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Expression of vector-based small interfering RNA against West Nile virus effectively inhibits virus replication

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Abstract

RNA interference is one of the effective emerging anti-viral strategies to inhibit virus infection in cells. In this study, a small interfering RNA expressing vector (pSilencer-NS5) targeting the NS5 gene of West Nile virus (WNV) was employed to target and destroy WNV transcripts. Real-time PCR revealed drastic reduction in WNV RNA transcripts in pSilencer-NS5-transfected Vero cells. The virus infectious titre was also significantly reduced by 90% as determined by plaque assays. The resulting decrease in virus replication was shown to be specific since both scrambled and nucleotide(s) mismatch siRNA against WNV NS5 gene did not have any effect on WNV productive yields. Furthermore, Western immunoblot analysis on the expression of viral NS5 and envelope (E) proteins showed significant down-regulation on the expression of viral NS5 and envelope (E) proteins in virus-infected cells that were pre-transfected with pSilencer-NS5. These data clearly supported the notion that the expression of vector-based siRNA against WNV NS5 gene is able to exert its silencing effect on WNV-infected cells without inducing cytotoxicity, hence holding promise in therapeutic treatment of this important emerging infectious disease.

Keywords: Flavivirus; Envelope protein; Non-structural NS5 protein; siRNA

1. Introduction

The phenomenon of RNA interference (RNAi) was observed in the nematode worm *Caenorhadbitis elegans* (Fire et al., 1998), plants (Waterhouse et al., 1998), *Drosophili*a (Kennerdell and Carthew, 1998) and other organisms. Presence of double-stranded RNA in the cell as synthetic RNAs, replicating viruses or transcribed from nuclear genes can trigger the silencing effects. It is initiated by the Dicer enzyme where it processes double-stranded RNA into approximately 22-nucleotide small interfering RNAs (siRNA). The processed duplex siRNAs are then incorporated into the multicomponent nuclease, RNA-induced silencing complex (RISC), through homologous binding, followed by the association of nucleases with RISC. Activated RISC complexes can act by promoting RNA degradation and translational inhibition of the targeted gene (Wadhwa et al., 2004).

Several methods have been employed to generate siRNAs: chemical synthesis, *in vitro* transcription, digestion of long dsRNA by an RNase III family enzyme, expression in cells from a siRNA expression plasmid or viral vector, and expression in cells from a PCR-derived siRNA expression cassette (Wadhwa et al., 2004). Plasmid-based expression systems make use of RNA polymerase (pol III) promoters such as the U6 promoter and the H1 promoter to produce short RNA species (Myslinski et al., 2001). Two approaches have been employed for the expression of siRNA species by constructs that are driven by RNA pol III. In the first approach, the sense and antisense strands of the siRNA are expressed from different U6 promoters. Alternatively, short hairpin RNAs are expressed by H1 promoters and processed by Dicer into siRNAs (Brummelkamp et al., 2002; Tuschl, 2002). The potential impact of RNAi on viral infections with diverse replication strategies had been demonstrated both *in vitro* and *in vivo* (Tan and Yin, 2004; Bai et al., 2005).

West Nile virus (WNV) is a mosquito-borne flavivirus belonging to the family *Flaviviridae*. It is the aetiological agent of West Nile fever and severe human meningoencephalitis (Solomon and Vaughn, 2002). Several parts of the world including Romania, Israel, France and Italy are experiencing

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outbreaks of WNV with associated human infection (Zeller and Schuffenecker, 2004). In the year 2004, through April 2005, there have been 2535 reported cases of West Nile virus infection in the United States of America and of which 44% were reported as West Nile meningitis or encephalitis (CDC statistics). Therefore, the emergence of WNV in several parts of the world has caused drastic increase in human infection.

Flavivirus virion contains a single plus-sense RNA genome of about 11 kb in length, which encodes a single long open reading frame (Rice et al., 1985). The encoded polyprotein is co- and post-translationally processed by viral and cellular proteases into three structural proteins [capsid (C), premembrane (prM) or membrane (M), and envelope (E)], and seven non-structural proteins [(NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) (Chambers et al., 1990)].

The NS5 protein is the largest (approximately 100-kDa) and the most conserved of the flavivirus proteins (Lindenbach and Rice, 2001). The N-terminal region of NS5 protein contains a methyltransferase domain and the C-terminal portion of this protein contains motif characteristics of an RNA-dependent RNA polymerase [(RdRps) (Koonin, 1991, 1993)]. NS5 protein interacts with the viral RNA, viral non-structural proteins and cellular proteins within the replication complex to facilitate the synthesis of viral RNA transcripts (Westaway et al., 2002). In this study, a region in the NS5 gene was selected to be a suitable silencing sequence and siRNA was designed and generated from an expression-based vector (pSilencer 3.0-H1, Ambion).

2. Materials and methods

2.1. Construction of pSilencer-NS5 vector

The coding sequence of WNV (Sarafend) NS5 protein (GenBank; AY688948) was used to design the hairpin siRNA to silent this gene using the software program siRNA Target Finder provided by Ambion, USA (http://www.ambion.com/techlib/misc/siRNA_finder.html). The algorithm selects siRNA targets by searching for AA dinulcleotides in the mRNA sequence and its downstream nucleotides with GC content ranging from 30 to 50%. The sequence 5′-AAGAACCACAACTGGTGCAGA-3′ at 8017–8035, located in the NS5 protein gene encoding region was selected by the siRNA target finder program as one of the most promising gene silencing sequence based on the selection criteria for generating siRNA target sequence. Oligonucleotides with sequence 5′-GATCCACCACAACTGGTGCAGAGCTTCAAGAGAGCTCTGCACCAGTTGTGGTTCTTTTTTGGAAA-3′ was use for cloning and its complementary strand was allowed to anneal before ligating to the linearized pSilencer 3.0-H1 vector (Ambion, USA) via the BamH I and Hind III restriction sites at the 5′ and 3′ end, respectively for overnight. This vector contains a human H1 RNA polymerase promoter site. This construct was designated as pSilencer-NS5. DNA sequencing revealed the vector constructs contained the particular insert in the correct orientation, which in turn is transcribed into short hairpin RNA (shRNA). Experimental control pSilencer vectors are pSilencer-scambled (consisting of scrambled nucleotide of the original WNV target sequence: 5′-GACGGAACGCCAGCCAATTGA-3′), pSilencer-M1 control that contains a nucleotide mismatch (nucleotide is underlined) from the original WNV target sequence (5′-AAGAACCACA CCTGGTGCAGA-3′) and pSilencer-M2 control containing two nucleotides mismatch (nucleotides are underlined) from the original WNV target sequence (5′-AAGAACCACA GATGGTGCAGA-3′) were also included in our study.

2.2. Cells and viruses

Vero cells (African green monkey kidney) used throughout all experiments were grown in Medium 199 (M199) containing 10% heat-inactivated FCS. WNV [(strain Sarafend, WNV lineage II human isolate from Israel in 1950s) (Scherret et al., 2002)], a kind gift from Emeritus Professor E.G. Westaway (Queensland, Australia), was propagated in Vero cells throughout this study. After infection at a multiplicity of infection (M.O.I.) of 10, cell monolayers were maintained in M199 with 2% FCS. To quantify virus titres, plaque assays were carried out as essentially described by Chu and Ng (2003).

2.3. Transfections

Unless stated otherwise, transfections were performed by using Lipofectamine Plus reagents (Invitrogen, California, USA) according to the manufacturer's protocol. In brief, 2 μg of the plasmid was complexed with 4 μl of Plus reagent in 25 μl of OPTI-MEM medium (Gibco, California, USA) for 15 min at room temperature. The mixture was then added to 25 μl of OPTI-MEM containing 2 μl of Lipofectamine. After incubation for another 15 min, the DNA-liposome complexes were added to the Vero cells grown in 24-well tissue culture plates (Nunc, Neerijse, Belgium). After incubation for 3 h at 37 °C, 1 ml of complete growth medium was added and incubated for another 24 h before virus infection was carried out.

2.4. Quantification of WNV transcript by RT-PCR

Total mRNA was extracted from the pSilencer-NS5-transfected cells at 12 and 24 h p.i. The mock-infected/pSilencer-NS5-transfected cells were used as negative control. As described in the protocol of Qiagen Rneasy Mini Kit (Hilden, Germany), the cells were lysed and mRNA was collected with the inclusion of an additional DNase digestion step to remove contaminating genomic DNA. The total mRNA isolated from WNV-infected cells (at 12 h p.i., not transfected), were used as positive control. Total mRNA collected from cells transfected with pSilencer-control vector and infected with WNV was also used in the study. WNV gene-specific primer, with sequence 5′-AGCATGGACGTTGACCGAAAGG-3′ that correspond to the WNV E gene sequence, was used in the reverse transcription PCR to generate its cDNA. Real time PCR (RT-PCR) was carried out to quantify the copy numbers of the E protein gene. A 25 μl reaction mixture contained 2 μl of cDNA, 12.5 μl of Platinum SYBR Green (Invitrogen, California, USA) and 1 μl each of the forward (5′-TCCCTGAACGACCTTACACC-3′) and reverse 5′-

ACTAAACGGGGGTTCGAGTT-3′ primers. The negative control contained all SYBR green reagents except DNA. Reactions were cycled at 50 °C for 2 min and then 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, followed by a melting curve analysis. WNV RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the protocol and serial diluted before subjected to the above reactions to construct a standard curve for comparisons of relative virus transcripts. The threshold cycles from the various samples were translated to relative virus transcripts using the calibration graph.

2.5. Antibodies

Rabbit polyclonal anti-WNV NS5 antiserum was a kind gift from Emeritus Professor Westaway (Australia). Monoclonal anti-WNV antibody and anti-actin antibody were purchased from Microbix Biosystem (Toronto, Canada) and Chemicon (California, USA), respectively. Secondary antibodies conjugated with fluorescein isothiocyanate (FITC) and alkaline phosphatase (Amersham Pharmacia, Buckinghamshire, UK) was used in indirect immunofluorescence microscopy and Western blot, respectively.

2.6. Indirect immunofluorescence microscopy

For immunofluorescence microscopy, cell monolayers were grown on coverslips and infected with WNV at an M.O.I. of 10. The procedure was similar to that described by Chu and Ng, 2003. Cells were incubated with the primary antibodies (at a 1:500 dilution for anti-NS5 antibody and 1:1000 for anti-envelope antibody) in a humidity chamber for 1 h at 37 °C, washed and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Amersham Pharmacia, Buckinghamshire, UK). The samples were viewed under 1X-81 microscope (Olympus, Tokyo, Japan) at an excitation wavelength of 480 nm for FITC using oil immersion objectives under 100× magnification with the Metamorph imaging software programme. Semi-quantitative measurement of the percentages of fluorescing cells was noted in each sample of 200 cells taken from five different fields.

2.7. SDS-PAGE and Western blot analysis

Cell lysates were subjected to SDS-PAGE before transfer to nitrocellulose membranes (Bio-Rad, San Francisco, USA) using the Phast system (Amersham Pharmacia, Buckinghamshire, UK) as described by (Chu and Ng, 2003). Primary antibodies, anti-NS5, anti-envelope at dilutions of 1:500, 1:1000, respectively, were used to detect these proteins. The blot was incubated overnight at room temperature on an orbital shaker. Alkaline phosphatase-conjugated secondary antibodies (Amersham Pharmacia, Buckinghamshire, UK) were used to detect antibody binding with the addition of substrate (*p*-nitroblue tetrazolium, NBT). Band intensity was measured using ImageQuant version 5.2 (Molecular Dynamics, Buckinghamshire, UK).

2.8. Ultrastructural studies

At 12 h p.i., Vero cells that were pSilencer-NS5-transfected and/or infected with WNV at an M.O.I. of 10 was washed twice with cold PBS and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde. Cells were then processed for embedding in low-viscosity epoxy resin as previously described by (Chu and Ng, 2003). Ultrathin sections (50–70 nm) stained with 2% uranyl acetate and post-stained with 2% lead citrate were viewed under a Philips electron microscope (CM 120, BioTwin, Enidhoven, The Netherlands).

3. Results

3.1. siRNA decreases WNV RNA transcripts

A plasmid-based siRNA expressing vector, pSilencer-NS5 was designed to specifically target the NS5 gene of the viral genome for destruction. Stringent selection of the target region was carried out (as described in Section 2) to ensure effective gene silencing. Firstly, to determine if the specific siRNA generated from pSilencer-NS5 construct has a direct effect on inhibiting WNV replication at gene level, the amount of WNV viral RNA transcripts in pSilencer-NS5 transfected cells was quantified by real-time PCR. Vero cells were transfected with pSilencer-NS5 and the cells were then subjected to WNV infection at a M.O.I. of 10. The infected cells were then harvested at 12 and 24 h p.i. and processed for real-time PCR. The cells transfected with pSilencer-NS5 showed a 2.57 and 2.895 log unit reduction in WNV viral transcripts when compared to the wild-type infection at 12 and 24 h p.i., respectively (Fig. 1). Therefore, pSilencer-NS5 is capable of reducing the amount of viral RNA transcripts significantly as compared to the control vector-transfected cells.

3.2. siRNA decreases the expression of viral NS5 and E proteins

Since pSilencer-NS5 is capable of causing destruction to the viral RNA transcripts, the expressional level of viral NS5 protein was further assessed to provide confirmation on the inhibitory effects of the siRNA on WNV infection. Expression of NS5 protein in WNV-infected cells were determined by immunofluorescene staining using rabbit polyclonal anti-NS5 anti-serum and followed by secondary antibody conjugated to FITC. Two hundred cells were counted in each sample and the percentages of fluorescing cells were noted. Typical perinuclear staining distribution pattern of NS5 protein was observed in Vero cells infected with WNV (Fig. 2a and b, arrows). For WNV-infected cells (not transfected with pSilencer-NS5), at 12 and 24 h p.i., more than 90% of the cell population were stained green using anti-NS5 protein antibody-conjugated to secondary antibodies with FITC (Fig. 2a and b). Cells transfected with either the pSilencer-scrambled control, pSilencer-M1 control or pSilencer-M2 control vectors and infected with WNV showed almost the same intensity and frequency of fluorescence as the non-transfected wild type WNV-infected cells (Fig. 2c–h). This showed that the

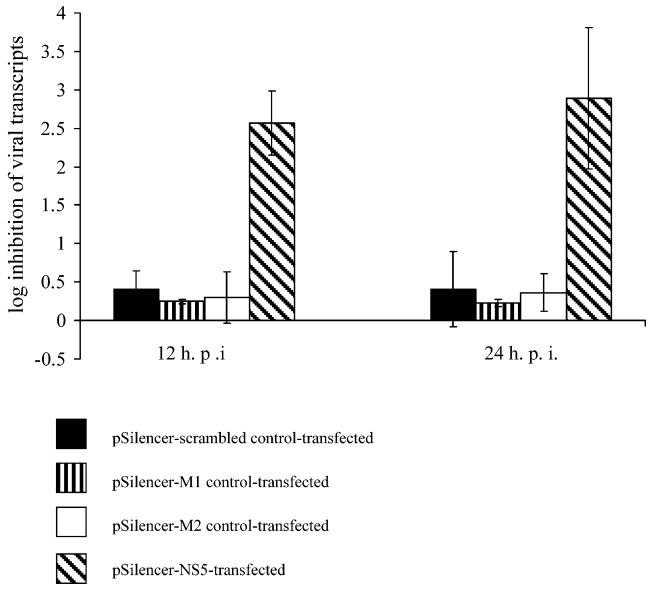

Fig. 1. There is a 2.57 and 2.895 log unit reduction the number of WNV transcripts from the pSilencer-NS5-transfected cells compared to WNV infection (without transfection) at 12 and 24 h p.i. respectively. Infection studies followed by the real-time PCR were carried out on three separated occasions. (■) Transfected with pSilencer-scrambled control vector; (▥) transfected with pSilencer-M1 (one nucleotide mismatch) control vector; (□) transfected with pSilencer-M2 (two nucleotides mismatch) control vector; (▨) transfected with pSilencer-NS5 vector.

vectors containing non-specific or mismatch sequences did not influence the virus replication. Mock-infected-pSilencer-NS5-transfected Vero cells acted as negative control yielded no green fluorescence at the two time points (Fig. 2i and j). This indicated that the anti-sera did not cross-react with the Vero cells.

In comparison to the wild-type WNV-infection (Fig. 2a), the infected Vero cells that were pre-transfected with pSilencer-NS5 construct displayed a marked decrease in intensity and frequency of staining, with less than 10% of the cell population emitting green fluorescence (Fig. 2k) at 12 h p.i. At 24 h p.i., about 40% of the pSilencer-NS5-transfected cells showed fluorescence but at much lower intensity when compared to the non-transfected cells (Fig. 2l). In addition, there was no visible sign of any cytotoxicity in the pre-transfected and infected cells. Thus, the lack of immunofluorescence was due to the effectiveness of the siRNA in terminating virus replication and not due to any possible cytotoxicity effects.

In addition, the protein expression level of the WNV major structural envelope (E) glycoprotein (as a representative of the degradation of the entire viral genome) was also investigated. Immunofluorescence staining was carried out with anti-E antibody. Vero cells transfected with the pSilencer-scrambled vector control plasmid and infected with WNV (Fig. 2o and p) showed similar infection patterns as the non-transfected wild type infection (Fig. 2m and n). Cells transfected with the plasmids producing mismatch siRNA and infected with WNV (Fig. 2q to t) also revealed similar infection rate as the non-transfected wild-type infection. Mock-infected-pSilencer-NS5-transfected cells were used as negative control (Fig. 2u and v). Consistent with the NS5 protein, WNV-infected Vero cells that were transfected with pSilencer-NS5 vector displayed a marked decrease in intensity and frequency of staining for viral E protein, indicating a reduction in the expression of the E protein. There was less than 10% of the cell population emitting green fluorescence (Fig. 2w) as compared to the non-transfected but WNV-infected cells (Fig. 2n) at 12 h p.i. Similarly, at 24 h p.i., there was a 30% increased in the number of pSilencer-NS5-transfected cells exhibiting strong fluorescence (Fig. 2x). A possible explanation for this observation is that transfection efficiency of pSilencer-NS5 is usually not 100% and this could possibly explain for the increase in the fluoresecene staining for NS5 and E proteins observed in the pSilencer-NS5-transfected cells at 24 h p.i.

To affirm the down-regulation on the expression of WNV viral protein by the plasmid-based expressed siRNAs, the expression levels of viral proteins (NS5 and E) were also determined by Western blot detection. In Fig. 3a, the pSilencer-NS5-transfected and infected cells showed a drastic decrease in the amount of NS5 protein at 12 h p.i. with WNV when compared to wild-type WNV infection that did not have transfection with the pSilencer-NS5. There was a four-fold decrease in the band intensity using ImageQuant version 5.2 (Molecular Dynamics, Buckinghamshire, UK). Similarly, the viral E protein concentration was also lower in pSilencer-NS5-transfected and infected cells at 12 h p.i. with a difference in band intensity of 18-folds compared to WNV-infected cells without transfection with the pSilencer-NS5. Although at 24 h p.i., there was an increase in the quantity of viral NS5 and E proteins, the pSilencer-NS5-transfected and infected cells still showed 2- and 1.7-folds lower amount of viral NS5 and E proteins, respectively when compared to the non-transfected WNV infected cells. Cell lysates from mock-infected-pSilencer-NS5 transfected cells and pSilencer-control vector-transfected cells were also included in our study. Actin was used as a loading control to ensure equal amount of proteins were loaded into each wells. The 12 and 24 h p.i. results correlated well with the previous data gathered from immunofluorescence detection of viral proteins suggesting that there was indeed an inhibition of viral proteins production.

3.3. Reduced production of WNV from pSilencer-NS5-transfected cells

To corroborate with the significant reduction in the viral RNA transcripts and viral proteins expression, the infectious titre of WNV from pSilencer-NS5-transfected cells were also determined by plaque assays. Supernatants (containing the released infectious virons) were collected from the pSilencer-NS5-transfected (infected) and WNV-infected (not-transfected) cell cultures and were used to determine the infectious virus titre. In the pSilencer-NS5-transfected and infected cells (12 h p.i.), there was a mark decrement of 90% in the number of infectious virus released as compared to the wild-type WNV virus-infected cells (Fig. 3b). Although at 24 h p.i., there was an increase in the number of virus particles produced by cells transfected with pSilencer-NS5, the virus titre was still lower than the non-tranfected WNV-infected cells by 65%. This corresponded well with the above data indicating that there was inhibition of virus replications.

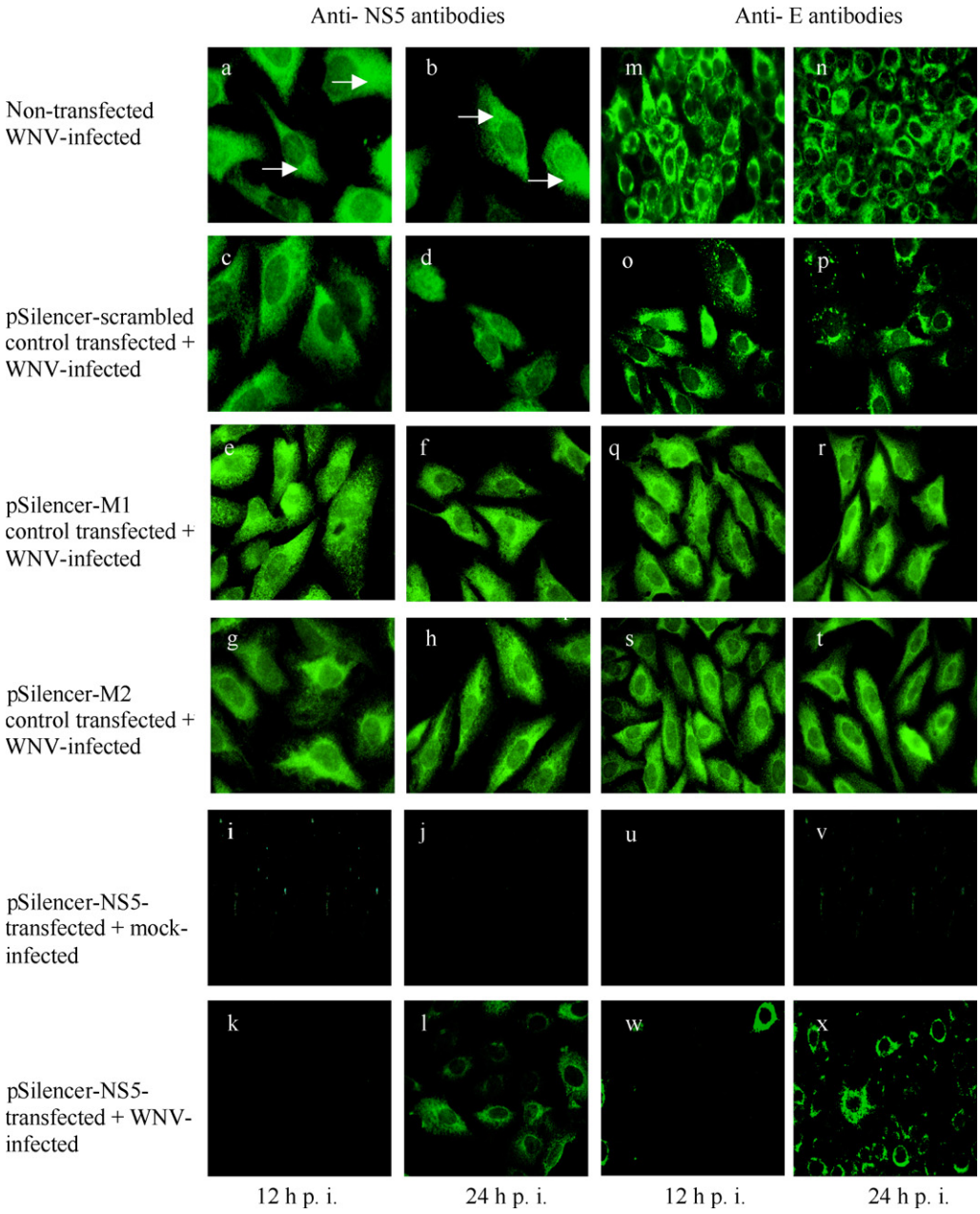

Fig. 2. Reduction of viral proteins expression in pSilencer-NS5-transfected cells. pSilencer-NS5-transfected cells show marked decrement in intracellular NS5 and E proteins after 12 h p.i. by WNV (M.O.I. = 10). Cells are stained with polyclonal anti-NS5 antibody (a–l) and monoclonal anti-envelope antibody (m–x) followed by secondary antibody-conjugated to FITC. Non-transfected wild-type WNV-infected cells WNV infection was used as positive control [(a, b, m and n)]. Cells transfected with pSilencer-scrambled control and infected with WNV are shown in (c, d, o and p). Cells transfected with nucleotide(s)-mismatch control vectors and infected with WNV are also included [pSilencer-M1 control (e, f, q and r); pSilencer-M2 (g, h, s and t)]. Mock-infected-pSilencer-NS5-transfected Vero cells act as negative control yielding minimal green fluorescence [(i, j, u and v)]. Cells transfected with pSilencer-NS5 showed minimal fluorescence, indicating the inhibition of viral replication [(k, l, w and x)].

In addition, transmission electron microscopy was also used to evaluate the ultrastructural effects of pSilencer-NS5 on WNV infection. pSilencer-NS5-transfected and infected cells showed minimal virus particles being released out of the cells. Screening of 25 cells showed only three virus particles extruded out of one cell (Fig. 4a). The pSilencer-NS5-transfected cells also appeared healthy with large number of intact mitochondria (Fig. 4b, arrowheads), indicating that siRNA did not interfere with the normal cellular physiology or cause any cytotoxic effects on the transfected cells. As expected, large number of virus particles was observed in all 25 cells of WNV-infected Vero cells in the absence of pSilencer-NS5 (Fig. 4c).

4. Discussion

This study demonstrated the effectiveness of using the siRNA expression-based vector targeting WNV NS5 gene to inhibit WNV replication, viral protein expression and the production of infectious viral particles. The results are consistent with previous studies that showed the effectiveness of gene silencing

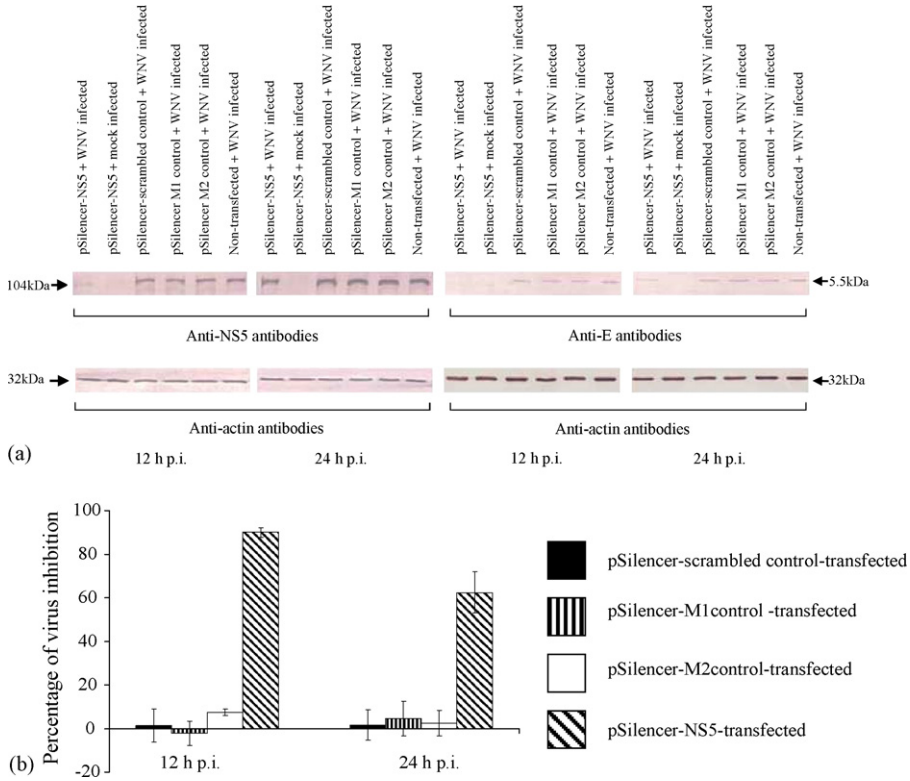

Fig. 3. Western blot and infectious titers in pSilencer-NS5-transfected cells. (a) SDS-PAGE and Western blots analyses of intracellular NS5 and E proteins. pSilencer-NS5-transfected and infected cells showed drastic decreases in NS5 and E protein at both 12 and 24 h p.i. Actin is used as a control to ensure equal amount of proteins are loaded. (b) There is a 90 and 65% decrease in infectious virus particles production from the pSilencer-NS5-transfected cells compared to WNV infection (without transfection) at 12 and 24 h p.i., respectively. Plaque assays are carried out using the supernatants from cells. (■) Transfected with pSilencer-scrambled control vector; (▥) transfected with pSilencer-M1 control vector; (□) transfected with pSilencer-M2 control vector; (▨) transfected with pSilencer-NS5 vector) at 12 and 24 h p.i. from three separate occasions.

technologies in preventing lethal WNV infections (Bai et al., 2005; McCown et al., 2003; Geiss et al., 2005).

Real-time PCR analysis of the pSilencer-NS5-transfected cells showed diminished level of viral RNA transcripts as compared to the WNV-infected cells in the absence of pSilencer-NS5 plasmid. Analysis of viral proteins expression also showed significant reduction in the intracellular viral E and NS5 proteins, as evident from the Western blot analysis (Fig. 3a). Furthermore, the pSilencer-NS5-transfected cells yielded a significantly lower virus titre as compared to the WNV-infected cells in the absence of pSilencer-NS5 (Fig. 3b). From the transmission electron microscopy, it was clearly demonstrated that the viral silencing effect using the transfected pSilencer-NS5 plasmid was specific for WNV RNA and did not disrupt cell morphology (Fig. 4b). In addition, cytotoxicity assay results showed that the transfection of the pSilencer-NS5 does not induce cytotoxicity by measuring the level of lactate dehydrogenase released when compared to non-transfected counterparts (data not shown). Therefore, siRNA inhibition of flavivirus infections could be an attractive therapeutic tool.

However, there are some important considerations about the therapeutic application of siRNA technology. Firstly, double-stranded RNA longer than 30 nucleotides (nt) can trigger interferon response leading to non-specific RNA transcripts degradation and a general shutdown of host cell protein translation (Sui et al., 2002). Even though this can be overcome by using shorter siRNA of about 21 nt, oligonucleotide-based siRNA is costly and their effect can be short-lived (Wadhwa et al., 2004). Hence, in this current study, a siRNA expression vector-based system was used. This system ensures continuous production of unimolecular siRNAs (Sui et al., 2002) in cells and does not trigger interferon inducible pathways at low doses of plasmids (Zhang et al., 2004). This enables a sustained silencing of the protein encoded by the targeted viral RNA without dose-dependability problem. Moreover, being a DNA vector it is also more stable and allows the introduction of siRNA vector intravenously or via liposomes and gene gun. In addition, the design of the siRNA sequence is important. It has to be completely complementary to the target sequence as a nucleotide change may either render the siRNA ineffective (as shown in our study) or result in siRNA-induced translational repression of target gene (Hammond, 2005).

To further assess the sustained gene silencing effect of pSilencer-NS5, virus replication was also determined at 24 h p.i. Although at 24 h p.i., there was an increment in the number of infectious virus from the pSilencer-NS5-transfected and infected cells (compared to 12 h p.i.), there was still a 65% reduction in infectious virus production compared to the wild type infection. The possible explanation for the slight increase in virus production could be attributed by the fact that transfection

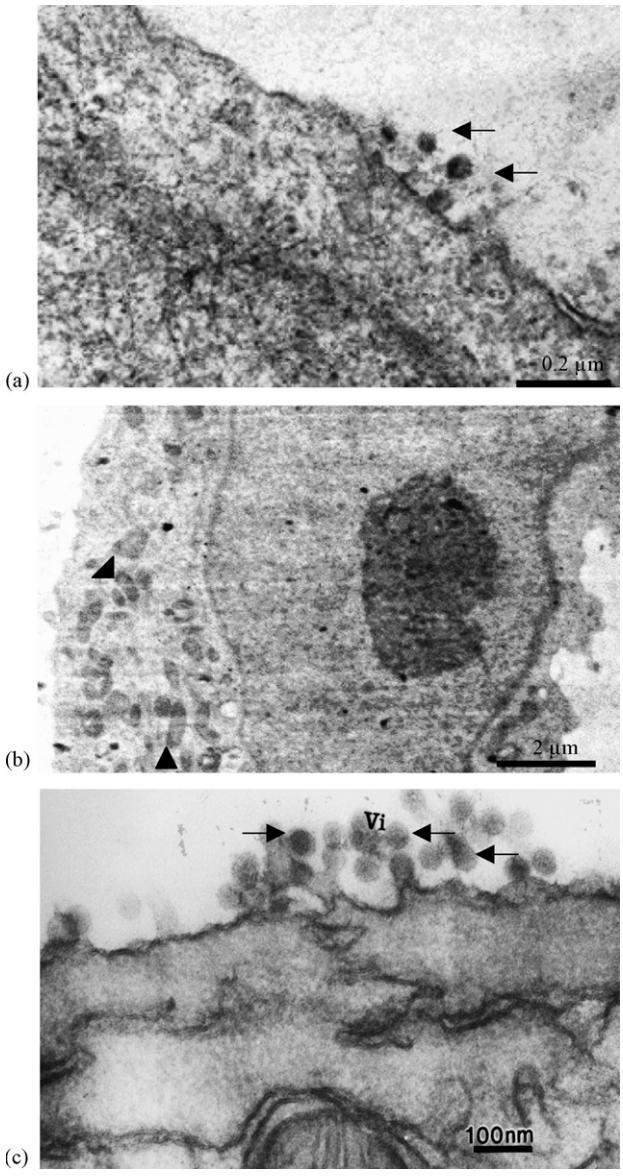

Fig. 4. Cells transfected with pSilencer-NS5 and infected with WNV exhibit normal morphology and reduced virus production at 12 h p.i. (a) Only three virus particles (arrows) are observed budding from one of the 25 cells screened from the pSilencer-NS5-transfected cells. (b) Transfected cells appear to maintain the normal physiological state as shown by the large number of mitochondria (arrowheads) in the cytoplasm. (c) Large number of virus particles (Vi-arrows) is seen budding from all 25 cells in the WNV-infection (not transfected).

of the pSilencer-NS5 constructs into Vero cells is not of 100% efficiency. In order to determine the transfection efficiency of the pSilencer-NS5 plasmid, a reporter plasmid (expresses GFP) was used to transfect Vero cells under the same conditions as the pSilencer plasmids. It was found only about 52% of the cells were transfected (data not shown). Thus, there were cells present in the population that were not transfected with the pSilencer-NS5 but were infected with the virus. These infected cells can produce high titres of infectious virus masking some of the silencing effects of the transfected cells. Nevertheless, these results indicated that the pSilencer-NS5 continued to exert its effect after 24 h p.i. (2 replication cycles) and the construct is stable over time without inducing any cytotoxic effects on the transfected cells.

The siRNA technology holds great promise in biological and medical fields. The potential of siRNA remains to be established in the area of gene therapy. To the present, siRNA has been successfully employed in inhibiting Hepatitis B and Hepatitis C virus replication in mice (McCaffrey et al., 2003; Wilson et al., 2003). This finding is particularly relevant to infectious disease as it is believed that endogenous RNA interference mechanisms evolved, at least in part, to protect cells against infectious pathogens such as viruses (Hannon, 2002). This study has demonstrated the potential of vector-based siRNA in inhibiting WNV replication. This study is now being extended to investigating the universal effect of this siRNA expression vector in other flavivirus infections. With more data on the mechanism of this inhibition process in flavivirus infections, a suitable animal model can be designed to investigate its suitability for in vivo study.

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