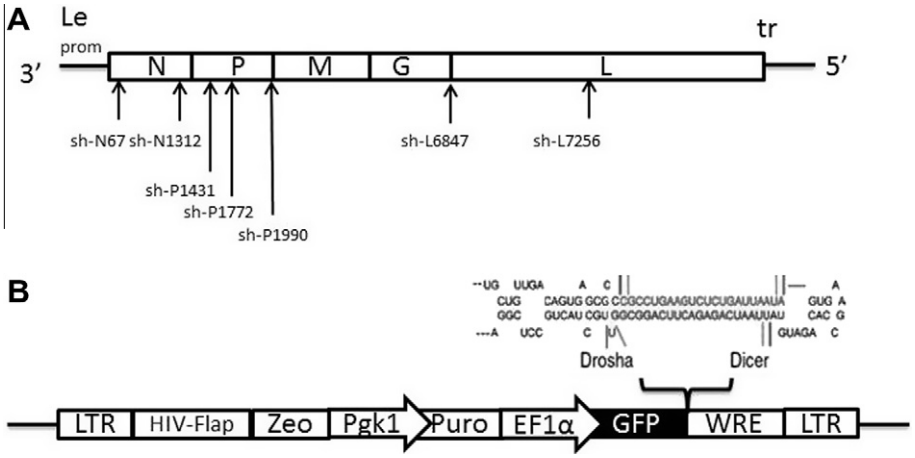

Fig. 1. Schematic diagrams of the genome organization of the viruses used in this study. (A) Vesicular stomatitis virus genome including 3′ leader sequences, promoter, and 5′ trailer sequence. Viral structural genes in their respective order of transcription: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and polymerase (L). Labels located below the 3′–5′ vRNA represent the name and target positions in the viral genome of the shRNAs used in this study. (B) Lentiviral unidirectional promoter construct used to transduce cell lines. HIV FLAP directs nuclear import of the construct, zeomycin (Zeo) is a bacterial selectable marker, Pgk1 denotes one promoter, puromycin is the eukaryotic selection cassette, EF1A is the promoter that drives the expression of the shRNA; WRE represents the Woodchuck regulatory element. The inset between the GFP and WRE ORFs exemplifies the sequence and structure of a shRNA including the processing sites for Dicer and Drosha enzymes during RNAi pathway.

et al., 1998; Grimm and Kay, 2007; de Fougerolles et al., 2007). The idea behind RNAi-based antiviral therapy is to activate RNAi machinery that targets specific viral transcripts inducing selective gene silencing of indispensable viral genes and ultimately leads to the reduction of viral titers in infected cells (Lopez-Fraga et al., 2008). The application of RNAi-based therapeutics has shown real promise in enhancing our ability to defend agriculture animal resources against viral disease (Lopez-Fraga et al., 2008; Shah and Schaffer, 2011). Successes in applying RNAi-based antiviral therapies in poultry (Chen et al., 2007, 2009; Hu et al., 2002; Sui et al., 2009) highlight the rationality of exploring its use in livestock species (Wise et al., 2008).

Barik (2004) reported the use of siRNA targeting G, P, and L genes to temporary target VSV genes (Barik, 2004). Otsuka et al. (2007) demonstrated the activation of the host RNAi pathway for targeting specific VSV genes and observed an increased VSV replication in mouse cells deficient of Dicer presumably ascribable to the lack of activation of endogenous RNAi (Otsuka et al., 2007). The purpose of this study was to test the silencing potency of different shRNA targeting the genes comprising the RNP complex of VSV. We utilized an established lentiviral vector system for delivery and stable expression of shRNA into target cell lines (Golding and Mann, 2011). We also aimed to evaluate the utility of this approach to produce an RNAi-induced antiviral effect in cultured mammalian cells before implementing *in vivo* systems.

2. Material and methods

2.1. Cell lines and viruses

Baby hamster kidney (BHK-21), Vero, and Mardin-Darby Bovine Kidney (MDBK) cell lines were used for the experiments. Cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin 10 μg/ml streptomycin and 0.25 μg/ml amphotericin B (GIBCO, Carlsbad, CA). A VSV strain serotype Indiana was kindly provided by Dr. Judith Ball (Texas A&M, USA). While two wild-type VSIV variants were isolated from a tissue collection of the Laboratory of Virology, School of Veterinary Medicine (Universidad Nacional, Costa Rica). Standard methods for viral isolation were employed (Wilson et al., 2009). Frozen bovine mucosa tissues with lesions compatible to VSV infection were the initial samples for viral isolation. In all cases, the serotype was confirmed by seroneutralization assay.

2.2. Challenge assays

Cells were trypsinized, counted, and seeded in duplicates into 24-well plates overnight prior to infection. Viral infections were carried out at MOI = 0.01 or 0.10 in FBS-free medium for 1 h. Supernantants were collected at 12 or 24 hpi. Viral supernatants were analyzed by microtitration in MDBK cells using standard methods for TCID50 determination. The titer was calculated using the method of Reed and Muench (Condit, 2007).

2.3. Design of shRNA

Sequences within N, P, or L genes of the VSIV were chosen after alignment of several published sequences using an online computer algorithm (RNAi codex) (Olson et al., 2006). Particular attention was paid to nucleotide sequences described in the literature as either being essential (Grdzelishvili et al., 2005), highly conserved (Ribeiro et al., 2008), or as locations where viral protein–protein interactions take place (Rodriguez et al., 2002). In this report position of shRNAs in the VSIV genome are given according to GenBank accession No. J02428.

2.4. Lentiviral constructs expressing shRNAs and generation of transgenic cell lines

A lentiviral shRNA-mir library (Silva et al., 2005) was used to clone each shRNA into the PEG unidirectional lentiviral construct (Golding and Mann, 2011) (Fig. 1A). Each shRNA was cloned using the PCR-based strategy described previously (Silva et al., 2005). Restriction enzyme analysis and DNA sequencing confirmed correct insertion and integrity of shRNAs. Self-inactivating (SIN) HIV-based recombinant lentiviral vectors were harvested after co-transfection of 293T cells with plasmids expressing the shRNA cassettes, VSV-G and the packaging construct (Miyoshi et al., 1998). These recombinant lentiviruses were used to transduce the Vero and BHK cells. Transgene expression was confirmed at 48 h post-infection by green fluorescent protein (GFP) expression, immediately followed by drug selection using puromycin. GFP expression in at least 90% of cell population was confirmed by flow

cytometry analysis (BD FACS Aria II, BD FAC DIVA Software) or cells were sorted until 90% GFP expression in each population was achieved.

2.5. Western blot

The VSV-G protein was detected using the primary antibody rabbit anti-VSV-G (Gentex, Irvine, CA) and the secondary antibody goat-anti-rabbit-horseradish peroxidase (Abcam, Cambridge, MA). In addition, detection of ß-actin (Abcam, Cambridge, MA) was performed as internal control. The amount of protein loaded was quantified by applying the Bradford method (Bradford assay kit, Thermo scientific,USA) in the Nanovue equipment (GE, USA). Cytoplasmic components of cell lysates treated with detergents, protease inhibitors and heat-inactivated cell lysates were separated by electrophoresis on 10% Mini-PROTEAN® TGX™ Precast Gel (Biorad, Hercules, CA) and electrophoretically transferred to polyvinylidene fluoride (PVDF) sheets. Blocking (Schmidt et al., 1987), washing, incubation with antibodies, and chemiluminesence visualization were performed following the manufacturer's instructions (Thermo scientific, Rockford, IL). Densitometry analysis was performed using using AlphaEase FC software (Alpha Innotech, San Leandro, CA).

2.6. Relative quantification of viral transcripts

Frozen cell lysates were thawed, homogenized (QIAshredder, Qiagen, Valencia, CA) and processed for RNA isolation using a commercially available extraction kit as suggested by the manufacturer's instructions (RNeasy kit, Qiagen, Valencia, CA) and treated with Dnase. Total RNA was quantified and used to produce cDNA with the qScript kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Real-time qPCR was carried out using the PerfeCTa® SYBR® Green FastMix, ROX (Quanta Biosciences, Gaithersburg, MD) and run in a StepOne thermocycler (Applied biosystems, Carlsbad, CA).

Relative quantification of viral N, P and L genes and cellular normalizing genes was evaluated using the comparative $\Delta\Delta C_T$ method as previously described (Livak and Schmittgen, 2001) and adjusting the method according to each primer set efficiency. Primers utilized for amplification of N (Wilson et al., 2009) and L (Hole et al., 2010) genes were previously described. For amplification of P genes the following primers were employed: 5′-ACTCCAGTGATG AACACACATCC-3′ (sense) and 5′- AGAGGCTGAAGACTTGCTTTCT-3′ (antisense). Glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) was used as cellular normalizing gene to account for variation in the cell number and quantity of RNA among groups. Standardization of each primer set was performed according to MIQE requirements (Bustin et al., 2009).

2.7. DNA sequencing analysis

Partial sequences of the viral genes were obtained from the VSIV field isolates and from the laboratory strain. Primers for PCR and sequencing reactions were designed based on the published sequences of VSIV. Multiple sequence alignments were performed using the online version of ClustalW (Thompson et al., 1994). Total genomic DNA from transgenic cell populations was isolated using DNAeasy (QIAGEN, USA) following the instructions of the manufacturer. Partial sequences of the region spanning the C-terminus of GFP and the 3' of the cloned shRNA were amplified and sequenced to confirm the expression of the shRNA in the cell lines.

2.8. Statistics and data analysis

Treatment differences were determined using Student's t-test or Wilcoxon-rank sum test as indicated in each figure legend. In figures, data represent a minimum of six observations over at least three experimental replicates (except by Fig. 3B which represent six observations over two experimental replicates). Analysis was performed using JMP software, version 8.0.2. In graphics, values are expressed as mean ± stand error (SEM), and statistical significance is indicated by an asterisk ($^{*}=p < 0.05$).

3. Results

3.1. ShRNAs trigger an antiviral response on Vero cells early after viral infection

The shRNAs were custom-designed to target regions of the VSIV mRNAs including the N gene, the P gene, the L subunit of RNA polymerase (Fig. 1A), or an irrelevant target control (also described as non-targeting control sh-NTC). Vero or BHK-21 cells were transduced with a self-inactivated lentiviral system (Fig. 1B) carrying the sequence of each shRNA (Table 1). After transduction, homogenous populations of cells expressing both GFP and each shRNA were drug-selected and sorted by flow cytometry. More than 90% of transduced cells were GFP positive, as determined by flow cytometry analysis. As an additional quality control, we confirmed the presence of the correct siRNA coding sequence in each cell line by sequencing the region containing the shRNA from genomic DNA. Mismatches in the siRNA (passenger strand, loop, or guide strand) coding regions were not detected for any group (data not shown). Additionally, microscopic evidence of malignant transformation (induced by lentiviral transduction) or cytotoxicity due to shRNA expression was not evident in any of the cell lines produced.

VSIV infection caused a marked cytopathic effect (CPE) resulting in cellular detachment, rounding, and cellular death. This effect was easily differentiated using light microscopy. In Vero cells infected with VSIV (MOI = 0.01), the CPE was severe between 12 and 24 hpi that correspond to 1 or 2 cycles of replication, respectively. At 12 hpi, many cells of the non-treated or shNTC control groups (Fig. 2B and C) were rounded and detached. Cell lines carrying N-shRNAs, however, showed remarkable reduction in CPE. In Particular, cells carrying sh-N67, which targets the 5′ of N (Fig. 1A), showed no CPE in two of the three experiments performed (Fig. 2D). In cell lines carrying shRNAs targeting viral P gene (Fig. 2F–H) or L gene (Fig. 2I and J), reduction in the CPE in a single microscopic field was not as evident as in groups expressing N-shRNAs. The microscopic evaluation suggested that expression of N-shRNAs had a protective effect against VSV infection at 12 hpi.

3.2. Transgenic cells system expressing shRNAs significantly decrease viral transcripts

Guided by the observed reduction of the CPE, we next quantified the reduction of viral transcripts and determined the specificity of the silencing induce by each shRNA tested. Total RNA from Vero cells infected at MOI = 0.01 or BHK-21 infected at

Table 1
Sequences of the VSIV gene regions targeted by the corresponding shRNAs.

Identification	Stem region of the shRNAs tested	Viral Gene targeted
sh-N67	5′-TCTGTTACAGTCAAGAGAATC-3′	N
sh-N1312	5′-AAGTCAGAATTTGACAAATGA-3′	N
sh-P1431	5′-TCCTATTCTCGTCTAGATCAG-3′	P
sh-P1772	5′-TGCCAGAGGGTTTAAGTGGAG-3′	P
sh-P1990	5′-TCTCTCAAAGACATCCATGAC-3′	P
sh-L6847	5′-GCTGTCAAAGTCTTGGCACAA-3′	L
sh-L7256	5′-TGCTAGACTCTTGTTGATGAT-3′	L
sh-NTC	5′-GCCTCTCCTTTGTATATTATT-3′	Null

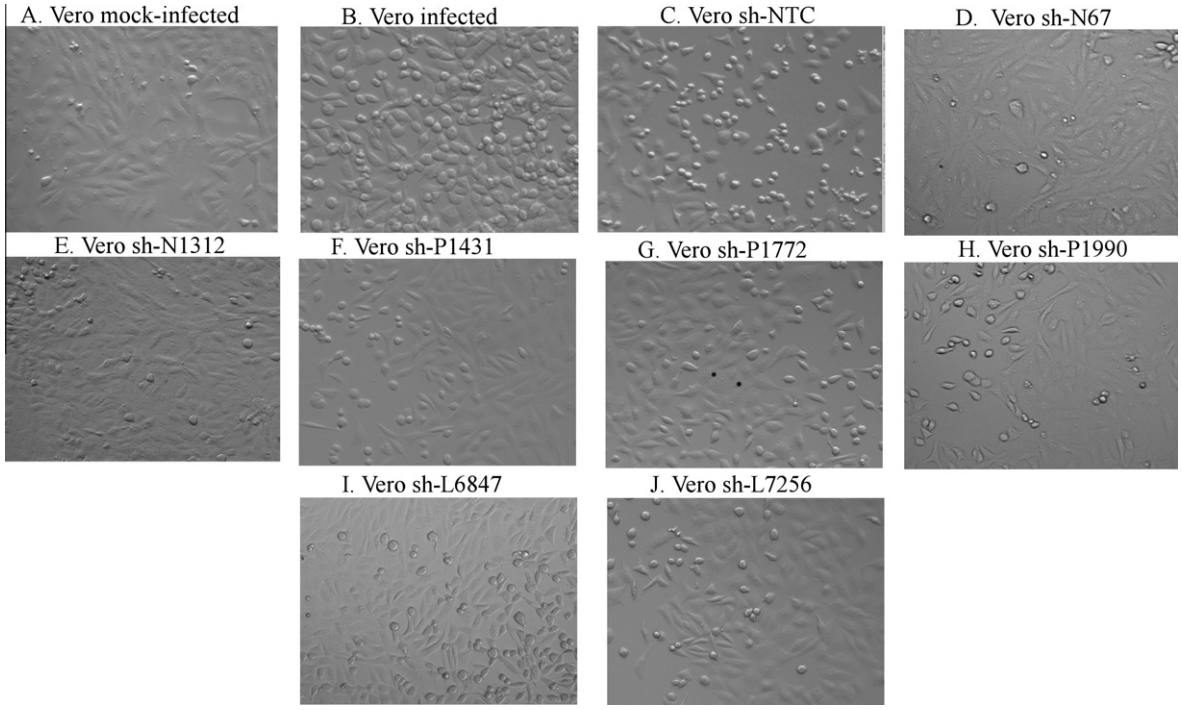

Fig. 2. ShRNAs targeting viral gene decrease the CPE in Vero cells. Transgenic Vero cells were infected at MOI = 0.01 and CPE was evaluated at 12 hpi using light microscopy. The experimental and control groups are denoted as (A) Mock infected cells, (B) CPE observed in wild-type infected Vero cells, (C) cell line expressing an irrelevant shRNA, (D and E) cell lines expressing shRNAs targeting two regions of N gene, (F–H) cell lines expressing shRNAs targeting different regions of P gene, and (I and J) cell lines expressing shRNAs targeting two regions of L gene.

MOI = 0.01 or MOI = 0.1 was collected at 12 or 24 hpi for relative quantification of viral transcripts using the RT-qPCR (Fig. 3).

In Vero cells, viral transcripts were reduced by all shRNAs ($p < 0.001$). In all cases the reduction in the viral transcripts corresponded to N, P, and L regardless of which mRNA was targeted (Fig. 3A). The effect was also significant in BHK cells expressing sh-N67 ($p < 0.001$; Fig. 3B) infected at the same MOI. Also, significant reduction of the N viral transcripts induced by sh-N67 and sh-N1312 ($p < 0.01$) was also evidenced in BHK-21 cells (Fig. 3C) when this cell line was challenged at 10 times higher MOI.

P- and L-shRNAs expressed in Vero cells were less effective than N-shRNAs, however, the reduction in viral transcription was at least 60% of the sh-NTC group (Fig. 3A). When combined, the results from all shRNAs expressed in transgenic cell lines sh-N1312 and sh-N67 yielded the greatest inhibition of N, P and L viral transcripts relative to sh-NTC control group.

3.3. ShRNAs targeting N gene but not L- or P genes reduced VSV- G protein levels

SiRNAs induce post-transcriptional silencing that may lead to translation repression of the viral protein encoded by the sequence targeted. Since commercial antibodies targeting N, P, or L VSV proteins are not available, we tested whether the expression of shRNAs had any effect on protein levels of a non-targeted gene. Inmunoblot analysis of cytoplasmic fraction of cell lysates for VSIV-G showed that at 12 hpi, transgenic Vero cell expressing sh-N1312 or sh-N67, but not P- or L- shRNAs, significantly reduced G viral protein levels as compared to sh-NTC ($p < 0.001$; Fig. 4A and B). In order to quantify this reduction, desitometric analysis was performed from three experiments and the averages of these observations are shown in Fig. 4C. After normalization to β-actin to account for variations in the amount of sample loaded, the differences corresponded to 40% or 30% less viral G protein than control cells expressing an irrelevant shRNA (Fig. 4C).

3.4. Transgenic cell expressing VSIV-shRNAs reduce viral yield

Having shown that N, P, and L-shRNAs were able to potently reduce viral transcripts and that N-shRNAs reduced viral protein expression, next we determined the virus yield released from the experimental and control cell lines after viral challenge. Supernatants from cells infected (MOI = 0.01 for Vero cells and MOI = 0.1 for BHK-21) were titrated at 12 hpi (Vero cells) or 24 hpi (BHK-21 cells). Viral yield, as determined by TCID50, recovered from Vero cells stably expressing sh-N1312 or sh-N67 was significantly lower than the sh-NTC control ($p < 0.001$; Fig. 4A). This difference in the viral titers corresponded to approximate 1 log. Importantly, there was no statistical difference between the titers recovered from cells treated with the sh-NTC as compared to non-transgenic infected cells ($p = 0.65$). Other shRNAs tested also yielded slightly lower titers than the sh-NTC but the reduction was not statistically significant. For this reason, the authors chose not to analyze P- or L-shRNAs in BHK-21 cells. Expression of sh-N67 in BHK-21 cells tends to reduce the infectious progeny virus at 12hpi and even at higher MOI (0.1) and at 24 hpi ($p = 0.069$; Fig. 5B).

3.5. Minor sequence mismatches in regions targeted by shRNA from VSIV field strains but not from the laboratory strain

To assess whether the difference in the potency of the shRNAs observed previously were attributed to mutations in the target regions of the shRNAs, we sequenced the viral target regions of N-, P- or L-shRNAs from the laboratory viral strain utilized for the challenge assays. Mutations were not found in the sequences targeted by shRNAs recovered from the laboratory-adapted strain. We next evaluated the sequence diversity in the target regions of sh-N1312

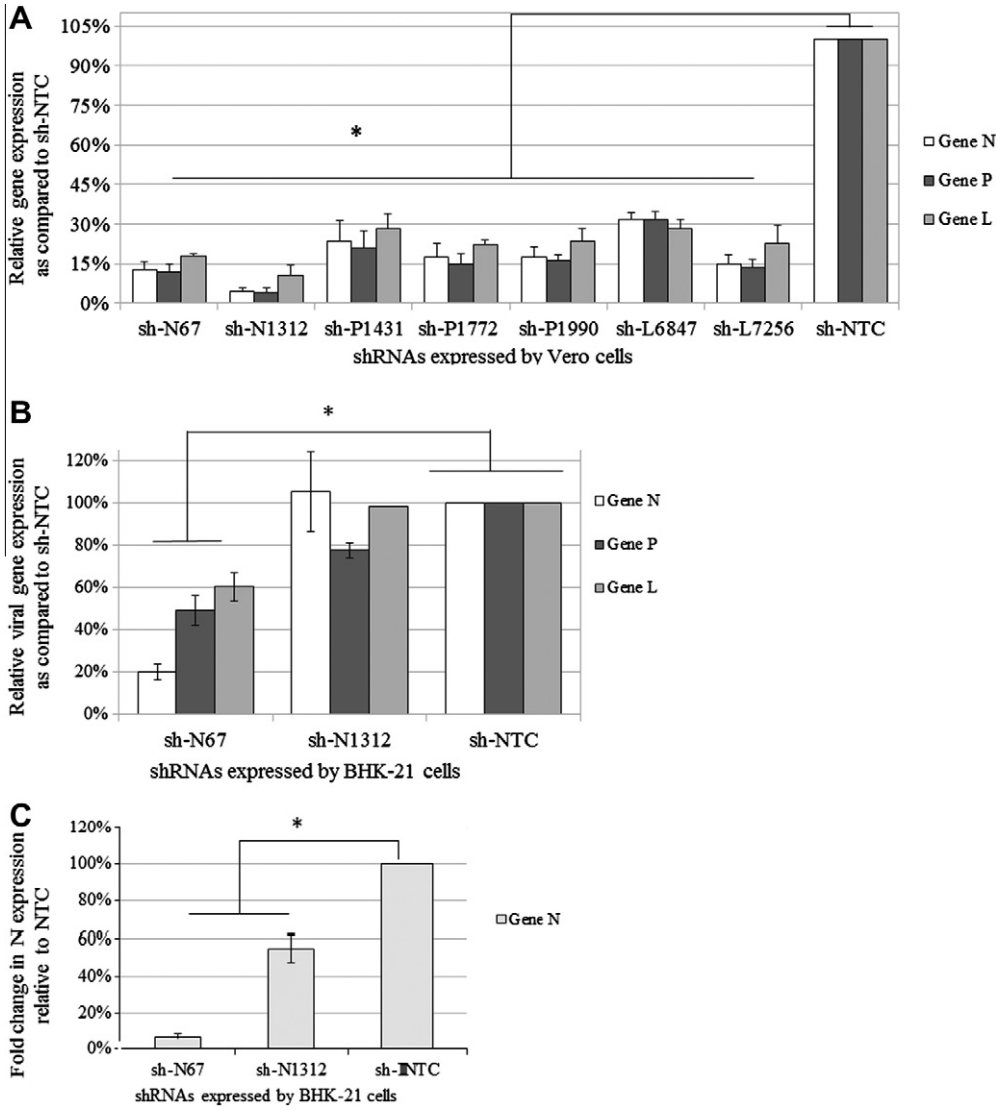

Fig. 3. Transgenic cell lines from different parental origin expressing shRNAs targeting RNP genes significantly reduced viral transcripts. Total RNA was collected at specified time post-infection, processed for qPCR, normalized to respective endogenous control(s) and viral transcript levels were compared among groups using the ΔΔ Ct method. (A) Fold change in N, P, and L transcripts in Vero cell lines expressing N-, P-, or L-shRNAs as compared to cells expressing sh-NTC. Vero-derived cell lines were infected at MOI 0.01 and total RNA was collected 12 hpi. Statistical analysis: Student *t*-test. (B) Fold change in N, P, and L transcripts in BHK-21 cell lines expressing N-shRNAs as compared to controls. BHK cell lines were infected at MOI 0.01 and total RNA was collected 12 hpi. Statistical analysis: Wilcoxon rank-sum test (C) Fold change in N transcripts in BHK-21 cell lines expressing N-shRNAs as compared to controls. BHK cell lines were infected at MOI 0.1 and total RNA was collected 24 hpi. Statistical analysis: Wilcoxon rank-sum test.

and sh-L6847 from two wild isolates circulating in past years in Costa Rica, a country in which VSIV infection is endemic. We found the same single point substitutions for the two field isolates in the target region of sh-N1312 (see GenBank Accession Nos. JQ425739, JQ425740) and sh-L6847 (Table 2).

4. Discussion

Proper selection of viral gene targets is a crucial aspect to induce activation of the RNAi pathway and develop antiviral strategies. In the case of VSIV, the activation of the endogenous cellular RNAi mechanism to limit cellular replication has been suggested (Otsuka et al., 2007). In order to achieve reduction of viral transcripts, protein levels, and VSV infectivity, this study characterized the potency of seven unique shRNAs expressed from integrated transgenes on the inhibition of viral genes that constitute the VSV RNP complex.

The genomes of NSNR viruses are templates for two different RNA synthetic processes: transcription to generate mRNAs and replication of the genome via production of a positive-sense anti-genome (Whelan et al., 2004). The anti-genome acts as a template to generate the negative-strand genomes of the progeny (Whelan et al., 2004). In this study, we found that all P- and L-shRNAs tested induced a significant ($p < 0.001$) reduction of viral RNA but had limited ability to reduce viral titer and no effect on decreasing non-targeted G protein levels. Similar studies from Barik (2004) or Bitko and Barik (2001) also indicated a reduction of siRNA targets accompanied by an unintended reduction in other viral transcripts. One interpretation of these results would be that these shRNAs may degrade the full-length genome and/or the intermediate anti-genome as well as the target viral mRNA (Whelan and Wertz, 2002; Li and Pattnaik, 1999). In this way, the reduction observed in the viral RNA detected by RT-qPCR (regardless of which viral mRNA was targeted) could be attributed in part to the degradation of genomic and antigenomic RNA complementary to the

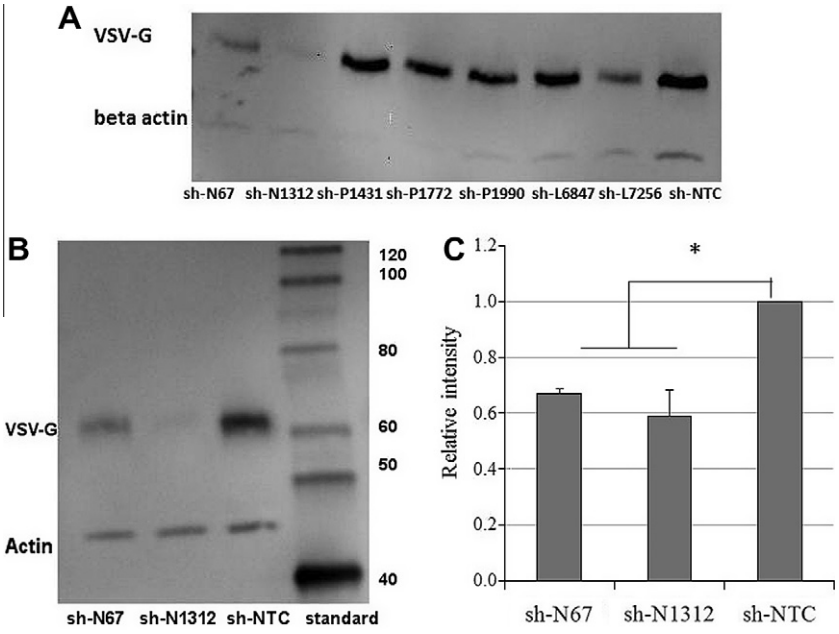

Fig. 4. Sh-N67 and sh-N1312 reduced VSV-G protein levels. (A) A representative westernblot image performed to detect VSV-G protein from cytoplasmic lysates of Vero infected cells expressing N-, P-, L-shRNAs at 12 hpi. (B) A representative image of the three immunoblots performed to detect VSV-G protein from cytoplasmic lysates of Vero infected cells expressing N-shRNAs at 12 hpi. (C). Densitometric analysis of the data from three experiments after normalization to β-actin. Protein levels are expressed as a percentage of sh-NTC.

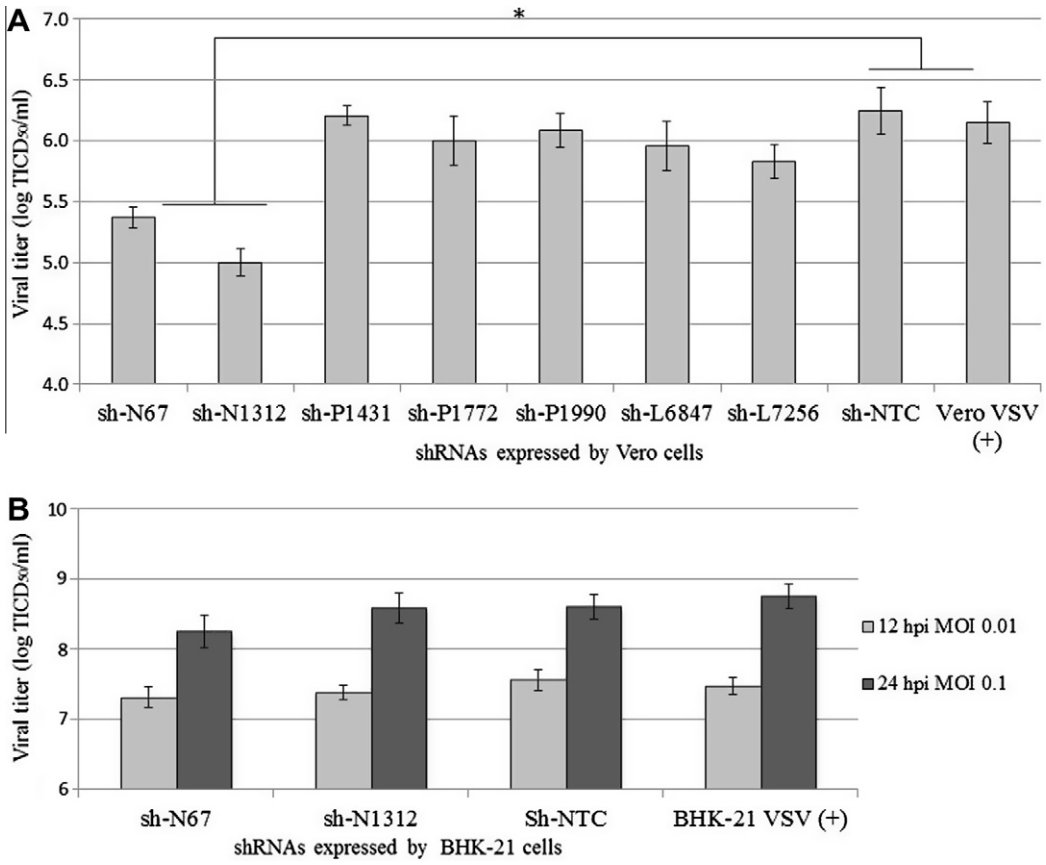

Fig. 5. Expression of shRNAs in cell lines reduced the viral infectivity titer. (A) Vero cell lines expressing shRNAs were infected at MOI = 0.01 and supernatants were collected at 12 hpi. Data is compared to the sh-NTC. (C) BHK-21 cell lines expressing shRNAs were infected at MOI 0.1 and supernantants were collected at 24 hpi. Statistical analysis: Student's *t*-test.

Table 2
Genetic variability in the sequence targeted by the shRNAs.

ShRNA		Gene	Sequence targeted by the guide strand	Position of the mismatch
sh-N1312	Target sequence	N	5′-UUCAGUCUUAAACUGUUUACU-3′	–
	Wild isolates[a,b]	N	5′-UUCAGUCUUAAGCUGUUUACU-3′	10
sh-L6847	Target sequence	L	5′-CGACAGUUUCAGAACCGUGUU-3′	–
	Wild isolates[a]	L	5′-CGACAGUUUCAAAACCGUGUU-3′	10

[a] Recovery sequences are shown as RNA sequences complementary to the DNA recovered experimentally.
[b] Access corresponding DNA sequences using GenBank Accession Nos. JQ425739, JQ425740.

shRNAs. This may explain the reduction in the RNA levels without changes in the levels of viral protein (as evidenced in western blot Fig. 4) and slight reductions in viral titers. Further studies using genome and anti-genome specific primers for qPCR are required to validate this hypothesis.

In contrast to our results, Barik (2004) reported high efficiency of siRNA transiently targeting L or P genes with partial loss of RNA synthesis in different viral models, including VSV and other NSNR viruses. The experimental data and corresponding sequences of the siRNAs employed in these experiments are not available for VSV, which makes it impossible to compare results (Barik, 2004).

We also found that expression of N-shRNAs had major effects reducing viral transcripts, viral protein, and viral titer (Figs. 3,4 and 5) resulting in transgenic phenotypes with reduced cytopathogenicity upon infection (Fig. 2). In past reports, it has been shown that N protein plays a crucial role in mediating interactions between viral RNA and viral polymerase (Rodriguez et al., 2002) during viral replication. Also, N protein regulates the transcription of downstream genes (Rodriguez et al., 2002). Other studies have suggested that the availability of unbound N protein determines the balance between replication and transcription of viral RNA (Arnheiter et al., 1985). Therefore, the viral RNA replication is proportional to the amount of N protein synthesized (Wertz et al., 1998; Patton et al., 1984). In accordance with our finding, post-transcriptional degradation of N induced by the N-shRNAs would also prompt a more acute reduction in viral transcription, translation, and replication as compared to other shRNAs targeting different regions of the viral genome.

Analogous with the western blot data in the current study, Fowler et al. (2005) also described a decreased production of other non-targeted viral proteins as a consequence of specific siRNA-mediated silencing of the Marburg virus nucleocapsid (another member of the Mononegavirales). Fowler et al. (2005) attributed this finding to the important role of N in virus replication and transcription.

We found that regions within the 3′ and 5′ regions of N mRNA are especially important targets for shRNA-mediated silencing. The region targeted by the sh-N1312 is included within the last 60 aa of the C-terminus of N, which has been reported as a highly conserved region required for interactions with the phosphoprotein (Rodriguez et al., 2002). In BHK cells infected at MOI 0.1 analyzed 24 hpi we found that sh-N67 induced a more potent reduction in N transcripts as compared to N-1312. This data is consistent with the experiments performed at MOI 0.01, 12 hpi in the same cell line. In contrast to our results in Vero cells, expression of sh-N1312 in BHK did not induce a significant reduction in transcripts or titers at 12 hpi. These findings might be explained by a variation in the expression of the N-1312 transgene between BHK and Vero cells.

We report that the analysis of the sequences from two VSV field variants revealed a single mismatch located 10 nt from the 5′ of the region target by sh-N1312 (Table 2). This mismatch is not expected to affect the canonical shRNA complementary sites within the seed region (Friedman et al., 2009) and hence the effectiveness of these shRNAs in blocking wild type VSV replication. Further experimental confirmation employing these strains is required to validate this prediction.

Although the N gene sequence is more conserved across VSV strains, making it an attractive target of RNAi based gene silencing, the location of N in close proximity to the viral promoter (Whelan et al., 2004) also favors its great abundance. Consequently, N transcripts represent a challenging target for the artificially induced RNAi system and may explain the failure of the siRNAs tested in this study to knockout N transcripts. The transcriptional features of N gene are shared by other NSNR viruses (Whelan et al., 2004) and should be considered when designed shRNAs targeting their genomes.

In the past, the induction of the IFN response has been suggested as a factor that bias the antiviral effects induced by RNAi-based strategies (Matskevich et al., 2009). Neither BHK-21 nor Vero cells, utilized in this study, induce effective IFN response (Emeny and Morgan, 1979; MacDonald et al., 2007). This feature makes them ideal *in vitro* models to evaluate the effect of specific RNAi-based antiviral therapies in the complete absence of the IFN response. Importantly, the net antiviral effect observed in our experiments as result of RNAi activation, should be potentiated in cells and organisms expressing a competent IFN-based response. Further experimentation is required to specify the threshold, in which the reduction in the number of viral particles produced by an antiviral therapy in combination with activation of the immune mechanisms is sufficient to prevent clinical manifestations of disease.

Other publications have suggested the combination of multiple shRNAs into a single expression vector as a worthy strategy to induce greater impairment of viral replication by silencing multiple target genes (Junn et al., 2010) and to prevent viral escape (ter Brake et al., 2008; Kim et al., 2010; McIntyre et al., 2011). The combination of the shRNAs identified here into a multiple shRNA vector may result in blockage of viral replication.

In summary, the information contained in this report contributes to broaden knowledge about the application of an RNAi-based antiviral approach for NSNR viruses. It is evident, however, that more research on this strategy is necessary to increase the effectiveness and durability particularly, when the systems will be used *in vivo*.

5. Conclusions

In this study, all transgene derived shRNAs targeting RNP genes of VSV reduced viral target messages but only N-shRNAs significantly reduced viral titers upon infection. The expression of N-shRNAs also resulted in reduction of VSV-G protein levels. This data demonstrate the antiviral potential of N-shRNAs in the absence of unintended stimulation of IFN response.

We also found that neither the L- nor P-shRNAs significantly impaired VSV replication even when one of L-shRNAs targeted a region highly conserved among NSNR viruses. This suggests that reduction in viral transcripts does not necessarily mean significant reduction in viral titers. The lack of potency of these shRNAs was not explained by mutations in target regions or shRNA context.

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