ESCAPE FROM SHORT-INTERFERING RNA-INDUCED SILENCING IN AN ORTHOBUNYAVIRUS, TENSAW VIRUS

By

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To my mother, father, and sister, for all their love and support

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Ribonucleic acid interference (RNAi) is a major component of antiviral immunity in dipteran insects, including mosquitoes. Virus-specific short-interfering RNA (siRNA) are being considered as a potential therapy to provide resistance to viral infection *in vivo*. However, due to the high mutation rate of ribonucleic acid (RNA) viruses, antiviral siRNA treatments often provide incomplete protection against viral proliferation, which results in a delayed reestablishment of infection. Yet, few studies have examined the infectivity profiles of viral escape populations, especially in arboviruses, where host alternation during arboviral transmission cycles constrains major genomic shifts. Tensaw virus (Family *Bunyaviridae*, Genus Orthobunyavirus) served as a model system for cell culture experiments examining the effects of inducing the antiviral RNA interference response *in vitro* on the fitness and sequence of an arbovirus.

Transfection with siRNA targeting the overlapping nucleoprotein/nonstructural protein coding regions of Tensaw virus significantly reduces viral titer at several time points in Vero (monkey) cells and HeLa (human) cells according to tissue culture infective dose assays. Virus from various siRNA-treated Vero cells were collected at 120 hours post-infection (hpi), diluted to uniform viral load, and used to infect both fresh

vertebrate (Vero, HeLa) and mosquito (C6/36) cells. Regression analysis of growth curves from all three second passage regimens indicate that the fitness of Tensaw virus from all siRNA treatment conditions that had established infection in Vero cells did not differ significantly from virus that emerged from Tensaw-infected Vero cells that were not treated with siRNA.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Arboviruses

Arthropod-borne viruses (arboviruses) are transmitted to hosts through the bite of an infected arthropod. Millions of people worldwide are exposed to arboviruses each year, and research into inner workings of arboviral relationships with their hosts and vectors have been at the forefront of entomological research for decades.

Arboviruses are unique insofar as they infect and thrive in both vertebrate and invertebrate hosts, and as such, not only have different pathogeneses and relationships with their respective hosts, but must accordingly be suited to handle the radically different environments of both. Interestingly, the immune responses and the manifestations of infection differ greatly between vertebrate hosts and invertebrate vectors, which may explain why arboviruses are not typically associated with pathology in the vector (Myles et al. 2008). Pathogenesis in vertebrate hosts ranges from harmless to highly virulent such as causing hemorrhagic fever or meningoencephalitis, amongst other major illnesses. On the other hand, arboviruses can infect tissue throughout a vector's entire body during an acute phase of efficient virus production with few pathologic effects. Infected vectors tend to have a persistent infection with low levels of virus that can persist for the lifetime of the vector (Brown 1984, Bishop 1996). Accordingly, in cell culture, infection of mammalian cells with arboviruses are often lytic, causing disruption of cellular replicative functions and cell apoptosis, while in insect cells, infection is typically noncytolytic and persistent (Borucki et al. 2002, Blakqori et al. 2007). It is generally supposed that it is more efficient from an evolutionary standpoint for viruses to minimize deleterious effects on vector health rather than to maximize

infection rates, which would result in consequent cost to the probability of the vector's daily survival of the vector and subsequent chances to spread the virus.

By gaining a better understanding of the molecular biology of arthropod vectors and their interactions with the viruses that inhabit them, scientists can effective arboviral control strategies. Cutting edge molecular approaches are providing considerable information about gene regulation and expression in both viral and host cellular mechanisms. In particular, technologies that involve assessing host immune response and the immune evasion mechanisms of viruses are at the forefront of novel approaches to controlling vector-borne viruses.

Bunyaviridae and Tensaw Virus (TENV)

Bunyaviridae

The *Bunyaviridae* family is a medically important family comprising five genera: *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus*. Of the over 300 described bunyaviruses, over 60 species from four genera have been reported to cause disease in humans or livestock, including Crimean-Congo hemorrhagic fever (CCHF), Hantavirus (HAN), La Crosse (LAC), and Rift Valley fever (RVF), amongst others (Elliott 1996). Of those five genera, nairoviruses, orthobunyaviruses, and phleboviruses are principally considered arboviruses.

Members of the *Bunyaviridae* family are icosahedral, enveloped viruses with a tripartite negative-sense ribonucleic acid (RNA) genome comprising small, medium, and large segments, named S, M, and L, respectively (see review in Elliott 1996). The genomes of all five genera encode four essential structural proteins: a nucleoprotein protein, coded for by the N open reading frame (ORF) of the S segment; two glycoproteins, encoded in the Gn and Gc ORFs on the M segment; and the RNA-

dependent RNA polymerase (RdRp) encoded by the L ORF on the L segment. Members of the *Hantavirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus* genera also contain a nonstructural protein encoded in the NSs ORF of the S segment (Jääskeläinen et al. 2007), while genomes of members of the *Orthobunyavirus* and *Tospovirus* genera and several members of the *Phlebovirus* genus, contain a nonstructural protein encoded by the NSm ORF of the M segment (Elliott 1996).

Some of the functions of the nonstructural proteins have been elucidated in recent years in reservoir hosts. The protein of the NSm segment has been implicated in inducing cells to create proteinaceous tubular structures that promote virion assembly in Bunyamwera virus (BUN) (Genus *Orthobunyavirus*) in vertebrate cell lines (Shi et al. 2006, Fontana et al. 2008), and that may facilitate cell-to-cell movement in tomato spotted wilt virus (TSW) (Genus *Tospovirus*) in plants (Storms et al. 1995). However, the NSm protein was deemed nonessential to viral maturation, replication, and infection in RVF (Genus *Phlebovirus*) (Gerrard et al. 2007), and dispensable to growth in Maguari virus (Genus *Orthobunyavirus*) (Pollitt et al. 2006).

As it stands, though the putative mode of action may vary or may not be fully characterized, the NSs protein serves at least one unambiguous and conserved function in all bunyaviruses studied thus far: attenuation of the reservoir (vertebrate) host's immune responses to the virus. Takeda et al. (2002) first demonstrated the immune suppression capabilities of the NSs protein in TSW, which attenuates antiviral RNA interference mechanisms of its plant host. Subsequent studies conducted on members of *Orthobunyavirus* (Blakqori et al. 2007, Hart et al. 2009), *Phlebovirus* (Billecocq et al. 2004, Perrone et al. 2007, Habjan et al. 2009), and *Hantavirus* genera

(Jääskeläinen et al. 2007) that infect vertebrate hosts indicate that the NSs protein inhibits the host's RNA polymerase II-mediated transcription, which in turn blocks the activation of IFN system, the major inducible antiviral response in vertebrates, as well as host cell protein synthesis. Furthermore, Soldan et al. (2005) indicate that expressing the NSs protein in human 293T cells globally suppresses RNA interference induced by short-interfering RNA (siRNA).

The function of the NSs protein in the mosquito vector is not fully understood. Soldan et al. (2005) demonstrated that siRNA-induced gene silencing targeting the overlapping N/NSs region of the S segment significantly attenuated viral expression in *Aedes* (*Ae.) albopictus* C6/36 cells. However, they could not take measures to examine the role of silencing the NSs gene alone because the entire NSs ORF overlaps with the N ORF. Blakqori et al. (2007) engineered a recombinant LAC orthobunyavirus that does not express the NSs protein, and they could not detect any advantage that the NSs protein conferred to LAC virus in infecting C6/36 mosquito cells, as the recombinant LAC did not replicate any less efficiently in C6/36 cells than wild-type LAC. Hart et al. (2010) demonstrated similar findings in infection of C6/36 cells, in which NSs deletion mutant of model orthobunyavirus Bunyamwera (BUN) had no effect on mosquito cell transcription or translation. However, more recently, Brackney et al. (2010) and Scott et al. (2010) determined that C6/36 cells mount an incomplete antiviral RNAi response due in part to a deletion in the Dicer-2 gene. Subsequently, Szemiel et al. (2012) demonstrated that the NSs deletion mutant could replicate effectively in C6/36 cells, no NSs deletion mutant could be detected in RNAi-competent *Ae. albopictus* U4.4 cells, indicating that the BUN NSs gene was essential to establish infection in immune-

competent cell systems. Furthermore, wild-type BUN was able to infect *Ae. aegypti* salivary glands three days quicker than BUN with the NSs deletion. To date, the specific mechanism by which NSs aids replication of orthobunyaviruses in mosquitoes is unknown, but NSs does appear to be required for efficacious and productive replication in the vector.

Tensaw Virus: Prevalence and Distribution

Tensaw virus, the model virus to be used in the experiments described herein, is an arbovirus belonging to the *Orthobunyavirus* genus of the *Bunyaviridae* family. TENV was initially isolated from *Anopheles (An.) crucians* mosquitoes in 1960 in the Tensaw delta valley in Alabama (Sudia et al. 1969). A number of studies in the 1960s, 1970s, and 1980s demonstrated the subsequent prevalence and pathogenicity in a wide array of vertebrates.

Coleman (1969) detailed animal experiments that exposed several immune-competent animals to high doses ($10^{6.0}$ particle forming units per milliliter, or PFU/mL) of TENV and examined immune response. Rabbits and hamsters generated a robust antibody-based response to the virus and did not die, while guinea pigs did not produce any demonstrable antibodies to TENV but still survived through the study. Young Swiss mice inoculated with virus at 1-2 days old and 10 days old died within 3-6 days of infection. Three-week old mice died within 6-9 days of infection when TENV was inoculated intracranially, but all survived and produced antibodies to TENV if inoculated intraperitoneally or subcutaneously. Ten-week old mice inoculated intracranially similarly also survived. Of the hosts studied, only rabbits are found in the wild in the Southeast US.

Subsequent tests by Sudia et al. (1969) demonstrated that rabbits, dogs, cats, cotton rats, and hamsters can amplify TENV and produce long-lasting viremic infections. They then found that experimentally infected dogs produce a high enough viremia that *An. quadrimaculatus* mosquitoes can take up enough virus to transmit it to immunodeficient suckling mice, indicating that dogs can potentially act as reservoir hosts. Interestingly, neither of the birds tested with experimental infection of TENV—sparrows or 1 day old chickens—produced high viremia, indicating that neither avian host is a particular threat as a reservoir.

Chamberlain et al. (1969), tested blood samples from several wild-caught and domestic vertebrates and screened them for antibodies to TENV. They found that 3 of 13 raccoons (23%), 10 of 14 dogs (71%), 1 of 16 cows (6%), and 13 of 150 humans in South Alabama (9%) tested had produced antibodies to TENV, suggesting prior exposure. Subsequent studies by others found that 15% (129 of 850 tested) (Castro et al. 1982), and 22% (65 of 300 tested) (Calisher et al. 1988) of human serum samples from people in Florida tested positive for antibodies to TENV in serological tests, suggesting a relatively high endemicity in Florida. Bigler and Hoff (1975) also conducted serological tests of sylvatic mammals, demonstrating that serum from 24% of raccoons (35 of 144 tested) and two of three marsh rabbits tested positive for TENV antibodies, while no rats mice, opossums, cotton rats, or black rats had antibodies against TENV. They suggested that raccoons and marsh rabbits are often exposed to TENV, but produce a high-grade immune response, and suggest that the mosquitoes associated with vectoring TENV do not prefer to feed on mice, opossums, or rats.

Calisher et al. (1986) isolated TENV from serum of four different animals, all in Florida—dogs, cotton rats, swamp rabbits, and cotton mice—some of which had not been known to produce antibodies to TENV in previous studies by others. Most importantly, however, virus was isolated from brain tissue of a gray fox in Florida, suggesting that the virus may have produced an encephalitic infection in the fox. As these produced infective virions in serum and brain tissue samples, all five animals, which are common in Florida, could potentially act as reservoirs for TENV if they produce high enough titers.

As for Tensaw-related human encephalitis cases, McGowan et al. (1973) reported that of the 3,739 arboviral-related encephalitis cases reported from 1955-1971, only one case in Indiana was attributed to TENV infection. Interestingly, however, no other information is provided on the patient, and no other arboviral surveillance studies have isolated TENV in Indiana.

Tensaw Virus Genome

As with other members of the *Orthobunyavirus* genus, the S segment encodes proteins for a nucleoprotein (N) and a nonstructural protein (NSs) in an overlapping (+1) open reading frame transcribed from the same messenger RNA (mRNA), while the M segment encodes two glycoproteins that flank a gene coding a nonstructural protein in non-overlapping reading frames (Figure 1-1) (Watts et al. 2009). The putative proteins associated with the genomic regions and closest match genome are listed in Table 1-2.

TENV was isolated from mosquitoes in Florida for the first time in 2006, and is believed to be distributed throughout the southeast United States (Watts et al. 2009). Little is known about the function of nonstructural proteins or the structural proteins of the Florida isolate of Tensaw virus. However, it is one of only five completely sequenced

viruses in the *Orthobunyavirus* genus (Watts et al. 2009). Future TENV research will not only provide a better understanding of its biological and molecular characteristics, but additionally, TENV can thus act as a model system for studies into the basic mechanisms of other medically important bunyaviruses.

Ribonucleic Acid Interference (RNAi)

Ribonucleic acid interference (RNAi) is a biological process responsible for regulation of endogenous genes as well as detection and destruction of foreign ribonucleic acid (RNA) (Fire et al. 1998). Many of the proteins involved in vertebrate RNAi pathways have been identified and their roles elucidated. Similarly, analogous proteins with similar functions and sequences have been identified in insects, where the short-interfering RNA (siRNA) degradation pathway serves as the primary major antiviral defense in insects (Campbell et al. 2008, Cirimotich et al. 2009, Blair 2011), but the proteins have been sequenced for only a few species.

RNA viruses generate partially double-stranded RNA structures as replicative intermediates in the course of invading and taking over host cellular mechanisms (Westaway et al. 1997). Much like the double-stranded RNA (dsRNA) triggered interferon (IFN) pathway in vertebrates, double-stranded RNA that is ~30 bp or more in length triggers the RNAi cascade. In insects, cytoplasmic RNase III enzyme Dicer-2, with the help of dsRNA-binding protein R2D2, binds and preferentially cleaves dsRNA from the ends of the strands to generate 21–25 nucleotide (nt) long dsRNA duplexes with characteristic 5’ phosphate groups and 2 nt 3’ overhangs (Liu et al. 2006). Virus derived siRNAs, called viRNAs, are then loaded into the multiprotein RNA-induced silencing complex (RISC). In the RISC, viRNAs are unwound by an RNA helicase into two single-stranded RNA strands. One of the two strands, deemed the passenger

strand, is degraded and discarded, while the remaining strand in the RISC, called the guide strand, that directs the RNA degradation machinery which targets mRNA sequences found throughout the cytoplasm that are complementary to the guide strand. (Paroo et al. 2007, Sanchez-Vargas et al. 2004). SiRNA-loaded RISCs repress mRNA translation through direct degradation of their mRNA targets (Hamilton et al. 2002). The RNAi signal can then spread between cells, thus inhibiting the replication of incoming viruses in neighboring cells by stimulating a preventative antiviral state prior to the dissemination of the virus (Attarzadeh-Yazdi et al. 2009).

Endogenously expressed small RNAs are responsible for controlling a number of key aspects of gene regulation and cellular development. These small, non-coding RNAs are transcribed from the host genome by RNA polymerase II and III from host deoxyribonucleic acid (DNA) (Peters and Meister 2007). Complementary sequences then fold back upon themselves to form double-stranded RNA structures, referred to as microRNAs or miRNAs, which trigger the posttranscriptional downregulation of the expression of protein coding genes within a cell and hence act as internal regulators of gene expression. MicroRNA processing proceeds in a manner similar to siRNA proessing, but with its own set of analogous proteins, including amongst others, separate miRNA-processing and cleavage proteins (Dicer-1 and Drosha) and a separate Argonaute protein, AGO1 (Lee et al. 2003, Lee et al. 2004, Okamura et al. 2004). MiRNA-loaded RISCs repress the expression of homologous mRNA by direct mRNA degradation and by expediting decay processes that reduce mRNA stability, including 3’ deadenylation and 5’ decapping of the mRNA (Peters and Meister 2007, Ghildiyal and Zamore 2009). A few studies have examined the ways that the miRNAs

may function in the immune response. Li et al. (2009) identified specific miRNAs expressed in bloodfed *Ae. aegypti* and suggest that they are involved in immune response. Winter et al. (2007) and Skalsky et al. (2010) also identified miRNAs that are specifically upregulated in response to *Plasmodium* infection in *Anopheles gambiae* and West Nile infection in *Culex* (*Cx*.) *quinquefasciatus*, respectively, suggesting that they may play a role in immune response. However, little is known about the specific function of these miRNAs in mosquito immunity, so further experiments need to be conducted.

Applications of RNA Interference

Elucidating Gene Function

As scientists are gaining a greater understanding of the vast scope of the RNAi cascade's possibilities, RNAi is rapidly developing into an important tool in manipulating host systems in a variety of ways. Systematic analyses of the effects of dsRNA length, secondary structure, nucleotide choice and sequence specificity have produced a bevy of information on how to synthesize siRNA duplexes that, when transfected into a variety of *in vitro* and *in vivo* systems, effectively trigger the RNAi cascade by mimicking the 21-25 nt Dicer cleavage substrate RNA duplex intermediates that are taken up and processed by the RISC (Hammond et al. 2000).

The earliest research into RNAi stem from experiments that utilizing dsRNA, and resulted in highly effective silencing of the expression of endogenous genes identical to the dsRNA introduced (Fire et al. 1998, Bass 2000, Cogoni and Macino 2000). Three landmark papers put out by Thomas Tuschl's lab in 2001 demonstrated that introducing 21-25 nt double-stranded RNA segments with 2 nt 3' overhangs into *in vitro* systems, mimicking the siRNA segments created by the Dicer protein, triggers the RISC to knockdown expression of homologous sequences (Elbashir et al. 2001a, b, c). Now, a

number of biotechnology companies – Dharmacon, Lifetech, Qiagen, and Sigma-Genosys, amongst others – that synthesize custom siRNA sequences have made these technologies commercially available and relatively affordable to the average researcher.

By introducing siRNA to silence specific sequences in a system, many studies can not only elucidate gene function, but can also examine how these systems change and operate when major components are effectively and precisely shut down. As such, siRNA-mediated knockdown of genes has become the hallmark of host gene silencing.

Specifically, a number of studies have examined introducing siRNA cognate to genes coding for host immune components, which have in turn elucidated many specific aspects of immune function and response as well as many characteristics of pathogenic invasion. Hammond et al. (2001) found that introducing siRNA that depleted the mRNA coding for Argonaute2 (AGO2), the essential Argonaute protein in plant and insect RISC complexes, led to a two-to-threefold increase in Flock House virus (FHV) RNAs in *Nicotiana benthamiana* leaves. Similarly, Li et al. (2004) knocked down *ago2* expression in *Drosophila melanogaster* and *Anopheles gambiae* cell lines, which in turn reestablished infection with recombinant FHV strains that were otherwise uninfective in the cell lines. Experiments have also been conducted that knocked down immune components *in vivo*. Keene et al. (2004) demonstrated that anopheline mosquitoes injected with dsRNA cognate to AGO*2* were more permissive to O'nyong-nyong virus replication and to viral dissemination throughout the mosquito's body. Similarly, Sanchez-Vargas et al. (2009) demonstrated that siRNA-mediated knockdown of *dicer-2*, *r2d2*, or *ago2* genes that encode essential sensor and effector proteins in the RNAi

cascade, increased Dengue virus titers and decreased the extrinsic incubation period required for transmission of Dengue in *Ae. aegypti* mosquitoes.

Antiviral RNAi

Mutations add diversity to a viral swarm and can enable a viral population to adapt to challenges imposed by the host's immune response. Theories on viral quasispecies suggest that most RNA viral populations maintain a fairly constant mutation rate, wherein mutations are tolerated at an equilibrium below a deleterious threshold (Domingo et al. 2001). However, Bull et al. (2006) suggest that elevating a viral population's mutation rate beyond this threshold presumably impairs the population's overall fitness, because viral progeny with too many mutations lose their ability to remain infective. The concept of lethal mutagenesis hence entails pushing a viral population within a host beyond that threshold into extinction using mutagens (Bull et al. 2006). Accordingly, many researchers have utilized several technologies, including monoclonal antibodies, ribavirin treatment, and the use of nucleoside analogues, both *in vitro* and *in vivo* with varying success (see Sanjuan et al. 2010 for review). In many instances, the high mutation rates of the viruses allowed for the emergence of viable virus even in the face of treatment (McKeating et al. 1989, Mo et al. 1997, Zharikova et al. 2005, Keck et al. 2009).

Since the advent of RNA interference technologies, researchers have explored introducing virus-specific dsRNA, siRNA, or DNA constructs or viral vectors coding for short-hairpin RNA (shRNA) into cells in order to interfere with the growth and proliferation of viruses. Li et al. (2002) made the landmark observation that viruses trigger RISC in invertebrates to reduce viral gene expression and in turn to halt viral replication. It became apparent through their work and subsequent work by others that

RNA interference is a major component of invertebrate antiviral immunity (Campbell et al. 2008, Cirimotich et al. 2009, Blair 2011). Similarly, later studies demonstrated that siRNA can reduce viral gene expression in vertebrate systems and that siRNA are too short to trigger an IFN response in some systems (Gitlin et al. 2002, Kapadia et al. 2003). Though the IFN system is a major component of vertebrate antiviral defenses, these studies suggest that RNAi may also play a role in halting viral proliferation. As such, virus-specific siRNA are being considered a potential therapy to provide refractoriness *in vivo* in invertebrates and vertebrates alike (Moens 2009).

Subsequently, a number of studies examined the possibilities of preventive inoculation with viral-specific siRNA as a means for silencing arboviral expression, both *in vitro* and *in vivo* in vertebrate and invertebrate systems (Keene et al. 2004, Bai et al. 2005, Murakami et al. 2005, Dash et al. 2008, Wu et al. 2010). Most of these studies revealed that exposure of cell lines to viral-specific siRNA prior to infection only transiently suppresses arboviral replication, typically for no more than 72 hours (Bai et al. 2005, Murakami et al. 2005, O'Brien 2007, Seyhan et al. 2007, Dash et al. 2008). Cell lines are at least transiently resistant to viral infection if they are transfected beforehand with siRNA homologous to regions of a viral genome (Li et al. 2004, Soldan et al. 2005, Wu et al. 2005). However, because RISC binds and/or cleaves viral genomic regions based highly sequence-specific binding to the viral RNA target, viruses that were not homologous to the transfected siRNA emerged either through *de novo* mutations in the targeted sequence (Boden et al. 2003, Das et al. 2004, Soldan et al. 2005) or through the selection for viral clones present in the initial virus population and selected out in the presence of siRNA (Gitlin et al. 2002, Wu et al. 2005). Dash et al.

(2008) found that viruses in escape populations had the same sequence as the initial wild-type virus, but did not examine mechanisms that otherwise allowed for escape from siRNA selection. Wu et al. (2010) also reported a siRNA-mediated reduction in cytopathology in Dengue virus (DEN) RNA in C6/36 *Ae. albopictus* cells, but RNA levels were decreased by about 40-fold by seven days post infection. In their experiments, antiviral siRNA was transfected into the cells prior to infection, and though the number of viral RNA copies were reduced by single-dose siRNA transfection, only 8.9% of cells contained antiviral siRNA by seven days after transfection. The authors go on to suggest that siRNA degradation and cell division reduce the concentration of siRNA over time, and thus weakens the antiviral siRNA's antagonistic effect.

In all of the aforementioned scenarios, viruses were able to circumvent the preventative siRNA treatment and established infection. However, none of these studies included experiments on whether siRNA selection caused any changes to the infectivity of the emergent viral populations.

Evasion of Host Defenses by Ribonucleic Acid (RNA) Viruses

RNA viruses are particularly prone to genomic drift for a number of reasons. RNA replication is particularly error-prone, as there are no known proofreading or mismatch repair mechanisms in RNA polymerases, and therefore, misinsertion errors arise at a rate of 10^{-3} to 10^{-5} substitutions per nucleotide, a much lower fidelity than DNA virus replication (Domingo and Holland 1997). While a vast majority of mutations are deleterious (Keightly and Lynch 2003, Sanjuan et al. 2004), this high mutation rate may also confer them with an advantage: an expeditious adaptability by viruses with beneficial mutations to environmental perturbations, particularly those of host immune responses.

Several theories have emerged attempting to explain how viruses evade destruction even in the face of robust immune responses. Sawicki et al. (1981) found that within 4-6 hours of infection, Sindbus-infected cells were releasing viable viruses into cell culture supernatant at rates of up to 2000 PFU per cell per hour, which suggests that high replication rates of viruses may overwhelm host immunities by sheer numbers. A bevy of research has also indicated that many arboviruses and other insect viruses have adapted to the barrage of host immunities by a variety of mechanisms that antagonize vector and host immune components. These mechanisms include proteins that antagonize the vertebrate interferon response (Billecocq et al. 2004, Blakqori et al. 2007, Jääskeläinen et al. 2007, Perrone et al. 2007, Habjan et al. 2009), or the antiviral RNAi response (Li et al. 2002, Aliyari et al. 2008, Nayak et al. 2010, Qi et al. 2012, van Mierlo et al. 2012), or by encoding subgenomic immunomodulatory RNAs that interfere by competing with the antiviral RNAi response (Schnettler et al. 2012, Schuessler et al. 2012).

Furthermore, as mentioned, viruses can also evade the host immune response by *de novo* mutations that produce viable viral progeny (Boden et al. 2003, Das et al. 2004, Soldan et al. 2005). Sanjuan et al. (2004) created a series of mutant viruses via site-directed mutagenesis of an infectious VSV complementary DNA (cDNA), and demonstrated that a majority of single-nucleotide substitution mutations, whether randomly chosen or previously described, were lethal or deleterious, but that previously described mutations were more likely to be neutral or beneficial than randomly-chosen single-nucleotide substitutions. Furthermore, several studies have indicated that identical point mutations emerge independently in separate lineages of cells infected

with identical viruses (Novella et al. 1999, Novella et al. 2004). Taken together, these findings suggest that patterns or motifs of mutation arise naturally as a result of parallel evolution, and that these mutations confer an advantage to the mutated clones by helping them evade host defenses.

Several studies have ventured to examine changes in the viability of siRNA-induced escape mutants in single-host studies with differing results. Senserrich et al. (2007) found that a second passage of human immunodeficiency virus (HIV-1) escape mutants were comparably as fit as their parent strains. Lv et al. (2009), on the other hand, found that FMDV escape mutants were susceptible to siRNA-induced degradation when subjected to a second round of selection with the same siRNA. Sabariegos et al. (2006) found that some HIV-1 escape mutants were more fit, and others less fit, than parent virus, depending on which position in the siRNA target region changes.

However, examples were not found where virus that escaped siRNA-mediated silencing was assayed for fitness in both the vector and the host. Therefore, it has not been thoroughly examined whether intracellular immunization with siRNA induces deleterious mutations in the special cases of arboviruses. Since artificially inducing the RNAi pathway with virus-specific siRNA can generate an accumulation of mutations and non-wild-type variations in siRNA targeted regions, it stands to reason that by simulating natural transmission back and forth between hosts, a majority of these escape mutants that may otherwise be able to persist within a single host will not survive when passed to another host.

Viral Infection in Alternating Hosts

Due in large part to low fidelity polymerases, RNA viruses tend to mutate at high rates compared to DNA viruses (Domingo and Holland 1997). However, the consensus sequences of arboviral genomes remain more stable over time and express highly variable phenotypes compared to RNA viruses that only infect closely related hosts (Holmes and Twiddy 2003). Though the consensus sequence of a virus may remain the same across time, RNA viral populations exhibit a high degree of genotypic diversity even within a single host. A particular viral variant may not exhibit the highest degree of fitness across all cell and host types, and hence a high degree of genomic diversity may confer fitness to the whole viral population and maintain phenotypic stability across time (Eigen and Biebricher 1988, van Nimwegen et al. 1999, Wilke et al. 2001). High fitness variants do not always outcompete less fit viral variants during the course of infection within a single host and often may aid in the maintenance of minority genomes through complementation, recombination, and reassortment within a cell infected with several variants (Aaskov et al. 2006, Li et al. 2011, Brackney et al. 2011, Ciota et al. 2012). As such, natural selection acts upon the entire swarm of variants rather than on any particular mutant and maintains genetic diversity within a population accordingly (Eigen and Biebricher 1988, Ciota et al. 2012).

Arboviruses are maintained in nature by continuous transmission between vertebrate and arthropod vectors. Whereas arboviruses tend to maintain a persistent, noncytolytic infection in mosquitoes, which reduces fitness costs associated with infection and allows the vector to transmit virus when feeding, arboviruses cause acute, cytolytic infection in mammals, which allows them to produce a high enough titer for transmission to the vector (Scott et al. 1994). Accordingly, these viruses are well suited

to very disparate fitness landscapes and must be able to evade highly divergent immune responses. While the phenotype of a virus varies across the range of vectors and hosts it infects, host alternation during the arbovirus life cycle constrains genetic diversity compared to virus that serially infects only one of the two hosts (Greene et al. 2005, Coffey et al. 2008, Vasilakis et al. 2009, Coffey and Vignuzzi 2011). These studies suggest that only a small minority of the clones that emerge in one host will be viable in the other host, and hence by back and forth passage between vertebrate and arthropod hosts, negative selection acts upon the viral population to provide bottlenecks that continuously purify out the majority of mutants (Domingo and Holland 1997). Experimental studies indicate that this is often the case, as different insertions, deletions, or synonymous and nonsynonymous mutations often arise when arboviral populations are subjected to regimens of infection in a vertebrate system only, in an arthropod systems only, or in alternating passage between the two (Novella et al. 1999, Weaver et al. 1999, Greene et al. 2005, Coffey et al. 2008, Vasilakis et al. 2009, Coffey and Vignuzzi 2011, McCurdy et al. 2011, Moutailler et al. 2011).

There is a general consensus in the arbovirus literature that an arbovirus infecting a single host cell line in serial passages will see improved fitness in that cell line passage compared to the parent virus in one-step growth curves and competition assays. Weaver et al. (1999), Coffey et al. (2008) and Vasilakis et al. (2009) demonstrate that serial passages of eastern equine encephalitis, Venezuelan equine encephalitis, and Dengue virus, respectively, in mosquito and/or vertebrate cell lines result in different mutations away from the wild-type genomic motif in serial passages, and more consensus mutations in serial passage than seen in alternating passage. This

suggests that where serial passage of arboviruses in a single host exhibit different patterns of genetic evolution over time from serial passage though the other host, but that host alternation again limits genomic variance of wild-type virus by selecting for viruses that are most suited to replicating at high rates in both mammalian and mosquito hosts.

However, there are several prevailing theories and lines of evidence within the literature about whether there are actual tradeoffs in fitness involved with infecting alternating hosts. One school of thought states that generalism by an arbovirus comes at a cost. The low fidelity of the polymerase allows some genetic drift through an accumulation of mutations over time, but host alternation constrains broad viral population evolution because of antagonistic pleiotropy, where fitness gains exhibited in a serial passage in one host usually results in diminished fitness in the other host (Elena et al. 2009). Accordingly, a virus population will be able to replicate across several hosts, but will not become optimally fit in any one of those hosts (Elena et al. 2009).

The unique and powerful selective pressures of alternating passage of arboviruses on viral variants and of the capabilities of harnessing inducible host immunities to knockdown viral expression through RNAi provide us with some novel opportunity to apply experiments that examine the interplay between these bottlenecks. However, there is a paucity of literature on whether conventional antiviral responses (e.g., monoclonal antibodies or ribavirin) or novel genetics-based treatments like RNAi induce different changes in fitness across a virus's host range. This knowledge is particularly important if these treatments are utilized as antiviral therapies with human patients or in controlling viral infection in vectors. Therefore, the experiments described

herein examine whether siRNA-based antiviral treatment causes mutations in a model orthobunyavirus, and how this antiviral treatment affects the fitness of the emergent virus across vertebrate and mosquito cell cultures.

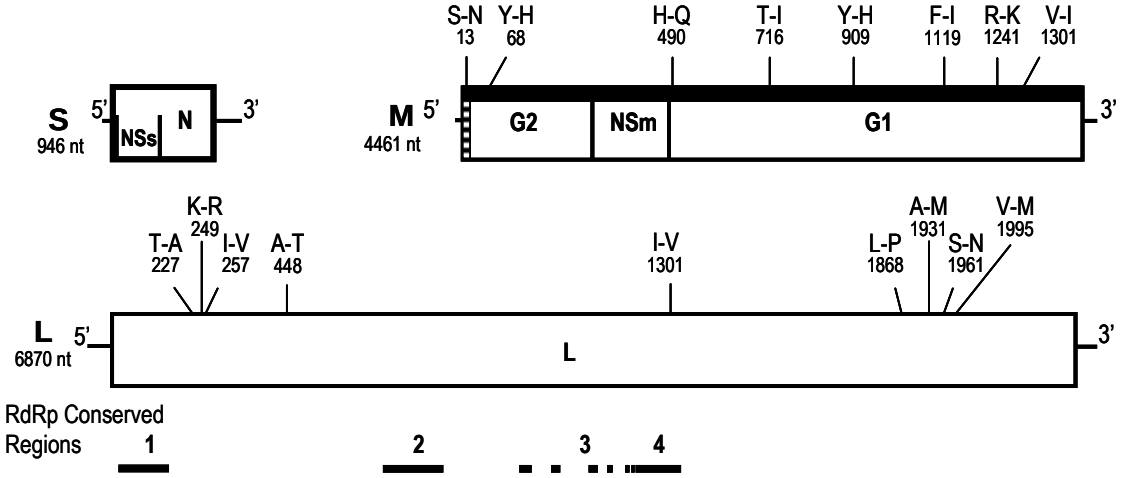

Figure 1-1. Schematic representation of the tripartite genome of Tensaw virus (TENV). All three segments are presented in the positive sense. The expected proteins coded by each segment, and the amino acid (aa) differences between two TENV isolates [TENV-FL06 (first aa) and TENV-FE3-66FB (second aa)] as well as the specific aa location are indicated. Checkered region at the beginning of the M coding region, represent the signal peptide. The M segment shows the translated polyprotein before (solid bar) and after cleavage (open boxes). Lines flanking the boxed areas represent the untranslated 5' and 3' regions (UTR). Lines under the L segment indicate the general location of the four conserved regions found in RdRp. Figure from Watts et al. (2009).

Table 1-1. Genomic organization of Tensaw (TENV) viral isolate TENV-FL06. From Watts et al. (2009)

Segment (nucleotide size)	Nucleotide Position of mRNA	Size in nucleotides	Size in amino acids	Protein encoded
Small (946)	75-776	702	233	Nucleocapsid
	94-399	306	101	Nonstructural protein (NSs)
	481-696	216	71	Uncharacterized ORF
Medium (4461)	44-4354	4311	1436	Polyprotein precursor
			17	Signal peptide
			286	Glycoprotein (G_n)
			175	Nonstructural protein (NSm)
			958	Glycoprotein (G_c)
Large (6864)	50-6766	6716	2238	RNA-dependent RNA polymerase

CHAPTER 2
THE EFFECTS OF ANTIVIRAL RIBONUCLEIC ACID INTERFERENCE ON TENSAW VIRUS REPLICATION

Introduction

Antiviral Ribonucleic Acid Interference (RNAi) against Arboviruses

Viruses from all three major families of mosquito-transmitted arboviruses – *Togaviridae*, *Flaviviridae*, and *Bunyaviridae* – are responsive to antiviral ribonucleic acid interference (RNAi). In general, these studies indicate that RNAi efficacy varies tremendously, based upon factors like viral target choice, the method of RNAi induction (i.e., whether inverted repeat ribonucleic acid [RNA], short-interfering RNA [siRNA], or short-hairpin RNA [shRNA] expressing plasmids are used), and model system. A major complication associated with this is escape from silencing, in which virus continues to proliferate in the face of antiviral RNAi therapy. Because of the mutability of RNA viruses, they can utilize a number of mechanisms to evade the RNAi response, including induction of or selection for mutations, insertions and deletions at the target site or in viral promoter and enhancer regions that abrogate the sequence-specific interference (see Shah and Schaffer 2011 for review). However, genomic changes are not responsible for all cases of viral escape (Gitlin et al. 2002, Soldan et al. 2005, Wu et al. 2005, Dash et al. 2008).

A majority of the antiviral RNAi literature pertaining to arboviruses focus on viruses in the *Togaviridae* and *Flaviviridae* families, and as such, there are only a few studies that investigate the therapeutic capacities of RNAi on medically important *Bunyaviridae*. In these studies, both structural and non-structural components of the *Bunyaviridae* have been targeted with varying degrees of success.

Levin et al. (2006) transfected three siRNA specific to Akabane orthobunyavirus S segment 24 hours prior to infection (hpi), and observed 99% reduction in viral proliferation according to quantitative reverse transcriptase polymerase chain reaction (RT-PCR), immunofluorescence, and endpoint virus titration up to 48 hpi. Soldan et al. (2005) pretreated human 293T cells and mosquito C6/36 cells with single siRNAs specific to all three segments of La Crosse orthobunyavirus (LAC), and reported a reduction in plaque-forming units (PFU) between 0-2.5 log knockdown for 12 of 13 siRNAs tested, but found up to a 3.8 log reduction in plaque-forming units (PFU) at 72 hpi in cells that were pre-inoculated with siRNA specific to the overlapping NSs/N reading frame. Interestingly, the authors found a considerably higher sequence diversity at the siRNA-target region at 72 hpi in LAC targeted with S-specific and M-specific siRNA, and observed viral variants in siRNA-treated samples that were not found in mock treated infections. This suggests that minority variants not seen in untreated cells were selected for in response to targeting the wild-type, or the *de novo* induction of variants not present in the viral population prior to treatment.

Flusin et al. (2012) also targeted all three segments with antiviral siRNA and found that S-specific siRNA most efficiently inhibited Hazara nairovirus nucleoprotein production up to 48 hpi, according to enzyme-linked immunosorbent assay (ELISA) and Western blot assays. Scott et al. (2012) designed plasmids coding for short-hairpin RNA (shRNA) specific to the non-overlapping NSs and N genes of Rift Valley Fever virus (RVF), in the genus *Phlebovirus*. Interestingly, the shRNA cassettes were able to significantly reduce the expression of plasmids coding for the NSs and N proteins and to abrogate the cytotoxic effects of the NSs protein. However, shRNA-induced therapeutic

prevention of RVF infection in cell culture was minimal. According to ELISA assays, N-specific shRNA only marginally reduced the expression of the putative nucleoprotein (15.5% by 72 hpi), whereas NSs-specific shRNA did not affect the production of nucleoprotein protein by RVF-infected cells. These results suggest that while shRNA-expressing plasmids may appear to effectively target bunyaviral RNA sequences for destruction, RNAi may not necessarily inhibit viral proliferation during active infection, where virus may eventually overcome antiviral treatment.

Because of the paucity of literature on how bunyaviruses respond to antiviral RNAi, questions about its efficacy remain unanswered. Most of these studies treated knockdown as a binary event; either knockdown was achieved in the first 48-72 hours following initial infection or it was not. Little is known about RNAi-induced knockdown of bunyaviruses at time points after 72 hours. Soldan et al. (2005) saw an increase in LAC titer beginning between 48 and 72 hours in many siRNAs tested, suggesting the reestablishment of infection after initially successful knockdown; however, none of their preventative siRNA assays provided data beyond 72 hpi. Similarly, in Levin et al. (2006), virus replication was reduced by siRNA pre-treatment but still apparent between 24 and 48 hpi according to RT-PCR and viral titration assays, but there was no information on the fate of virus replication thereafter. Because few studies have examined the effects of antiviral siRNA on bunyaviruses beyond 72 hours, it is not known whether these siRNA halt infection altogether or whether treatment simply delays the onset of infection, or whether emergent virus is perhaps immune to the targeting siRNA. Furthermore, of these studies, only Soldan et al. (2005) examined the effect of siRNA treatment on the sequence of the virus.

Fitness of Escape Populations

Antiviral RNAi has been shown to affect the viability of emergent virus in several model systems, and those fitness differences depended primarily on whether RNAi selection was maintained following escape from silencing. Gitlin et al. (2005) utilized viral competition assays to demonstrate that poliovirus that escaped siRNA silencing in HeLa cells was only more fit than wild-type poliovirus when siRNA selection remained in place; otherwise, RNAi-resistant variants were no more fit than wild-type virus in untreated cells.

Beyond the Gitlin et al (2005) study, most of the work on the viability of escape mutants has been performed in HIV research, where there is a history of utilizing siRNA and shRNA-induced silencing to reliably select for or induce mutations that have reduced fitness. Some escape mutants in HIV-1 have been well-characterized and can be reliably induced. Accordingly second-generation shRNA have been designed to anticipate and suppress potential escape mutants (Sabariegos et al. 2006, Schopman et al. 2010, Shah et al. 2012, Schopman et al. 2012). Similarly, after identifying common escape variants in poliovirus, Gitlin et al. (2005) designed pooled siRNA that included siRNA against those variants, which resulted in efficacious suppression of viral replication. Hence, there are precedents for using patterns by which a virus escapes RNAi-induced silencing to create other effective treatments.

However, where antiviral RNAi in HIV-1 has been studied thoroughly, no research could be found about the fitness of escape populations in the arboviral families. Furthermore, antiviral RNAi studies have focused largely on one-host systems (e.g., HIV-1, poliovirus), where escape from silencing has focused on the viability of emergent virus in the same cells. Apparently, no studies have examined the effects of

antiviral RNAi on fitness across a two or more host system that requires host alternation to maintain transmission. Indeed, the stabilizing selection of alternating passage has been demonstrated *in vitro* and *in vivo* (Greene et al. 2005, Coffey et al. 2008, Vasilakis et al. 2009, Coffey and Vignuzzi 2011).

The research in this chapter examines how preventative antiviral siRNA treatment affects the siRNA-targeted region and the infectivity of Tensaw virus (TENV), an orthobunyavirus, for up to 120 hpi in several cell culture model systems. These findings will provide insight into how bunyaviruses are affected by antiviral RNAi therapy over a longer period of infection that described in previous studies. Moreover, this study analyzes how antiviral RNAi affects the fitness of TENV emerging from siRNA-treated monkey kidney cells (Vero) in three model systems: two vertebrate (Vero, human hepatocellular carcinoma HeLa cells) and one mosquito (*Ae. albopictus* C6/36) cell lines.

Materials and Methods

Model Systems

Tensaw virus

Tensaw virus isolate TENV-FL06 was isolated from mosquitoes collected in Alachua County, Florida in 2006 as described in Watts et al. (2009). The initial stock was generated when mosquito homogenate was inoculated in 1 X 10^5 Vero cells growing in Leibovitz's L-15 media containing 5% fetal bovine serum (FBS), gentamycin, penicillin-streptomycin sulfate solution, and amphotericin B. Following visible cytopathology at 6 days post-infection (dpi), viral supernatant was used to infect 7 x 10^5 Vero cells in T-25 flasks. Twenty-four hours later, viral supernatants were collected and frozen at -70°C. Virus titer was then determined with endpoint dilution titration to

determine mean tissue culture infectious dose per milliliter ($TCID_{50}$/mL) (Appendix A). Tensaw virus stocks generated by this method contained approximately 1 X 10^7 $TCID_{50}$/mL.

Cell lines

Continuously multiplying "immortal" cell lines provide a powerful tool for assessing the effects of viral replication in an *in vitro* model. Three cell lines will act as models for host systems in my studies.

HeLa cells, a human cancer cell line isolated from Henrietta Lacks in 1951, have stood in as a popular in vitro model for human cancer and virology studies for more than 50 years (Lucey et al. 2009). Cantell (1961) demonstrated that HeLa cells display a robust IFN response to viral infection, and as such, our lab has utilized these cells in a number of experiments to assess the effects of viruses in immune-competent vertebrate cell lines. HeLa cells were maintained at 37°C incubators without carbon dioxide (CO_2) buffering in Leibovitz's L-15 media with 5% fetal bovine serum (FBS) and 50 micrograms per milliliter (µg/mL) gentamycin unless otherwise stated.

Vero cells are a continuous aneuploid derived from the epithelial kidney cells of African green monkey, *Cercopithecus aethiops*. Vero cells cannot produce IFN-β because of a genetic deletion, and as such have an attenuated interferon-based antiviral response (Desmyter et al. 1968, Emeny and Morgan 1979, Chew et al. 2009). Vero cells are used by our lab because they are highly permissive to a range of viruses and because of their observable cytopathologic effects (CPE) caused by infection. Vero cells were maintained at 37°C incubators without CO_2 buffering in Leibovitz's L-15 media with 5% FBS and 50 µg/mL gentamycin unless otherwise stated.

C6/36 cells, derived from larval *Ae. albopictus* mosquitoes by Singh (1967), are used in many studies as models for viral infection, as they are susceptible to infection by a wide range of viruses (Chen et al. 2004a, Cirimotich et al. 2009). Brackney et al. (2010) demonstrated that C6/36 cells have an incomplete RNA interference immune response to viruses. Scott et al. (2010) have identified a single deletion at nucleotide (nt) 2460 in the complementary deoxyribonucleic acid (cDNA) of the C6/36 Dicer-2 gene– a major component of the antiviral RNAi response – causing a frame shift and accordingly an early termination codon. This paper demonstrates that the putative Dicer-2 protein is dysfunctional, suggesting that this dysfunction may contribute to C6/36 cells producing high titers of virus (Brackney et al. 2010, Scott et al. 2010). However, ~22 nt anti-GFP siRNA is introduced, there is a reduction in GFP expression in C6/36 cells, suggesting that RNAi machinery downstream of the Dicer-complex is functional, and that transfected siRNA can still knockdown gene expression (Brackney et al. 2010). C6/36 cells were maintained in 26°C incubators without CO_2 buffering in Leibovitz's L-15 media with 5% FBS and 50 µg/mL gentamycin unless otherwise stated.

SiRNA Design and Construction

Candidate virus specific siRNA were provided according to criteria laid out by Tuschl et al. (2004) by Sigma-Aldrich using algorithms that take into account secondary structure, G-C content, and particular purine-pyramidine preferences at specific positions in the siRNA. SiRNAs were chosen for use in our work from candidate siRNA sequences provided by Sigma-Aldrich that we further analyzed with Mfold, a web-based program that predicts secondary folding of target mRNAs according to thermodynamics (Zuker et al. 2009), and according to genomic sequence conservation across viruses in the *Bunyaviridae* family (Elliott 1996), in order that we may specifically target less

mutable viral sequences that are required for the function of putative viral proteins. The two Tensaw-specific siRNA sequences are:

NSs-siRNA: targeted region (cDNA): 5' GTCTTACCCTCCACAGGCT 3'; sense siRNA: 5' GUCUUACCCUCCACAGGCUdTdT 3'; antisense siRNA: 5' AGCCUGUGGAGGGUAAGACdTdT 3'.

RdRp-siRNA: targeted region (cDNA): 5' AGCTTGAAATAAATGCAGA 3'; sense siRNA: 5' AGCUUGAAAUAAAUGCAGAdTdT 3'; antisense siRNA: 5' UCUGCAUUUAUUUCAAGCUdTdT 3'.

NSs-siRNA is homologous to nt 340-358 on the S-segment, and lies on a conserved region of the overlapping N/NSs open reading frames according to alignment with other viruses in the Bunyamwera serogroup. Similarly, RdRp-siRNA is homologous to nt 3141-3159, and aligns with one of four polymerase motifs that are also conserved amongst orthobunyaviruses (Poch et al. 1989).

A negative control (nonsense) siRNA was purchased from Lifetech, and was analyzed by Lifetech technical support to ensure that the siRNA had no homology to sequences in the Tensaw viral genome or the genomes of any cell lines used in our studies.

Seeding and Transfection of Adherent Cells

Vero, HeLa, or C6/36 cells were seeded in 24 cluster well plates at 6 X 10^4 cells per well approximately 24 hours prior to transfection. TENV-specific or nonsense siRNAs were transfected into Vero or HeLa cells with the lipid-based cationic liposome-mediated siRNA transfection reagents RiboJuice™ (EMD Chemicals) or siQuest™ (Mirus Bio) per manufacturer instructions, approximately 24 hours prior to infection with TENV.

Initial Infection with TENV

Cells were infected with 150 µL of media containing TENV at multiplicity of infection of 0.01 (MOI 0.01) in Vero cells or at MOI 0.1 in HeLa cells for 1 hour. Viral supernatant was then removed from the cells, and the cells were washed twice with 1X Hank's Balanced Salt Solution (Gibco). 1 mL of fresh media was then added to cells and then incubated at 37°C. Cell supernatants and whole cell lysates were collected separately at 0, 24, 48, 72, 96, and 120 hpi, and stored at -70°C until subsequent assays were performed.

Sequencing of Emergent TENV Populations

RNA was extracted with TRIzol reagent from supernatants collected at 24, 48, 72, 96, and 120 hpi from TENV-infected Vero and HeLa cells that had been transfected with TENV NSs siRNA prior to infection. RT-PCR was performed on extracted RNA with primers that amplified the entire NSs gene, which contains the NSs-siRNA target region. The resultant amplicon was cloned into the pGEM®-T Easy Vector (Promega) and transformed into Max-Efficiency® DH5α™ *E. coli* cells (Lifetech), which were grown overnight on selective media. Colonies containing the cloned plasmid were grown in selective media, and the siRNA target region of the plasmid was sequenced at the UF Interdisciplinary Center for Biotechnology Research (ICBR) using BigDye® Terminator Chemistry v1.0 (ABI).

Determination of Infectious Viral Load

Infectious viral load was determined by endpoint dilution titration to determine the mean tissue culture infectious dose per mL ($TCID_{50}$/mL) in viral supernatants (see Appendix A for protocol and calculation of titer).

Confirmation of Sustained RNAi-induced Knockdown

The Dual-Luciferase® Reporter Assay System (Promega) allows for the quantitation of the expression of two exogenous luminescent signals, firefly luciferase and *Renilla* luciferase, from cellular lysates. Both luciferase enzymes are active without post-translational modification, retain enzymatic activity in the cellular lysate, and produce a luminescent signal when exposed to their respective substrates. This signal can be quantified by a microplate reader. This assay was modified and optimized in Skalsky et al. (2007) and Haecker et al. (2012) to examine knockdown efficiency in mammalian cell cultures. To do this, cells are cotransfected with firefly and *Renilla* luciferases. The modification and validation of the pGL3 vector containing the firefly luciferase is described in full in Skalsky et al. (2007). In short, nucleotides 658 to 2495 of the 3' UTR from murine BACH-1 gene containing four miRNA binding sites for the K12-11 microRNA mimic (miR-K12-11) was cloned downstream of the firefly luciferase gene coding region. Therefore, miR-K12-11, which utilizes the cellular RNA interference machinery similar to the antiviral siRNA in this study, suppresses the expression of firefly luciferase. Accordingly, miR-K12-11 is co-transfected with the two luciferases in the treatment condition. At the appropriate time point following transfection, cells are lysed with a lysis buffer, and the ratio of the expression of the firefly and *Renilla* luciferases are compared in the presence and in the absence of the microRNA to quantitate the relative reduction of the firefly luciferase expression.

Transfection of Vero cells for the luciferase knockdown assay is described in full in Appendix B. Because the transfection-infection protocols examined virus knockdown at 132 hours after the initial transfection – i.e., 24 hours for transfection, followed by 120 hours of infection – cells in the luciferase knockdown assay were harvested accordingly

with Passive Lysis Buffer (Promega), and the activity of the two luciferases were quantified in succession using the Dual-Luciferase® Reporter Assay kit (Promega) Dr. Rolf Renne's laboratory using an MLX Microplate Luminometer (Dynex).

Infection with Viral Escape Populations

In order to assess the infectivity of virus emerging from siRNA-treated cells across several hosts, Vero, HeLa, or C6/36 cells were seeded in 24 cluster well plates at 8 X 10^4 cells per well approximately 24 hours prior to infection. Once the $TCID_{50}$/mL was determined for cell supernatants from each of the four Vero cell treatments from Passage 1 (NSs-siRNA, RdRp-siRNA, nonsense siRNA, and no treatment with siRNA or transfection reagent), stocks stored at -70°C were then thawed and diluted accordingly with L-15 Leibovitz's media in order to infect untreated Vero cells at MOI 0.01 or untreated HeLa or C6/36 cells at MOI 0.1 for 1 hour with emergent virus from all four Vero P1 treatments using methods described above for initial infection with TENV. Cell supernatants for all second-passage infections were collected every 24 hours up to 120 hpi, and stored at -70°C until endpoint dilution assays ($TCID_{50}$/mL) to assess titer were performed as described in Appendix A. Three or four replicates of infection of Vero, HeLa, C6/36 with Vero P1 escape populations were performed.

Statistical Analysis

A mixed effects model was used for analysis of all viral growth data using the lme4 package and the lmer function in the R software environment, version 2.15.2, with time and siRNA treatment condition predictor variables. Time was treated as continuous, and linear and quadratic terms were included in the model. Interaction effects for siRNA treatment condition with both linear and quadratic time trends were also included. Random effects for replicate and treatment batch are included to account

for random variability due to these factors. For the analysis of the growth curve of Vero escape populations in C6/36 cells, a likelihood ratio test comparing a linear-only model with the quadratic model failed to reject the null hypothesis that data fit both models equally well, so the simpler (i.e., linear) model was used. For all other growth curves, a quadratic model was used for analysis. To account for multiple comparisons, the method described by Benjamini and Hochberg (1995) was applied to the pairwise differences analyses to adjust for false discovery rate. Therefore, the values have been adjusted accordingly, so that a p-value of 0.05 or lower is still considered significant for all growth curve analysis data.

A student's t-test calculated with Microsoft Excel was used to analyze the luciferase repression assay data, and a p-value of 0.05 or lower was considered significant.

Results

Reduced TENV Titer in HeLa and Vero Cells

To assess how TENV viral replication is affected by transfection with TENV-specific siRNA, Vero and HeLa cells previously transfected with NSs-siRNA or RdRp-siRNA or with nonsense siRNA were infected with TENV at MOI 0.01 and MOI 0.1, respectively. Results from the primary TENV infection (referred to as Passage 1 or P1) are shown in Figure 2-2 and 2-3. In Vero cells infected with TENV, there were no significant differences in viral titers in cells previously transfected with nonsense siRNA and untransfected cells ($p > 0.082$) at any time point, which indicated that the transfection reagent and nonsense siRNA did not affect viral proliferation in Vero cells. Transfection of Vero cells with NSs-siRNA resulted in a reduction in TENV titers that were significantly lower than the no-siRNA condition at all time points ($p < 0.002$) and

significantly lower than nonsense siRNA-time points at all time points except 96 hpi, in which there was a borderline significance ($p < 0.048$ for 24, 48, 72, and 120 hpi; $p = 0.082$ for 96 hpi). However, though significantly lower than the untreated control at all time points, viral titer in NSs-siRNA treated cells did increase at each point after t = 24 hpi, and in fact showed an increase in TENV titer of nearly four logs between 48 and 120 hpi. This increase was the highest increase seen across conditions in that span of time. However, regression analysis of the time trends of the four growth curves from 0-120 hpi did not indicate that there were significant differences between any treatments in the rate of TENV growth over time ($p > 0.908$).

Whereas TENV titer was lower as a result of NSs-siRNA treatment throughout the 120 hours of exposure, TENV titer in RdRp-siRNA treated Vero cells was significantly lower than the infection-only cells at all points 48 hpi and later ($p < 0.040$), but was never significantly different from the nonsense siRNA-treated Vero cells at any time point ($p > 0.154$), suggesting that there is no additive silencing effect induced by RdRp-siRNA in Vero cells.

HeLa cells were transfected with the same three siRNA (NSs-siRNA, RdRp-siRNA or nonsense siRNA) prior to infection with TENV-FL06, and the resulting TENV-P1 supernatants, collected from 0 to 120 hpi then titered in Vero cells. TENV-infected HeLa cells that were not treated with transfection reagent or siRNA had a significantly higher titer than cells treated with NSs-siRNA, RdRp-siRNA or nonsense siRNA at all time points ($p < 0.033$) (Figure 2-3). This indicated that there was a significant effect of treatment with siRNA and transfection reagent, because it was higher at all time points for all conditions than even the nonsense siRNA control. Transfection of HeLa cells with

RdRp-siRNA did not result in a significant reduction in viral titer compared to viral infection in nonsense siRNA-treated HeLa cells at any time point, suggesting again that RdRp-siRNA does not confer any significant antiviral resistance. On the other hand, NSs-siRNA treatment resulted in viral titer significantly lower than that of infected cells without siRNA treatment at all time points ($p < 0.0002$) and lower than nonsense siRNA-treated cells up to 96 hpi ($p < 0.003$ up to 96 hpi; $p = 0.11$ at 120 hpi). In fact, TENV titer increased only 1.5 log during the 120 hours of infection when treated with NSs-siRNA, compared to a 2.3 or more log increase in all other RdRp-siRNA treatments. Finally, regression analysis indicated that the rate of TENV growth differed between all conditions ($p < 0.039$), aside from the RdRp-siRNA and nonsense siRNA growth curves, which were not significantly different ($p = 0.861$). In other words, where transfection with siRNA nonspecifically reduces the rate of TENV growth in HeLa cells compared to infection in untransfected HeLa, NSs-siRNA reduces the rate of TENV growth even more.

These results showed that NSs-siRNA was capable of causing a decrease in viral titer in both vertebrate cell lines across time, though NSs-siRNA only significantly reduced the rate of growth in HeLa cells.

Sequence Analysis of Emergent TENV in Supernatant

To determine if the increase in the number of infectious virions that followed at 72 hours after NSs-siRNA treatment in Vero cells was due to the selection for non wild type variants, the NSs gene was sequenced and compared to the TENV-P1 virus that was collected from siRNA untreated cells (TENV-P1V). The results from a small number of clones ($n = 9, 10, 27$, in the three replicates tested) showed that only one TENV isolate from NSs-siRNA treated cells had sequence modifications that alter the siRNA target

sequence. One had a point mutation (from GTCTTACCCTCCACAGGCT to GTCTCACCCTCCACAGGCT) that would result in no amino acid replacement for the NSs gene, but would cause a replacement in the nucleoprotein (N) gene from a leucine to a proline. All the viral clones belonging to the untreated controls tested (n=10) showed no nucleotide modification in the NSs-siRNA target region. The altered amino acid presumably should not affect the function of the TENV nucleoprotein, since other orthobunyaviruses have been sequenced containing a proline in the same location of the translated protein (Yandoko et al. 2007). Moreover, in all three instances where TENV NSs was sequenced, there were no consensus mutations in the regions flanking the NSs-siRNA target site that would indicate changes to viral RNA secondary structure.

Subsequent analysis of seven to ten sequences of TENV emerging from TENV NSs siRNA in infected HeLa or Vero cells at 24, 48, 72, 96, and 120 hpi did not reveal any changes to the target region of siRNA-treated TENV at any time point, suggesting that changes to the siRNA target sequence were not always associated with escape from silencing with antiviral siRNA in this system.

Luciferase Assay

In order to confirm that RNAi-induced expression persists in Vero cells for the length of the experiment, a dual luciferase repression assays were performed in which plasmids coding for firefly luciferase and *Renilla luciferase* were co-transfected with a microRNA mimic (miR-K12-11) that suppresses expression of firefly luciferase. At 132 hours after transfection, firefly luciferase expression was reduced 96.35% compared to cells that were transfected with the luciferase reporter plasmids only (See Figure 2-1). However, because of the high variation in the no microRNA condition, the differences between conditions were only borderline significant ($p = 0.052$).

Infection of Vero, HeLa, and C6/36 Cells with Viral Escape Populations

To assess whether antiviral siRNA treatment affects replication in several cell culture systems, TENV supernatants collected from the four treatments described – NSs-siRNA, RdRp-siRNA, nonsense siRNA, and no siRNA treatments – at 120 hpi were diluted to uniform viral loads, and used to infect Vero, HeLa, or C6/36 cells.

Growth curves and $TCID_{50}$/mL data for all three second-passage infection regimens are shown in Figures 2-4, 2-5, and 2-6. All the information concerning the TSV-P1 virus that had been used as inoculum is presented in parenthesis including the specific siRNA used to transfect cells prior to the first infection and the time of the TSV-P1 collection. For example “TENV (P1V NSs 120) P2H” represents TENV Passage 2 (P2) virus in HeLa cells (H) that were infected with a P1 inoculum collected from infected Vero cells (P1V) at 120 hpi after those cells had been transfected with the NSs-siRNA.

In all three passage regimens, no significant differences were apparent in growth rate of P1 virus from either TENV-specific siRNA treatments compared to virus from untreated or nonsense siRNA-treated Vero cells, according to regression analysis of viral growth over time. ($p > 0.717$ for Vero to Vero passage; $p > 0.103$ for Vero to C6/36 passage; $p > 0.596$ for Vero to HeLa passage). Moreover, there were no significant differences in viral load between the four treatments at any time points in Vero to C6/36 passage ($p > 0.538$) or Vero to HeLa passage ($p > 0.199$). In the Vero to Vero passage, the titer of TENV from RdRP-siRNA treated cells was lower than TENV from untreated cells at 24 hpi ($p = 0.035$), but this difference disappeared in all subsequent time points. Otherwise, no other pairwise comparisons achieved statistical significance in Vero to Vero passage regimen ($p > 0.057$).

Discussion

Initial Escape from Silencing

To date, no studies have examined whether siRNA escape populations retained infectivity across hosts, and so our study examined how transfection of two siRNA homologous to conserved regions of TENV (NSs-siRNA and RdRp-siRNA) into Vero monkey cells affected viral growth in vertebrate and mosquito cell cultures, using change in viral titer in fresh cell cultures over time as a metric for fitness.

RdRp-siRNA did not inhibit viral replication any more than control siRNA in either Vero or HeLa cells. This is interesting for several reasons. It has been demonstrated that the L mRNA exists in the lowest quantity in other orthobunyavirus-infected cells (Rossier et al. 1988), and that the L segment product (the viral polymerase) is necessary for replication and transcription of the rest of the segments. Therefore it was believed that interfering with the typically low expression of the L segment would efficiently reduce general virus expression. Furthermore, sequence analysis determined that the RdRp-siRNA target region was highly conserved among orthobunyaviruses, While the function of this domain remains unclear in orthobunyaviruses, conservation of sequence would suggest that it is a functional domain, and therefore would be expected to be resistant to mutations that would otherwise allow for loss of sequence-specific interference. That said, other RNAi studies targeted other conserved domains on the bunyaviral L-segment, and therefore there is no other precedent for antiviral RNAi against the putative functional domain chosen in this study. Where the L-segment was targeted other studies, the efficiency of antiviral RNAi-induced knockdown varied widely, depending on the target chosen, even in highly conserved viral coding regions. For instance, depending on which region of the L segment they targeted with siRNA, Soldan

et al. (2005) observed between two-fold and forty-fold reduction in La Crosse titer in 293T and C6/36 cells compared to control siRNA-treated cells at 48 hours. Similarly, Flusin et al. (2012) saw significant knockdown of Akabane infection with only one of four L-specific siRNAs tested in human A549 cells. Several explanations are possible. Bunyaviruses express nucleoproteins that form ribonucleoprotein complexes with the genomic and antigenomic RNA (Elliott 1996). This may have prevented access of the RNA interference silencing complex (RISC) machinery to naked viral RNA. Additionally, because the guide strand of the RISC complex must bind to viral RNA to activate translational repression or mRNA degradation, secondary structures in or near the target region viral RNA may have similarly prevented adequate binding of the RISC complex to the target site.

On the other hand, siRNA against the S segment of Tensaw was effective at reducing viral titer in both Vero and HeLa cells. These findings were consistent with the findings of others in S-specific antiviral RNAi against bunyaviruses (Soldan et al. 2005, Levin et al. 2006, Flusin et al. 2012). Other studies have demonstrated that transfection in HeLa cells induces a non-specific antiviral interferon response to transfection reagents and siRNA (Li et al. 1998, Reynolds et al. 2006). Therefore, because RdRp-siRNA treatment did not reduce viral titer any more than nonsense siRNA treatment, this suggests that the RdRp-siRNA-related reduction in TENV titer was not a result of increased virus specific knockdown of viral gene expression by the RISC, but the result of general induction of the interferon response. NSs-siRNA reduced TENV growth even further than the nonspecific siRNA response, suggesting that NSs-siRNA provided additional antiviral protection.

Vero cells were also highly responsive to NSs-siRNA. Similar to results seen in Soldan et al. (2005), Levin et al. (2006), and Flusin et al. (2012), viral replication in Vero cells was significantly inhibited early in infection, as titer increased only about 0.5 log for the first 48 hpi, and remained significantly lower at later time points. However, by allowing infection to continue up to 120 hpi, our study suggests that antiviral RNAi merely delays the onset of infection, as viral titer increased dramatically after 48 hpi and eventually reached $10^{5.63}$ $TCID_{50}$/mL (or 4.26 X 10^5 $TCID_{50}$/mL) by 120 hpi.

These data suggest that short-interfering RNA targeting the overlapping open reading frames for TENV nucleoprotein and small-segment nonstructural protein temporarily inhibits TENV proliferation. In addition to the protecting naked RNA from recognition and cleavage by RISC, the nucleoprotein has several functions in facilitating the replication cycle for viruses in the *Bunyaviridae*. The nucleoprotein forms ribonucleoprotein complexes that induce and signal the transition between replication and transcription by the viral polymerase, forms multimers with other copies of the nucleoprotein, and interacts with viral RNA, viral glycoproteins, and possibly cellular proteins, though the nature and function of these interactions are unclear (Leonard et al. 2005, Eifan and Elliott 2009, Longhi 2009). Furthermore, the region of the S segment targeted by NSs-siRNA in this study was found to be highly conserved among orthobunyaviruses, and was found to be necessary for nucleoprotein-nucleoprotein interactions in Bunyamwera, another orthobunyavirus in the same serogroup as TENV (Leonard et al. 2005, Eifan and Elliott 2009). In light of the many putative functions of TENV nucleoproteins, it makes sense that antiviral RNAi that targets and effectively disrupts nucleoprotein synthesis has been shown inhibit or delay bunyaviral replication.

Moreover, the NSs gene also lies within the target region for NSs-siRNA. Soldan et al. (2005) suggest that the NSs protein suppresses cellular RNA silencing machinery in orthobunyaviruses, which is the very network that our study induces to stop viral replication. Considering the interferon response is defective in Vero cells, reducing the expression of the viral NSs gene may rescue the antiviral RNAi response. It makes sense that detection and downregulation of viral RNA translation by the RNAi response, which is active in vertebrate systems, remains a major antiviral response in the absence of an interferon response. Moreover, in HeLa cells, where TENV NSs purportedly antagonizes both the interferon response and the antiviral RNAi response, siRNA-induced knockdown of the overlapping reading NSs/N region of TENV appears to effectively suppress TENV replication, as is apparent in light of the inhibition that NSs-siRNA provides against TENV infection beyond the general protection conferred by the nonsense siRNA.

The persistence of antiviral RNAi therapy varies widely across the model system and the virus studied. Several studies have indicated that the degradation of siRNA and cell division may dilute siRNA dose over time (Novina et al. 2002, Chen et al. 2004b). However, while there were no measures taken to examine whether NSs-siRNA and miR-K12-11 stability differed significantly across time, we found that RNAi appeared to persist in our cell culture system for 132 hours following single-dose treatment with 50 pmol, or approximately 300 micromolar (μM), miR-K12-11 at 96.35% knockdown of the firefly luciferase target. However, statistical analysis of the knockdown response can only be considered borderline significant at 132 hpi ($p = 0.052$), likely because of the highly variable response in the no miR-K12-11 control. Alkhalil et al. (2009) observed

viral gene knockdown for up to 7 days in Vero E6 cells transfected with 10 μM of antiviral siRNA, a considerably lower concentration than used here, and therefore there is some precedence for extended antiviral RNAi in Vero cells. However, the lack of efficacious knockdown at 132 hours post-transfection would help explain why the differences in antiviral response of NSs-siRNA did not remain statistically different from nonsense siRNA treatment up to 132 hours post-transfection (i.e., 120 hours post-infection) in Vero cells.

Interestingly, despite successful inhibition by NSs-siRNA at 72 hpi in Vero cells, TENV that emerged from NSs-siRNA-treated Vero cells following escape from silencing at 72 hpi typically did not show any mutations at the siRNA target site, as we saw only one instance of a non-target sequence in the 46 samples that were sequenced at 72 hpi. This suggests that mutations were not wholly responsible for evasion of the induced immune response, and that alternate mechanisms are thus responsible for evasion of silencing. Viral titer in the supernatant increases even in the initial stages of infection in all treatment conditions, suggesting that siRNA inhibition is not 100% efficacious. Of the small sample of TENV sequenced from the supernatant of NSs-siRNA treated Vero cells at 24 and 48 hpi (n = 10 for both), none showed differences from the NSs-siRNA target sequence. It is possible that viral replication persists and eventually overwhelms the intracellular antiviral response, even when RNAi is induced by preventative inoculation with siRNA to the virus, and that even at low levels, replicating virus may be able to antagonize the cellular antiviral response.

Fitness of Escape Populations

Pairwise analyses of the growth rates of NSs-siRNA and RdRp-siRNA escape populations were not statistically different from virus grown in nonsense siRNA-treated

or no siRNA-treated cells. Furthermore, the titers of NSs-siRNA and RdRp-siRNA escape populations did not differ from the control infections in HeLa or C6/36 cells at any time point. While the titer of TENV from RdRp-siRNA-treated Vero cells was significantly lower than TENV from untreated cells in Vero cells at 24 hpi, this difference disappeared in all subsequent samples taken. Moreover, RdRp-siRNA did not reduce the infectivity of TENV any more than nonsense siRNA did at any time points. Therefore, our results suggest that the fitness of TENV collected from Vero cells at 120 hpi did not change as a result of siRNA selection in any of the three cell culture systems tested.

Going into this study, we hoped to use siRNA selection to select for non-target TENV variants or to select for or induce mutations that were deleterious to TENV fitness, in hopes that these variants might be less fit in other cell culture systems. However, our data suggests that RdRp-siRNA did not affect TENV replication or fitness by any metrics we used. Conversely, where NSs-siRNA did delay the onset of infection, our data suggest that mutations or selection for non-wild-type variants were not responsible for escape from silencing with NSs-siRNA, and that TENV virions with the NSs-siRNA target sequence persisted. Because NSs-siRNA treatment did not appear to change the sequence or infectivity of emergent virus, it does not appear that we were able to truly test whether inducing mutations or selecting for non-target viral variants would affect TENV fitness.

There may be several possible explanations why targeted viral sequences persisted. Firstly, there may be little diversity in the TENV target region, and hence there may not be a variety of non-target variants to select from. To that end, deep

sequencing of TENV prior to the study may have identified sequences with more variation. Similarly, siRNA targeting other NSs sequences may have identified more mutable regions of the TENV genome. Furthermore, mutations that would abrogate NSs or nucleoprotein function may not have been viable or may have had lower replication rates than targeted TENV, in which case only targeted variants would remain by 120 hpi.

Maintenance of NSs integrity may be advantageous to TENV fitness in Vero, where NSs presumably antagonizes the antiviral RNAi. However, bunyaviral NSs is not required for efficient replication in C6/36 cells (Blakqori et al. 2007, Szemiel et al. 2012), likely because of the dysfunctional RNAi response (Brackney et al. 2010, Scott et al. 2010). By applying siRNA selection pressure in C6/36 cells, where there may not be positive selection for the maintenance of NSs, TENV may have been more susceptible to selection for non-consensus sequence variants in the C6/36 cell culture system. Presumably, we may have been able to induce or to select for variants in C6/36 cells that were less viable when re-introduced to immune-competent cell systems. However, our attempts at siRNA-induced antiviral silencing in C6/36 cells were unsuccessful, so this possibility could not be examined here.

Incomplete silencing may also have been responsible for allowing siRNA-targeted TENV to escape silencing and persist in fresh cells. Berkhout and Das (2012) suggest that wild-type virus will always outcompete other escape mutants in the absence of siRNA selection. Indeed, this corroborates findings by Gitlin et al. (2005), where RNAi-resistant poliovirus that differed from wild-type variant only maintained a fitness advantage over the wild-type when siRNA selection was in place. NSs-siRNA

and RdRp-siRNA were designed according to the consensus sequence of TENV, and therefore, incomplete silencing would allow virions with the consensus sequence to escape siRNA selection and outcompete less fit variants. Holz et al. (2012) demonstrated that mutations in morbillivirus subjected to several rounds of RNAi selection were not evident until as many as 20 serial passages through siRNA-treated cells, suggesting that extended exposure to antiviral RNAi selection beyond the timeframe tested in our study may eventually induce mutations or select for non-target variants.

Even though we did not see mutations in siRNA target region, no further tests were conducted to examine whether siRNA selection caused other changes to viral populations that were not apparent in sequencing (e.g., change of particle-to-infectivity ratio, induction of formation of defective-interfering particles, etc.).

Conclusions

The research herein used Tensaw virus (Family *Bunyaviridae*, Genus Orthobunyavirus) as a model system for cell culture experiments examining the effects of inducing the antiviral RNA interference response *in vitro* on the infectivity and genome of an arbovirus. While one TENV-specific siRNA did delay the onset of infection in Vero cells, the siRNA sequences chosen did not induce or select for mutations that affected the viability of the virus that emerged from siRNA-selection, nor did they appear to affect the emergent viral population's fitness. However, this study provides valuable insight into the mechanisms underlying how arthropod-borne viruses evade the immune response and suggests future avenues for research into antiviral RNAi. For instance, it was supposed in many other bunyaviral studies that an initial reduction in titer was indicative of successful knockdown. However, our results suggest

that successful knockdown is marked by prolonged suppression of viral replication, and that even successfully targeted by RNAi at first can eventually overcome treatment. This suggests that factors other than target site mutations and selection for non-target variants need to be explored thoroughly. Similarly, a thorough examination of transcript levels, protein expression, and enzyme activity involved in antiviral RNAi in the face of virus-mediated suppression of the immune system by bunyaviral NSs protein expression in Vero or HeLa cells would uncover the timing and the interplay in the antagonistic host-pathogen relationship.

Future efforts should also examine whether using different siRNAs or multiple siRNAs will result in sustained, efficacious knockdown of bunyavirus production, as has been seen in other systems using siRNA therapies against bunyaviruses. Hepatitis C, and HIV (Henry et al. 2006, ter Brake et al. 2006, Kumar et al. 2008, Shah et al. 2011, Flusin et al. 2012). Furthermore, Flusin et al. (2012) demonstrated that siRNA therapy is enhanced when combined with treatment with ribavirin, a guanosine analogue that also inhibits bunyaviral replication *in vivo* and *in vitro* (Watts et al. 1989, Bente et al. 2010). This suggests that where single-dose regimens of siRNA do not work alone, combination with other antiviral treatments may make RNAi therapy a reality.

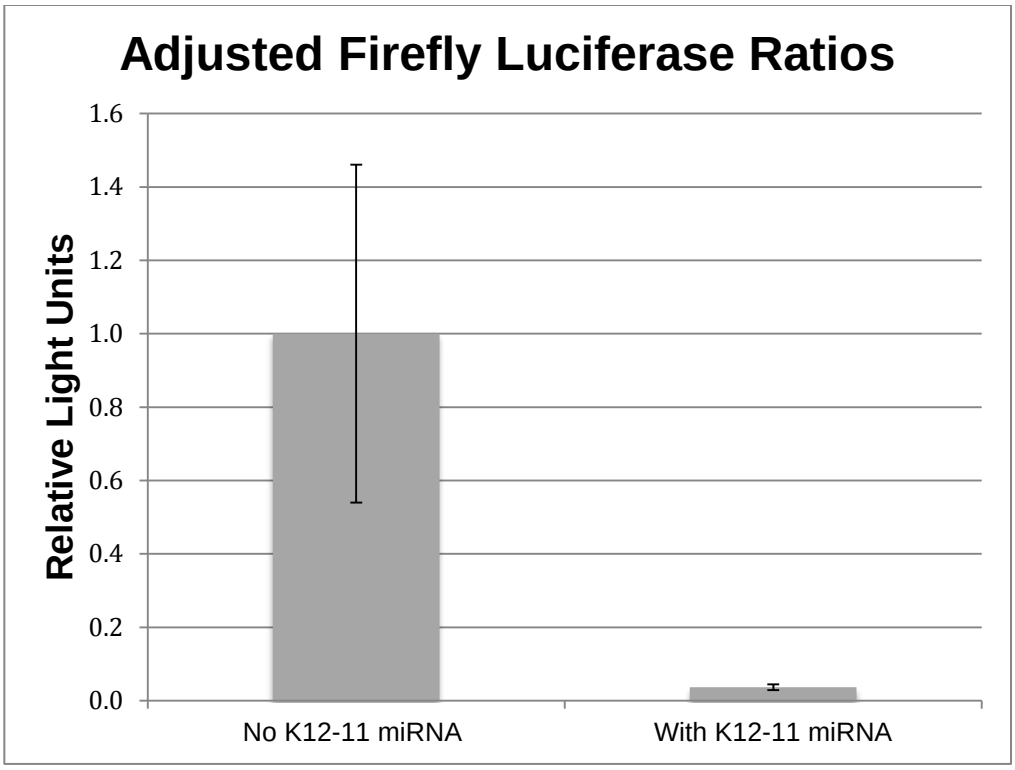

Figure 2-1. Adjusted firefly luciferase ratios**.** In order to confirm that RNAi-induced knockdown is sustained,Vero cells were co-transfected with three plasmids (one expressing firefly luciferase, one expressing *Renilla* luciferase, and an empty vector to equalize the volume transfected). In one condition, miR-K12-11, which suppresses the expression of firefly luciferase, was also transfected. The ratio of firefly luciferase to *Renilla* luciferase was measured at 132 hours post-transfection, and the ratio of the "No miR-K12-11" condition was adjusted to 1 to show differences as a measure of relative light units. Error bars indicate standard error.

Table 2-1. Growth of Tensaw Passage 1 (TENV-P1) isolates from siRNA-treated Vero cells

Hours Post Infection	TENV-P1-NSs	TENV-P1-RdRp	TENV-P1-Nonsense	No siRNA
0	1.50	1.50	1.50	1.50
24	1.65 a	1.59 b	2.57 b	3.00 b
48	2.06 a	3.36 ab	3.57 bc	4.61 c
72	4.02 a	4.50 ab	5.15 bc	5.33 c
96	5.15 a	5.88 a	6.29 ab	7.31 b
120	5.63 a	6.69 ab	6.58 bc	7.18 c

Vero cells were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) prior to infection with TENV-FL06 at MOI of 0.01. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. Error bars indicate standard error.

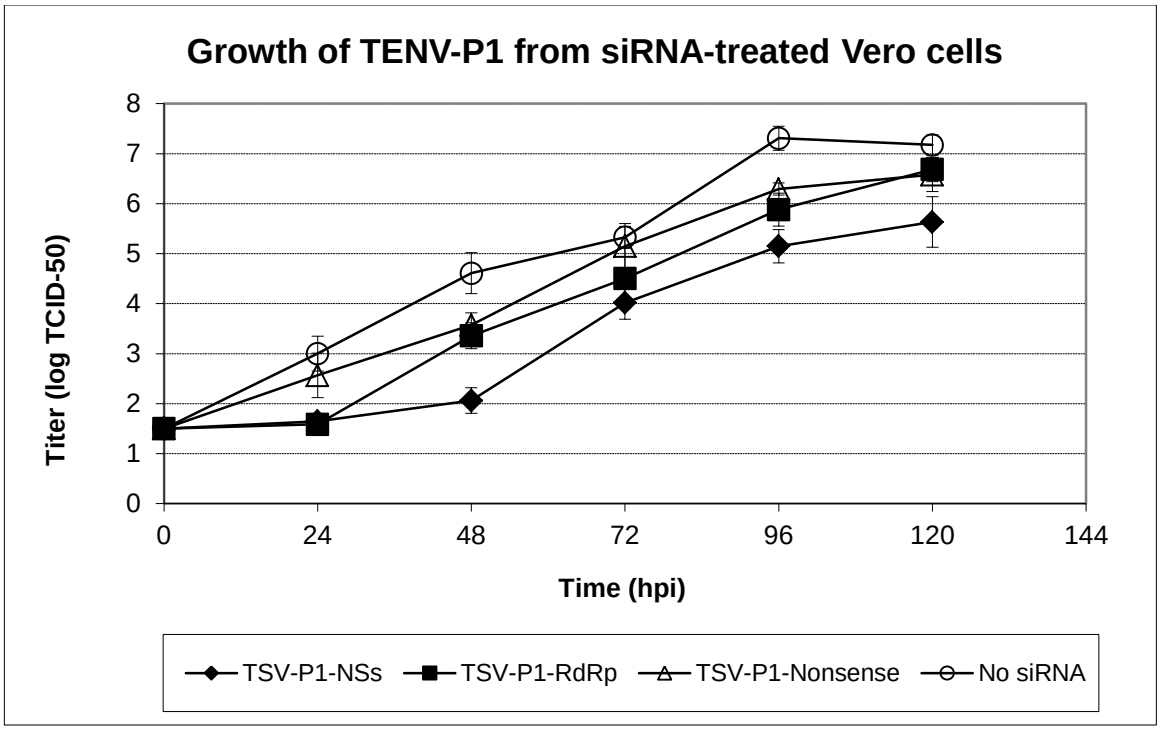

Figure 2-2. Growth of TENV-P1 isolates obtained after infecting short-interfering (siRNA) treated Vero cells. Vero cells were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) prior to infection with TENV-FL06 at MOI of 0.01. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. Error bars indicate standard error. See Table 2-1 for values.

Table 2-2. Growth of TENV-P1 isolates from siRNA-treated HeLa cells

Hours Post Infection	TENV-P1-NSs	TENV-P1-RdRp	TENV-P1-Nonsense	No siRNA
0	2.09	1.86	1.91	2.15
24	2.75 a	3.05 b	3.17 b	4.28 c
48	2.78 a	3.39 b	3.61 b	4.99 c
72	2.60 a	3.79 ab	3.88 b	6.05 c
96	3.27 a	4.16 b	4.41 b	6.69 c
120	3.50 a	4.18 b	4.30 b	5.93 c

HeLa cells were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) prior to infection with TENV-FL06 at MOI of 0.01. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. Error bars indicate standard error.

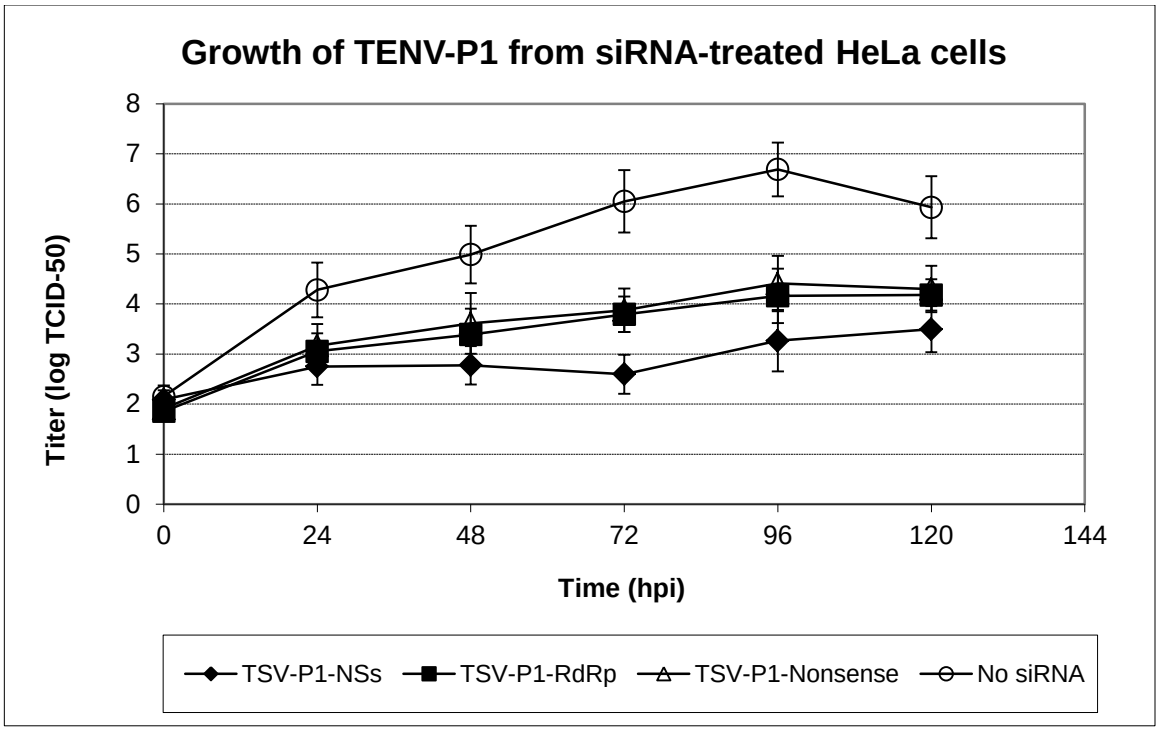

Figure 2-3. Growth of TENV-P1 isolates obtained after infecting siRNA-treated HeLa cells. HeLa cells were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) prior to infection with TENV-FL06 at MOI of 0.01. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. Error bars indicate standard error. See Table 2-2 for values.

Table 2-3. Growth of TENV from siRNA-treated Vero cells in Vero cells

Hours Post Infection	TENV (P1V NSs 120) P2V	TENV (P1V RdRp 120) P2V	TENV (P1V nonsense 120) P2V	TENV (P1V no siRNA 120) P2V
0	1.83	1.54	1.58	1.71
24	2.74 a	2.86 ab	2.57 ab	3.76 b
48	4.55 a	4.28 a	4.73 a	5.17 a
72	5.93 a	5.84 a	5.93 a	5.67 a
96	6.93 a	6.72 a	5.90 a	5.22 a
120	6.61 a	7.15 a	6.34 a	5.50 a

TENV from Vero cells that were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) was collected at 120 hpi, and diluted to MOI of 0.01 for infection of fresh Vero cells. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. (P1V ____ 120) indicates the siRNA treatment for the first passage of TENV in Vero cells. Error bars indicate standard error.

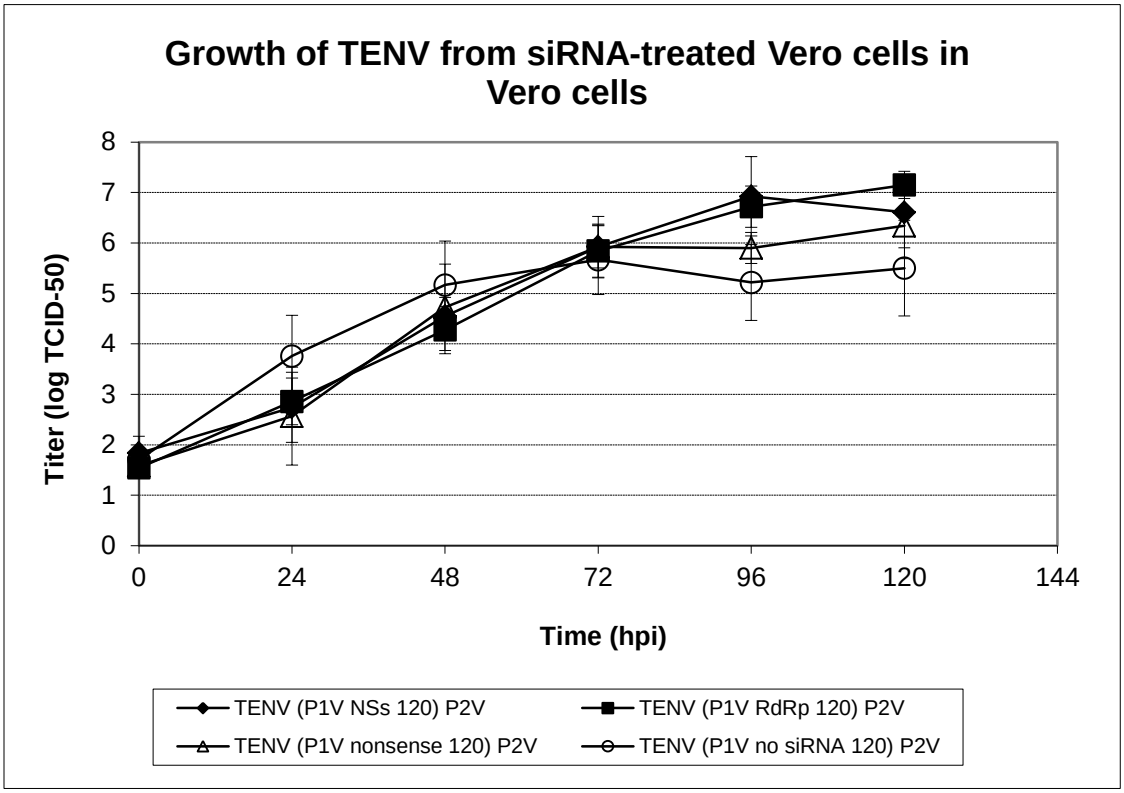

Figure 2-4. Growth of TENV from siRNA-treated Vero cells in Vero cells. TENV from Vero cells that were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) was collected at 120 hpi, and diluted to MOI of 0.01 for infection of fresh Vero cells. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. (P1V ____ 120) indicates the siRNA treatment for the first passage of TENV in Vero cells. Error bars indicate standard error. See Table 2-3 for values.

Table 2-4. Growth of TENV from siRNA-treated Vero cells in HeLa cells

Hours Post Infection	TENV (P1V NSs 120) P2H	TENV (P1V RdRp 120) P2H	TENV (P1V nonsense 120) P2H	TENV (P1V no siRNA 120) P2H
0	1.83	1.76	1.72	1.92
24	3.07 a	3.33 a	3.46 a	3.82 a
48	4.37 a	5.54 a	5.25 a	5.39 a
72	5.87 a	6.24 a	6.63 a	6.23 a
96	5.74 a	6.78 a	7.05 a	6.75 a
120	5.97 a	6.57 a	6.73 a	6.58 a

TENV from Vero cells that were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) was collected at 120 hpi, and diluted to MOI of 0.1 for infection of fresh HeLa cells. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. (P1V ____ 120) indicates the siRNA treatment for the first passage of TENV in Vero cells. Error bars indicate standard error.

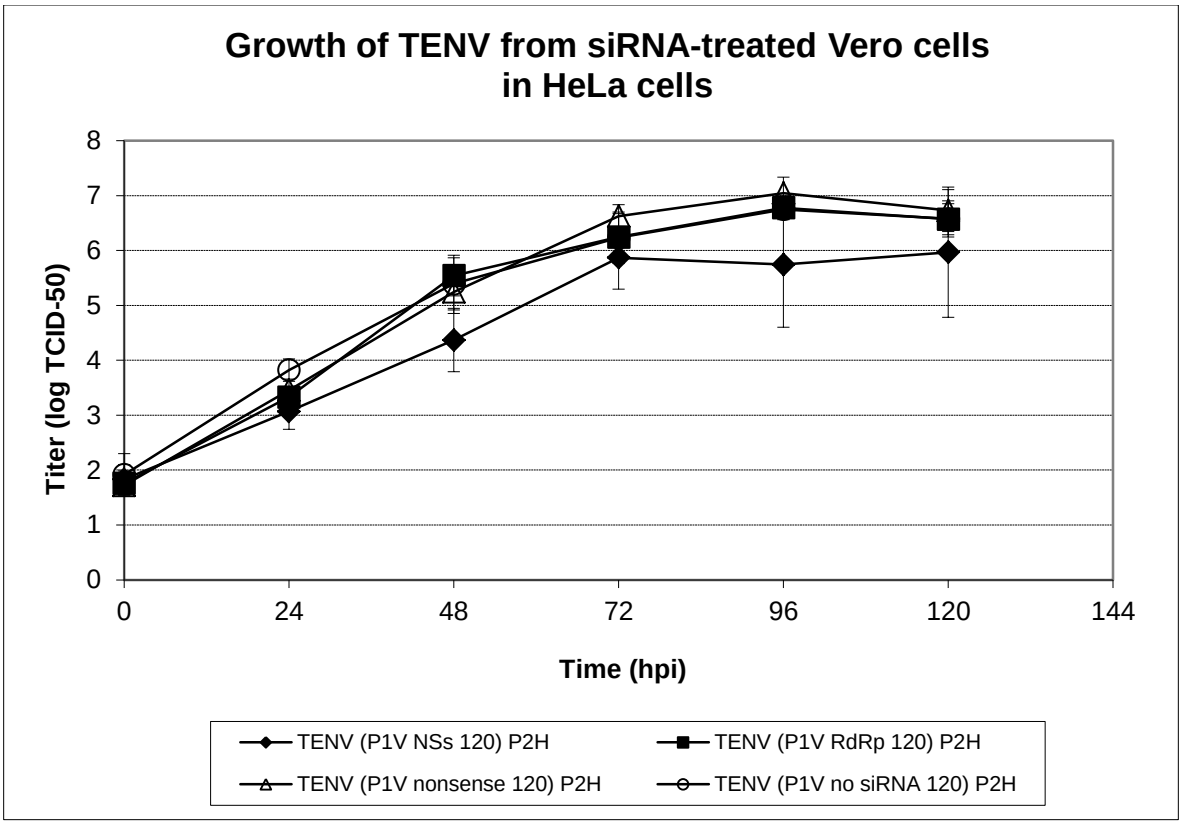

Figure 2-5. Growth of TENV from siRNA-treated Vero cells in HeLa cells. TENV from Vero cells that were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) was collected at 120 hpi, and diluted to MOI of 0.1 for infection of fresh HeLa cells. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. (P1V ____ 120) indicates the siRNA treatment for the first passage of TENV in Vero cells. Error bars indicate standard error. See Table 2-4 for values.

Table 2-5. Growth of TENV from siRNA-treated Vero cells in C6/36 cells

Hours Post Infection	TENV (P1V NSs 120) P2C	TENV (P1V RdRp 120) P2C	TENV (P1V nonsense 120) P2C	TENV (P1V no siRNA 120) P2C
0	1.50	1.60	1.80	1.94
24	4.12 a	2.93 a	3.21 a	3.27 a
48	3.53 a	3.53 a	3.60 a	4.20 a
72	3.94 a	4.38 a	5.44 a	4.84 a
96	5.67 a	5.47 a	6.05 a	6.15 a
120	6.18 a	6.15 a	6.91 a	6.86 a

TENV from Vero cells that were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) was collected at 120 hpi, and diluted to MOI of 0.1 for infection of fresh C6/36 cells. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. (P1V ____ 120) indicates the siRNA treatment for the first passage of TENV in Vero cells. Error bars indicate standard error.

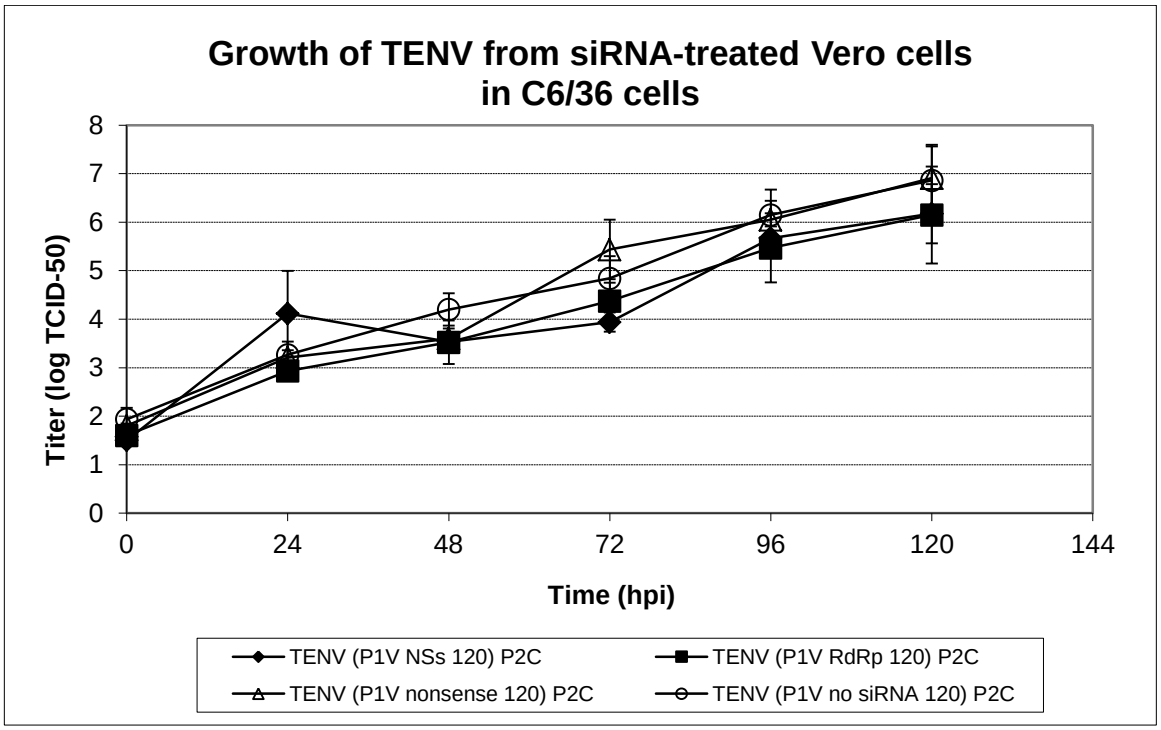

Figure 2-6. Growth of TENV from siRNA-treated Vero cells in C6/36 cells. TENV from Vero cells that were pre-treated with NSs-siRNA, RdRp-siRNA, nonsense siRNA or were not transfected (no siRNA) was collected at 120 hpi, and diluted to MOI of 0.1 for infection of fresh C6/36 cells. Infectivity was measured by analysis of the $TCID_{50}$/mL values of tissue culture fluids collected at the time points indicated (hpi). Letters after data points express levels of statistical difference within a data set for that time point. (P1V ____ 120) indicates the siRNA treatment for the first passage of TENV in Vero cells. Error bars indicate standard error. See Table 2-5 for values.

APPENDIX A
DETERMINING VIRAL TITER BY MEDIAN TISSUE CULTURE INFECTIOUS DOSE ($TCID_{50}$/MILLILITER)

Modified from Reed and Muench (1938) and Aissa Doumbouya's dissertation (2007)

All cells were be maintained at 37°C without carbon dioxide (CO_2). Leibovitz's L-15 media containing 5% fetal bovine serum (FBS) and 50 micrograms per milliliter (µg/mL) gentamycin will be used for cell maintenance and virus dilution.

1. Calculate correct number of 96 cluster well (cw) plates needed for the titration. Up to eight rows of serial dilutions can be titered in each 96 cw plate with ten replicates per row. 5×10^3 cells will be seeded in each well in 50 microliters (µL) of media. Therefore, to achieve this concentration, prepare 6×10^5 cells in 6 mL of media. Grow enough Vero cells accordingly. Each T-25 flask holds approximately 5×10^6 Vero cells at confluency.

2. Twenty-four hours prior to infection, trypsinize the Vero cells, add one mL media for each mL trypsin used to loose cells. Determine concentration with hemocytometer.

3. Seed cells in 96 cw well with repeat pipettor.

4. Prepare 1/10 dilutions by filling sterile Starstedt tubes with 1.8 mL of media.

5. Pipet 200 microliters (µL) of initial supernatant into the first tube with a P-200 pipettor. This is the 10^{-1} dilution. Make serial dilutions with 200 µL of each tube per dilution up to 10^{-8} as needed.

6. Once dilutions are made for all time points and conditions, inoculate ten wells with 100 µL of the most diluted dilution using the repeat pipettor. Repeat for each stronger dilution until the highest dilution is reached for that condition. Repeat this step for all treatments and time points, using a different disposable repeat pipettor tip per dilution set.

7. Once all cells have been inoculated, use sterile 5 milliliter (mL) pipettor to add two drops of sterile mineral oil to each well to prevent desiccation. Incubate cells for one week at 37°C.

8. Examine cells under microscope. Identify wells where virus caused observable cytopathologic effects (CPE) and mark the well with X.

9. Calculate viral titer in a 96 cluster well as follows (see Figure A-1):

Reed and Muench equation:

$TCID_{50}/mL = 10^{A} + 10^{B/C} + 10^{1} + 10^{0.5}$

A = row where CPE is seen in all inoculated wells
B = number of infected wells below the row with cytopathologic effects (CPE) in every well (i.e., after the row used to calculate variable A)
C = number of wells inoculated for each dilution (i.e., 10 in the above example)
10^{1} = correction for the dilution. (i.e., 100 µL is used for every dilution, whereas $TCID_{50}$ is calculated in 1 mL)
$10^{1/2}$ = correction based upon Poisson distribution of units of infective particle per well

Calculation of above example:

$TCID_{50}/mL = 10^{A} + 10^{B/C} + 10^{1} + 10^{1/2}$
$= 10^{5} + 10^{6/10} + 10^{1} + 10^{1/2}$
$= 10^{7.1}$ or 1.26×10^{7}

Row number (Dilution)	Inoculated Wells										Uninfected	
	1	2	3	4	5	6	7	8	9	10	11	12
Row 1 (10^{-1})	X	X	X	X	X	X	X	X	X	X		
Row 2 (10^{-2})	X	X	X	X	X	X	X	X	X	X		
Row 3 (10^{-3})	X	X	X	X	X	X	X	X	X	X		
Row 4 (10^{-4})	X	X	X	X	X	X	X	X	X	X		
Row 5 (10^{-5})	X	X	X	X	X	X	X	X	X	X		
Row 6 (10^{-6})	X			X	X	X		X				
Row 7 (10^{-7})					X							
Row 8 (10^{-8})												

Figure A-1. Graphical representation of 96 cluster well (cw) plate infected. X indicates a well with visible cytopathologic effects (CPE).

APPENDIX B
LUCIFERASE DEREPRESSION ASSAY

I. Luciferase Transfection and Expression

1. Seed Vero cells at 6 X 10^4 cells per 24 cluster well at 24 hours before transfection.

2. Co-transfect 2 nanongrams (ng) of pCMV-Renilla luciferase vector (Promega), 20 ng of the Firefly luciferase pGL3 reporter construct (Promega), and 200 ng of an empty pcDNA 3.1/V5/HisA in triplicate (e.g., technical replicates) for 24 hours using Lipofectamine® LTX (Lifetech) transfection reagent per instructions. Additionally, for the treatment condition, transfect those three plasmids as well as 50 picomoles (pmol) of K12-11 microRNA mimic in triplicate.

3. After 24 hours, replace the transfection media with fresh media.

4. After 120 hours, remove media from cells and wash twice with 1X Hank's Balanced Salt Solution to remove trace amounts of media.

5. Place 100 microliters (µL) of Passive Lysis Buffer (Promega) into each well, and gently rock the cells for 15 minutes.

6. Scrap cells with pipet tip to facilitate detachment and lysis, and transfer cell-containing buffer to 1.5 milliliters (mL) microcentrifuge tube.

7. Vortex each tube for 10 seconds. To remove excess cellular debris, centrifuge tubes at 12,000 rotations per minute (rpm) for 1 minute, and transfer supernatant to fresh 1.5 mL microcentrifuge tube. Freeze at -70°C until quantitation steps.

II. Luciferase Quantitation

1. Thaw cells prior to quantitation. Meanwhile, have the microplate luminometer program ready to measure the samples, because the respective luciferase signals decay quickly after the Luciferase Assay Reagent and the Stop & Glo® Buffer are added. Measure luminescent signal for each luciferase for 24 intervals at 1 second per interval.

2. Add 20 µL of tube of the cell-containing buffer to a clean well in the 96 well microplate. Add 100 µL of 1X Luciferase Assay Reagent to the cells.

3. Immediately place microplate in the luminometer reader to measure firefly luciferase luminescence signal in each well.

4. Remove microplate from reader, and add 100 µL of Stop & Glo® Buffer to quench the firefly luciferase reaction and complete the activation the *Renilla* luciferase signal.

5. Immediately place microplate in the luminometer reader to measure the *Renilla* luciferase luminescence signal in each well.

6. Remove the first two and the last two measurement intervals from the calculation of each luminescent signal. The computer will then take the average measurement of the remaining 20 intervals.

III. Calculation of Relative Light Units and Example Equation

1. Normalize the signal of the firefly luciferase expression signal by dividing the value of each replicates of the firefly luciferase signal by the value of each replicate from *Renilla* luciferase signal, for each technical replicate in all control and treatment samples. This will yield a value for the relative firefly luciferase signal for each technical replicate.

2. Calculate the average firefly luciferase signal for the control and the treatment samples by taking the average of the three technical replicates. Adjust the control value to a relative light unit of 1 in order to determine the relative knockdown of the firefly luciferase in the treatment condition as a percentage.

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BIOGRAPHICAL SKETCH

Daniel Mark Fitzpatrick was born in 1984 in Naples, FL, and at age 6, his family relocated to Gainesville. He knew from a young age that he would be a member of the Gator Nation, and in 2006, he graduated from the University of Florida with a dual Bachelor of Science degree in microbiology and psychology. After a brief stint a baker and a waiter, Daniel took a research and development internship at the biotechnology company Regeneration Technologies Inc. in Alachua, FL, and rekindled his love for biological research. Daniel started graduate school in 2008 in Dr. James Maruniak's Insect Virology lab at the University of Florida Entomology and Nematology Department. He received his Master of Science in 2013.