AN RNA INTERFERENCE-BASED APPROACH TO COMBAT VIRAL INFECTIONS: VESICULAR STOMATITIS VIRUS GROUP-PROTOTYPE

A Thesis

by

LISBETH RAMIREZ CARVAJAL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Biomedical Sciences

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Approved by:

Co-Chairs of Committee,	Charles R. Long
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ABSTRACT

RNA Interference-Based Approach to Combat Viral Infections: Vesicular Stomatitis Virus Group Prototype. (August 2011)

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Co-Chairs of Advisory Committee: Dr. Charles R. Long
Dr. Susan Payne

Vesicular stomatitis virus (VSV) is considered a prototype for studying non-segmented negative-stranded ribonucleic acid (RNA) viruses. Livestock are naturally infected by VSV, causing severe economic impact due to lack of any effective treatment. RNA interference (RNAi)-based therapeutics are promising alternatives to control viral infections. Lentiviral vector systems deliver artificial short hairpin RNA (shRNA) into the genome of cells to activate the RNAi pathway. In this study, an RNAi-based approach to generate cell lines with reduced susceptibility to VSV (Indiana) infection was tested. First, eight shRNAs targeting either the nucleocapsid (N), phosphoprotein (P), or the polymerase (L) viral genes were designed and introduced into cell systems. To test the potency of the shRNAs for silencing the target viral transcripts, semi-quantitative polymerase chain reaction (PCR) analysis of viral N, P, and L transcripts was performed. Then, supernatants from infected groups were evaluated by microtitration and immunoblot. Finally, the effect of VSV genomic variability in the target region of shRNAs was predicted by partial sequencing field and laboratory-adapted strains.

Viral transcripts were significantly reduced in cells stably expressing shRNAs targeting the N viral gene (nucleotides 67-97 or 1312-1332; $p<0.05$) or P gene (nucleotides 1772-1792; $p<0.05$). Reduction in viral transcripts was not observed by other VSV-shRNAs tested. Reduction of viral transcripts by the N-shRNA (sh-1312) was accompanied by a decrease in viral protein. Also, a reduction in the viral particles shed from cells expressing N-shRNAs (nucleotides 67-97, $p<0.05$) was noted. The results also showed complementarity of target gene sequences for shRNAs in the sequence from the laboratory-adapted strain and single base substitutions in the corresponding regions from VSV field isolates. However, these mismatches did not occur within the seed region of the shRNAs.

In conclusion, partial silencing of viral transcripts by a single shRNA does not block VSIV replication; however, partial impairment of VSIV replication was observed in N-shRNAs expressing cells. During infection, the naturally high level of N gene transcription may have modulated the sh-RNA effect. The combination of the most potent shRNAs identified here into a multiple shRNA vector may result in further reduction of viral replication. These data contribute to ongoing development of effective RNAi-based technologies to combat viral diseases.

DEDICATION

To my family

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Charles Long, for his support and trust. I also extend my gratitude to him for opening the doors to the Reproductive Science Laboratory (RSL) and to Aggieland. I deeply thank my committee members, Dr. Susan Payne and Dr. Michael Golding, for their guidance throughout the course of this research, especially for their comments on the experimental design. I thank the faculty and staff of the Department of Veterinary Physiology and Pharmacology and the University Writing Center.

I also extend my thanks to my labmates, Greg, Mike, Paulette, Kim T, Ali, Kim G, and Raven, for all the help they provided in the lab and also for making my time at Texas A&M University a great experience. Additionally, I thank Dr. Carlos Jimenez for supporting my work in Costa Rica.

I especially thank my husband, Elías, for his patience, love, and enthusiasm. I also want to extend my deepest gratitude to my mother, Ligia Carvajal Castro, and father, Franklin Ramírez-Vargas, for their encouragement and unfailing support.

I thank the Fulbright Academic Exchange Program from the U.S. Department of State's Bureau of Educational and Cultural Affairs for their sponsorship. This work was partially funded by the College of Veterinary Medicine- Texas A&M University (CVM-TAMU) graduate trainee research award and the CVM-TAMU Link Equine Research Endowment.

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1. INTRODUCTION

Around the world, numerous viral diseases of veterinary importance are economically devastating, but also many viral pathogens are important from a zoonotic medical perspective. These aspects justify the development of innovative strategies aimed to confer innate resistance to viral diseases. Recently, the generation of genetically modified animals that constitutively triggers the RNA interference (RNAi) pathway as an antiviral response has been suggested, particularly, in mice (Wise et al., 2008;Otsuka et al., 2007) and avian models (Chen et al., 2009).

Several approaches can be adopted to test the applicability of an RNAi-based strategy to impair the viral replication cycle. This work aimed to utilize RNAi-based strategies to generate stable cell lines with reduced capacity for viral infection by using Vesicular stomatitis virus (Serotype Indiana; VSIV) as a prototype. The specific objectives for this investigation included: 1) To test the effectiveness of several short-hairpins RNAs (shRNAs) targeting VSIV genes, 2) To evaluate an RNAi–based approach to reduce VSIV replication *in vitro,* 3) To analyze the sequence variability in VSIV genomic regions targeted by the designed shRNAs.

In the first section, general aspects of the RNA interference pathway will be addressed followed by a discourse of the recombinant lentiviral delivery systems used to integrate shRNAs into cell genome. Lastly, relevant hallmarks of non-segmented negati-

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This thesis follows the style of Antiviral Research.

ve strand RNA viruses, particularly features of VSV, the group-prototype virus used in this study, will be described.

The experimental design and results presented in the second section will describe the suitability of RNAi-based approaches to reduce susceptibility to VSIV *in vitro*. Comparable and/or improved methodologies using *in vivo* models could be developed in future to combat similar viral infections. Also in this section, the analysis of the sequence variation of the target regions of the shRNAs in wild strains will help to gain insights about the possible effect of high viral mutational rate on RNAi-based viral control. In section three, the author will conclude this thesis by summarizing the contributions of this study and addressing possibilities for future work.

1.1 RNA interference (RNAi)

RNA interference-based therapeutics utilize methods based on the ability of complementary double strand RNA (dsRNA) to specifically suppress the expression of disease-causing genes from cellular or pathogen origin (Sliva and Schnierle, 2010). These methods constitute an innovative and promising strategy with broad applications in molecular medicine.

In a broad sense, RNA interference (RNAi) is used to describe a diverse set of pathways in which short double-stranded RNA (dsRNA) fragments recognize and manipulate the processing of complementary regions of the messenger RNA (mRNA) (Obbard et al., 2009). This is an ancient mechanism described in plant and animals cells, initiated by short dsRNA, that results in sequence-specific silencing of messenger RNA (mRNA) at the post-transcriptional level (Elbashir et al., 2001).

The first description of RNAi was provided by Fire and Mello (1998). In their key experiment, dsRNA was injected into a nematode, *Caenorhabditis elegans*, resulting in the diminished expression of genes that had sequences complementary to those introduced. This conclusion was supported by a significant decrease in the corresponding endogenous messenger RNA (mRNA) (Hannon and Rossi, 2004; Fire et al., 1998). The RNAi mechanism was previously described in plants as post-transcriptional gene silencing and co-suppression, however, it was not clear that dsRNA causes the initiation of the effect. Some years later, this mechanism was seen in routinely-used mammalian cell culture systems such as Human Embryonic Kidney (HEK) 293 cells and HeLa (Human epithelial cervical cancer) (Elbashir et al., 2001). In 2006, Fire and Mello received the Nobel Prize for their first description of the RNAi pathway and further elucidation of its molecular complexities (Sliva and Schnierle, 2010).

The RNA interference pathway is initiated by small double stranded RNAs (dsRNAs) that include siRNAs and microRNAs (miRNA or mir). SiRNA can induce gene silencing through sequence-specific cleavage of perfectly complementary mRNA (de Fougerolles et al., 2007). The miRNA pathway is endogenously triggered by short sequence of RNA, derived from imperfectly paired non-coding hairpin RNA structures, that recognizes complementary mRNA and targets it for degradation or translation suppression in the case of imperfectly complementary targets (Fig. 1) (Obbard et al., 2009). The microRNA is initially encoded by the host genome and then transcribed by a RNA polymerase II. While it is located in nucleus, the primary miRNAs is processed by

the Drosha family members leading to pre-miRNAs formation. Then, the pre-miRNAs are transported from nucleus to the cytoplasm using the cellular exportin-5 and are further processed by a member of Dicer family to generate the mature miRNA (Fig.1) (Obbard et al., 2009;Ma et al., 2007).

In cytoplasm, the miRNA is loaded into a multi-enzyme RNA-induced silencing complex (RISC), which includes Argonaute 2 (Ago2). Next, one of the strands is discarded to form an activated complex containing the guide strand. Then, the interactions between the complex and the target mRNA takes place; perfect or imperfect matching between the short RNA and its target mRNA leads to either mRNA degradation or blockage of the translation, respectively. MicroRNAs may mediate mRNA degradation in processing bodies (P-bodies), a cytoplasmic compartment (Fig. 1) (Obbard et al., 2009; de Fougerolles et al., 2007; Lopez-Fraga et al., 2008).

The RNAi mechanism is a key component of the post-transcriptional control of gene expression. Notably, these pathways are employed by both the host cell and the viral pathogens. From the host cell perspective, microRNAs provide a mechanism for regulating the cell's gene expression as well as defense against viruses and transposable elements (Obbard et al., 2009). In contrast, some viruses commandeer the host RNAi mechanism to take control of the cell machinery for their benefit and to prevent host mechanisms targeted to combat viral infection (Li and Ding, 2005).

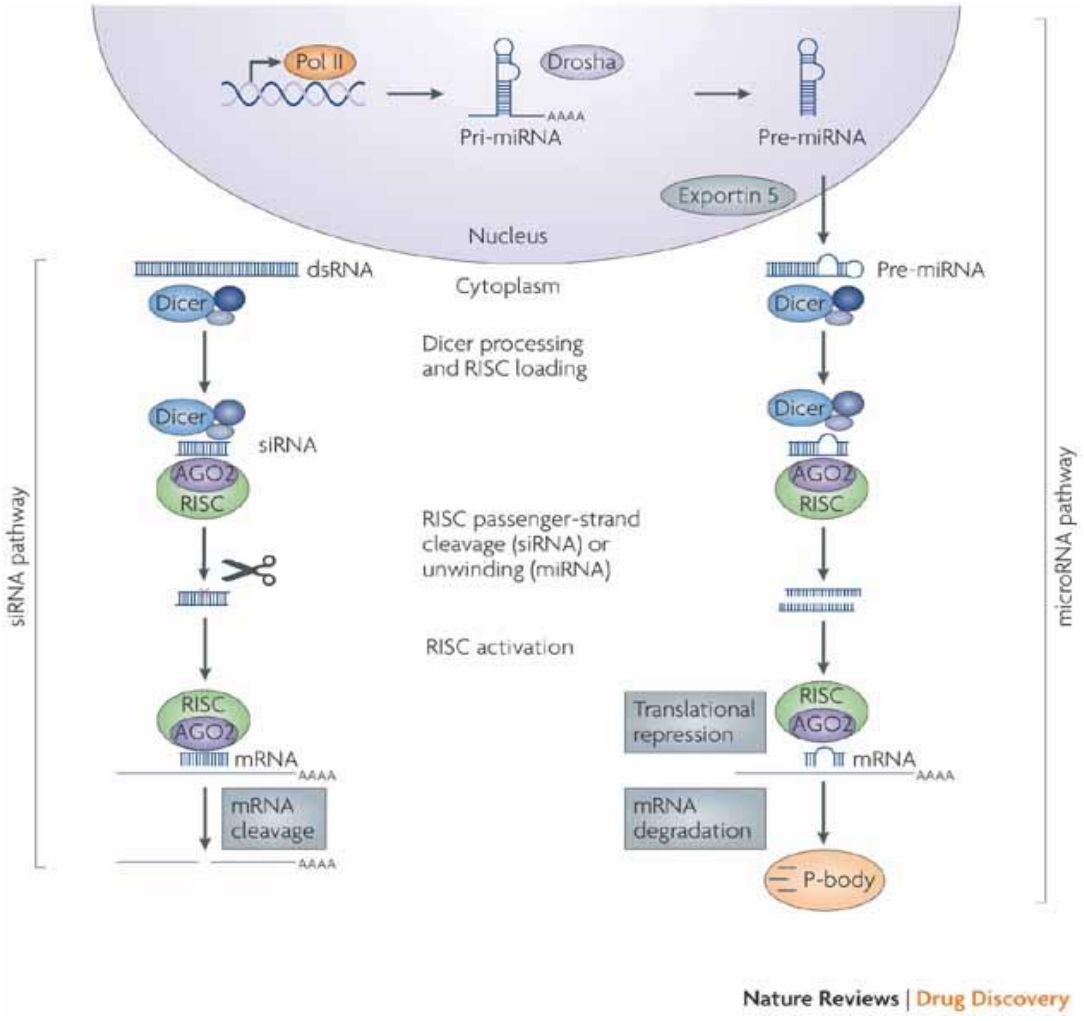


Fig. 1. The RNAi pathway in mammalian cells. More details of the components of the pathway are described in the text (de Fougerolles et al., 2007).

At first glance, the molecular components of RNAi response and the experiments conducted by Fire and Mello (1998) seemed contradictory with previous knowledge of cellular nonspecific responses targeting foreign dsRNA to destruction. During activation of the innate response, RNA molecules located in the cytoplasm of mammalian cells trigger a mechanism, in which two cellular enzymes known as dsRNA-dependent

protein kinase (PKR) and 2',5'-oligoadenylate synthetase (2',5'-AS) become active (Elbashir et al., 2001). PKR leads to inhibition of translation of mRNA and 2',5'-AS induces mRNA degradation by a ribonuclease (Elbashir et al., 2001; Hannon and Rossi, 2004;Manche et al., 1992).

In this scenario, introduction of dsRNAs will inevitability lead to degradation of the nucleic acid and repression of protein synthesis regardless of sequence complementarity. However, further biochemical analysis of the RNAi pathway revealed that dsRNAs smaller than 30 base pairs (bp) could induce the RNAi response in mammals without activating this innate immune response (Hannon and Rossi, 2004). Therefore, RNAi was envisioned as a new tool for studying gene function (Elbashir et al., 2001).

After discovery of endogenous encoded microRNAs, scientists explored the possibility of triggering the RNAi pathway by expressing or introducing artificial RNA sequences that mimic endogenous microRNAs. Further investigations led to the establishment of two robust systems to evoke RNAi response and produce selective gene silencing: in one system, the short double-stranded interfering RNA oligonucleotides (siRNA) are delivered to the cytoplasm, and in the other system, short hairpin RNA (shRNA) cassettes are expressed in the nucleus and further processed in the cytoplasm.

ShRNAs are processed like microRNAs and lead to stable gene down-regulation of genetic targets. ShRNAs are transcribed by the cell from a DNA template, originating a single-stranded molecule of RNA. The structure encompasses two complementary regions separated by a short loop that serves to fold the transcript on itself and produce

the characteristic dsRNA hairpin structure. ShRNAs vary in size, design, and structure, and the degree of similarity to natural miRNAs (Hannon and Rossi, 2004).

Conversely, siRNAs are artificially synthesized and introduced into cell. There are accessible tools and commercial manufactures that offer a variety of pre-validated siRNAs targeting mainly human, mice and rat genes (Hannon and Rossi, 2004). As it is the case with other nucleic acids, siRNAs do not freely penetrate hydrophobic cellular membranes due to their negative charge (Lopez-Fraga et al., 2008). For this reason, chemically synthesized siRNA duplexes are usually incorporated into mammalian cells using transfection methods; as a result, the gene silencing effect is only temporary. In addition, siRNAs can be easily degraded by cellular RNases (Sliva and Schnierle, 2010), which also accounts for their transient silencing effect. Likewise, the potency and duration of the silencing response depends on the efficiency of transfection and the differences in the cell lines chosen (Hannon and Rossi, 2004). ShRNAs present several advantages when compared to siRNAs; shRNAs induce longer and more stable silencing, are lower in cost, and there are various delivery strategies to introduce them into the target cell (McIntyre and Fanning, 2006). After reviewing the limitations of siRNA systems and considering the above-mentioned advantages of shRNA, the current investigation employs shRNAs to activate RNAi.

Currently, RNAi is accepted versatile tool for applications such as gene therapy (Grimm and Kay, 2007), cancer therapeutic (Grimm and Kay, 2007; Takeshita and Ochiya, 2006), reverse genetics (Sliva and Schnierle, 2010), regulation of chromatin structure (Lavrov and Kibanov, 2007), elucidation of components of molecular

pathways, screening of drug targets (Sliva and Schnierle, 2010), and as antiviral agents (Grimm and Kay, 2007). Beyond these applications of RNAi in science and medicine, it is important to remember that RNAi is an essential and ubiquitous biological phenomenon which is part of cellular physiology. Complete shutdown or saturation of the cellular miRNA pathway can led to cell dysfunction and death (Barik, 2006).

Some advantages of an RNAi-based therapeutic approach include: i) high potency and persistence, ii) high selectivity and specificity, and iii) low immunogenicity (Lopez-Fraga et al., 2008). Additionally, RNAi therapeutics can eventually be developed in large scale production (Lopez-Fraga et al., 2008). This strategy has promising applications for developing stable, specific and heritable gene silencing.

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). Recently, siRNA- and shRNA-based strategies have been shown to affect the viral replication of a large number of viruses. Their effects on viral cycle range from reduction in titers or suppression of replication to protection against clinical manifestation or even lethal infection. Some examples of studies using this RNAi antiviral approach include: bovine viral diarrhea virus (Lambeth et al., 2007), rabies virus (Israsena et al., 2009), foot and mouth disease virus (FMDV) (Liu et al., 2005), influeza A viruses (Sui et al., 2009) hepatitis B and C viruses, West Nile Virus, SARS-Coronavirus (Lopez-Fraga et al., 2008) and many others.

Conversely, a significant problem of most antiviral therapies is the appearance of

resistant strains (Gitlin et al., 2005). This problem has also been described previously in the case of siRNA- and shRNA-based strategies. Indeed, reports have demonstrated the emergence of human immunodeficiency virus type 1 (HIV-1) and poliovirus variants resistant to RNAi. These resistant variants could carry as little as a single point mutations in the region targeted by the siRNA to produce the failure of silencing (Gitlin et al., 2005)(Boden et al., 2003).

Some researchers argued that mismatches within the central position of the siRNA, around nucleotides 9 to 11 of the target RNA, might result in improper silencing (Gitlin et al., 2005). Others (Grimson et al., 2007; Friedman et al., 2009;Jackson et al., 2006) mantain the critical role of the matching between nucleotides 2 to 7 of the 5' region (called the seed region) of the miRNA and its target. Canonical miRNA complementary sites within seed region, namely 6mer site, 7mer-m8 site, 7mer-A1 site, and 8mer site have been defined (Friedman et al., 2009). Jackson et al. (2006) reported that single base mismatches at positions distal to the seed region had minimal effect on the off-target signature or on the silencing (Jackson et al., 2006).

However, a general consensus regarding the most critical region of complementarity between the siRNA and its target mRNA has not been established (Gitlin et al., 2005). Other researchers argue that siRNAs might not require complete and contiguous base pairing to their target mRNA to lead its degradation because it is known that miRNAs are able to recognize and silence their targets even in the presence of imperfect complementarity (Hannon and Rossi, 2004).

For these reasons, correct selection of target sequences in the viral genome and careful testing of shRNAs and their targets are indispensable to demonstrate the relevant applicability of the RNAi-based antiviral strategy with the chosen viral model. Additionally, establishment of *in vitro* models before launching experiments with animal models is important to reduce costs and provide the proof of concept.

1.2 Recombinant viral vectors for delivery of shRNA

One common approach to incorporate shRNA-cassettes into target cells involves the use of viral vectors as DNA delivery vehicles. The discovery of the promoter-driven expression of shRNAs made possible the use of RNAi-delivery viral vectors to induce the RNAi machinery. Using this strategy, nuclear integration of the shRNA-carrying viral genome is accomplished. Then, shRNA is processed and exported into the cytoplasm to form the corresponding siRNA. Finally, the post-transcriptional silencing cascade is activated.

Members of several viral families have been employed as delivery vehicles of shRNA-expression cassettes. These recombinant viruses vary in several aspects such as transgene carrying capacity, immunogenicity, replication ability, the range of cells in which they replicate, and the pathogenicity of the non-recombinant virus of origin. All these characteristics convey different advantages and disadvantages that narrow their usage in gene and shRNA-based therapies. Some existing viral vector systems include: adenovirus, adenovirus-associated viruses, baculoviruses and retroviruses –including lentiviruses (Sliva and Schnierle, 2010).

Currently, replication-defective retroviral vectors are broadly employed tools for gene-transfer studies due to their ability to introduce and stably incorporate transgenes in mammalian genomes (Ramezani and Hawley, 2002). In fact, recombinant retroviruses are among the first transfer vehicles used to demonstrate specific hairpin-RNA mediated activation of the RNAi pathway (Sliva and Schnierle, 2010).

Retrovirus vectors are derived from members of the family *Retroviridae*, which comprises seven genera of different genetic complexity. Their genome is a single-stranded RNA (ssRNA) that replicates through a double-stranded DNA intermediate, produced by the viral retrotranscriptase (RT) (Freed, E. and Martin, M., 2007).

The simplest genome organization found among retroviruses consists of at least three genes: *gag* (codes for core proteins, but the acronym corresponds to the group antigen), *pol* (codes for the reverse transcriptase) and *env* (codes for envelope protein). However, numerous accessory proteins are found in more complex retroviruses (Freed, E. and Martin, M., 2007).

As part of their genome structure, retroviruses contain long terminal repeats (LTRs) at each end. These sequences serve as promoter/enhancer regions and are involved in integration and help to initiate the copying of the viral genome into the host DNA by the RT (Sliva and Schnierle, 2010).

Retroviruses have remarkable features that make them exceptional gene delivery agents. First, this type of vector is able to stably integrate into the host cell genome leading to long-term expression of the inserted cassette. Second, a single retroviral promoter can conduct the expression of all genes encoded within the limits of its

genome. Third, most viral genes can be removed from the coding region of the genome and be replaced by the constructs of interest. Also, when packaging cells are employed to supply viral proteins *in trans*, the particles released are free from any contaminating helper particles or replication-competent recombinant viruses. Finally, recovered replication-defective recombinant particles do not synthesize viral protein in transduced cells which avoid inducing immune responses against the vector and prevent dissemination (Sliva and Schnierle, 2010).

Commonly used recombinant oncoretroviral vectors are based on murine leukemia viruses (MLV), which are members of gammaretrovirus subclass. These viruses also integrate into the host chromosomes and provide long-term gene expression. However, these oncoretroviral vector systems require cell division for transduction and integration which restrict gene therapy applications to dividing target cells (VandenDriessche et al., 2002). As it is the case with other retroviral vectors, this group has been genetically manipulated to increase safety and transgene delivery capacity resulting in efficient non- replicating vectors.

Reports also describe replicating MLVs as transfer vehicles for shRNAs. ShRNA cassettes are usually short sequences; their insertion in viral genome does not interfere significantly with viral replication, generating genetically stable vectors that are able to induce efficient silencing of target gene expression (Sliva and Schnierle, 2010).

Lentiviruses (LV) constitute another diverse and complex subclass of non-oncogenic retroviruses. The members of this class can be classified based on host species into primate-human immunodeficiency viruses, and non-primate groups. Non-

primate lentiviruses-based vectors such as feline immunodeficiency virus, bovine immunodeficiency virus, caprine arthitis and encephalitis virus, Visna virus, and equine infectious anemia virus has been described (Ramezani and Hawley, 2002; Trono, 2002). However, the most established and best studied lentivirus traditionally used as vector is the HIV-1 (Trono, 2002).

Lentivirus have more complex genomic structures, encoding not only the basic *Gag*, *Pol* and *Env* polyproteins but also additional proteins that function such as regulatory and virulence factors that are not essential for viral replication but are associated with pathogenesis in host cell(Freed, E. and Martin, M., 2007). HIV encodes the following polyproteins: (i) ***Gag*** that is cleaved to form nucleocapsid, capsid, matrix, P6, P2, and P1, (ii) ***Env*** (also known as gp160) that is cleaved into surface glycoproteins (gp120) and the transmembrane (TM or gp41), and (iii) ***Pol*** that is processed into reverse transcriptase, integrase, and protease. Also other accessory proteins are *Rev*, *Tat*, *Vif*, *Vpr*, *Vpu*, and *Nef* (Ramezani and Hawley, 2002; Freed, E. and Martin, M., 2007).

In contrast with replicating oncoretroviral vectors, HIV-1-based vectors have agreater packaging limit (roughly 8kb for a third generation HIV-vector). This transgene carrying capacity is calculated after subtracting an average-sized internal promoter, the *cis*-acting sequences, and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), from the maximum carry capacity of the vector(Ramezani and Hawley, 2002). However, some reports demonstrated that viral titer is reduced significantly when the length of the insert is increased (Ramezani and Hawley, 2002).

Lentiviruses have the ability to transduce dividing and non-dividing cells (Ramezani and Hawley, 2002) because the DNA provirus in association with the integrase (called the pre-integration complex), crosses the intact nuclear membrane of the target cell. This important characteristic makes them unique candidate vectors for nervous system gene therapy and other slow or non-dividing cells (Naldini et al., 1996).

To obtain a recombinant lentiviral vector, the reporter genes or shRNA coding cassettes should be cloned into a vector sequence flanked by LTRs and containing the packaging signal. As with other retroviruses, LTRs are required for integration of the dsDNA copy of the virus into its host chromosome. The psi (Ψ) sequence is necessary for packaging of RNA in virions. Additionally, *gag*, *pol* and *env* are genes necessary in viral replication and should be provided by the helper plasmids (Friedmann, T. and Rossi, J., 2007).

Currently, lentiviral vectors are created in transient packaging systems in which cells are cotransfected with three separate plasmids. Virus particles released from these systems are replication deficient, so are unable to continue to infect their host after delivery of transgene. The three plasmids employed are the following; (i) the transgene construct that contains *cis*-acting genetic sequences and an exogenous promoter necessary to express the gene of interest, (ii) the packaging construct that provides the backbone of the virus system and supplies the structural and regulatory genes *in trans*, and (iii) the envelope plasmid that specifies what type of cell to target and infect (Amado and Chen, 1999).

In general, when lentivirus vectors bud from the host cell they acquire cell membrane-derived lipids that contain the *Env* proteins embedded. During initial stages of the infections, *Env* protein also serves for attachment and fusion between the host and viral membranes. This permits the release of capsid containing the genetic material into the cytoplasm.

Since *Env* protein interacts with a specific cellular receptor, this viral protein determines viral tropism. Thus, when the env gene is altered, the target cell range can be manipulated (Sliva and Schnierle, 2010). Knowing that, recombinant lentivirus has been VSV-G pseudotyped, which means that the retroviral *Env* has been replaced with glycoprotein (G) of VSV. This change broadens lentiviral tropism, supports uptake into otherwise refractory cells, and permits the vector to be concentrated to higher titers.

Although the classification of lentiviral vectors in different generations is not completely adopted by some experts, authors utilize this designation to categorize the constructs depending on first time of engineering and the safety modifications that were adopted in constructs as more aspects of HIV molecular biology and pathogenesis was elucidated.

First generation lentivirus vector systems consist of HIV-1 genome with some modifications. Replication-defective pseudotyped vector particles are recovered by transient co-transfection of the three mentioned expression plasmids into the packaging cell line. Recovery titers are around 10^5 transducing units (TU) /milliliter (Ramezani and Hawley, 2002). In the transfer vector, the original LTRs and *cis*-acting sequences of HIV, including the Ψ packaging signal and the R repeats (RRE) are maintained. The

expression of the transgene of interest is driven by an internal enhancer-promoter such as human cytomegalovirus (CMV) (Ramezani and Hawley, 2002).

The packaging plasmid contains all of the HIV-1 coding regions except the packaging signal, *Vpu* and *Env* that are deleted. Also, the 3'LTR is modified by addition of a polyadenylation (poly-A) site. The 5' LTR is replaced with the promoter that controls expression of other viral proteins (Ramezani and Hawley, 2002; Naldini et al., 1996).

Second generation HIV-1 vector systems utilize an attenuated packaging construct containing fewer genes *(Gag*, *Pol*, *Rev*, and *Tat* genes). This system was engineered by elimination of the rest of accessory genes. It was shown that this change did not affect recovery titers of pseudotyped vector particles in 293T cells but might alter transduction efficiencies in other cell lines (Ramezani and Hawley, 2002).

Current third generation HIV-delivery systems include modifications intended to increase biosafety of second generation systems by reducing significantly the content of original HIV genes. In these constructs, so-called self-inactivating (SIN) HIV-1 vectors, part of the U3 region of the LTR is deleted resulting in almost complete inactivation of 5' LTR of the integrated vector (Miyoshi et al., 1998). This modification decreases the risk of generating replication competent wildtype-like viruses, it also reduces the possibility of activation of proto-oncogenes by insertional mutagenesis but it does not affect vector recovery titers (Ramezani and Hawley, 2002; Miyoshi et al., 1998).

Additional modifications usually introduced in third generation vectors without affecting the recovered titer include: removal of the *Tat* gene from the packaging

construct, introduction of genetic elements such as WPRE that stimulate transgene expression, re-storage of Rev/RRE element and reintroduction of preintegration complex (PIC) nuclear import signals such as central Polypurine Tract (cPPT) and central termination sequences of pol open reading frame (ORF) (Ramezani and Hawley, 2002). In more recent versions of HIV-transfer vectors, the U3 region of the 5' LTR has been replaced with the LTR promoters from other retroviruses to avoid reestablishment of U3 deleted sequences by homologous recombination with an unmodified HIV-1 (Ramezani and Hawley, 2002).

Novel internal promoters drive transcription of the transgene in SIN constructs. Many cellular and viral promoters have been tested in HIV-1 vector backbones to obtain stronger transgene expression and a wider range of susceptible cells. Some of these commonly used promoters are the human elongation factor 1α (EF1α) promoter, the CMV early enhancer/chicken beta actin (CAG) promoter, the human X chromosome phosphoglycerate kinase-1 promoter (PGK), and various exogenous LTRs from different retroviruses (Ramezani and Hawley, 2002).

Although lentiviral vectors are useful delivery vehicles for gene therapy, their common origin with HIV raised considerable safety concerns. One of the main concerns is that during manufacturing of the vector in the packaging cell line, viral recombination could lead to generation of a lentivirus that is able to self-replicate, and potentially transferring its genetic material to germ line cells. For this reason, non-replicating viruses are currently used for transgene expression (Amado and Chen, 1999).

Another great safety concern is the possibility that integration of retroviruses may activate nearby proto-oncogenes in cellular host which may promote malignant cell phenotype. However, when using replication-incompetent retroviral vectors, this risk has been estimated to be small, 10^{-7} per insertion under the assumption that integration is random and at rate of a single hit per cell (Li et al., 2002; Calmels et al., 2005). Since tumor development requires the accumulation genetic lesions, a single copy of a transgene is not expected to produce grave side effects (Li et al., 2002). However, considering the complexity of eukaryotic cell genomes, it is hard to predict the functional consequences of vector insertions (Modlich et al., 2009).

Retroviral-induced dysregulation of neighboring cellular genes is related to the nature of the vector's enhancer–promoter and the retroviral insertion pattern (Modlich et al., 2009). A study from Modlich et al (2009) suggests that the pattern of insertion of lentiviral vectors is less likely to trigger malignant transformation of hematopoietic cells than pattern observed in gammaretroviruses (Modlich et al., 2009). Contrastingly, in this investigation the alteration of the vectors' enhancer–promoter elements within the viral construct had more profound consequences on safety than simple the pattern of insertion of each virus. To exemplify this finding, it was shown that lentiviruses carrying strong enhancer–promoter sequences in the LTRs induced insertional transformation of cells, but SIN-Lentivirus constructs containing the same enhancer–promoter sequences as an internal promoter position did not result in malignancy (Modlich et al., 2009). However, the risk of transformation induced by SIN-LV carrying strong internal enhancer–

promoter sequences must be determined in each experimental scenario (Modlich et al., 2009; Romano et al., 2009).

Reports of potent lentiviral promoters that enhance tumor development in animal models (Modlich et al., 2009) have raised concern regarding the safety of these tools. Further experimentation is required to generate systems that allow efficient delivery for shRNA expression cassettes with minimum biological hazard to ensure that the benefits of the therapy would greatly overcome any risk associated.

In summary, lentiviral vector systems have been used efficiently to introduce shRNA expression cassettes into target cells for several years. Increases in safety and versatility of recombinant lentiviral vectors, particularly their ability to transduce quiescent cells, make them remarkable choices for transgene delivery into a myriad of *in vitro* and *in vivo* systems. Therefore, current interest in improving last generation of lentiviral-based systems and using lentiviral-mediated activation of RNAi pathway for cancer therapeutic and antiviral applications has increased enormously.

1.3 Vesicular stomatitis virus

For several reasons, VSV has historically been considered a model for studying non-segmented negative-strand RNA viruses. Some of the characteristics that make it a group prototype are: the simple structure and genomic organization, the rapid disease course in several vertebrate hosts, the high replication rate in different *in vitro* models, and the elevated error rate with lack of proofreading of its polymerase (Lyles and Rupprecht, 2007). In fact, there are extensive studies on the molecular biology,

epidemiology (Rainwater-Lovett et al., 2007), evolution (Rodriguez et al., 1996; Smith-Tsurkan et al., 2010) and functional analysis of its genome (Rodriguez et al., 2002).

The first description of lesions compatible to VSV disease dates back to 1800's in army horses (Letchworth et al., 1999), although official reports correspond to 1916 and 1939 in the United States and South America, respectively (Lyles and Rupprecht, 2007; Rodriguez et al., 2000). VSV disease is distinguished by the presence of vesicles on the tongue and lips and ulcerative lesions in coronary band, teats, and prepuce (Martinez et al., 2003). Lameness may be present (Letchworth et al., 1999). These lesions disturb the animals feeding behavior, limit gain of weight and also cause acute reduction in milk production (Martinez et al., 2003). In addition, ulcerative lesions are frequently accompanied by secondary bacterial infections that delay healing and required antibiotic therapy. In humans, acute febrile disease has been described sporadically (Letchworth et al., 1999).

The disease is transmitted by contact inoculation of the virus beneath the skin and mucous membranes, or it can be inoculated by insect vectors. There is no evidence suggestive of viremia, but the virus can be found in vesicular fluid at high titers. In natural infections the incubation period lasts from 2 to 9 days but lesions develop between 2-5 days after infection (Lyles and Rupprecht, 2007).

Importantly, the clinical manifestations of VSV in livestock are remarkably similar to the disease caused by FMDV (Martinez et al., 2003;Rodriguez, 2002).This clinical homology is relevant since FMDV is highly contagious—for animals or even humans. Also, foot and mouth disease (FMD) has been recognized as the most important

constraint to international trade in animal products, the presence of FMD translates into negative economic consequences to the livestock industry (Grubman and Baxt, 2004). FMD is enzootic in all regions, except a few countries. For this reasons countries that are free of the disease, including United States (USA), have established strict measures to retain this status (Grubman and Baxt, 2004). Since VSV and FMD are indistinguishable solely by clinical manifestation, the control or the eradication of VSV disease from a region will translate into better diagnostic capacity for FMD disease.

VSV natural cycles in livestock, humans, wild animals and insect vectors have been documented (Letchworth et al., 1999). VSV disease is endemic areas extending from northern South America to southern Mexico, where seasonal outbreaks of clinical disease occur. Two main serotypes of VSV have been described using neutralizing antibodies: Indiana and New Jersey (Rodriguez, 2002). The current investigation focus on VSV serotype Indiana (VSIV).

In the USA, VSV cases have been continuously reported. Several communications have associated VSV disease with outbreaks during 1995, 1997, 1998, 2004, and 2005 in the western region of the United States (Howerth et al., 2006). Also, the Animal Health Monitoring and Surveillance (APHIS) agency confirmed cases in Texas and New Mexico during 2009, and Arizona during 2010.

In order to control VSV cases, non-specific measures have been employed such as water and feed cleaning, milking equipment disinfection and even spraying cattle with insecticide early during a VSV outbreak (Letchworth et al., 1999). Besides, supportive

care (soft feeds and electrolyte therapy), antiseptic mouthwashes, and antibiotics are often been used to treat the infections.

During great outbreaks, particularly in South America, livestock has been immunized with vaccines based on live, unmodified virus (Flanagan et al., 2001). However, the presence of neutralizing antibodies is not sufficient to prevent clinical disease in animals under natural conditions and animals can be re-infected following recovery (Letchworth et al., 1999). In recent years, scientists have reported limited success in the development of new subunit- or DNA-mediated vaccines for VSV. However, several rational approaches to attenuate VSV growth and virulence have been described (Flanagan et al., 2001; Clarke et al., 2007).

VSV is a member of the order Mononegavirales and family Rhabdoviridae. Viruses in this family possess enveloped virions with helical nucleocapsids. In this group, there are pathogens that affect plants, animals and insects. Animal rhabdoviruses include four members: Lyssavirus, Vesiculovirus, Ephemerovirus and Novirhabdoviruses which are approximately 180 nm long and 80 nm wide, but the length can vary depending on the size of the genome (Lyles and Rupprecht, 2007; Fu, 2005).

VSV belongs to the vesiculovirus genus, its genome encompasses 11kb of non-segmented, negative polarity single-stranded RNA (Fig. 2C) which is encapsidated by approximately 1200 copies of the nucleocapsid protein. The virion has a characteristic bullet shape as shown by electron microscopy (Fig. 2B). The genome encodes five protein coding genes: Nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein

(G) and polymerase (L) (Fig. 2C) (Lyles and Rupprecht, 2007). Gene junctions separate each gene and the junctions have three components: a sequence that specify the end of the upstream gene, an intergenic sequence, and the start sequence for the next gene.

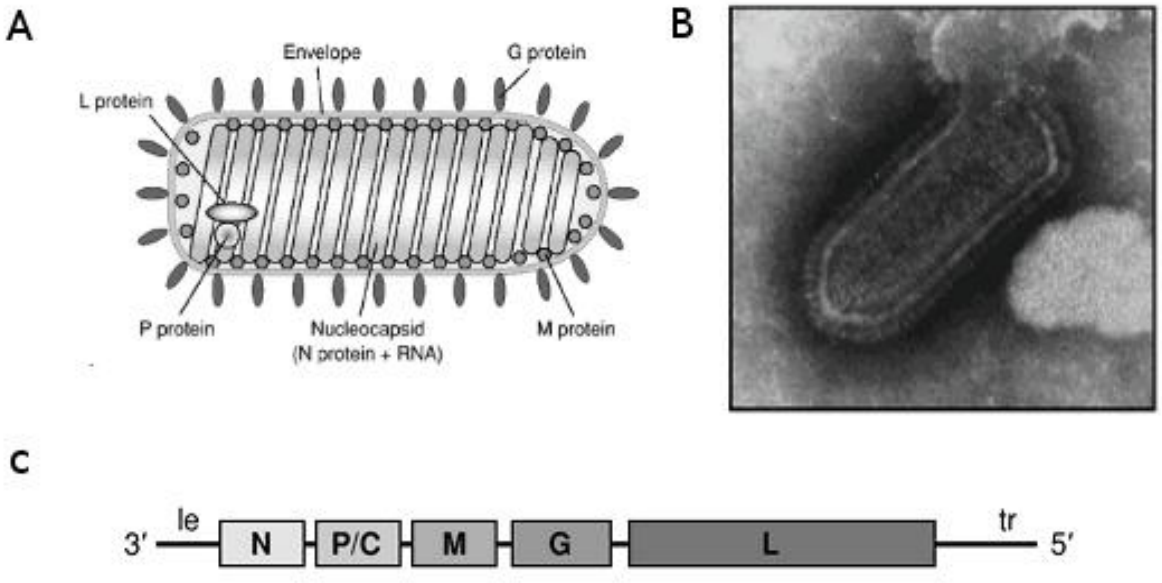


Fig. 2. Diagram of vesicular stomatitis viral particle and genome. (A) Structural proteins and characteristic "bullet" shape of the virion (B) Negative stain of electron micrograph (C) Genomic organization (Lyles et al., 2007).

Additionally, the genome has leader and trailer sequences that consist of 50 partially complementary nucleotides at the 5' and 3' ends, respectively which play crucial roles for transcription, replication and encapsidation (Lyles and Rupprecht, 2007).

The negative strand genome lacks mRNA function and requires transcription by the viral RNA dependent RNA polymerase. After transcription, capped and

polyadenylated mRNAs are translated by cellular machinery to produce the structural proteins. The following proteins are transcribed by VSV genome (Fig. 2C).

1.3.1 Nucleocapsid protein (N)

N protein is the most conserved and abundant viral protein expressed in VSV infected cells (Rodriguez et al., 2002). It wraps the negative-strand genome RNA along its full-length to protect it from nuclease-mediated degradation. Structurally, the N protein seems to be composed by two lobes with the RNA located between these lobes (Lyles and Rupprecht, 2007). This protein is associated with two other viral proteins; P and L. In particular, the last 60 aminoacids (aa) of the C-terminal end of N, are critical for interactions with the P and also for encapsidation (Rodriguez et al., 2002).

During VSV infection, N protein distributes between two pools; (i) a cytoplasmic-diffuse and dynamic pool which represents newly synthesized material not associated with RNA yet and (ii) a punctate-like staining mostly corresponding to N protein assembled into nucleocapsids (Arnheiter et al., 1985).

Importantly, studies using microinjection of antibodies targeting N protein have shown that the availability of free N-protein is one of the factors controlling RNA synthesis i.e. the synthesis of all forms of genome-length RNA, whether encapsidated or not, is restricted during N protein deficiency (Arnheiter et al., 1985).

1.3.2 Phosphoprotein (P)

The phosphoprotein, formerly called non-strucural (NS) protein, is the smallest subunit of the RNA-dependent RNA polymerase (RdRP). It mediates the binding of L protein to the N protein-RNA complex and it functions as an essential transcription

factor for the viral polymerase (Bitko and Barik, 2001). The P protein permits the RdRP holoenzyme to exit the promoter and subsequently to sustain elongation (Bitko and Barik, 2001). There are structural sites in the protein that should be phosphorylated by a cellular kinase to generate an active oligomer that mediate viral transcription (Lyles and Rupprecht, 2007).

1.3.3 Matrix protein (M)

The M protein interacts with the viral envelope and with N (Lyles and Rupprecht, 2007). It mediates binding of N to the cytoplasmic surface of the host plasma membrane during virus assembly. In fact, functional M protein is essential to impart the characteristic bullet-like shape of the virion by joining N and M proteins into a complex (Lyles et al., 1996). After uncoating, the majority of M stays associated with vesicular structures in close proximity to recycling endosomes. Recently Mire et al. (2010) showed that a small portion of the protein is released and it moves toward the nuclear envelope in a process that is independent of microtubule or actin cytoskeleton components (Mire et al., 2010). Also, Carey et al. (2008) reported that mutations in the M protein eliminate the capacity of the virus to shut off host translation, consequently infected cells combat better viral infection by the expression of interferon-I (IFN-I)-based response and other antiviral cytokines produced in response to the pathogen (Carey et al., 2008).

1.3.4 Glycoprotein (G)

The glycoprotein forms trimers that are anchored in the viral envelope and arrange into 300-400 spikes. G protein mediates the attachment of the virus to cell

membrane of host cell (Fu, 2005) as well as fusion between the endosomal and viral membranes to trigger the release of the ribonuclear particles (RNP) to the cytoplasm (Mire et al., 2010).

1.3.5 Polymerase or L protein (L)

The large (L) protein is the major subunit of the RdRP. It is a 250-kDa multifunctional protein that has enzymatic activities for genome replication and for each step of mRNA processing, including cap formation (addition and methylation) and polyadenylation (Rahmeh et al., 2010).

The enzymatic activities of L protein have been well studied. The knowledge generated after studying this protein in VSV group prototype is mostly shared among non-segmented negative-strand RNA viruses (Fu, 2005). In the case of VSV-L the characterization is available at the amino acid level. When considering the primary sequence of L, six conserved regions (CR) or blocks can be recognized (Fu, 2005). Separate locations of polymerase functions suggest that L may be organized as a series of independent structural domains (Rahmeh et al., 2010). For instance, CR-II may play a role in RNA recognition (Fu, 2005) and CR-III (Fu, 2005) participates in RdRP and polyadenylation activities. CR-III has four (A-D) highly conserved motifs and a mutation of core sequence of C-motif represses transcription and replication of rabies virus (Fu, 2005; Schnell and Conzelmann, 1995). Also, CR-IV may be involved in nucleotide binding and CR-V may play a catalytic role via metal binding. The RNA-Guanine diphosphate (GDP)-transferase encoded within CR-V plays a role during

mRNA capping and the methyl-transferase located in CR-VI modifies the mRNA cap (Rahmeh et al., 2010).

1.3.6 VSV replication cycle

The steps in the replication cycle of VSV are typical for most viruses. The infectious cycle last around 16-20 h, but maximum rate of transcription occurs 8-10 hours post-infection (hpi) (Lyles and Rupprecht, 2007; Carey et al., 2008). The first step in the cycle is probably driven by low affinity interactions between cellular molecules and the G protein. The low specificity of this mechanism makes it difficult to identify a unique receptor, but phosphatidyl serine has been traditionally proposed as cellular receptor. (Lyles and Rupprecht, 2007; Carey et al., 2008). Next, penetration is achieved by activation of clathrin-dependent endocytic pathway. Uncoating occurs approximately 20 minutes post-entry (Mire et al., 2010). In this process, G protein, potentiated by low pH mediates fusion of the viral envelope with endosomal membranes causing release of internal virion components into the cytoplasm. Following, M protein dissociates from N and allows viral transcription (Lyles and Rupprecht, 2007). The attachment, penetration, and primary transcription occur within the first 2 hpi. These steps are sensitive to the number of input viral particles, or multiplicity of infection (MOI) (Carey et al., 2008).

Following host cell infection, the virus-encoded RdRP starts sequential primary transcription of each mRNA at the single 3' proximal site (Whelan et al., 2004). Viral RNA polymerase is fully able to generate all of the viral mRNA without synthesis of additional host or viral proteins. The mechanism of transcription is called stop-start. The ends of viral genes contains *cis*-acting elements (the end sequence for the upstream gene

3’AUACUUUUUUU5’, the intergenic 2 nt, and the start sequence for the next gene 3’ UUGUC5’) that regulate processing steps of mRNA such as polyadenylation, termination of the previous mRNA, and initiation, capping, and methylation of the downstream mRNA (Lyles and Rupprecht, 2007).

After polyadelylation of mRNA, the transcriptase complex can either resume transcription of the downstream gene or dissociate from the template leading to gradual attenuation of the expression of the downstream gene at each junction. This strategy controls the viral gene expression by the conserved order of the genes relative to the transcriptional promoter and also by the *cis*-acting sequences (Whelan et al., 2004). In this singular mechanism, a single promoter controls the expression of all genes in an orderly fashion, this means; genes located in close proximity to 3′ promoter site are transcribed at higher levels whereas those at distal positions are transcribed progressively less abundantly. Thus, the N gene which is required in greater amounts during viral replication is located at or near the 3′ terminus, whereas L gene that encodes the catalytic products of the RNA polymerase is more distant from the promoter (Barik, 2004). The importance of this gene regulation is evidence by the observation that the VSV RNA replication *in vitro* and in cultured cells is proportional to the amount of N protein synthesized (Wertz et al., 1998).

The viral proteins are rapidly produced using cellular machinery meanwhile hindrance of host translation occurs early in the infectious cycle. Viral mRNAs do not contain *cis*-acting sequences to promote their translation. Instead, restriction of host protein synthesis appears to result from the action of M protein (Lyles and Rupprecht,

2007;Carey et al., 2008). The efficient and rapid accumulation of viral protein occurs from 2 to 6 hpi or until late in the viral cycle, when the translation is inhibited as part of the antiviral mechanism of the cell (Lyles and Rupprecht, 2007).

The accumulation of new viral protein and encapsidation of nascent RNA are key signals required by viral RNA polymerase to generate full-length complementary RNA, called antigenomes (Lyles and Rupprecht, 2007) that will be used as templates for generating progeny genomes. The 5' end of progeny genomes contain sequences that target encapsidation. Additionally, another step of transcription occurs when progeny genomes accumulate and are used as templates for secondary transcription (Lyles and Rupprecht, 2007; Barik, 2004).

Assembly of progeny virions starts at the same time as secondary transcription initiates, and it peaks at 8-10hpi (Lyles and Rupprecht, 2007). All components of virions are synthesized in different compartments of the cell and they come together in final steps of assembly. Lastly, the viral envelope is derived from host cell membrane during virus budding (Lyles and Rupprecht, 2007). Virions are released in a process that is mediated by interaction of M protein with the plasma membrane proteins (Lyles and Rupprecht, 2007).

2. IN VITRO IMPAIRMENT OF VSV REPLICATION USING RNA i

2.1. Introduction

A broad spectrum of viral diseases threatens a wide range of organisms; in certain cases these diseases may be able to produce pandemics or lead to serious public health problems (Nichol et al., 2000). New emerging biological threats, on one hand, rapidly raise alarm which causes scientist to focus their efforts to understand the pathogenesis of the infectious agents and to establish control measures. On the other hand, non-emerging viral diseases, such as VSV, have accompanied livestock industry for decades and cases still recur occasionally. Over the years, these pathogens have repeatedly overcome eradication efforts and cause continuous economic impact.

Interest in the development of new antiviral strategies is fueled by several considerations: (i) the severity and importance of the viral infection of concern, (ii) the availability of a specific and efficient treatment to combat the virus infection, and (iii) the rationality of the antiviral approach and its applicability to control a wider range of viral infections (De Clercq, 2004).

In this context, the presence of VSV in herds has economic relevance including: costs of veterinary care and symptomatic treatments (Letchworth et al., 1999), delay in gain of weight, rejection of meat and dairy products, closures of livestock markets and animal quarantines, and cancellation of shows and auctions (Rodriguez, 2002;Howerth et al., 2006). Also, efforts to control VSV disease in livestock are limited to early detection of the disease and unspecific recommended sanitary measures. The effectiveness of the available vaccines is limited by the fact that antibodies are usually

not protective against clinical manifestations of disease (Letchworth et al., 1999). Additionally, VSV is an important and extensively studied virus that has been considered the prototype for non-segmented negative-strand RNA viruses,

The application of RNAi-based therapeutics is beginning to show real promise in enhancing our ability to defend agriculture animal resources against viral disease. Recent successes in applying RNAi-based antiviral therapies in poultry highlight the rationality of using this approach for other livestock species. For instance, bird cells have now been genetically engineered to resist viral diseases such as Marek's disease, infectious bursal disease, avian leukosis, and avian influenza (Chen et al., 2009;Sui et al., 2009;Chen et al., 2007;Hu et al., 2002). For several reasons, it is clear that VSV is a significant pathogen, which justifies the necessity to develop a new antiviral approach for combating VSV and other similar viral diseases.

Studies from Otsuka et al. (2007) in Dicer1-deficient mice demonstrated the activation of the host RNAi pathway for targeting specific VSV genes. They described that miR24 and miR93 from the host cell origin targeted the L and P proteins of VSV for silencing. In fact, this research group observed an increased VSV replication in Dicer1d/d cells presumably ascribable to the lack of activation of endogenous microRNAs (Otsuka et al., 2007). Additionally, a study from Barik (2004) reported the use of siRNA to successfully but temporary target VSV genes (Barik, 2004).

Based on the literature described, the hypothesis was that shRNA targeting essential VSIV genes and stably expressed by a cell system can be utilized to diminish

the expression of the viral genes resulting in the impairment of VSIV replication and subsequent generation of resistance in an otherwise VSIV-permissive cell line.

To obtain durable and effective antiviral therapies, it is essential to identify viral proteins that can be disabled. Ideally, these targets should be essential viral factors and share conserved sequences across many different isolates or even among different strains. Remarkable features of the N, L, and P proteins of VSIV make them the most appropriate targets (Wilson et al., 2009). Given the importance of these structural proteins in the viral life cycle and/or conservation between several strains, they represent perfect candidate sequences to target shRNAs for antiviral purposes. This investigation could form a valuable foundation upon which to build experiments leading to the ultimate long-term goal of combating this and other important viral diseases.

2.2 Material and methods

2.2.1 Cell lines

Baby hamster kidney (BHK-21) and Vero cell lines acquired from the American Tissue Culture Center (ATCC) were employed. Both 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/streptomycin and amphotericin B at 0.25 µg/ml.

2.2.2 Viruses and challenging assays

A laboratory-adapted VSIV strain was kindly provided by Dr. Judith Ball (VTPP, Texas A&M). Additionally, wild-type VSIV variants were isolated from a tissue collection owed by the Laboratory of Virology, School of Veterinary Medicine, National University of Costa Rica, following standard protocols (Wilson et al., 2009). The samples corresponded to frozen bovine mucosa tissues with lesions compatible to VSV infection. For processing, small tissue samples were washed with PBS and macerated with a sterile pestle into medium consisting of DMEM with 10% FBS and antibiotics. The samples were then centrifuged and the supernatant was homogenized, collected, and inoculated on a sub-confluent culture of Vero cells. Cells were observed microscopically for CPE once per day for up to three days. VS Indiana serotype was confirmed by seroneutralization assay following a home-standardized protocol (available upon request).

For challenge assays, either transgenic cells stably expressing shRNAs targeting viral genes (denoted as experimental groups) or control groups were trypsinized, counted, and seeded in replicates into 24-wells plate overnight before infection. Then, viral infections were carried out at an MOI=0.01 or 0.10 in FBS-free medium using the laboratory-adapted strain of VSIV. In all experiments control groups included infected cells transduced with an irrelevant shRNA (denoted as sh-NTC), infected wild-type cells that do not express any shRNA, and non-infected wild-type cells.

2.2.3 Design of shRNA

Sequences of N, L, or P genes of VSIV were chosen after alignment of several published sequences. Additionally, nucleotide regions considered were described in 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). These regions were used to design shRNAs using an online computer algorithm (RNAi codex) (Olson et al., 2006) and ordered as DNA oligonucleotides from a commercial manufacturer (Invitrogen, USA). Position of shRNAs in the VSIV genome are given according to GeneBank accession no. J02428. In this text, the viral gene targeted by each set of shRNA is denoted as N-shRNA, P-shRNA, L-shRNA to indicate N, P, or L genes, respectively.

2.2.4 Lentiviral constructs expressing shRNAs

A lentiviral shRNA-mir construct (containing the backbone of the primary mir-30 miRNA) (Silva et al., 2005) was used to clone each shRNA into the PEG unidirectional lentiviral construct (Golding and Mann, 2011) (Fig. 3A and Table 6, appendix). Each shRNA was cloned using the PCR-based strategy described previously (Silva et al., 2005). Broadly, these oligonucleotides were converted to double-stranded DNA and restriction sites were incorporated by PCR. The insert and the vector were ligated and transformed into competent Stabl3 bacterial cells. Positive clones were confirmed by restriction enzyme analysis and DNA sequencing.

VSIV-G-pseudotyped SIN HIV-based recombinant lentiviral vectors were harvested after co-transfection (Lipofectamine, Invitrogen) of 60-80% confluent 293T

cells with three plasmids containing: the construct expressing the shRNA cassettes, the VSIV-G-expressing construct, and the packaging construct (Miyoshi et al., 1998). Transgene expression was confirmed 48 h later by green fluorescent protein (GFP) expression. Next, cells seeded in six-well plates at approximately 50% confluency were transduced with the lentivirus carrying a distinct shRNA for each well.

2.2.5 Selection of transgenic cell lines

Cells carrying the transgene were drug selected using puromycin. The optimal dose was determined prior to selection and was considered to be the concentration displaying the best selection without detectable toxic effects on GFP-expressing cells. GFP expression in at least 90% of cell population was confirmed by flow cytometry analysis (BD FACS Aria II, BD FAC DIVA Software). Alternatively, GFP-labeled cells were sorted until at least a 90% GFP expression in each population was reached (Table 5, appendix).

2.2.6 Microtitration and plaque assays

Viral microtitration of VSIV was performed using standard methods for TCID50 determination. Briefly, 10-fold serial dilutions of the virus material were prepared, and 100 microliters (µl) of this dilution was added to each well of confluent cells previously seeded in a 96-well plate using four replicates for each dilution and non-infected controls. Cells were incubated at 37° Celsius (C) in 5% CO2 for 2 to 3 days for development of CPE. The titer was calculated using the method of Reed and Muench (Condit, 2007).

For plaque assays, confluent cell monolayers in six-well plates were infected using 0.1 mililiters (ml) aliquots from serial 10-fold dilutions of virus material. An additional 0.4 ml of medium was added to each well to prevent cell desiccation. The virus was adsorbed for 60 minutes at 37° C, followed by inoculum removal. The monolayers were overlaid with 4% (wt/vol) agar diluted in growth medium. Cells were incubated at 37 °C in 5% CO2 for 1 to 3 days for plaque development. After that, cells were fixed with 10% formaldehyde and stained using crystal violet. Finally, plaques were counted, and morphology and size was observed (Condit, 2007).

2.2.7 Western blot

Western blot assays for detection of the VSIV-G protein were conducted using the primary antibody rabbit anti-VSIV-G (GeneTex, USA) and the secondary antibody goat-anti-rabbit-horseradish peroxidase (Abcam, USA). Additionally, detection of ß-actin (Abcam, USA) was performed as loading control.

The amount of protein loaded was quantified using the Bradford method with Coomassie Plus (Bradford assay kit, Thermo scientific,USA) in the Nanovue (GE, USA). Five micrograms of the cytoplasmic components of cell lysates, previously fractionated and treated with protease inhibitors, were used. Detergent treated, heat-inactivated (100°C for 5 minutes) cell lysates were separated by electrophoresis on tris (hydroxymethyl) aminomethane (Tris) sodium dodecyl sulfate (SDS) 10% gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) sheets. Nonspecific interactions were minimized by blocking with a 5% solution of fat free powdered milk in Tris Buffered Saline (TBS) and incubated at 4°C overnight (Schmidt et al., 1987). Also

three washing steps with TBS-tween 0.05% were performed between steps. To visualization, a commercial chemiluminesence assay was used following the instructions of the manufacturer (Thermo Scientific SuperSignal West Pico, USA). Densitometry analysis was performed using using AlphaEase FC software (Alpha Innotech).

2.2.8 Quantification of viral transcripts

2.2.8.1 RNA isolation and reverse transcription (RT)

Frozen cell lysates were thawed, homogenized (QIAshredder, Qiagen, USA) and processed for RNA isolation using a commercially available extraction kit as suggested by the manufacturer's instructions (RNeasy kit, Qiagen, USA) and treated with Dnase I (RNase-Free DNase Set Qiagen, USA) to reduce genomic DNA contamination.

RT was carried out using random hexamers included in the qScript kit (Quanta Biosciences, USA) according to the manufacturer's instructions. Total RNA was quantified using Nanovue (GE, USA) and 200 ng of RNA were used in each reaction to produce cDNA.

2.2.8.2 Real-time qPCR reactions

Real time qPCR was carried out using the PerfeCTa® SYBR® Green FastMix, ROX (Quanta Biosciences, USA) following the manufacturer's instructions, and run in a StepOne (Applied biosystems, USA) thermocycler. Relative quantification of viral N, P and L genes and cellular normalizing genes was evaluated using the comparative ΔΔCT method as previously described (Livak and Schmittgen, 2001) and adjusting the method according to each primer set efficiency. Cellular genes that were tested for normalization were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β2-microglobulin, and β-

actin for BHK-21 cells. For Vero cell lines GAPDH gene was used for normalization. The primers for normalizing genes were designed to expand exon-exon boundaries of the target gene and based on the published sequences. Optimal primer concentration was assessed and standard curves for each set of primers was calculated following the MIQE requirements (Bustin et al., 2009) (Tables 8 and 9, appendix).

2.2.9 DNA sequencing analysis of VSIV variants and shRNAs

Partial sequences of the viral genes were obtained from the VSIV field isolates and from laboratory adapted strain. Primers for PCR and sequencing reactions were designed based on the published sequences of VSIV (Table 7, appendix). Multiple sequence alignments were performed using the online version of ClustalW (Thompson et al., 1994) or Bioedit (Hall, 1999). Homology of recovered sequences with available sequences was determined using BLAST (Altschul et al., 1997).

To confirm the presence of the shRNAs in the genome of transgenic Vero cell lines, total genomic DNA from each cell population 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, sequenced and analyzed (Table 7, appendix).

2.2.10 Statistics and data analysis

JMP software version 8.0.2 was used for statistical analysis. Statistical significance was determined using Wilcoxon-rank sum for qPCR experiments. Analysis was performed for each experimental group compared to NTC control group. Fisher's t-test was used for comparing viral titers among groups. In graphics, values are expressed

as experimental mean ± stand error (SEM), and statistical significance is indicated by an asterisk ($p < 0.05$).

2.3. Results

2.3.1 Establishment of cell systems expressing shRNAs

ShRNAs induce sequence specific post-transcriptional silencing of genes after being processed by cellular machinery. For this investigation, nine different shRNAs were custom-designed and cloned into a lentiviral vector (Table 1). The shRNAs targeted either different regions of the viral genome including the N gene, the P gene, the L subunit of RNA polymerase, or an irrelevant target control (also described as null shRNA or non-targeting control sh-NTC).

Table 1

Tested shRNA targeting genes of VSIV.

Identification	Stem region of the shRNAs tested	Viral Gene targeted
sh-N67	TCTGTTACAGTCAAGAGAATC	N
sh-N1312	AAGTCAGAATTTGACAAATGA	N
sh-P1431	TCCTATTCTCGTCTAGATCAG	P
sh-P1772	TGCCAGAGGGTTTAAGTGGAG	P
sh-P1990	TCTCTCAAAGACATCCATGAC	P
sh-L6859	TGGCACAA**GGTGATAATCAAG**[1]	L
sh-L6847	GCTGTCAAAGTCTTGGCACAA	L
sh-L7256	TGCTAGACTCTTGTTGATGAT	L
sh-NTC	ACGGTACAAGGTATACTGGAA	Null

[1] Bold sequence encodes the conserved amino acid residues GDNQ of L protein (Schnell and Conzelmann, 1995).

ShRNA cassettes were expressed into cell systems. Vero cells were transduced with a PEG unidirectional lentiviral system (Fig. 3A,C) carrying the sequence of each shRNA.

BHK-21 cells (Fig. 3B) were transduced with lentivirus carrying each of the N-shRNAs, the sh-L6859, or the sh-NTC. After transduction, homogenous populations of GFP-shRNA-expressing cells were drug-selected and sorted by flow cytometry. GFP expression accounted for more than 90% of transduced cells, as determined by flow cytometry analysis in both cell groups (Table 5, appendix A).

2.3.2 ShRNAs trigger an antiviral response on transgenic cells

Once transgenic cell lines stably expressing each shRNA were generated, the cells were challenged with standardized doses of VSIV to determine the transgene effect on VSIV infection in experimental groups as compared to control groups.

A

Fig. 3. Transgenic cell lines expressing GFP and shRNAs after transduction with the SIN lentivirus vector. (A) Diagram of the 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 another promoter; WRE represents the Woodchuck Regulatory Element (Golding and Mann, 2011). (B)(C). Representative confocal images of BHK-21(B) or Vero cells (C) UV light (left) or at bright field (right).

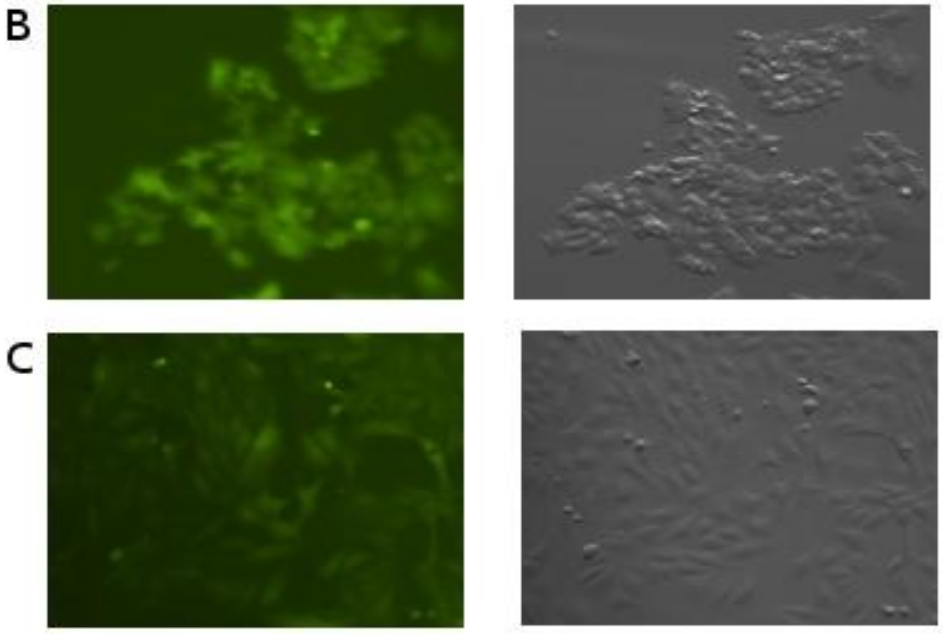


Fig. 3. Continued.

VSIV infection causes a marked CPE resulting in cellular detachment, rounding, and finally leading to cellular death. This effect can be easily differentiated using light microscopy (Fig. 4B) and can be compared to mock infected cells (Fig. 4A). In cells infected with VSIV, the CPE first appeared approximately 10-12 hpi and it was particularly severe between 12 to 24 hpi. At 12 hpi, many cells of the control groups (Fig. 4B-C) were rounded and started to detach from the flask. However, cell lines carrying any of the two shRNAs targeting the N viral gene (Fig. 4D-E) had reduced CPE relatively to controls. Transgenic cells carrying sh-N67, which targets the 5' of N mRNA, showed no CPE in two of the three experiments performed (Fig. 4D).

However, at lower microscopic magnification fewer spots of CPE per confluent monolayer were observed in cells expressing shRNA targeting viral N or P genes but not

in cells expressing L-shRNAs. At 24 hpi cells were mostly dead, and therefore, no difference was easily distinguished between experimental groups expressing any shRNA targeting any viral gene and the control groups (data not shown).

Although not quantitative, the data from these experiments suggest that the expression of shRNAs targeting VS viral genes has some degree of protective effect against infection at 12 hpi. Particularly, cells expressing shRNAs targeting the N gene showed significant reduction in CPE.

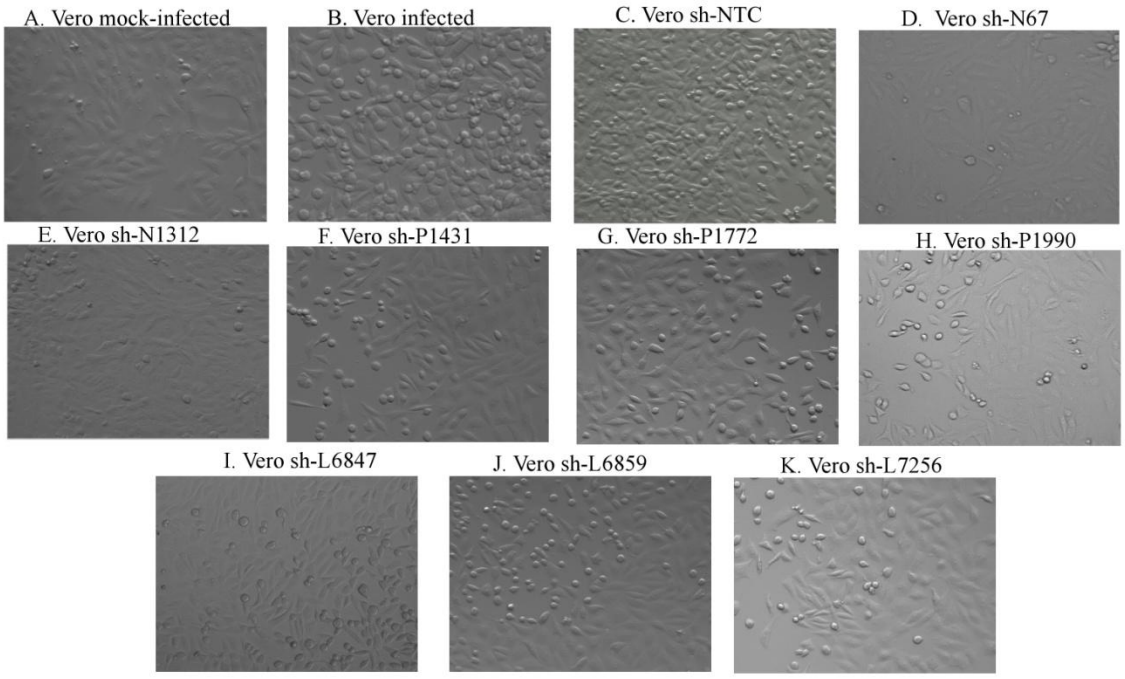


Fig. 4. ShRNAs targeting viral gene decrease the CPE in Vero cells. Transgenic Vero cells were infected at MOI=0.01 and CPE was evaluated at 12 hpi using light microscopy. The experimental and control groups are denoted as (A)Mock infected cells, (B) CPE observed in wild-type Vero cells, (C) cell line expressing an irrelevant shRNA, (D-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-K) cell lines expressing shRNAs targeting three regions of L gene.

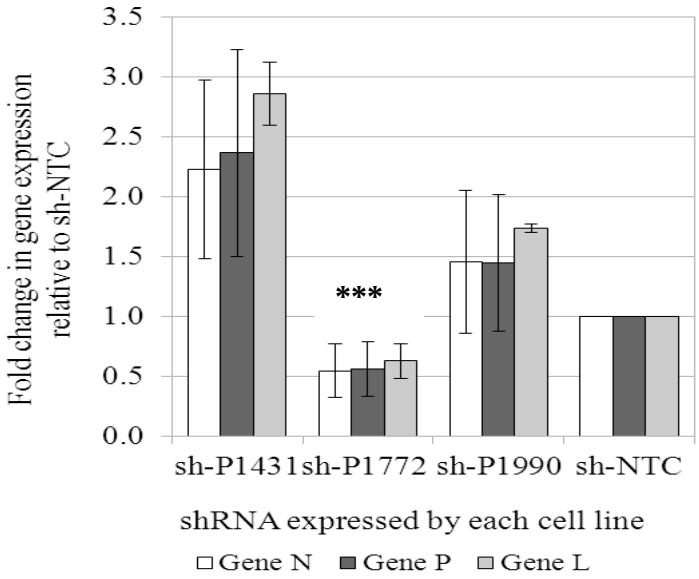
2.3.3 P-shRNA, but not L-shRNAs, decrease viral transcripts.

VSIV L and P subunits of viral RdRP are essential for viral transcription and replication (Das and Pattnaik, 2004). Thus, we tested the potency of shRNAs targeting these genes. For this purpose, experimental and control groups were infected at MOI=0.01 and RNA was collected at 12 hpi for relative quantification of viral transcripts using the RT-qPCR. RT-qPCR was validated according to MIQE guidelines (Bustin et al., 2009) (Tables 8 and 9, appendix).

From the three P-shRNAs tested, shP-1772 significantly reduced N, P and L transcripts ($p < 0.01$ for all viral transcripts) as compared to the NTC control or wild-type control group (not shown). Other P-shRNAs analyzed, sh-P1431 or sh-P1990, did not significantly reduce any of the viral transcripts tested when compared to sh-NTC.

As compared to sh-NTC, viral transcripts were not reduced by any of the L-shRNAs tested (Fig. 5B) at 12 hpi. Indeed, when viral transcriptional levels of sh-L7256, sh-L6847, or sh-L6859 were analyzed, it was evident that viral transcripts were increased in the experimental groups as compared to NTC control. However, viral transcriptional levels again varied significantly between the two control groups (sh-NTC and sh-None) (Fig. 5B).

A



B

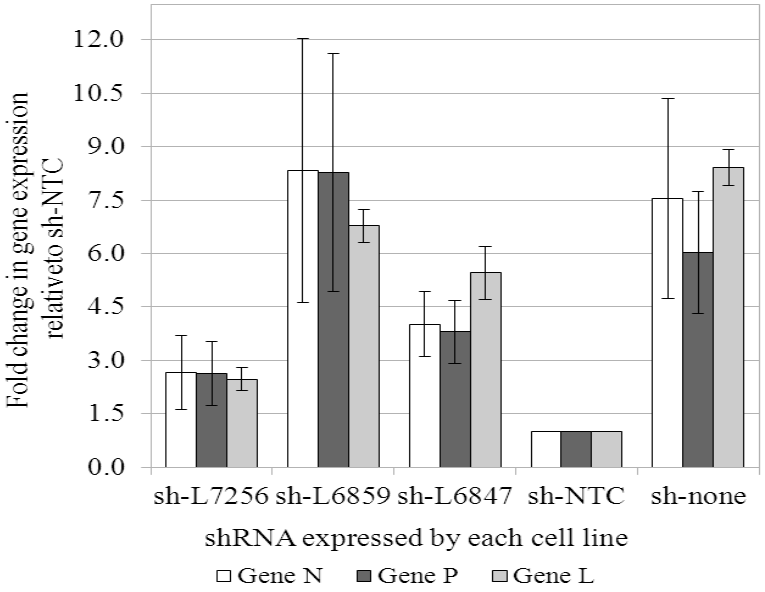


Fig. 5. Fold change in virus transcripts when shRNAs targeted P and L genes. Vero derived cell lines were infected at MOI= 0.01. Total RNA was collected at 12 hpi, processed for qPCR, normalized to respective endogenous control, and viral transcripts levels were compared among groups using the ΔΔ Ct method. (A) N, P, and L transcripts as percentages of the NTC in cell lines expressing P-shRNAs. (B) N, P, and L transcripts as a percentage of the NTC in cell lines expressing L-shRNAs. * Values are different ($p < 0.05$, Wilcoxon rank-sum test) as compared to sh-NTC control.

2.3.4 N-shRNAs significantly decrease N viral transcripts.

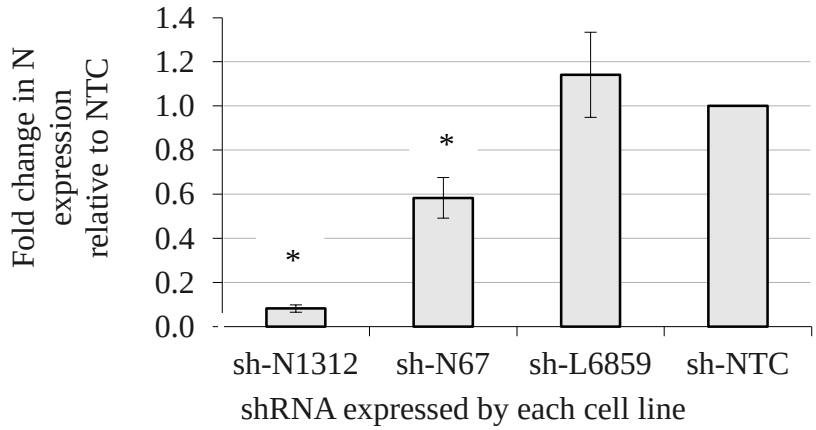
Guided by the reduction of the CPE observed in Fig. 4 (D, E) and the effects of L- or P-shRNAs on viral transcripts, quantification of the potency and durability of shRNAs targeting an additional viral target was done. N is a conserved viral gene which encodes the most abundant viral transcript in the VSIV infection. Structurally, N is part of the RNP and plays an important role in mediating interactions between viral RNA and viral polymerase (Rodriguez et al., 2002).

At 12hpi, (Fig. 6A) N, P, and L transcripts were significantly reduced by sh-N67 ($p<0.01$ for viral all transcripts) and sh-N1312 ($p<0.01$ for all viral transcripts) in Vero-derived cell lines as compared to either the sh-NTC or sh-none controls.

The reduction of the N viral transcripts induced by sh-N67 and sh-N1312 was also observed in transgenic BHK-21 cells (Fig. 6B) as compared to sh-NTC. Significantly, the reduction in N transcripts was apparent at 12, 24, and 36 time points after infection (Fig. 6C) even when experimental groups were challenged at 10 times higher MOI (Fig. 6B).

Although both N-shRNAs induced a significant reduction of N transcripts, the reduction observed by BHK-21-derived cells expressing sh-N1312 was more significant ($p<0.01$) than the response observed by sh-N67 as compared to transgenic cells derived from Vero cell line (Fig. 6A).

A



B

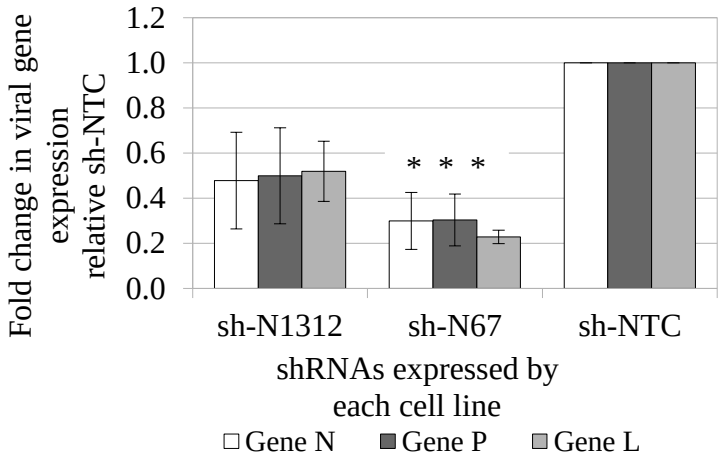


Fig. 6. Transgenic cell lines from different parental origin expressing N-shRNAs significantly reduced viral transcripts. Total RNA was collected at specified time post-infection, processed for qPCR, normalized to respective endogenous control(s) and viral transcript levels were compared among groups using the ΔΔ Ct method. (A) Fold change in N, P, and L transcripts in cell lines expressing N-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. (B) Fold change in N transcripts in cell lines expressing N-shRNAs and sh-L6859, as compared to cells expressing sh-NTC. BHK-derived cell lines were infected at MOI 0.1. Total RNA was collected at 24hpi. (C) Transcriptional levels of N gene in BHK-21-derived transgenic cell lines or wild type cells (sh-none) over time. BHH-21-derived cell lines were infected at MOI 0.1. Total RNA was collected 12, 24, and 36 hpi. * (Wilcoxon rank-sum test) compared to sh-NTC control.

C

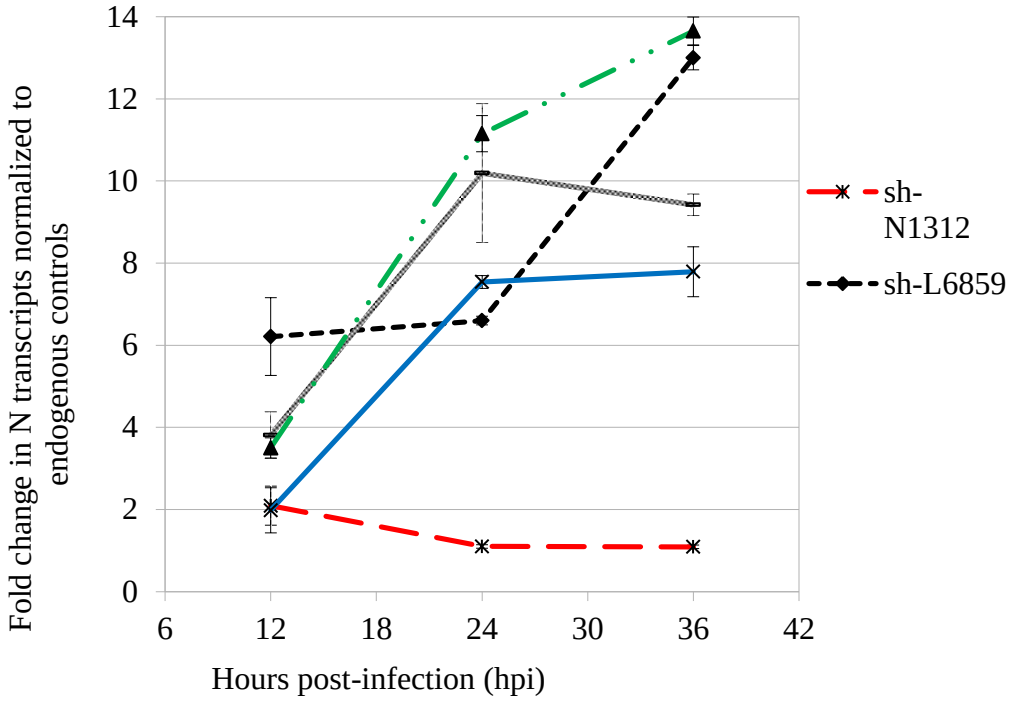
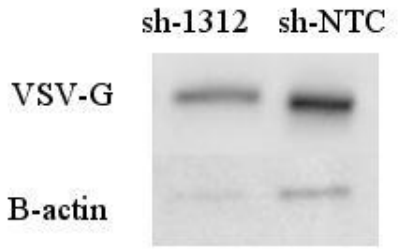


Fig. 6. Continued.

When combined, the results from all shRNAs expressed in transgenic cell lines (including shRNAs that target N, P, and L viral genes), sh-N67 and sh-N1312 yielded the highest inhibition of N, P and L viral transcripts relative to sh-NTC control group, followed by sh-P1772 (Fig. 5A and 6A). Conversely, none of L-shRNAs tested reduced N, P and L viral transcripts (Fig. 5B).

A



B

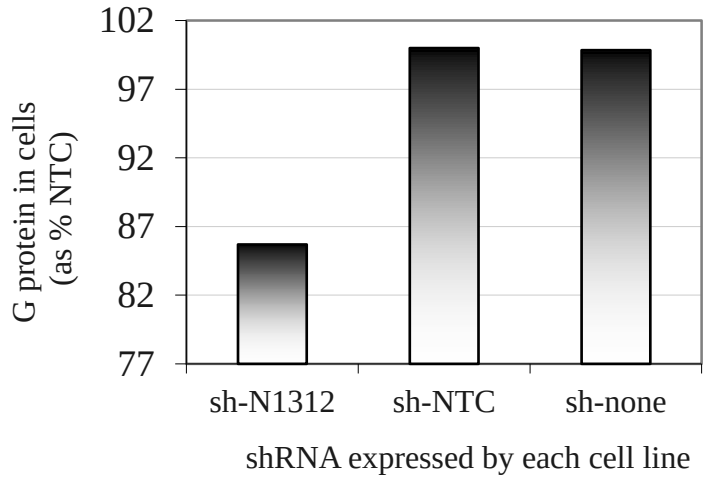


Fig. 7. Sh-N1312 reduced VSIV protein levels. (A)Western blot analysis to detect VSIV-G protein from cytoplasmic lysates of infected cells expressing N-shRNAs or control groups collected at 24 hpi. (B). Densitometric analysis of the bands observed in A. Protein levels are expressed as a percentage of null control.

2.3.5 Sh-N1312 reduced VSIV protein levels

*S*hRNAs induce a post-transcriptional silencing that may lead to translation repression of the viral protein encoded by the sequence targeted (see Fig. 1). To test whether or not the expression of N-shRNAs had an effect on viral protein levels during

VSIV infection, an immunoblot analysis for detection of VSIV-G protein from cytoplasmic lysates was performed. As shown in Fig. 7, at 24 hpi, transgenic Vero cell expressing sh-N1312 but not sh-N67 reduced G protein levels as compared to sh-NTC or wild-type infected cells. Cell lines expressing sh-N1312 showed approximate 15% less viral protein than controls as evidenced by densitometric analysis.

Table 2

VSIV titers recovered at 12 hpi from transgenic Vero cells (MOI=0.01).

shRNA	Log(Titration1)	Log(Titration 2)	Log(Titration 3)	Mean	SEM
sh-67	5.400	5.080	5.415	5.284	0.109
sh-1312	5.785	5.450	6.500	5.838	0.310
sh-1431	6.500	6.500	6.415	6.479	0.028
sh-1772	5.610	6.000	5.885	5.825	0.116
sh-1990	6.500	5.830	6.280	6.194	0.197
sh-7256	7.000	6.250	6.500	6.594	0.220
sh-6847	6.830	6.415	6.750	6.654	0.127
sh-6859	6.750	7.000	6.915	6.885	0.073
sh-NTC	6.000	5.585	6.000	5.844	0.138

* Statistical significance ($p<0.05$, Fisher t-test) indicates reduction in viral titers as compared to sh-NTC control.

2.3.6 Vero cells expressing VSIV-shRNAs release fewer infectious particles

Having shown that N-shRNA and P-shRNA were able to potently and specifically reduce viral transcripts and protein, next the virus yield released from the experimental and control groups was determined. To do this, supernatants from infected cells were titrated at either 12 hpi (Vero cells) or 24 hpi (BHK-21 cells), and the approximate number of viral particles released was calculated based on the titer. The

results from titrations of Vero- or BHK-derived experimental groups infected under different experimental conditions are presented in Tables 2 and 3, respectively.

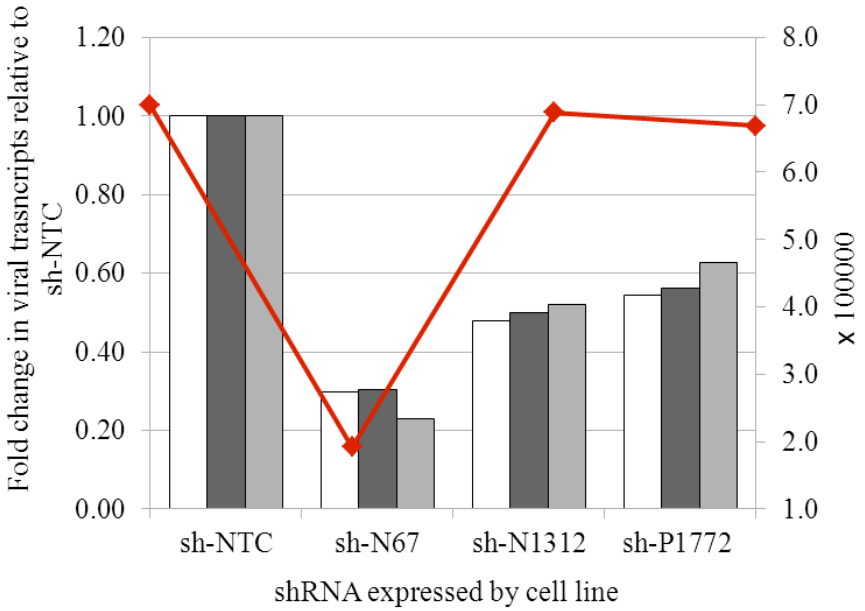
Table 3

VSIV titers recovered at 24hpi from BHK-21 cells expressing shRNAs (MOI=0.1).

shRNA	Log(Titration1)	Log(Titration 2)	Log(Titration 3)	Mean	SEM
sh-67	8.000	8.670	8.330	8.333	0.193
sh-1312	7.500	7.830	8.330	7.887	0.241
sh-6859	8.000	8.000	8.670	8.223	0.223
sh-NTC	8.700	7.670	8.670	8.347	0.338

Consistent with previous results (Fig. 6), at 12 hpi Vero cell lines expressing sh-N67 and sh-P1772 yielded lower titers than the sh-NTC control group (see Table 2) when infection was performed at lower MOI. However, the reduction observed was statistically significant ($p<0.01$) only for sh-N67. Accordingly, sh-N67 expressing cells released approximate 5×10^5 less infectious progeny virus than the sh-NTC control (Fig. 8A).Although sh-N1312 and sh-P1772 significantly reduced viral transcripts, they yielded reduced titers than the sh-NTC, but the reduction was not statistically significant (Table 2, Fig. 8).

A



B

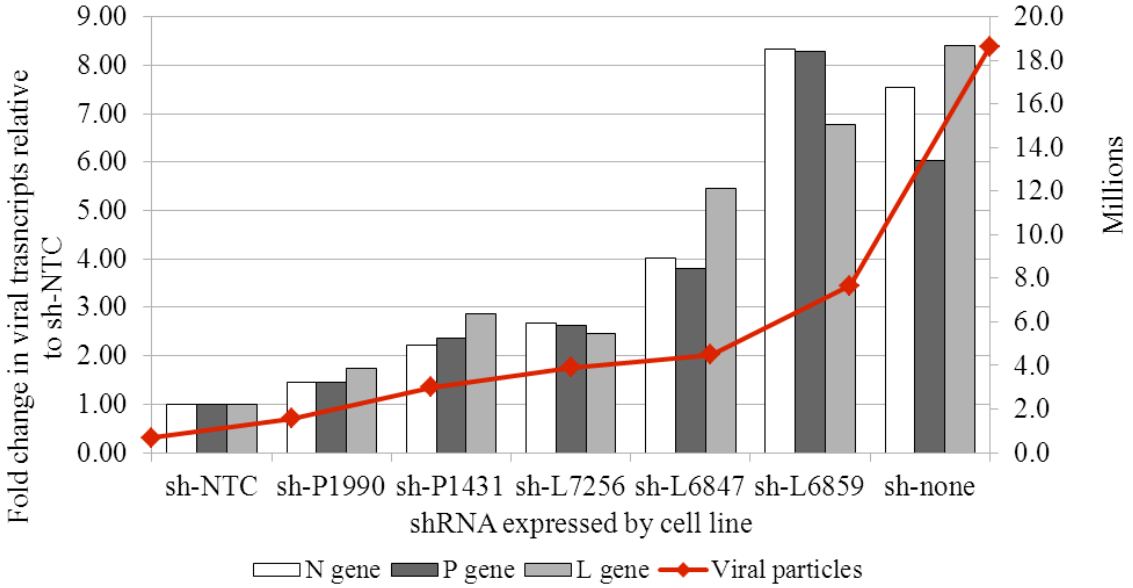


Fig. 8. The effect of expression of shRNAs in cell lines over the number of viral particles shed followed the tendency of the viral transcripts. Bar graph represents the fold change in N, P, or L viral transcripts in cell lines expressing each shRNA. Line represents the particles released calculated as an anti-log of the titer. (A)(B) Vero-derived experimental and controls groups were infected at MOI=0.01 and supernantants were collected at 12 hpi. Data is compared to the sh-NTC. (C) BHK-21-derived experimental and controls groups were infected at MOI 0.1 and supernantants were collected at 24 hpi.

C

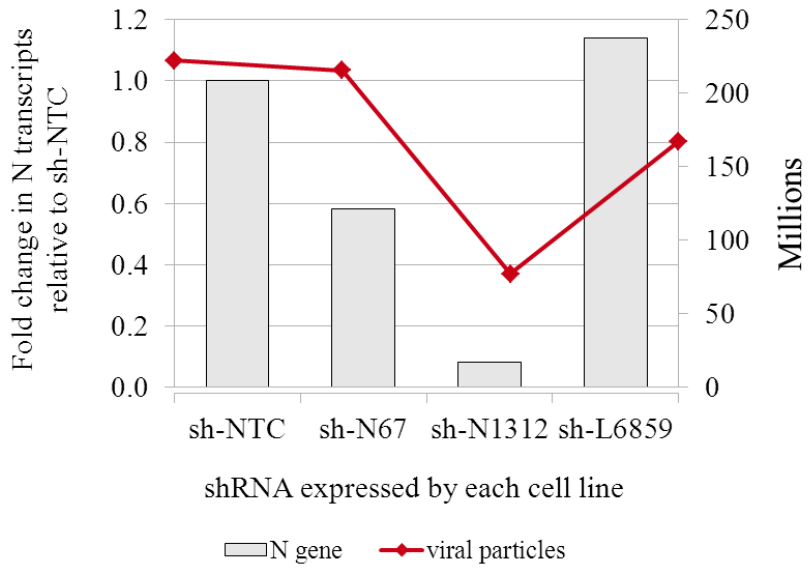


Fig. 8. Continued.

When transgenic BHK-21 cells that expressed sh-N67, sh-N1312, sh-L6859, or sh-NTC were challenged with VSIV at 10 times higher MOI, cells expressing sh-N1312 produced a lower titer and released roughly 100 million less infectious progeny virus as compared to sh-NTC (Table 3, Fig. 8C). The difference in the viral titer released between cells expressing shRNA and controls expressing an irrelevant shRNA was not statistically significant.

Additionally, the results from titration at 24 hpi were corroborated by performing plaque assays from the supernatants collected from BHK-21 cells at 24hpi. As shown in Fig. 9, the number of plaques and their morphology produced from the supernatants of infected cells expressing either VSIV targeting or non-targeting shRNAs were roughly

the same. Plaque assays showed the similarity in lytic activity of released viruses from N-shRNAs expressing cells and control groups (Lyles and Rupprecht, 2007).

The data from above-mentioned experiments suggests that at early time points following infection, VSIV replicated significantly less viral RNA (Fig. 4) and shed less infectious progeny virus (Table 3 and Fig. 5A) in Vero cells expressing sh-N67 as compared to sh-NTC. In BHK-21 cells expressing sh-N67 challenged at higher MOI, the antiviral effect was also observed but was not significant at 24 hpi. A reduction in the number of viral particles released by cells expressing sh-N1312 was observed.

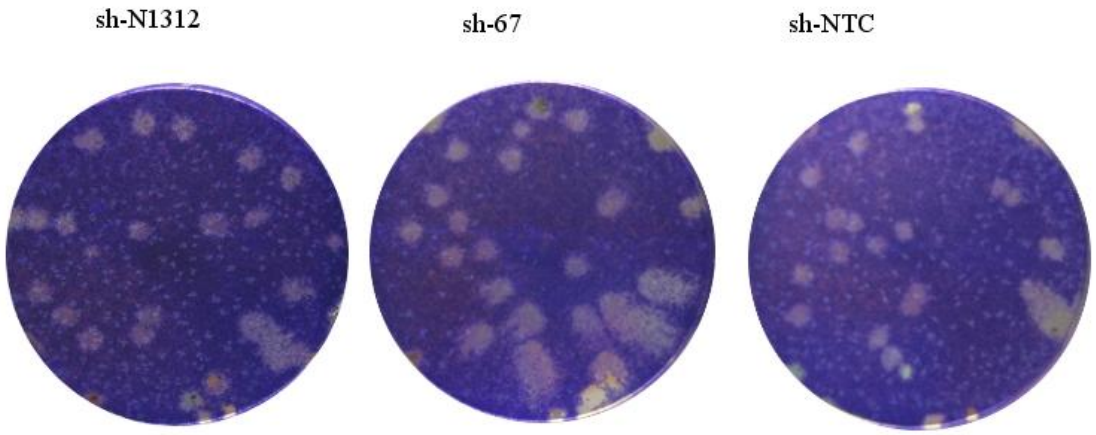


Fig. 9. Plaque assay of the VSIV-infected BHK-21 transgenic and controls at MOI 0.1, supernatants collected at 24hpi. Plaques formed by VSIV detected in supernatants collected from cells expressing sh-N67, -N1312 or -NTC.

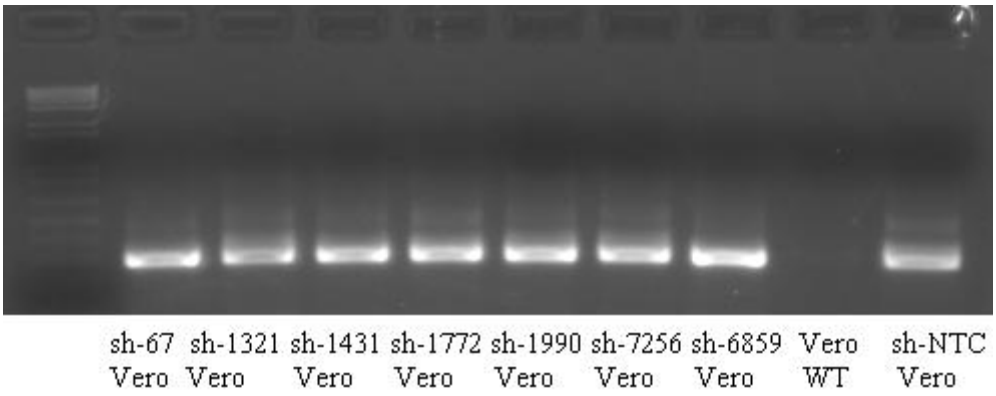
2.3.7 Minor sequence diversity in regions targeted by shRNA

In order to evaluate the sequence diversity in the target regions of the shRNAs, we isolated and analyzed partial DNA sequences from 3 field strains circulating in Costa Rica, a country in which VSIV infection is endemic. Thirteen tissue samples referenced to the virology laboratory were used for virus isolation. Eight samples resulted positive for tissue culture viral isolation and three out of these eight samples corresponded to Indiana strain as revealed by a seroneutralization test (data not shown). Next, we analyzed the partial sequences of the target region of sh-N1312, sh-L6847, and sh-L6859 from the VSIV wild isolates. Also sequences from the regions targeted by sh-N1312, sh-P1772, sh-P1990, sh-L6847, sh-L7256 and sh-L6859 from the laboratory-adapted strain were analyzed. As shown in Table 4, single substitutions were found in the sequence targeted by sh-N1312, sh-L6859 that was conserved for the laboratory-adapted viruses. Also, the mutations detected were located in sites 10 or 12 of the region of the guide strand of the shRNA. Mutations in the regions targeted by shRNAs were not found in the laboratory-adapted strain.

2.3.8 VSV-shRNA sequences retrieved from genomes of transgenic cells

To confirm the presence of the corresponding shRNA coding region in each cell line, the genomic sequence spanning the C-terminus of GFP and the shRNA (Fig. 10A) was sequenced for the experimental cell population and the cell line expressing the irrelevant control.

A.



B.

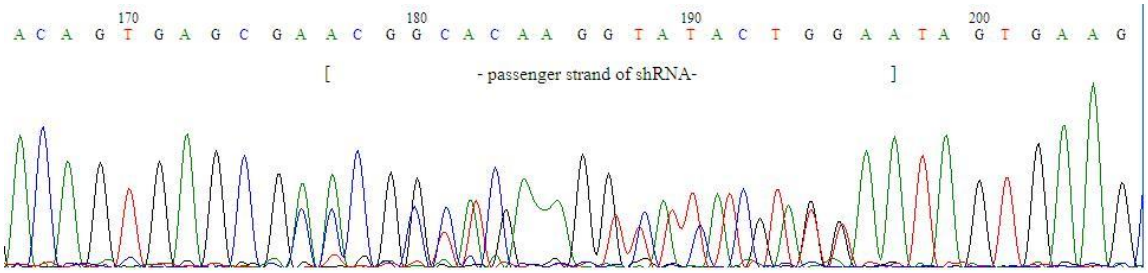


Fig. 10. Expected shRNA sequences were amplified from genomic DNA from transgenic cell lines and sequenced. (A) Total genomic DNA was extracted from each population of cells (labeled in picture) and the region spanning the C-terminus of GFP and the shRNA amplified, each primary band of the corresponding size was sliced and further sequenced. WT= wild type cell line. (B) Sequence analysis of chromatograms revealed some spots of duplication in the passenger and guide sequence of the sh-NTC.

Mismatches in the shRNA (passenger strand, loop or guide strand) coding region were not detected for any of our experimental groups. However, when the chromatograms of the NTC group were analyzed, some spots of sequence duplication in the regions encoding the sh-NTC were evidenced (Fig. 10A).

To explore a putative target region for sh-NTC within VSIV genome, we searched using Blast and confirmed that only sequences from irrelevant genes matched the query at 100%. We found partial matching between sh-NTC stem region and 2 different regions of VSIV genome when the alignment was performed using two distinct bioinformatic resources (Table 4).

Table 4

Genetic variability in the sequence targeted by the shRNAs.

Identification of sequences	Gene	Sequence targeted by the guide strand of denoted shRNA[1]	Mis-match[1]	Posi-tion
Predicted sequence contained in guide strand of sh-N1312	N	3'- UUCAGUCUUAAACUGUUUACU-5'	n/a[1]	-
Target region[2] of sh-N1312 in laboratory-adapted strain	N	5'-UUCAGUCUUAAACUGUUUACU-3'	0	-
Target region[2] of sh-N1312 in VSIV wild isolates	N	5'- UUCAGUCUUAAGCUGUUUACU-5'	1	10
Predicted sequence contained in guide strand of sh-L6859	L	3'-ACCGUGU U**CCACUAUUAGUUC-5'**	n/a	-
Target region[2] of sh-L6859 in laboratory-adapted strain	L	5'- ACCGUGU U**CCACUAUUAGUUC**-3'	0	-
Target region[2] of sh-L6859 in VSIV wild isolates	L	5'-ACCGUGU UCCACUGUUAGU UC-3'	1	8
Predicted sequence contained in guide strand of sh-L6847	L	3'-CGACAGUUUCAGAACCGUGUU-5'	n/a	-
Target region[2] of sh-L6847 from laboratory-adapted strain	L	5'-CGACAGUUUCAGAACCGUGUU-3'	0	-
Comp[2] target region of sh-L6847 in VSIV wild isolates.	L	5'-CGACAGUUUCAAAACCGUGUU-5'	1	10
Predicted sequence contained in guide strand of sh-L7256	L	3'-ACGATCTGAGAACAACTACTA-5'	n/a	-

Table 4 Continued.

Identification of sequences	Gene	Sequence targeted by the guide strand of denoted shRNA[1]	Mis-match[1]	Position
Target region[2] of sh-L7256 from laboratory-adapted strain	L	5'-ACGATCTGAGAACAACTACTA-3'	0	-
Predicted sequence contained in guide strand of sh-P1772	P	3'-ACGGACUCCCAAAUUCACCUC-5'	n/a	-
Target region[2] of sh-P1772 from laboratory-adapted strain	P	5'-ACGGACUCCCAAAUUCACCUC-5'	0	-
Predicted sequence contained in guide strand of sh-P1990	P	3'-AGAGAGUUUCUGUAGGUACUG-5'	n/a	-
Target region[2] of sh-P1990 from laboratory-adapted strain	P	5'-AGAGAGUUUCUGUAGGUACUG-5'	0	-
Predicted sequence contained in guide strand of sh-NTC		3'-UGCCAUGUUCCAUAUGACCUU-5'	n/a	-
Putative target region[2] 1 for sh-NTC within VSIV genome[4]	P	5'UGC*UAAUUUCGUCAG*CACGUUCCA C*GGUU*UAUGACCUU-3'[6]	2	-
Putative target region[2] 2 for sh-NTC within VSIV genome[5]	G	3'-UAACCUGUACCAUACAACCUG-5'[6]	7	-

[1]Nucleotide substitutions are shown underlined, gaps are shown in italics, n/a= non-applicable.
[2]Sequences shown are RNA sequences complementary to the DNA recovered experimentally.
[3]Bold region denotes region coding for the aminoacid residues GDNQ which is the core region in the C motif of the L protein (Schnell and Conzelmann, 1995)
[4]The sequence of the Gene bank accession no. J02428.1 was used as reference for multiple alignments using Clustal W (Thompson et al., 1994).
[5]The sequence of the Gene bank accession no. J02428.1 was used as reference for multiple alignment using Bioedit(Hall, 1999).
[6]In red matching nucleotides of sh-NTC and genomic VSIV from 1816-1836 nt or 4284-4304 nt for first and second alignment, respectively.

2.4. Discussion

Selection of the viral gene targets is a crucial aspect in developing antiviral therapies. In the case of VSIV, the activation of the endogenous cellular RNAi mechanism to limit its replication has been suggested (Otsuka et al., 2007). Also, the proof of concept of utilization of temporal siRNA-mediated silencing targeting VSIV genes has also been reported (Barik, 2004). Based on these publications and evidence of successful application of RNAi to reduce viral transcripts mentioned in the previous section, this investigation was designed to test if reduction in VSIV transcription, translation, and hence, viral replication can be accomplished by potent shRNA-mediated inhibition of genes that form the RNP complex.

Following this deduction, shRNAs were designed after selecting appropriate regions within N, P and L viral genes. Because P and L are genes transcribed in a lower amount than N during naturally-occurring viral infection, it was expected that shRNAs targeting regions of P or L genes, would have a notable effect to impair VSIV replication. Surprisingly, only sh-P1772 out of the three P-shRNAs tested significantly reduced viral transcripts. The expression of sh-P1172 did not result in a significant reduction in viral titers at 12hpi. In accordance to Bitko and Barik (2001), the inhibition observed by the effective P-shRNA not only led to a reduction of P transcripts but also led to a reduction of other viral transcripts (Fig. 5A). We hypothesized that the passenger strand of the shRNA could be affecting the viral RNA anti-genomes instead of passively being degraded by RISC. Further testing of any effect of the passenger strand could be

assessed using primers specifics for each strand of viral RNA produced namely viral transcripts and anti-genomes.

Contrastingly, Barik (2004) observed that siRNA-based silencing of either L or P resulted in partial loss of all RNA synthesis in three different viral models (RSV, VSIV, and HPIV-3). However, Barik (2004) did not publish the experimental data nor the sequences of the siRNAs employed when using VSIV model, which makes it impossible to compare the viral regions targeted.

Although the viral polymerase contains sequence motifs conserved throughout the non-segmented negative-strand RNA viruses (Schnell and Conzelmann, 1995), none of the L-shRNAs tested resulted in a reduction in viral transcripts (Fig. 5B). The lack of any suppressive effect in the viral transcriptional levels shown by sh-P6859 was particularly unexpected because the region targeted by this shRNA belongs to a highly conserved block (CR-III, C) of the polymerase (Schnell and Conzelmann, 1995). The unique amino acid residues "GDNQ", included in the region targeted at RNA level by sh-L6859 have been recognized as the invariant core of the C-motif of L protein. Schnell et al. (1995) found sequence conservation in these core residues (but not necessarily the flanking amino acids) from several non-segmented negative-strand RNA viruses including VSIV, Borna disease virus, human parainfluenza type 3, Sendai virus, measles virus, canine distemper, human parainfluenza type 2, simian virus 5, Newcastle disease virus, human respiratory syncytial virus, and Marburg virus. Also, in past it has been shown that replacement of an amino acid within the "GDN" core resulted in loss of the functionality of L (Schnell and Conzelmann, 1995).

The lack of potency of sh-L6847 or sh-L6859 cannot be explained by mutations in the target sequence from the laboratory adapted strain used for challenging assays (Table 4). When the corresponding target sequences from wild viruses were analyzed, a single nucleotide substitution in the three field strains sequences analyzed was found (Table 4). But this mutation does not result in any change to the amino acid sequence (GDNQ) of the core of the C-motif from L protein. Together these results suggest that absolute amino acid sequence conservation does not guarantee potency and effectiveness of the shRNA designed. This can probably be explained by the degeneracy of the genetic code which ensures protein sequence conservation even at the high mutation rate observed during replication of RNA viruses.

Nucleocapsid was targeted due to its natural abundance and degree of conservation among the VSIV strains (Rodriguez,L.L. 2002). The shRNAs that target the regions 67-87 nt and 1312-1332 of the N gene reduced the number of N viral transcripts to a statistically significant degree (Fig. 6). This data suggests that both 3' and 5' regions of N mRNA are acceptable targets for shRNA-mediated post-trascriptional silencing. According to literature, the region targeted by the sh-N1312 is included within the last 60 aa of the C-terminus of N, which has been considered a highly conserved region required for interactions with P (Rodriguez et al., 2002). Also, we found that expression of N-shRNAs reduced N, P and L transcripts. In contrast, previous literature (Arnheiter et al., 1985)) suggested that the functionality of polymerase or viral RNA is not altered when the function of N protein is disturbed.

When the sequence variability in the target region of several of the tested shRNAs was determined, results suggested that shRNAs targeting the same gene, overlapping regions of the same gene, or different genes had different potencies for knockdown. Literature emphasizes the importance of the matching between the stem region of shRNA at the seed region of 5' of the corresponding miRNA and its target (Grimson et al., 2007; Friedman et al., 2009;Jackson et al., 2006). In this regard, all the target sequences recovered from the laboratory-adapted strain employed for challenge assays showed normal 'perfect' matching with the corresponding stem regions of the hairpins (Table 4). This data suggests that variation in the effectiveness of the different shRNAs could be associated with features beyond complementarity of the seed region. Features such as structural thermodynamic differences and shRNA target site context may permit better processing or better matching with the template.

Features of the shRNA targeting region can increase binding site efficacy of miRNAs. These features include: positioning outside of the center of a long 3'UTRs; proximity to sites where miRNAs are co-expressed; AU-rich nucleotide context; and positioning at least 15 nt from the stop codon (Grimson et al., 2007). In general, the shRNA target region is frequently found in an "AU" rich context because "AU" nucleotides represent almost 60% of the viral genome. Also, only one of the shRNAs (sh-N1312) targeted a region relatively close to the stop codon of N gene but definitely at a longer distance than 15 nt. Therefore, none of these characteristics of shRNA target context may explain the differences in the potency observed among shRNAs.

Furthermore, based on analysis of sequences from VSIV wild-type isolates it is predicted that single substitutions observed in target messages of sh-N1312 or sh-L6847 will produce a mismatch at 10 nt from the 5' of the shRNA. These mismatches are not expected to affect the canonical shRNA complementary sites within seed region (Friedman et al., 2009). Only the substitution observed in the target message of sh-L6859 may affect the 8mer but not the other canonical miRNA complementary sites.

Moreover, although the passenger strand of the shRNA is degraded during RNAi activation (see Fig. 1.), McIntyre et al. (2006) demonstrated that hairpins with mismatches in the sense stem are less potent gene suppressors (McIntyre and Fanning, 2006). In this regard, we did not detect mismatches in the shRNA coding region (passenger strand, loop or guide strand) of our experimental groups. Together, the above-presented data fails to explain the differences in the potency of the shRNA directed to silence various genomic regions of VSIV. Detailed research should be performed to improve *in silico* prediction capability of the effectiveness and potency of designed shRNAs.

Genomic DNA isolated from the population of cells expressing the non-targeting control was analyzed and duplication in the regions encoding the sh-NTC was observed. This data suggests that the NTC population may be expressing not only the sequence contained in the irrelevant shRNA but also other sequences. In agreement with this hypothesis, the sequence duplication were only found in sequences isolated from genomic DNA of transduced cells but not in sequences recovered from transformed

bacterial cells. Importantly, this finding could explain a portion of the off-target antiviral effect induced by the irrelevant sh-NTC (Fig. 3b).

The differences in the results from infected sh-NTC cells and wild-type cells could also be attributed to several other factors such as: an artifact induced by drug selection; the effect of stringent sorting by flow cytometry that resulted in very different cell populations; or any off-target effects induced by insertion of the lentiviral vector. Further research is required to test these hypotheses.

Alternatively, although very unlikely, 5 out of 8 shRNAs tested could induce up-regulation of viral transcription and replication as an off-target effect. In this regard, high levels of viral transcripts and titers recovered from infected cell lines carrying sh-L6859 and sh-L6847 (which target overlapping regions of L gene) did not differ from the untreated wild-type infected control, and this could suggest these are the levels normally observed in untreated viral infections.

Alignment of the VSIV genome and the stem region of sh-NTC predicted two putative sides for hybridization of the shRNA (Table 4). In the first alignment, which included a 16 nt gap and 2 mismatches, the putative target message is located in the viral G protein. The 5' region of the NTC-shRNA, including the shRNA positions 2-7 of the seed region, would potentially pair with the putative targets. This supports the notion that sh-NTC may cause an off-target effect on viral transcripts through activation of RNAi.

The second alignment (Table 4), which suggests P as sh-NTC target, evidenced mismatches in only 7 out of 21 nts without inclusion of any gap in the alignment. The

matched regions were the 5' and middle of the stem sequence; however, two mismatches occurred in the seed region. These predictions may also explain the off-target effect induced by the shRNA sequence on the viral transcripts and replication. However, Saxena et. al (2003)(Saxena et al., 2003) reported that translational repression was not observed when five or more mismatches were present. In contrast to our results, Saxena et al. (2003) incorporated mismatches within the middle region of the siRNA, and in our experiments, the mismatches predicted are distributed in all the stem sequence.

Cytopatic effect determination suggested that the expression of tested shRNAs did not block VSIV replication. However, expression of N-shRNAs generated transgenic phenotypes with a reduced susceptibility to the infection (Fig. 4). Cells expressing N-shRNAs yielded less viral particles and displayed an antiviral effect at the early time point after VSIV infection (MOI=0.01). As the exponential viral growth rate increased at later points after infection, the expression of N-shRNA had limited effectiveness to impair viral replication and cytopathogenicity. In this regard, N gene is a difficult target for post-transcriptional silencing because it displays a higher level of transcription as compared to the other viral genes (Whelan et al., 2004). Due to the N gene's proximity to the promoter within the viral genome, it shows a higher transcriptional efficiency that may ensure protein expression of N even in the presence of RNAi-induced silencing or translational repression. This may limit the effectiveness of shRNAs at later points of infection. Importantly, this transcriptional feature is shared by many other non-segmented negative-strand RNA viruses that are important human, animal, and plant pathogens (Whelan et al., 2004).

Barik et al. (2004) also mentioned that at a higher MOI, the degree of primary transcription contributing to the bulk of viral mRNA and protein synthesis is increased (Barik, 2004). Primary transcription is catalyzed by RdRP already carried by in infecting virions (Bitko and Barik, 2001), and therefore, it remains unaffected by shRNA targeting RdRp, which can explain basal viral protein and transcripts levels even in the presence of L-shRNAs.

Overall, interpretation of our data suggested that the RNAi pathway was activated in transgenic cell lines after transduction with the lentiviral vector (Fig. 3) that carried one of the relevant shRNAs (sh-N67, sh-P1312, and sh-P1772). The transcription of the artificial shRNAs already integrated in the nucleus led to the activation of the RNAi mechanism and achieved the ability to partially down-regulate viral mRNA produced during infection (Fig. 5A and 6). It is likely that the RNAi activation also caused translational repression as evidenced by reduction in protein levels of VSIV-G (Fig. 7). Finally, the expression of relevant shRNAs (N-shRNAs) by cell lines exerted an antiviral effect early after VSIV infection. This antiviral effect was manifested as a reduction in viral particles released in supernatants (Fig. 8A, Table 2) and, consequently, reduction in CPE (Fig. 4). However, the antiviral effect was less obvious as the number of viral replication cycles increased (Fig. 8B, Table 3). Considering the role of VSV as a prototype for non-segmented negative-strand viruses (Letchworth et al., 1999), the findings of this study could be broadly applied to predict the applicability and limitations of the RNAi-based approach to other important viral pathogens.

Lastly, the proper selection of a cell line is indispensable for generating relevant transgene expression systems and testing potency of shRNAs for impairing viral replication. VSIV can infect a wide range of cells by being rapidly cytolytic in most of them (Hill et al., 1986). In this study, we chose two different parental cell lines, BHK-21 and Vero cells, to derivate our transgenic populations. Both cell lines are routinely used for VSIV infection as well as for plasmid transfection, lentiviral transduction and drug selection (Smith-Tsurkan et al., 2010;Wilson et al., 2009;Dudek et al., 2010;Yasuhara-Bell et al., 2010;Arshed et al., 2011).

Malignant transformation of lentiviral-transduced cell lines has been reported. The Vero cell line has been used to study cell transformation because of its well-established growth pattern in culture (Goncalves et al., 2006). In this investigation, microscopic evidence of transformation of the cell line was not observed.

Finally, the primary innate immune defense against viral infections is mediated by the IFN response (Noser et al., 2007). Matskevich et al. (2009) suggested that the presence of the IFN response can bias the antiviral effects observed in cells treated with RNAi-based therapies (Matskevich et al., 2009). Vero cells do not produce IFN α or β (Emeny and Morgan, 1979) which make them more susceptible to viral infection. For this reason, Vero cells are good models to evaluate the effect of specific RNAi-based antiviral therapies in the complete absence of the IFN response. Therefore, it is expected that the antiviral effect observed in our experiments (Fig. 8A, Table 2) as result of RNAi activation, will be potentiated in organisms expressing a competent IFN-based antiviral response.

3. CONCLUSIONS AND FUTURE WORK

3.1 Conclusions

In the past, molecular strategies such as gene shuffling, truncation of viral proteins and isolation of mutants have been employed for studying VSV replication cycle, impairing VSV growth, and attenuating its virulence (Flanagan et al., 2001; Clarke et al., 2007). Fewer reports, however, (Bitko and Barik, 2001;Barik, 2004) have explored the applicability of exogenously activated RNAi as an alternative to combat VSV and other viral infections caused by non-segmented negative- strand RNA viruses.

In the current investigation, we provided an experimental approach to generate stable transgenic cell lines with reduced susceptibility to VSIV infection by using an RNAi-based strategy. In objective 1, we tested the effectiveness, potency, and durability of several shRNAs specifically targeting viral genes. From the experimental data the following six conclusions were reached:

(i) The three shRNAs (targeting the N gene or a particular region of the P gene) reported in this study are good candidates to reduce viral target messages.

(ii) Viral transcriptional reduction was observed up to last point tested and in cells infected at low MOI.

(iii) Expression of a relevant N-shRNA also resulted in reduction of VSV-G protein levels. This is attributed to shRNA-mediated transcriptional and translational repression of the viral transcripts.

(iv) None of the L-shRNAs tested significantly reduced viral transcription or impaired VSV replication even when one of these targeted a region highly conserved among non-segmented negative-strand RNA viruses.

(v) The cell line expressing sh-NTC reduced viral transcripts as compared to wild-type infected cells. This off-target effect could be due to several reasons including partial complementarity with the viral genome, stringent antibiotic selection or flow cytometric sorting of the transgenic cell lines.

(vi) There is a gap between theoretical optimization of shRNAs and experimental validation. It was necessary to test at least eight shRNAs and three different viral genomic regions to find three shRNAs that significantly reduced viral transcripts.

For objective 2, we evaluated *in vitro* the applicability of a shRNAi–based approach to reduce VSIV titers and consequently reduce the amount of shed virions. From the experimental data we reached the following four conclusions:

(vii) Transgenic cell lines that expressed N-shRNAs displayed reduced CPE after VSV infection. However, the inhibition of CPE was not well-defined in cell lines expressing P- or L-shRNAs.

(viii) Cell lines that expressed N-shRNAs shed reduced amount of viral particles in supernatants after VSIV infection.

(ix) Reduction in viral transcripts does not necessarily mean reduction in viral titers. Cell lines expressing a P-shRNA that reduced viral transcripts did not impaired VSIV replication.

(x) Partial silencing of viral transcripts by a single shRNAs is not sufficient to block VSIV replication; however some antiviral activity was evident in cells expressing N-shRNAs.

(xi) The antiviral effect induced by relevant shRNAs is expected to be potentiated in organisms expressing active IFN response. Moreover, Vero cell line constitutes a good model to study *in vitro* new antiviral approaches due to the lack of interference of IFN-based antiviral response and its well-established growth pattern.

For objective 3, we sought to analyze the sequence variability of VSIV genomic regions targeted by some of the shRNAs designed. The following four conclusions were found:

(xii) The lack of potency of some shRNAs was not associated with mutations in target regions or features of shRNA context.

(xiii) The sequence conservation in the target messages (from the laboratory-adapted strain) of shRNAs reinforced the results of the experimental validation of the shRNA designed.

(xiv) Single substitutions founded in target sequences of shRNAs from wild strains are not predicted to affect the matching of the shRNAs designed to target VSIV.

(xv) *In silico* predictions of the effectiveness of shRNAs offer guidelines for designing shRNAs, but experimental validation is still required. More detailed knowledge about shRNA processing and matching preferences should be clarified to improve the predictive capability.

In general, this study utilizes a stable long-term expression system that differs from past investigations (Barik, 2004). This feature of the research design enables the researcher to experimentally test the potency and durability of the RNAi effects for longer infection periods. Also, the application of lentiviral vectors constituted a reliable strategy to accomplish the expression of shRNA-cassettes in a diversity of cell systems and in a cost-effective way. Additionally, this approach reinforces the applicability of these and other genetic engineering techniques for establishing a form of "intracellular immunity" that can be extended for producing transgenic animals resistant to viral infections.

Together, our data and the above-mentioned literature support the hypothesis that RNAi-based strategies have the potential for reducing viral transcription and impairing viral replication. Our experiences using VSIV prototype provide new insights into the challenges faced when developing innovative antiviral strategies for non-segmented negative strand RNA viruses.

3.2 Future work

While the information contained in this report contributes to broaden knowledge about the application of an RNAi-based antiviral approach for non-segmented negative-stranded RNA viruses. It is evident, however, that much more can and must be learned about this strategy to increase the effectiveness, durability, and safety, particularly, when the systems are envisioned to be used *in vivo*. In the following section, we summarize potential avenues of study that need to be expanded and identify future projects to achieve the long-term goal of integrating research into practice:

- The effects of shRNAi-mediated silencing in the remaining viral gene targets (G and M) could be investigated.
- The potential use of combination of multiple shRNA into a single expression vector should be tested to potentially induce a more significant impairment of viral replication and to prevent viral escape from mutations. Also, we anticipate that the combination of the most potent shRNAs reported in this study will increase the antiviral effect observed.
- The current investigation revealed the critical role of proper selection of the non-targeting control. In the future, it would be interesting to compare the effects of the viral-shRNAs tested in comparison to a scrambled, completely irrelevant shRNA control.
- Specific assays can be performed to better assess any transforming effect induced by the SIN-lentivirus construct used in the transgenic cell systems. Rigorous viral vector testing is required to ensure low frequency of insertional adverse events.

- To reduce promoter-associated off-target effects, it is important to consider not only the capacity of the promoter to drive gene expression but also the physiological aspects of endogenous microRNA pathway such as saturation (Barik, 2006) produced by overexpression of artificially introduced siRNAs. Additionally, tissue-specific promoters and inducible promoters could be employed to reduce off-target effects.
- Further investigations are needed to reveal the effect of the RNAi-based antiviral approach described here to impair the replication of wild-type viral strains.

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APPENDIX A

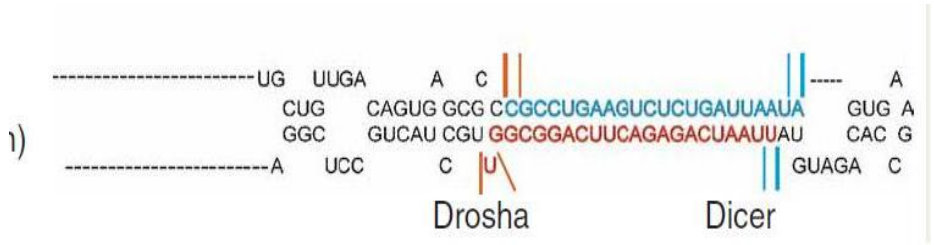

Fig. 11. Secondary structure of the model shRNA including the processing sites.

Table 5

GFP expression confirmed by FAC in transgenic cell lines.

shRNA	Transgenic cells derived from Vero	
	%	M. Fluores.
sh-N67	95.5	70126
sh-N1312	97.6	41377
sh-P1431	92.0	35968
sh-P1772	98.8*	39946
sh-P1990	95.9*	35262
sh-L6847	99.0*	25780
sh-L6859	96.5	36128
sh-L7256	94.0	10942
sh-control	100*	37216

* Post-sorting GFP-expression.

Table 6

Sequence of the shRNA used for cloning into the lentiviral vector.

	ShRNAs sequences
sh-N67	CAGAAGG**CTCGAG**AAGGTATATTGCTGTTGACAGTGAGCGATCTGTTA CAGTCAAGAGAATCTAGTGAAGCCACAGATGTAGATTCTCTTGACTGT AACAGACTGCCTACTGCCTCG**GA ATT**CAAGGGGCTACTTTAG
sh-N1312	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCAAGTCA GAATTTGACAAATGA*TA*GTGAAGCCACAGATG*TA*TCATTTGTCAAATTC TGACTTATGCCTACTGCCTCG**GA ATT**CAAGGGGCTACTTTAG
sh-P1431	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGATCCTAT TCTCGTCTAGATCAGTAGTGAAGCCACAGATGTACTGATCTAGACGA GAATAGGACTGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAG
sh-P1772	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCTGCCAG AGGGTTTAAGTGGAGTAGTGAAGCCACAGATGTACTCCACTTAAACC CTCTGGCAATGCCTACTGCCTCG**GAATTC**AAGGGGCTACTTTAG
sh-P1990	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGATCTCTC AAAGACATCCATGACTAGTGAAGCCACAGATGTAGTCATGGATGTCT TTGAGAGAGTGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAG
sh-L6847	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGCTGTC AAAGTCTTGGCACAATAGTGAAGCCACAGATGTATTGTGCCAAGACT TTGACAGCATGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAG
sh-L6859	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCTGGCAC AAGGTGATAATCAAGTAGTGAAGCCACAGATGTACTTGATTATCACC TTGTGCCAATGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAG
sh-L7256	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCTGCTAG ACTCTTGTTGATGATTAGTGAAGCCACAGATGTAATCATCAACAAGA GTCTAGCAATGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAG
sh-NTC	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAACGGTA CAAGGTATACTGGAATAGTGAAGCCACAGATGTATTCCAGTATACCT TGTACCGTCTGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAGG

Key:
- =5'mir-30 and Xho I restriction site
- =3'mir-30 and EcoRI restriction site
- =spacer nucleotides
- =guide strand of shRNA
- =passenger strand of shRNA

Table 7

Primers for cloning and sequencing.

ID	Target	Sequence 5'-3'
3'mir30-R	cloning vector	CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA
5'mir30-F	cloning vector	CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAG-TGAGCG
EGFP-C 240-F	GFP	CATGGTCCTGCTGGAGTTCGTG
VSIV-N-F	VSIV-N	AGGCCGATTCATACATGCCT
VSIV-N1307-R	VSIV-N-seq	AATAGGTGATCTGAGAATTATAGGG
VSIV-P320-F	VSIV-P-seq	GGAAAGACCTTACGGTTGACA
VSIV-P639-R	VSIV-P seq-	AGAGGCTGAAGACTTGCTTTCT
VSV-L2029-F	VSV-L-seq	GAGGGTGGACTGGAAGGTCT
VSV-L2609-R	VSV-L-seq	CTGTGCAAGCCCGGTATCTT

Table 8

Summary of optimized RT-q-PCR parameters for BHK-21 cells.

Gene	Concentration[1]	Efficiency[2](%)	R^2[3]	Slope[4]	Ct range[5]	A.base[6]
VSIV-N[7]	666	98	1	-3.36	9 to 29	1.988
VSIV-L[8]	666	95	0.96	-3.46	14 to 31	1.945
VSIV-P	666	98	0.92	-3.38	15 to 33	1.976
β2-MG	666	96	0.99	-3.39	19 to 36	1.972
GAPDH	666	97	0.99	-3.38	17 to 35	1.976
β-Actin	666	98	0.99	-3.35	16 to 36	1.988

[1] Forward and reverse primers concentrations tested: 100, 200, 666 and 1000 nM. The combination with the lowest Ct and highest ΔRn was chosen.
[2] Single melt curve and the ideal value should range between 90-110%.
[3] R^2 should be closer to 1 to ensure the maximum predictive value of the ΔΔCt method.
[4] Slope should be close to -3.3 to ensure linear regression.
[5] Linear prediction and ΔΔCt method are valid under this range.
[6] Amplification base for each cycle adjusted considering the efficiency of the primers
[7] Protocol adapted from Wilson et al. (2009).
[8] Protocol adapted from Hole et al. (2010).

Table 9

Summary of optimized RT-q-PCR parameters for Vero cells.

Gene	Concentration[1]	Efficiency[2]	R [3]	Slope[4]	Ct range[5]	Amp.base[6]
VSIV-N[7]	666	95	0.99	-3.44	9 to 17	1.953
VSIV-L[8]	666	95	0.96	-3.45	12 to 29	1.949
VSIV-P	666	98	0.92	-3.38	12 to 28	1.976
GAPDH	666	91	0.99	-3.53	21 to 32	1.920

[1] Forward and reverse primers concentrations tested: 100, 200, 666 and 1000 nM. The combination with the lowest Ct and highest ΔRn was chosen.
[2] Single melt curve and the ideal value should range between 90-110%.
[3] R^2 should be closer to 1 to ensure the maximum predictive value of the ΔΔCt method.
[4] Slope should be close to -3.3 to ensure linear regression.
[5] Linear prediction and ΔΔCt method are valid under this range.
[6] Amplification base for each cycle adjusted considering the efficiency of the primers
[7] Protocol adapted from Wilson et al. (2009).
[8] Protocol adapted from Hole et al. (2010).

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