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CHAPTER FOUR

# Arthropod-Borne Flaviviruses and RNA Interference: Seeking New Approaches for Antiviral Therapy

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## Contents



## Abstract

Flaviviruses are the most prevalent arthropod-borne viruses worldwide, and nearly half of the 70 Flavivirus members identified are human pathogens. Despite the huge clinical impact of flaviviruses, there is no specific human antiviral therapy available to treat infection with any of the flaviviruses. Therefore, there is a continued search for novel therapies, and this review describes the current knowledge on the usage of RNA interference (RNAi) in combating flavivirus infections. RNAi is a process of sequence-specific gene silencing triggered by double-stranded RNA. Antiviral RNAi strategies against arthropod-borne flaviviruses have been reported and although several hurdles must be overcome to employ this technology in clinical applications, they potentially represent a new therapeutic tool.

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## 1. INTRODUCTION

The genus *Flavivirus* of the family Flaviviridae comprises more than 70 enveloped RNA viruses and depending on the virus can cause disease and mortality in humans and animals. Flaviviruses are arthropod-borne viruses (arboviruses) and most of them are transmitted to vertebrates by mosquitoes or ticks (Gubler, Kuno, & Markoff, 2007). Important human pathogens are Dengue virus (DENV), West Nile virus (WNV), Yellow fever virus (YFV), and Japanese encephalitis virus (JEV; Gould & Solomon, 2008). These viruses cause major outbreaks with a variety of disease symptoms, including encephalitis and hemorrhagic fever. In severe cases, the mortality rates range from below 1% to 50% (WHO, 2012a, 2012b, 2012c, 2012d).

Currently, effective vaccines are in use for YFV (Barrett & Teuwen, 2009), JEV (Halstead & Thomas, 2010), and tick-borne encephalitis virus (TBEV) (Heinz, Holzmann, Essl, & Kundi, 2007) but not for other flaviviruses. Furthermore, there is no specific antiviral therapy available for any of them. For the rational design of vaccines and therapeutic drugs, it is imperative to understand the molecular interplay between the host and the pathogen. In recent years, extensive progress has been made in this area and one of the important discoveries is the role of RNAi in controlling flavivirus infections.

RNAi is a conserved process of gene silencing, and due to its ability to control viral and host gene expression, it is considered a key determinant in the replication machinery of viruses and consequently in the pathogenesis of disease (Sidahmed & Wilkie, 2010). The two primary effector molecules of the RNAi pathway are microRNAs (miRNAs) and small interfering RNAs (siRNAs; Carthew & Sontheimer, 2009; Kim, Han, & Siomi, 2009). RNAi can act to reduce viral loads by inhibition of viral protein expression and transcription of viral genomes (Sidahmed & Wilkie, 2010). The RNAi strategy has been shown to be effective against multiple mammalian host viruses such as Coronavirus (Shi et al., 2005), vesicular stomatitis virus (Otsuka et al., 2007), human immunodeficiency virus (HIV; Ahluwalia et al., 2008), H1N1 influenza A virus (Song, Liu, Gao, Jiang, & Huang, 2010), and hepatitis C virus (HCV; Pfeffer & Baumert, 2010).

In this review, we will discuss a compilation of papers in which RNAi has been suggested as a potential therapy against the most commonly distributed flaviviruses. We will begin with the description of the RNAi mechanism and its antiviral features, as well as some general characteristics of the

genus *Flavivirus*. Then, we will summarize the studies that have been reported in the field of RNAi against flaviviruses and its vectors. Finally, the challenges that need to be overcome in order to improve siRNA delivery and its applicability to prevent or treat infections by flaviviruses will be discussed.

## 2. THE RNA INTERFERENCE MECHANISM AND ITS ANTIVIRAL EFFECTS

The effector molecules of the RNAi mechanism are small RNA molecules of 20–30 nucleotides in length. There are several categories of small RNAs, and in this review, I will focus on the two most described molecules, miRNAs and siRNAs. The processing and generation of miRNAs and siRNAs is depicted in Fig. 4.1. Within the nucleus, pri-miRNA molecules are generated by RNA polymerase II transcription (Lee et al., 2004). These pri-miRNA molecules have a hairpin-shaped structure and are about 70 nucleotides in length. The double-stranded RNA structure within the hairpin is subsequently recognized by a nuclear protein called DiGeorge syndrome critical region 8 (DGCR8; Landthaler, Yalcin, & Tuschl, 2004). Thereafter, DGCR8 interacts with the RNA-cutting enzyme Drosha to generate miRNA precursors (pre-miRNAs). The pre-miRNAs are then transported to the cytoplasm by Exportin-5 (Lee, Jeon, Lee, Kim, & Kim, 2002; Lund, Guttinger, Calado, Dahlberg, & Kutay, 2004). Once in the cytoplasm, the pre-miRNAs are cleaved into 22-nt mature miRNAs by an RNase III-like enzyme termed Dicer (Bernstein, Caudy, Hammond, & Hannon, 2001). The mature miRNA duplex then undergoes an ATP-dependent unwinding process and a single strand is loaded onto the RNA-induced silencing complex (RISC). The miRNA binds to miRNA recognition elements (MRE) which are mostly located in the 3′ untranslated region (3′UTR) of mRNAs (Bartel, 2009). Binding of a miRNA to its mRNA target typically leads to translational repression and exonucleolytic mRNA decay, although highly complementary targets can be cleaved endonucleolytically as well (Filipowicz, Bhattacharyya, & Sonenberg, 2008).

Several methodologies have been developed to exploit the RNAi mechanism. For example, miRNA functioning can be mimicked through the introduction of a plasmid or viral vector encoding short hairpin RNA (shRNA) molecules into the cell (Amarzguioui, Rossi, & Kim, 2005). Furthermore, it has been shown that exogenous dsRNA molecules can also be incorporated in the RNAi pathway. The dsRNA molecules can be

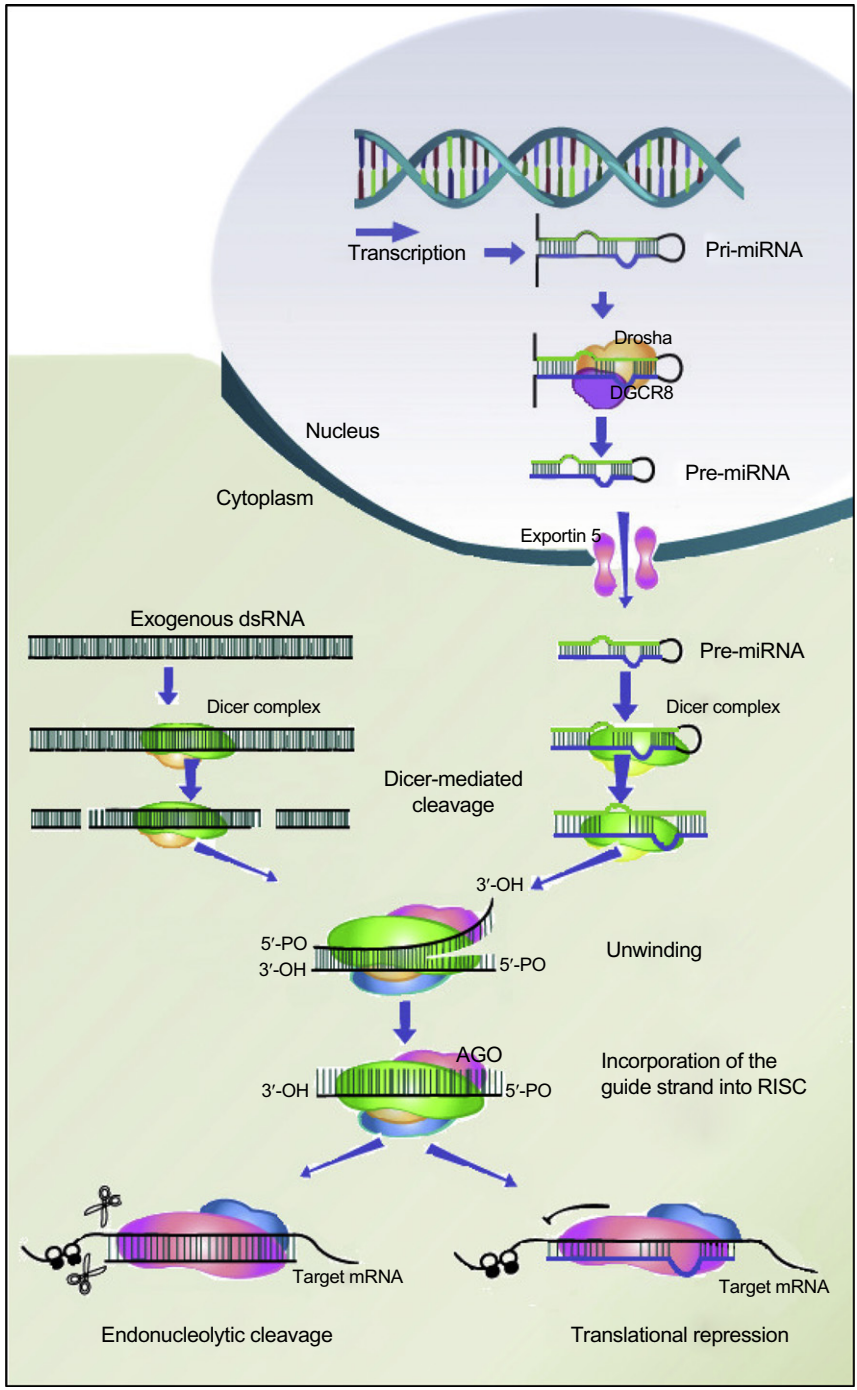


**Figure 4.1** RNA interference (RNAi). RNAi is a post-transcriptional gene silencing mechanism that regulates gene expression and mediates resistance against pathogenic nucleic acids. The main effector molecules of RNAi are miRNAs and siRNAs. MiRNAs

transfected into the cell cytoplasm, taken up from the environment, or produced in virus-infected cells as RNA replicative intermediates (Hammond, Caudy, & Hannon, 2001). These dsRNA molecules are then processed by Dicer into siRNAs. Alternatively, exogenous synthetic 21–29 nt siRNAs can be transfected directly into the cytoplasm (Elbashir et al., 2001). The siRNA molecules undergo an ATP-depending unwinding process and a single strand is incorporated in the RISC complex, similarly as with the processing of miRNAs. In contrast to miRNAs, siRNAs only induce degradation of the perfectly base-paired target mRNA (Dykxhoorn, Novina, & Sharp, 2003).

Besides regulation of gene expression, RNAi in plants, insects, nematodes, and fungi play an essential role in the antiviral response via virus-specific siRNAs (Segers, Zhang, Deng, Sun, & Nuss, 2007; Voinnet, 2001; Wilkins et al., 2005). In virus-infected mammalian cells, small RNAs have been identified (Parameswaran et al., 2010; Yeung et al., 2009) but it remains unclear whether they contribute to an antiviral response (Haasnoot & Berkhout, 2011; Sidahmed & Wilkie, 2010). In contrast, mammalian-encoded miRNAs have been reported to target and inhibit viral gene expression. For example, miRNA miR-32 targets two different sequences (located in the 3′UTR and the open reading frame 2) in the genome of primate foamy virus type 1, thereby inhibiting viral translation and replication (Lecellier et al., 2005). Furthermore, similar findings were reported for miR-24 and miR-93 in vesicular stomatitis virus infection (Otsuka et al., 2007); miR-29a during HIV infection (Ahluwalia et al., 2008); and miR-323, -491, and -654 in influenza H1N1 infection (Song et al., 2010). These findings are indicative for the importance of the host RNAi

are encoded in the genome and transcribed into primary miRNA transcripts (pri-miRNAs). Pri-miRNAs are processed to miRNA precursors (pre-miRNAs) in the nucleus by the RNase III-like enzyme Drosha and the DiGeorge syndrome critical region 8 (*DGCR8*). Pre-miRNAs are transported to the cytoplasm by *exportin 5*, where they are cleaved by Dicer to yield ~19–23 nt miRNA duplexes. One strand is then selected and loaded in the RNA-induced silencing complex (RISC). The key components of RISC are proteins of the Argonaute (AGO) family which mediate translational repression or cleavage of target mRNAs. Furthermore, dsRNA molecules are targets for the RNAi pathway. These dsRNA molecules are artificially introduced in the cell cytoplasm or are virus RNAs. Like miRNA precursors, long dsRNA are processed by Dicer into ~21 nt siRNA duplexes. One strand of the siRNA is selected and loaded into RISC. The binding of an siRNA to its target mRNA typically induces degradation.

in modulating the replication efficiency of the infecting viruses and point out that harnessing this pathway could represent a powerful antiviral method.

## 3. ARTHROPOD-BORNE FLAVIVIRUSES

Flaviviruses are spherical particles with a diameter of approximately 50 nm. The viral genome consists of a single-stranded positive-sense RNA molecule which is approximately 11 kb in length. The RNA molecule contains highly structured 5′ and 3′UTRs and has one open reading frame (Lindenbach, Thiel, & Rice, 2007; Markoff, 2003). The RNA is packaged by multiple copies of the capsid (C) protein (nucleocapsid) and is surrounded by a host-derived lipid membrane in which two structural transmembrane proteins are inserted, the envelope glycoprotein E and the membrane protein M.

Infection with one of the arthropod-borne flaviviruses begins when the vector takes a blood meal thereby injecting the virus into the dermis of the host. The reproductive cycle of flaviviruses starts with attachment of the virus to cell-surface receptors. Upon virus–receptor interaction, the particle is internalized by receptor-mediated endocytosis and delivered to endosomes (Stiasny, Fritz, Pangerl, & Heinz, 2011). Within the acidic lumen of the endosome, fusion of the viral and cellular membranes is triggered by the viral E glycoprotein allowing the release of the nucleocapsid into the cell cytoplasm (Smit, Moesker, Rodenhuis-Zybert, & Wilschut, 2011). Following nucleocapsid uncoating, the RNA is translated into a polyprotein of about 3400 amino acids in length. The polyprotein is subsequently processed by viral and host cellular proteases yielding the three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, 2A, 2B, 3, 4A, 4B, and NS5) (Lindenbach et al., 2007; Urcuqui-Inchima, Patino, Torres, Haenni, & Diaz, 2010). The prM and E proteins are translocated across the endoplasmic reticulum (ER) membrane and form heterodimers that are oriented into the lumen of the ER. The NS proteins assemble and form a viral RNA replication complex (Mackenzie, Jones, & Westaway, 1999; Urcuqui-Inchima et al., 2010). RNA synthesis is regulated by the 5′–3′CS and 5′–3′UAR inverted complementary sequences that allow the circularization of the genome (Villordo & Gamarnik, 2009). After genome cyclization, the RNA-dependent RNA polymerase, NS5, synthesizes the complementary minus-strand RNA molecule from the genomic RNA, which subsequently acts as a template for the synthesis of new positive-sense viral RNA (Davidson, 2009; Maeda, Maeda, Takagi, &

Kurane, 2008). The newly synthesized positive-sense RNA molecule interacts with multiple copies of the C protein to form a nucleocapsid (Ivanyi-Nagy & Darlix, 2010). The nucleocapsid then buds into the ER thereby acquiring a lipid bilayer containing heterodimers of the prM and E proteins. The prM protein acts as a scaffold to prevent premature fusion of the virus during its transport out of the cell (Perera & Kuhn, 2008). During the secretory pathway, virion maturation occurs in the trans-Golgi network through furin-mediated cleavage of the prM protein to M and a pr peptide (Fernandez-Garcia, Mazzon, Jacobs, & Amara, 2009). The pr peptide dissociates after the release of the virion into the extracellular milieu and allows new progeny virus particles to bind and infect a new permissive host cell via receptor-mediated endocytosis. In figure 4.2 the life cycle of flaviviruses is depicted.

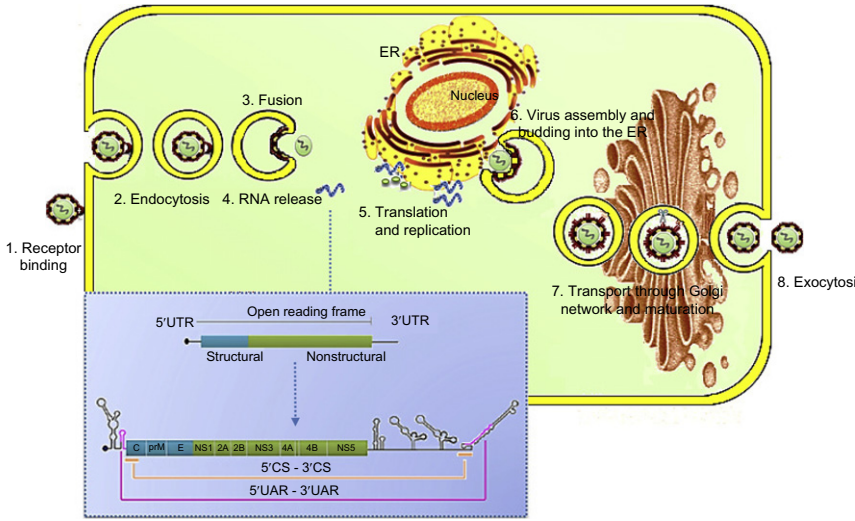


**Figure 4.2** Flavivirus life cycle. Flaviviruses bind to cellular receptors on the surface of susceptible host cells (1). Virions are internalized by endocytosis (2). The acidic environment within the endosomes induces fusion (3) between the viral and cell membranes resulting in the release of the RNA genome (4). The RNA genome of flaviviruses contains a single open reading frame and highly structured 3′ and 5′UTRs ends. The RNA is translated into a polyprotein precursor that is processed into three structural proteins and seven nonstructural proteins (5). The complementary sequences CS (cyclization sequence) and UAR (upstream AUG regions) at both ends of the genome allow its circularization a required step for replication. Replication is mediated by the nonstructural proteins. Virus assembly occurs in the endoplasmic reticulum (ER) (6). The resultant immature particles are transported through the Golgi network where furin-mediated cleavage of prM to M generates mature infectious particles (7) that are released by exocytosis (8).

### 3.1. West Nile virus

The natural hosts of WNV are birds, but humans, horses, and other mammals can also be infected (Ulbert, 2011). The virus is transmitted by a vast range of mosquitoes and depending on the geographical region, different species are responsible for the transmission of the virus. The most important vectors are bird-feeding mosquitoes of the *Culex* genus (Ulbert, 2011). Approximately 20% of the infected people develop a classical West Nile fever (flu-like symptoms with high fever), and in about 1% of the cases, severe neurological complications, such as encephalitis, meningitis, or flaccid paralysis are seen. In the latest group, the mortality rate can reach 20% (WHO, 2012c).

The prophylactic potential of exogenous siRNAs against WNV infection was highlighted several years ago. McCown et al. constructed DNA vectors encoding siRNAs targeting the *C* and *NS5* genes of WNV and transfected these into 293T cells 24 h prior to WNV infection. The number of E-positive cells was 30% and 83% lower in cells transfected with the plasmid encoding the siRNA against C and NS5, respectively. In addition, a decrease in RNA levels (65%) and virus particle production (60%) was achieved in cells transfected with the siRNA against C (McCown, Diamond, & Pekosz, 2003). Similar results were observed in Vero cells using siRNAs targeting the *NS5* gene (Ong, Choo, Chu, & Ng, 2006) and the *E* gene (Bai et al., 2005) and in HTB-11 cells when the *C* gene was targeted (Yang et al., 2008). Also, WNV replication and virus particle production was found attenuated when siRNAs were applied targeting the 3′UTRs of the viral genome (Anthony, Bai, Krishnan, Fikrig, & Koski, 2009). Taken together, these observations indicate that pretreatment of cells with siRNAs directed against the WNV genome results in a significant inhibition of WNV replication.

The therapeutic effect of siRNAs to WNV infection is limited and appears to be dependent on the transfection methodology applied. Geiss, Pierson, and Diamond (2005) observed that siRNAs targeting the *C* gene had no effect on virus replication when transfected into cells 10 h after WNV infection using lipid-based reagents. In addition, no significant reduction in viral protein or RNA levels was seen in WNV replicon-expressing cells transfected with siRNAs targeting the *NS3* gene using lipid-based reagents. On the other hand, however, a 10-fold reduction in NS3 protein expression and a 7.5-fold decrease in RNA levels were observed when the siRNA molecules were delivered to the cell cytoplasm by electroporation. These results indicate that transient opening of membrane structures is

required to induce RNAi during active WNV replication. However, a word of caution is in place here as siRNA transfection with oligofectamine, also a lipid-based reagent, was found to reduce HCV protein expression and RNA replication in HCV replicon-expressing cells (Takigawa et al., 2004).

The different efficacy of siRNA treatment before and after WNV infection is supported by *in vivo* data in mice. Bai et al. reported that mice are protected from fatal infection with WNV when siRNAs targeting the *E* gene are administered 24 h before the virus challenge, but failed to protect when the siRNA is given 24 h after virus challenge (Bai et al., 2005). Furthermore, Ye et al. observed that the administration of siRNAs toward *NS5* and *C* genes failed to protect mice when given 2 days postinfection with WNV (Ye, Abraham, Wu, Shankar, & Manjunath, 2011).

## 3.2. Dengue virus

Dengue is the most prevalent vector-borne disease in the world with an estimated 50 million cases each year in (sub)tropical regions (WHO, 2012a). The virus is predominantly transmitted to humans through the mosquito *Aedes aegypti*, but *Aedes albopictus* can act as a vector as well. Four antigenically distinct viruses designated from 1 to 4 (DENV 1–4) have been identified so far. Infection with any of the four DENV serotypes can give a broad spectrum of clinical manifestations ranging from acute self-limiting febrile illness to life-threatening complications like hemorrhagic fever and dengue shock. Individuals that develop severe disease often have preexisting anti-dengue immunity generated during previous heterologous DENV infections or received anti-dengue antibodies from their mother (Ubol & Halstead, 2010). The immunopathogenesis of severe disease is complex and see references for reviews on this topic (Green & Rothman, 2006; Ubol & Halstead, 2010) In the past few decades, dengue disease has become a major health problem due to increased endemic activity and emergence to previously nonendemic regions (Gould & Solomon, 2008).

The first evidence of an RNAi-mediated antiviral mechanism during DENV infection was obtained by Olson and colleagues. The authors showed that transient or stable expression of long dsRNAs cognate to the DENV-2 genome in *A. albopictus* (C6/36) cells inhibited subsequent infection of these cells with DENV-2 (Adelman, Blair, Carlson, Beaty, & Olson, 2001; Adelman et al., 2002; Gaines et al., 1996; Olson et al., 2002). Similar results were observed in dsRNA transduced female *A. aegypti* mosquitoes (Adelman et al., 2001, 2002; Gaines et al., 1996; Olson et al., 2002).

Furthermore, Wu et al. found that a synthetic siRNA molecule targeting a sequence in the prM protein-coding region reduced the cytopathic effect in C6/36 cells and increased cell survival more than twofold (Wu et al., 2010).

The therapeutic potential of siRNA for DENV infection in mammalian cells has also been described. For example, adeno-associated virus vectors carrying siRNA against the CS1 region (a conserved sequence in the 3′UTR of all four DENV serotypes) decreased the percentage of DENV-infected human monocyte-derived dendritic cells (MDDC) and reduced apoptosis of the infected cells. The observed attenuated but prolonged virus particle production may be beneficial as activation of humoral and cellular immune responses is considered to help with the clearance of DENV particles (Zhang et al., 2004). In addition, Subramanya et al. reported 80% reduction in infection of MDDC when cells were treated with siRNAs targeting *E* gene after DENV infection (Subramanya et al., 2010). In this study, the authors used a novel peptide-based delivery system that allows targeting of siRNAs to human DCs both *in vitro* as well as in *in vivo* models (Subramanya et al., 2010).

Importantly, the effectiveness of RNAi against DENV infection has also been verified *in vivo* (Stein et al., 2011). Retro-orbital intravenous injection of three doses of siRNA molecules (targeting a conserved sequence in the 5′CS region) in mice at 24 h pre- and 24 and 72 h-postchallenge with DENV revealed that siRNA treatment increases the average survival, and significantly reduces viral loads in several tissues and at different time-points postinfection (Stein et al., 2011).

In addition to the use of exogenous siRNAs to restrict DENV replication, the antiviral function of endogenous miRNAs has also been described. In these studies, engineered viruses were used that contain MREs for cellular miRNAs within the viral genome (Heiss, Maximova, Thach, Speicher, & Pletnev, 2012; Lee et al., 2010). It was found that the insertion of the MRE for the hepatic-specific miR-122 in the 3′UTR of DENV genome suppresses the viral replication in transfected cells (Lee et al., 2010). Moreover, using a chimeric DENV/TBEV virus (C, prM, E from TBEV), it was shown that the inclusion of the MRE for the brain-expressed miR-9 and miR-124a, reduces the access of the virus to the central nervous system and consequently the development of lethal encephalitis in mice (Heiss et al., 2012).

Another approach that has been explored is to reduce DENV replication by modulating host gene expression. For instance, downregulation of the heat shock protein 60 by RNAi in DENV-infected U937 cells decreased viral replication and particle production significantly (Padwad et al., 2009).

In another study, the cell-surface receptor GRP78 and the clathrin-mediated endocytosis pathway were silenced by RNAi, and this approach resulted in a 90% reduction of DENV-infected cells (Alhoot, Wang, & Sekaran, 2012). Also, a recent report showed that siRNA toward the *TNF-α* gene reduced cytokine response in DENV-infected DCs, highlighting the potential of targeted RNAi-based approaches to simultaneously decrease viral replication and the detrimental host immune response (Subramanya et al., 2010).

### 3.3. Japanese encephalitis virus and Yellow fever virus

Although effective vaccination against JEV and YFV are currently available, these viruses remain a major public health problem that threat hundreds of millions of people living in endemic regions and to people traveling to these regions. The annual estimated incidence of JEV and YFV infections is 50,000 and 200,000 cases with a mortality rate of 25% and 15%, respectively (Misra & Kalita, 2010; WHO, 2012d).

JEV is transmitted through a zoonotic cycle between mosquitoes, pigs, and water birds. Humans are only accidentally infected. The primary mosquito vector of JEV is *Culex tritaeniorhynchus*, but species such as *Cx. gelidus*, *Cx. fuscocephala*, and *Cx. annulirostris* are important secondary or regional vectors (van den Hurk, Ritchie, & Mackenzie, 2009). For YFV, several species of the *Aedes* and *Haemogogus* mosquitoes are important for transmission. The vertebrate hosts of YFV are monkeys and humans, and the mosquitoes transmit the virus from one host to another (WHO, 2012d). The continuous geographical expansion of the mosquito vectors of JEV and YFV and the difficulties to implement a worldwide vaccination program highlights the need for a specific antiviral therapy.

The effectiveness of RNAi to inhibit YFV replication was described by Pacca and coworkers (Pacca et al., 2009). Vero cells stably transfected with a shRNA targeting the *NS1* or the *E* gene decreased YFV infection in 97% and 68%, respectively. Administration of these shRNAs to mice 12 h prior to YFV challenge increased the survival rate in 40% (siRNA toward NS1) and 20% (siRNA toward E) (Pacca et al., 2009).

The effect of RNAi in JEV replication has been examined *in vitro* and *in vivo*. JEV replication was attenuated in BHK-21 and HEK-293T cells transfected with a shRNA targeting the *NS5* gene 5 h prior to infection (Liu et al., 2006; Qi et al., 2008). In addition, Kumar and coworkers reported that JEV infection is almost completely abolished in Vero cells and Neuro 2a cells prior transduced with a shRNA directed to the *E* gene. Importantly, all mice survived JEV infection when treated with shRNA

0.5 or 6 h postchallenge (Kumar, Lee, Shankar, & Manjunath, 2006). The use of several siRNAs to target multiple sites along the genome of JEV has also been reported. Wu et al. constructed a single polycistron to simultaneously express nine siRNAs-targeting eight genes of JEV (*PrM*, *NS1*, *NS2A*, *NS2B*, *NS3*, *NS4A*, *NS4B*, and *NS5*). Transfection of BHK-21 cells with this construct inhibited replication of three wild-type JEV strains belonging to two different genotypes (Wu, Xue, Wang, Du, & Jin, 2011). Furthermore, efficient inhibition of infection was seen using recombinant JEV carrying MREs. Yen et al. inserted two copies of the MRE for miR-124 in the 3′UTR of JEV. None of the mice challenged with this virus died from infection, whereas virus challenged with the wild-type strain or with a virus carrying a mutated MRE was lethal for all mice. Interestingly, immunization with the JEV carrying the MRE for miR-124 elicited protective immunity against subsequent lethal challenge with wild-type JEV (Yen et al., 2011).

## 4. RNAi AND THE MOSQUITO VECTOR

The vast majority of mosquitoes become infected with an arbovirus when they take a blood meal from a viremic vertebrate host. Viral amplification begins with the infection of midgut epithelium cells which is followed by dissemination of the virus to the salivary glands and other tissues. Once the virus is present in saliva, a female mosquito transmits the virus to a new host while taking blood meal (Kuno & Chang, 2005). Despite efficient virus replication, minimal pathology is seen in the mosquito vector (Lambrechts & Scott, 2009). This suggests the presence of evolutionary mechanisms that allow virus replication without jeopardizing the host and transmission. Indeed, it has been suggested that RNAi is a key component of the innate immune response of mosquitoes to viral infections (Blair, 2011). In previous sections, we have described that an exogenous trigger of the RNAi pathway in mosquito-cultured cells (Adelman et al., 2001, 2002; Gaines et al., 1996; Olson et al., 2002) and *A. aegypti* mosquitoes (Adelman et al., 2001, 2002; Gaines et al., 1996; Olson et al., 2002) can mediate resistance against flaviviruses. In addition, it has been shown that WNV (Chotkowski et al., 2008) and DENV (Mukherjee & Hanley, 2010) infection (Mukherjee & Hanley, 2010) of *Drosophila* cell lines induce functional virus-specific siRNAs that promote a protective RNAi response. Although the production of DENV-specific siRNAs has also been reported in Aag2 cells and orally infected *A. aegypti* mosquitoes, it seems that the virus

can somehow circumvent the natural RNAi response, since persistent infection is observed in both cases (Brackney et al., 2010; Sanchez-Vargas et al., 2009). Furthermore, impairment of the RNAi pathway was found to increase viral replication (Brackney et al., 2010; Sanchez-Vargas et al., 2009). Taken together, these observations suggest that the RNAi response in mosquitoes controls virus replication to such an extent that it prevents pathology in the infected host but is sufficiently high to allow transmission of the virus to a new host.

Furthermore, the exploitation of RNAi to reduce vector competence for Flavivirus infection has been suggested, especially since traditional approaches for vector control have failed (Beaty, 2005). Significant progress has been made in this area with *A. aegypti*, the major vector of DENV. Female mosquitoes genetically manipulated with germ line transgenes to express dsRNA derived from the *prM* gene of DENV-2 in their midgets have a high level of resistance toward DENV-2 infection and a reduced ability to transmit the virus (Franz et al., 2006). The resistant phenotype of the transgenic mosquitoes was maintained for 13 generations; however, from G14 it became weaker and eventually it was lost in G17. The loss of resistance was caused by the inhibition of the transgene expression, but the explanation for this phenomenon remains unclear (Franz et al., 2009). As the authors pointed out, considerations in the transgene design—such as the potential risk for the modified mosquitoes and the ability of the transgene to be inherited and expressed—should be taken into account if a strategy of population replacement will be applied in the field.

## 5. THE INTERPLAY BETWEEN ARTHROPOD-BORNE FLAVIVIRUSES AND THE ENDOGENOUS HOST CELL RNAi PATHWAY

So far we have described the antiviral effect of the RNAi mechanism induced by exogenous delivery of siRNA or precursors, and how cellular miRNA can target sequences artificially introduced within the genome of flaviviruses. In recent years, the naturally occurring interactions between viruses and the host-cellular RNAi pathway have also been highlighted. Viral infections induce profound changes in the miRNA expression profile of the host cell. These changes may represent an additional mechanism of the innate immune response and/or are induced by the virus in order to promote reproduction (Skalsky & Cullen, 2010). There are only a few reports available describing how arthropod-borne flaviviruses influence the

miRNA expression of their host cells. Skalsky, Vanlandingham, Scholle, Higgs, and Cullen (2010), observed downregulation of miR-989 and overexpression of miR-92 in *Cx. quinquefasciatus* mosquitoes infected with WNV when compared to noninfected mosquitoes. Unfortunately, there is no evidence on the functional role of these miRNAs during WNV infection (Skalsky et al., 2010). It has also been reported that WNV induces upregulation of miRNA Hs_154 in infected neuronal cells and in the central nervous system tissue of infected mice. Hs_154 targets the mRNA of the antiapoptotic proteins, CCCTC-binding factor (CTCF), and EGFR-coexpressed protein (ECOP). The authors hypothesize that the upregulation of Hs_154 is a cellular antiviral response to the infection since downregulation of CTCF and ECOP contributes to cell death (Smith, Grey, Uhrlaub, Nikolich-Zugich, & Hirsch, 2012).

In addition to cellular miRNAs, many virally encoded miRNAs have been identified. Viral miRNAs have been described to regulate both, cellular and viral gene expression (Grundhoff & Sullivan, 2011). Although initially cytoplasmic RNA viruses such as flaviviruses were not believed to express miRNAs (Pfeffer et al., 2005), Hussain et al. have reported the generation of a mature miRNA derived from WNV in infected Aag2 and C6/36 cells. The discovered miRNA (KUN-miR-1) upregulates the mosquito GATA4 mRNA level and promotes replication of the virus (Hussain et al., 2012). Furthermore, as a proof of concept it was shown that functional viral miRNAs can be produced if the pre-miRNA sequence is incorporated into the genome of the virus. For example, TBEV was able to express an Epstein–Barr viral miRNA when the precursor was integrated into the 3′UTR of TBEV genome (Rouha, Thurner, & Mandl, 2010).

## 6. CONCLUSIONS AND CHALLENGES

Flavivirus infections represent a severe global public health problem with major individual, social, and economic consequences; therefore, preventive and therapeutic strategies are urgently needed. As we reviewed here, RNAi can be used as a prophylactic and therapeutic agent against flavivirus infections and its associated diseases. Furthermore, we highlighted the possibility to use RNAi in vector control and pathogen transmission. However, many hurdles must be overcome in order to translate the positive *in vitro* and *in vivo* data to the clinic and into the fields.

RNAi is based on sequence homology and therefore virtually any sequence can be targeted. Indeed, this review described that many flavivirus genes can be successfully targeted. In addition, mRNAs encoding cellular cofactors for viral replication can be targeted to suppress viral replication. However, careful design of the small RNA sequence must be done since partial homology between small RNAs and unintended mRNA transcripts might result in nondesirable gene silencing (Jackson et al., 2006). In addition, if the activation of the innate immune response is not desirable, it is critical to avoid sequences that can be recognized by Toll-like receptors (Robbins, Judge, & MacLachlan, 2009). Moreover, during RNAi silencing of viral replication, escape mutants often arise and therefore targeting of conserved regions is preferable as well as the use of multiple RNA molecules targeting distinct regions (Sugiyama, Habu, Ohnari, Miyano-Kurosaki, & Takaku, 2009; Wu et al., 2011; Zhang et al., 2012). Bioinformatic approaches followed up with experimental validation should be applied to determine optimal siRNA sequences that are complementary to the target mRNA, induce minimal immune response, and avoid the appearance of escape mutants. In the case of flaviviruses, highly conserved sequences within the C, *NS5* genes and within the 3′UTR might represent excellent targets for RNAi therapies. Furthermore, future research should continue to focus on the complex interplay of arthropod-borne flaviviruses and the RNAi pathway in different host cells to search for novel targets for RNAi-based intervention.

Perhaps, the biggest challenge in the development of successful siRNA-based drugs lies within the design of a safe and effective delivery system targeting biological relevant cell types. This is a complex task with multiple demands, including slow excretion, high stability in blood serum, limited nonspecific accumulation in tissues, efficient cellular uptake, and intracellular release. The most commonly applied delivery systems to date are lipid-based carriers, antibodies, peptides, and nanoparticles conjugates (Shim & Kwon, 2010). Recently, a clinical trial with patients having solid cancers showed inhibition of gene expression using siRNA-targeted nanoparticles that were administered systemically (Davis et al., 2010). This technology was also found effective in a cell culture model of HCV infection (Chandra et al., 2012). Alternatively, as was mentioned above, Subramanya and coworkers successfully linked an siRNA molecule to a peptide able to bind specifically to human MDDC, one of the main targets of DENV (Subramanya et al., 2010). Future research is required to further characterize the potential of these and other delivery systems to combat flavivirus infections.

In addition, we must keep in mind that for the elimination of flavivirus infections, we most likely need an integrated approach that includes vaccination, enhanced mosquito control, and improved clinical management.

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## REFERENCES

Adelman, Z. N., Blair, C. D., Carlson, J. O., Beaty, B. J., & Olson, K. E. (2001). Sindbis virus-induced silencing of dengue viruses in mosquitoes. *Insect Molecular Biology*, *10* (3), 265–273.

Adelman, Z. N., Sanchez-Vargas, I., Travanty, E. A., Carlson, J. O., Beaty, B. J., Blair, C. D., et al. (2002). RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. *Journal of Virology*, *76*(24), 12925–12933.

Ahluwalia, J. K., Khan, S. Z., Soni, K., Rawat, P., Gupta, A., Hariharan, M., et al. (2008). Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology*, *5*, 117.

Alhoot, M. A., Wang, S. M., & Sekaran, S. D. (2012). RNA interference mediated inhibition of dengue virus multiplication and entry in HepG2 cells. *PLoS One*, *7*(3), e34060.

Amarzguioui, M., Rossi, J. J., & Kim, D. (2005). Approaches for chemically synthesized siRNA and vector-mediated RNAi. *FEBS Letters*, *579*(26), 5974–5981.

Anthony, K. G., Bai, F., Krishnan, M. N., Fikrig, E., & Koski, R. A. (2009). Effective siRNA targeting of the 3' untranslated region of the West Nile virus genome. *Antiviral Research*, *82*(3), 166–168.

Bai, F., Wang, T., Pal, U., Bao, F., Gould, L. H., & Fikrig, E. (2005). Use of RNA interference to prevent lethal murine west nile virus infection. *The Journal of Infectious Diseases*, *191*(7), 1148–1154.

Barrett, A. D., & Teuwen, D. E. (2009). Yellow fever vaccine—How does it work and why do rare cases of serious adverse events take place? *Current Opinion in Immunology*, *21*(3), 308–313.

Bartel, D. P. (2009). MicroRNAs: Target recognition and regulatory functions. *Cell*, *136*(2), 215–233.

Beaty, B. J. (2005). Control of arbovirus diseases: Is the vector the weak link? *Archives of Virology*, (Suppl. 19), 73–88.

Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, *409*(6818), 363–366.

Blair, C. D. (2011). Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission. *Future Microbiology*, *6*(3), 265–277.

Brackney, D. E., Scott, J. C., Sagawa, F., Woodward, J. E., Miller, N. A., Schilkey, F. D., et al. (2010). C6/36 *Aedes albopictus* cells have a dysfunctional antiviral RNA interference response. *PLoS Neglected Tropical Diseases*, *4*(10), e856.

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and mechanisms of miRNAs and siRNAs. *Cell*, *136*(4), 642–655.

Chandra, P. K., Kundu, A. K., Hazari, S., Chandra, S., Bao, L., Ooms, T., et al. (2012). Inhibition of Hepatitis C virus replication by intracellular delivery of multiple siRNAs by nanosomes. *Molecular Therapy*, *20*(9), 1724–1736.

Chotkowski, H. L., Ciota, A. T., Jia, Y., Puig-Basagoiti, F., Kramer, L. D., Shi, P. Y., et al. (2008). West Nile virus infection of *Drosophila melanogaster* induces a protective RNAi response. *Virology*, *377*(1), 197–206.

Davidson, A. D. (2009). Chapter 2. New insights into flavivirus nonstructural protein 5. *Advances in Virus Research*, *74*, 41–101.

Davis, M. E., Zuckerman, J. E., Choi, C. H., Seligson, D., Tolcher, A., Alabi, C. A., et al. (2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*, *464*(7291), 1067–1070.

Dykxhoorn, D. M., Novina, C. D., & Sharp, P. A. (2003). Killing the messenger: Short RNAs that silence gene expression. *Nature Reviews Molecular Cell Biology*, *4*(6), 457–467.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, *411*(6836), 494–498.

Fernandez-Garcia, M. D., Mazzon, M., Jacobs, M., & Amara, A. (2009). Pathogenesis of flavivirus infections: Using and abusing the host cell. *Cell Host and Microbe*, *5*(4), 318–328.

Filipowicz, W., Bhattacharyya, S. N., & Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? *Nature Reviews Genetics*, *9*(2), 102–114.

Franz, A. W., Sanchez-Vargas, I., Adelman, Z. N., Blair, C. D., Beaty, B. J., James, A. A., et al. (2006). Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(11), 4198–4203.

Franz, A. W., Sanchez-Vargas, I., Piper, J., Smith, M. R., Khoo, C. C., James, A. A., et al. (2009). Stability and loss of a virus resistance phenotype over time in transgenic mosquitoes harbouring an antiviral effector gene. *Insect Molecular Biology*, *18*(5), 661–672.

Gaines, P. J., Olson, K. E., Higgs, S., Powers, A. M., Beaty, B. J., & Blair, C. D. (1996). Pathogen-derived resistance to dengue type 2 virus in mosquito cells by expression of the premembrane coding region of the viral genome. *Journal of Virology*, *70*(4), 2132–2137.

Geiss, B. J., Pierson, T. C., & Diamond, M. S. (2005). Actively replicating West Nile virus is resistant to cytoplasmic delivery of siRNA. *Virology Journal*, *2*, 53.

Gould, E. A., & Solomon, T. (2008). Pathogenic flaviviruses. *Lancet*, *371*(9611), 500–509.

Green, S., & Rothman, A. (2006). Immunopathological mechanisms in dengue and dengue hemorrhagic fever. *Current Opinion in Infectious Diseases*, *19*(5), 429–436.

Grundhoff, A., & Sullivan, C. S. (2011). Virus-encoded microRNAs. *Virology*, *411*(2), 325–343.

Gubler, D., Kuno, G., & Markoff, L. (2007). Flaviviruses. In D. Knipe & P. Howley (Eds.), *Fields virology* (pp. 1155–1252). (5th ed.). Philadelphia: Lippincott Williams & Wilkins.

Haasnoot, J., & Berkhout, B. (2011). RNAi and cellular miRNAs in infections by mammalian viruses. *Methods in Molecular Biology*, *721*, 23–41.

Halstead, S. B., & Thomas, S. J. (2010). Japanese encephalitis: New options for active immunization. *Clinical Infectious Diseases*, *50*(8), 1155–1164.

Hammond, S. M., Caudy, A. A., & Hannon, G. J. (2001). Post-transcriptional gene silencing by double-stranded RNA. *Nature Reviews. Genetics*, *2*(2), 110–119.

Heinz, F. X., Holzmann, H., Essl, A., & Kundi, M. (2007). Field effectiveness of vaccination against tick-borne encephalitis. *Vaccine*, *25*(43), 7559–7567.

Heiss, B. L., Maximova, O. A., Thach, D. C., Speicher, J. M., & Pletnev, A. G. (2012). MicroRNA targeting of neurotropic flavivirus: Effective control of virus escape and reversion to neurovirulent phenotype. *Journal of Virology*, *86*(10), 5647–5659.

Hussain, M., Torres, S., Schnettler, E., Funk, A., Grundhoff, A., Pijlman, G. P., et al. (2012). West Nile virus encodes a microRNA-like small RNA in the 3' untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells. *Nucleic Acids Research*, *40*(5), 2210–2223.

Ivanyi-Nagy, R., & Darlix, J. L. (2010). Intrinsic disorder in the core proteins of flaviviruses. *Protein and Peptide Letters*, *17*(8), 1019–1025.

Jackson, A. L., Burchard, J., Schelter, J., Chau, B. N., Cleary, M., Lim, L., et al. (2006). Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA*, *12*(7), 1179–1187.

Kim, V. N., Han, J., & Siomi, M. C. (2009). Biogenesis of small RNAs in animals. *Nature Reviews. Molecular Cell Biology*, *10*(2), 126–139.

Kumar, P., Lee, S. K., Shankar, P., & Manjunath, N. (2006). A single siRNA suppresses fatal encephalitis induced by two different flaviviruses. *PLoS Medicine*, *3*(4), e96.

Kuno, G., & Chang, G. J. (2005). Biological transmission of arboviruses: Reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends. *Clinical Microbiology Reviews*, *18*(4), 608–637.

Lambrechts, L., & Scott, T. W. (2009). Mode of transmission and the evolution of arbovirus virulence in mosquito vectors. *Proceedings of the Royal Society B Biolological Sciences*, *276* (1660), 1369–1378.

Landthaler, M., Yalcin, A., & Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. *Current Biology*, *14*(23), 2162–2167.

Lecellier, C. H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., et al. (2005). A cellular microRNA mediates antiviral defense in human cells. *Science*, *308* (5721), 557–560.

Lee, Y., Jeon, K., Lee, J. T., Kim, S., & Kim, V. N. (2002). MicroRNA maturation: Stepwise processing and subcellular localization. *The EMBO Journal*, *21*(17), 4663–4670.

Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., et al. (2004). MicroRNA genes are transcribed by RNA polymerase II. *The EMBO Journal*, *23*(20), 4051–4060.

Lee, T. C., Lin, Y. L., Liao, J. T., Su, C. M., Lin, C. C., Lin, W. P., et al. (2010). Utilizing liver-specific microRNA-122 to modulate replication of dengue virus replicon. *Biochemical and Biophysical Research Communications*, *396*(3), 596–601.

Lindenbach, B. D., Thiel, H. J., & Rice, C. M. (2007). Flaviviridae: The viruses and their replication. In D. M. Knipe & P. M. Howley (Eds.), *Fields virology*. (5th ed.). Philadelphia: Lippincott-Raven.

Liu, X., Cao, S., Zhou, R., Xu, G., Xiao, S., Yang, Y., et al. (2006). Inhibition of Japanese encephalitis virus NS1 protein expression in cell by small interfering RNAs. *Virus Genes*, *33*(1), 69–75.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., & Kutay, U. (2004). Nuclear export of microRNA precursors. *Science*, *303*(5654), 95–98.

Mackenzie, J. M., Jones, M. K., & Westaway, E. G. (1999). Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. *Journal of Virology*, *73*(11), 9555–9567.

Maeda, A., Maeda, J., Takagi, H., & Kurane, I. (2008). Detection of small RNAs containing the 5'- and the 3'-end sequences of viral genome during West Nile virus replication. *Virology*, *371*(1), 130–138.

Markoff, L. (2003). 5'- and 3'-noncoding regions in flavivirus RNA. *Advances in Virus Research*, *59*, 177–228.

McCown, M., Diamond, M. S., & Pekosz, A. (2003). The utility of siRNA transcripts produced by RNA polymerase i in down regulating viral gene expression and replication of negative- and positive-strand RNA viruses. *Virology*, *313*(2), 514–524.

Misra, U. K., & Kalita, J. (2010). Overview: Japanese encephalitis. *Progress in Neurobiology*, *91* (2), 108–120.

Mukherjee, S., & Hanley, K. A. (2010). RNA interference modulates replication of dengue virus in *Drosophila melanogaster* cells. *BMC Microbiology*, *10*, 127.

Olson, K. E., Adelman, Z. N., Travanty, E. A., Sanchez-Vargas, I., Beaty, B. J., & Blair, C. D. (2002). Developing arbovirus resistance in mosquitoes. *Insect Biochemistry and Molecular Biology*, *32*(10), 1333–1343.

Ong, S. P., Choo, B. G., Chu, J. J., & Ng, M. L. (2006). Expression of vector-based small interfering RNA against West Nile virus effectively inhibits virus replication. *Antiviral Research*, *72*(3), 216–223.

Otsuka, M., Jing, Q., Georgel, P., New, L., Chen, J., Mols, J., et al. (2007). Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. *Immunity*, *27*(1), 123–134.

Pacca, C. C., Severino, A. A., Mondini, A., Rahal, P., D'Avila, S. G., Cordeiro, J. A., et al. (2009). RNA interference inhibits yellow fever virus replication in vitro and in vivo. *Virus Genes*, *38*(2), 224–231.

Padwad, Y. S., Mishra, K. P., Jain, M., Chanda, S., Karan, D., & Ganju, L. (2009). RNA interference mediated silencing of Hsp60 gene in human monocytic myeloma cell line U937 revealed decreased dengue virus multiplication. *Immunobiology*, *214*(6), 422–429.

Parameswaran, P., Sklan, E., Wilkins, C., Burgon, T., Samuel, M. A., Lu, R., et al. (2010). Six RNA viruses and forty-one hosts: Viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathogens*, *6*(2), e1000764.

Perera, R., & Kuhn, R. J. (2008). Structural proteomics of dengue virus. *Current Opinion in Microbiology*, *11*(4), 369–377.

Pfeffer, S., & Baumert, T. F. (2010). Impact of microRNAs for pathogenesis and treatment of hepatitis C virus infection. *Gastroentérologie Clinique et Biologique*, *34*(8–9), 431–435.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., et al. (2005). Identification of microRNAs of the herpesvirus family. *Nature Methods*, *2*(4), 269–276.

Qi, W. B., Hua, R. H., Yan, L. P., Tong, G. Z., Zhang, G. H., Ren, T., et al. (2008). Effective inhibition of Japanese encephalitis virus replication by small interfering RNAs targeting the NS5 gene. *Virus Research*, *132*(1–2), 145–151.

Robbins, M., Judge, A., & MacLachlan, I. (2009). siRNA and innate immunity. *Oligonucleotides*, *19*(2), 89–102.

Rouha, H., Thurner, C., & Mandl, C. W. (2010). Functional microRNA generated from a cytoplasmic RNA virus. *Nucleic Acids Research*, *38*(22), 8328–8337.

Sanchez-Vargas, I., Scott, J. C., Poole-Smith, B. K., Franz, A. W., Barbosa-Solomieu, V., Wilusz, J., et al. (2009). Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway. *PLoS Pathogens*, *5*(2), e1000299.

Segers, G. C., Zhang, X., Deng, F., Sun, Q., & Nuss, D. L. (2007). Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(31), 12902–12906.

Shi, Y., Yang, D. H., Xiong, J., Jia, J., Huang, B., & Jin, Y. X. (2005). Inhibition of genes expression of SARS coronavirus by synthetic small interfering RNAs. *Cell Research*, *15* (3), 193–200.

Shim, M. S., & Kwon, Y. J. (2010). Efficient and targeted delivery of siRNA in vivo. *The FEBS Journal*, *277*(23), 4814–4827.

Sidahmed, A. M., & Wilkie, B. (2010). Endogenous antiviral mechanisms of RNA interference: A comparative biology perspective. *Methods in Molecular Biology*, *623*, 3–19.

Skalsky, R. L., & Cullen, B. R. (2010). Viruses, microRNAs, and host interactions. *Annual Review of Microbiology*, *64*, 123–141.

Skalsky, R. L., Vanlandingham, D. L., Scholle, F., Higgs, S., & Cullen, B. R. (2010). Identification of microRNAs expressed in two mosquito vectors, Aedes albopictus and Culex quinquefasciatus. *BMC Genomics*, *11*, 119.

Smit, J. M., Moesker, B., Rodenhuis-Zybert, I., & Wilschut, J. (2011). Flavivirus cell entry and membrane fusion. *Viruses*, *3*(2), 160–171.

Smith, J. L., Grey, F. E., Uhrlaub, J. L., Nikolich-Zugich, J., & Hirsch, A. J. (2012). Induction of the cellular microRNA, Hs_154, by West Nile virus contributes to virus-mediated apoptosis through repression of antiapoptotic factors. *Journal of Virology*, *86* (9), 5278–5287.

Song, L., Liu, H., Gao, S., Jiang, W., & Huang, W. (2010). Cellular microRNAs inhibit replication of the H1N1 influenza A virus in infected cells. *Journal of Virology*, *84*(17), 8849–8860.

Stein, D. A., Perry, S. T., Buck, M. D., Oehmen, C. S., Fischer, M. A., Poore, E., et al. (2011). Inhibition of dengue virus infections in cell cultures and in AG129 mice by a small interfering RNA targeting a highly conserved sequence. *Journal of Virology*, *85* (19), 10154–10166.

Stiasny, K., Fritz, R., Pangerl, K., & Heinz, F. X. (2011). Molecular mechanisms of flavivirus membrane fusion. *Amino Acids*, *41*(5), 1159–1163.

Subramanya, S., Kim, S. S., Abraham, S., Yao, J., Kumar, M., Kumar, P., et al. (2010). Targeted delivery of small interfering RNA to human dendritic cells to suppress dengue virus infection and associated proinflammatory cytokine production. *Journal of Virology*, *84*(5), 2490–2501.

Sugiyama, R., Habu, Y., Ohnari, A., Miyano-Kurosaki, N., & Takaku, H. (2009). RNA interference targeted to the conserved dimerization initiation site (DIS) of HIV-1 restricts virus escape mutation. *Journal of Biochemistry*, *146*(4), 481–489.

Takigawa, Y., Nagano-Fujii, M., Deng, L., Hidajat, R., Tanaka, M., Mizuta, H., et al. (2004). Suppression of hepatitis C virus replicon by RNA interference directed against the NS3 and NS5B regions of the viral genome. *Microbiology and Immunology*, *48*(8), 591–598.

Ubol, S., & Halstead, S. B. (2010). How innate immune mechanisms contribute to antibody-enhanced viral infections. *Clinical and Vaccine Immunology*, *17*(12), 1829–1835.

Ulbert, S. (2011). West Nile virus: The complex biology of an emerging pathogen. *Intervirology*, *54*(4), 171–184.

Urcuqui-Inchima, S., Patino, C., Torres, S., Haenni, A. L., & Diaz, F. J. (2010). Recent developments in understanding dengue virus replication. *Advances in Virus Research*, *77*, 1–39.

van den Hurk, A. F., Ritchie, S. A., & Mackenzie, J. S. (2009). Ecology and geographical expansion of Japanese encephalitis virus. *Annual Review of Entomology*, *54*, 17–35.

Villordo, S. M., & Gamarnik, A. V. (2009). Genome cyclization as strategy for flavivirus RNA replication. *Virus Research*, *139*(2), 230–239.

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends in Genetics*, *17*(8), 449–459.

WHO (2012—Dengue). Dengue and dengue haemorrhagic fever. Fact sheet N. 117.

WHO (2012—Japanese encephalitis). Water-related diseases. Japanese encephalitis.

WHO (2012—West Nile). West Nile virus. Fact sheet N. 354.

WHO (2012—Yellow Fever). Yellow fever. Fact sheet N. 100.

Wilkins, C., Dishongh, R., Moore, S. C., Whitt, M. A., Chow, M., & Machaca, K. (2005). RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans*. *Nature*, *436* (7053), 1044–1047.

Wu, X., Hong, H., Yue, J., Wu, Y., Li, X., Jiang, L., et al. (2010). Inhibitory effect of small interfering RNA on dengue virus replication in mosquito cells. *Virology Journal*, 7, 270.

Wu, Z., Xue, Y., Wang, B., Du, J., & Jin, Q. (2011). Broad-spectrum antiviral activity of RNA interference against four genotypes of Japanese encephalitis virus based on single microRNA polycistrons. *PloS One*, *6*(10), e26304.

Yang, Y., Wu, C., Wu, J., Nerurkar, V. R., Yanagihara, R., & Lu, Y. (2008). Inhibition of West Nile Virus replication by retrovirus-delivered small interfering RNA in human neuroblastoma cells. *Journal of Medical Virology*, *80*(5), 930–936.

Ye, C., Abraham, S., Wu, H., Shankar, P., & Manjunath, N. (2011). Silencing early viral replication in macrophages and dendritic cells effectively suppresses flavivirus encephalitis. *PloS One*, *6*(3), e17889.

Yen, L. C., Lin, Y. L., Sung, H. H., Liao, J. T., Tsao, C. H., Su, C. M., et al. (2011). Neurovirulent flavivirus can be attenuated in mice by incorporation of neuron-specific microRNA recognition elements into viral genome. *Vaccine*, Epub ahead of print.

Yeung, M. L., Bennasser, Y., Watashi, K., Le, S. Y., Houzet, L., & Jeang, K. T. (2009). Pyrosequencing of small non-coding RNAs in HIV-1 infected cells: Evidence for the processing of a viral-cellular double-stranded RNA hybrid. *Nucleic Acids Research*, *37* (19), 6575–6586.

Zhang, T., Cheng, T., Wei, L., Cai, Y., Yeo, A. E., Han, J., et al. (2012). Efficient inhibition of HIV-1 replication by an artificial polycistronic miRNA construct. *Virology Journal*, *9*, 118.

Zhang, W., Singam, R., Hellermann, G., Kong, X., Juan, H. S., Lockey, R. F., et al. (2004). Attenuation of dengue virus infection by adeno-associated virus-mediated siRNA delivery. *Genetic Vaccines and Therapy*, *2*(1), 8.