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Insertion of MicroRNA Targets into the Flavivirus Genome Alters Its Highly Neurovirulent Phenotype▽

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Flaviviruses such as West Nile, Japanese encephalitis, and tick-borne encephalitis (TBEV) viruses are important neurotropic human pathogens, causing a devastating and often fatal neuroinfection. Here, we demonstrate that incorporation into the viral genome of a target sequence for cellular microRNAs expressed in the central nervous system (CNS) enables alteration of the neurovirulence of the virus and control of the neuropathogenesis of flavivirus infection. As a model virus for this type of modification, we used a neurovirulent chimeric tick-borne encephalitis/dengue virus (TBEV/DEN4) that contained the structural protein genes of a highly pathogenic TBEV. The inclusion of just a single target copy for a brain tissue-expressed mir-9, mir-124a, mir-128a, mir-218, or let-7c microRNA into the TBEV/DEN4 genome was sufficient to prevent the development of otherwise lethal encephalitis in mice infected intracerebrally with a large dose of virus. Viruses bearing a complementary target for mir-9 or mir-124a were highly restricted in replication in primary neuronal cells, had limited access into the CNS of immunodeficient mice, and retained the ability to induce a strong humoral immune response in monkeys. This work suggests that microRNA targeting to control flavivirus tissue tropism and pathogenesis might represent a rational approach for virus attenuation and vaccine development.

There are more than 70 single-stranded, positive-sense RNA viruses in the arthropod-borne flavivirus genus of the *Flaviviridae* family, many of which are important human pathogens that cause a devastating and often fatal neuroinfection (21). Flaviviruses are transmitted in nature to various mammals and birds through the bite of an infected mosquito or tick; they are endemic in many regions of the world and include mosquito-borne yellow fever (YFV), Japanese encephalitis (JEV), West Nile (WNV), St. Louis encephalitis virus (SLEV), dengue viruses, and the tick-borne encephalitis (TBEV) viruses. During the past 2 decades, both mosquito- and tick-borne flaviviruses have emerged in new geographic areas of the world where previously they were not endemic and have caused outbreaks of diseases in humans and domestic animals (TBEV in Northern Europe and Japan, JEV in Australia and Oceania, and WNV in North and South America).

There are only two successful live attenuated flavivirus vaccines that protect against diseases caused by flaviviruses, one for yellow fever and one for Japanese encephalitis. These vaccine viruses were generated using the classical method of repeated passage of virus in cell cultures (10, 26). Long-term experience with these two vaccines has demonstrated that live attenuated virus vaccines are an efficient approach to prevent diseases caused by flaviviruses since just a single dose of the vaccine virus provides a long-lasting protective immunity in humans that mimics the immune response following natural infection (26). For many years, a number of new flavivirus vaccine strategies have been developed or are under way (34), but they have not yet led to licensed human vaccines against neurotropic flaviviruses such as TBEV, SLEV, or WNV. The discovery of microRNAs (miRNAs), small regulatory noncoding RNAs that regulate the expression of cellular genes at the posttranscriptional level, has enabled a novel strategy to control virus tissue tropism and may provide opportunities for developing live attenuated virus vaccines (20).

Mature miRNAs regulate diverse cellular processes in many plant and animal species through the assembly of an miRNA-induced silencing complex (RISC), which binds the complementary targets in mRNA and subsequently catalytically cleaves or transcriptionally represses the targeted mRNA (4, 7, 11). In addition, recent studies suggest that miRNAs also play a role in the regulation of virus infections (5, 8, 13). Since the pattern of miRNA expression is cell and tissue specific, it would be a disadvantage for viruses to contain sequences in their genomes that are complementary to cellular miRNAs present in tissues in which they would otherwise replicate efficiently and cause disease. Several miRNAs have recently been shown to modulate the tissue tropism of a number of viruses from different families (3, 15–17). Many flaviviruses cause neurologic disease such as meningitis and/or encephalitis, and we sought to design a flavivirus that would be selectively attenuated for the central nervous system (CNS) since this is a target of wild-type neurotropic virus. In the present study, we explored the ability of the cellular miRNAs expressed in brain tissue to control the neurotropism of a flavivirus bearing complementary miRNA target sequences. We anticipated that these viruses would replicate in peripheral non-CNS tissues and induce a strong adaptive immune response but would be restricted in their ability to replicate in the CNS since the

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CNS-expressed miRNAs would recognize the introduced complementary target sequences in the viral RNA genome and limit its translation, replication, and assembly into a virion.

The miRNA target sequences that were selected for insertion into the viral genome were complementary to let-7c, mir-9, mir-124a, mir-128a, and mir-218 miRNA, which have evolutionarily conserved sequences among mammalian species, including mice and humans (38). With the exception of mir-218a, which was shown to be exclusively expressed in cranial motor nuclei and spinal motor neurons of zebrafish (14), all of the other selected miRNAs were previously found to be highly expressed in the brain of adult mice and humans (1, 19, 38). mir-124a is highly upregulated in neuronal cells as are mir-9 and mir-128a, but the latter two are also found in peripheral tissue cells (29, 38). The brain-enriched let-7c miRNA is a member of the let-7 family of miRNAs that are found to be widely expressed in many tissues of various species and also act as tumor suppressors (2, 41).

The flavivirus genome is a positive-sense single-stranded RNA that is approximately 11 kb in length and contains 5′ and 3′ noncoding regions (NCR) flanking a single open reading frame (ORF) encoding a polyprotein that is processed by viral and cellular proteases into three structural proteins (capsid [C], premembrane [prM], and envelope [E]) and seven nonstructural proteins (21). The five miRNA targets that we selected were individually introduced into the 3′ NCR of the viral genome since the 3′ NCR targeting of cellular mRNAs was found to be more frequent and effective than 5′ NCR or ORF targeting (4, 7, 9). As a model virus for modification of flavivirus neurotropism, we selected a chimeric tick-borne encephalitis/dengue type 4 virus (TBEV/DEN4) that was constructed by replacing the structural prM and E protein genes of the nonneuroinvasive, mosquito-borne dengue type 4 virus (DEN4) with the corresponding genes of the highly virulent Far Eastern strain of TBEV (30). TBEV/DEN4 retains a high level of neurovirulence from its TBEV parent (a biosafety level 4 agent) in mice inoculated intracerebrally; however, consistent with the phenotype of its other parent, a DEN4 virus, the chimeric TBEV/DEN4 virus is nonneuroinvasive in immunocompetent mice and monkeys following a peripheral route of inoculation (37). Here, we demonstrate that the incorporation into the TBEV/DEN4 genome of a single copy of a target for an miRNA highly expressed in brain tissue (mir-9, mir-124a, or let-7c) was sufficient to restrict the neurotropism of the engineered chimeric viruses, resulting in attenuation of the TBEV/DEN4 virus for the CNS of adult mice. Importantly, the miRNA target TBEV/DEN4 viruses retained the ability to replicate in non-CNS tissues of rhesus monkeys and induce a robust immune response.

MATERIALS AND METHODS

Cells and viruses. Mosquito C6/36 cells (ATCC) were maintained in Eagle's minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (Lonza), 2 mM L-glutamine, 2 mM nonessential amino acids, and 50 μg/ml gentamicin (Invitrogen) at 32°C in an atmosphere of 5% CO_2 (32). Simian Vero cells were cultured as previously described (6). Primary rat cortical neurons (Gibco) isolated from day 18 Fisher 344 rat embryos were maintained in Neurobasal medium supplemented with 0.5 mM GlutaMAX-I and 2% B27 supplement (Invitrogen) at 37°C in 5% CO_2.

Chimeric TBEV/DEN4 cDNA (GenBank accession number FJ28986) contains the prM and E protein genes of Far Eastern subtype TBEV strain Sofjin, with the remaining sequence derived from recombinant DEN4 virus (30). This chimeric cDNA was used to generate recombinant viruses containing a large deletion in the 3′ NCR or the insertion of an miRNA target sequence. To generate the 3′ NCR deletion mutant virus, the DNA PstI-BstBI fragment (from nucleotides [nt] 8171 to 10280) and BstBI-KpnI fragment (from nt 10523 to 10664) were amplified by PCR and used for replacement of the corresponding sequence (from nt 8171 to 10664) in the full-length cDNA genome of TBEV/DEN4. The resulting cDNA clone (TBEV/DEN4Δ243) differed from parental cDNA by the insertion of the new BstBI cleavage site located immediately after the TAA stop codon and the deletion of 243 nt of the TBEV/DEN4 genome (from nt 10281 to 10523). To generate viruses carrying a single target for let-7c, mir-9, mir-124a, mir-128a, or mir-218 miRNA, the inserted sequence was 5′-TTCGAAAACCATACAACCTACTACCTCACTCGAG-3′, 5′-TTCGAATCATACAGCTAGATAACCAAAGACTCGAG-3′, 5′-TTCGAATGGCATTCACCGCGTGCCTTAACTCGAG-3′, 5′-TTCGAAAAAAGAGACCGGTTCACTGTGACTCGAG-3′, or 5′-TTCGAAACATGGTTAGATCAAGCACAACTCGAG-3′, respectively. Each miRNA target sequence flanked with a BstBI site at the 5′ end and an XhoI site at the 3′ end was synthesized by Blue Heron Biotechnology and inserted immediately after the TAA stop codon into the TBEV/DEN4 genome between nt 10280 and 10281. RNA transcripts derived from the modified TBEV/DEN4 cDNA clones were generated by transcription with SP6 polymerase and used to transfect Vero or C6/36 cells in the presence of Lipofectamine (Invitrogen) as described previously (6, 33). Transfected cells were examined by immunofluorescence assay (IFA) for the presence of TBEV proteins using TBEV-specific antibodies in hyperimmune mouse ascetic fluid (ATCC). When 80 to 100% of cells were positive by IFA, the recovered viruses from the cell culture medium were collected, biologically cloned by two terminal dilutions, and then amplified by two passages in Vero or C6/36 cells. The 3′ NCR deletion mutant (TBEV/DEN4Δ243) and four TBEV/DEN4 viruses carrying miRNA target sequences (designated mir-9T, mir-124aT, mir-128aT, and mir-218T) were recovered from Vero and C6/36 cells while a virus containing the target sequence for miRNA let-7c (designed let-7cT) was rescued only from C6/36 cells. To verify the presence of the introduced miRNA target insertion into the genome, viral RNA for each virus was isolated, and the consensus sequence of the genome was determined.

Virus infection in cell cultures. The levels of replication of parental and derivative viruses were compared in a Vero or C6/36 cell line. Cells grown on six-well plates were inoculated with virus at a multiplicity of infection (MOI) of 0.01 PFU/cell and were allowed to adsorb virus for 1 h. Inoculum was then replaced with fresh medium. Virus in culture medium was harvested on day 4 postinfection (p.i.), and its titer was determined in Vero or C6/36 cells using a plaque-forming assay (PFA) as described previously (32, 33). To initiate let-7cT virus replication in Vero cells, cells were infected with the C6/36 cell-derived let-7cT virus at an MOI of 5 and examined on every third day postinoculation by IFA. In two separate experiments, on days 12 and 15 postinfection, when ~100% of cells became virus antigen positive, two viruses (let-7cTΔ and let-7cT*) were isolated, purified, sequenced, and used for studies in cell cultures. Primary rat cortical neuronal cells were grown in chambers (10^5 cells/chamber) of BD BioCoat glass slides (BD Biosciences) for 7 days and then infected with viruses at an MOI of 0.5. Cell supernatant was collected daily, and virus titer was determined in Vero or C6/36 cells.

Immunocytochemistry. Virus-infected cells on glass slides were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 for 5 min. Cells were incubated for 1 h with 5% goat serum (Invitrogen) in Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen) and then treated sequentially with 1:100 diluted TBEV-specific antibodies for 1 h and with 1:500 diluted fluorescein-labeled antibodies to mouse IgG (KPL) for 1 h. All incubations were followed by extensive washing with D-PBS (Invitrogen). To stain nuclei, we used mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Fluorescence microscopy was performed with a Nikon 90i epifluorescence microscope equipped with a digital imaging system (Roper Scientific camera and Nikon NIS-Elements software).

RNA isolation, reverse transcription, sequence analysis, and quantitation. Viral RNA from cell culture medium or mouse brain homogenates was isolated using a QiaAmp Viral RNA Mini kit (Qiagen), and one-step reverse transcription-PCR (RT-PCR) was performed using a Superscript One-Step kit (Invitrogen) with DEN4- or TBEV-specific primers. The nucleotide consensus sequences of the virus genomes were determined through direct sequence analysis of the PCR fragments on a 3730 Genetic Analyzer using TBEV or DEN4 virus-specific primers in BigDye terminator cycle sequencing reactions (Applied Biosystems) and were analyzed using Sequencher, version 4.7, software (Gene Codes Corp.).

Total RNA from 4×10^6 Vero or C6/36 cells was first isolated using a Qiagen

miRNeasy kit (Qiagen) and then used to determine the copy number of let-7c, mir-9, mir-124a, mir-128a, and mir-218 microRNA molecules by TaqMan microRNA quantitative RT-PCR (qRT-PCR; Applied Biosystems) according to the manufacturer's protocols. Briefly, 1 ng of each RNA sample was reverse transcribed, and the subsequent product was amplified and measured in triplicate using an ABI TaqMan microRNA assay on a validated ABI 7900HT real-time thermocycler. To determine each miRNA copy number per ng, the absolute quantity of each miRNA was calculated using a standard curve that was independently generated with known quantities of the corresponding synthetic miRNA oligonucleotide (Asuragen, Inc.).

Evaluation of viruses in mice. Studies in mice were conducted according to federal and NIAID Animal Care and Use Committee regulations. The neurovirulence of parental TBEV/DEN4, TBEV/DEN4Δ243, let-7cT, and let-7cT* viruses was evaluated in 3-day-old Swiss Webster mice (Taconic Farms) by intracerebral (i.c.) inoculation. Suckling mice in litters of 10 were inoculated with 1, 10, or 10^2 PFU of virus and monitored for morbidity and mortality up to 21 days postinoculation. The 50% lethal dose (LD_{50}) was determined by the Reed and Muench method (35). Moribund (paralyzed) mice were humanely euthanized. To determine the neurovirulence of TBEV/DEN4 and all miRNA target TBEV/DEN4 viruses, 6-week-old Swiss mice in groups of five were inoculated i.c. with 10-fold serial dilutions of virus ranging from 1 to 10^3 PFU of TBEV/DEN4 or from 10 to 10^5 PFU of each engineered miRNA target virus. Mice were monitored for signs of encephalitis for 21 days, and the brains of mice which received a dose of 10^5 PFU of virus were harvested on day 22 and were assayed for virus by PFA on Vero and C6/36 cells (6, 37) and by RT-PCR. To investigate the neuroinvasiveness of TBEV/DEN4 and its derivatives, 3-week-old SCID mice (ICRSC-M; Taconic Farms) in groups of five were inoculated intraperitoneally (i.p.) with 10^5 PFU of virus and observed for 49 days for signs of morbidity typical for CNS involvement, including paralysis. Moribund mice were humanely euthanized, and their brain homogenates were prepared as previously described (6) to assess the level of virus replication using the PFA on Vero or C6/36 cells. Also, viral RNA from brain homogenates was extracted, and the sequence of the genomic region containing the engineered miRNA target was determined.

Evaluation of viruses in rhesus monkeys. Studies in monkeys were performed in accordance with federal and NIAID Animal Care and Use Committee regulations. Twelve *Macaca mulatta* monkeys, weighting 2.5 to 5 kg, were screened for neutralizing antibody to TBEV and DEN4 and found to be seronegative. Groups of four monkeys were subcutaneously inoculated in each shoulder (0.5 ml/site) with 10^5 PFU of TBEV/DEN4, mir-9T, or mir-124aT virus. Monkeys were bled daily for 7 days for detection of viremia and on days 21 and 28 for measurement of neutralizing antibody titer. The amount of virus in serum was determined by direct titration on Vero cells using the PFA (33, 37). The TBEV-specific neutralizing antibody titer was determined by a plaque reduction assay for individual serum samples using TBEV/DEN4 virus. Serum samples from another group of four monkeys that received three doses of a commercial TBEV inactivated vaccine (Encepur; Chiron/Behring) in our previous study (37) were used for comparison.

Statistical analysis. One-way analysis of variance (ANOVA) followed by a Tukey posthoc test was used for multiple comparisons of the viruses based on the level of their replication in C6/36, Vero, or primary neuronal cells. Significance was assumed for P values of <0.05.

RESULTS

Generation and growth of miRNA target TBEV/DEN4 viruses in cell cultures. The 3′ NCR of flaviviruses varies from 380 to 800 nucleotides (nt) in length, and the terminal 120 nt (core element), which are essential for viral replication (23), are more conserved among all flavivirus genomes than the variable region located between the stop codon of the ORF and the core element. The 3′ NCR of TBEV/DEN4 is 384 nt in length, and we introduced a large (243-nt) deletion into the genome that started at the first nucleotide (at nt position 10281) following the TAA stop codon and extended into the entire variable element of the 3′ NCR. The engineered deletion mutant virus was not considerably different from the unmodified TBEV/DEN4 virus with respect to replication in simian Vero cells (mean peak virus titer of 3×10^6 and 8×10^6 PFU/ml, respectively) and neurovirulence in suckling mice inoculated via the intracerebral (i.c.) route (the 50% lethal dose was approximately 1 PFU for both viruses). Consistent with previous observations, these data suggest that the variable region of a flavivirus genome can tolerate large insertions or deletions without a significant effect on virus replication *in vivo* and *in vitro* (12, 23, 28).

The selected miRNA target sequences for brain tissue-specific or enriched miRNAs (let-7c, mir-9, mir-124a, mir-128a, and mir-218) were inserted into the variable element of the 3′ NCR of the TBEV/DEN4 genome between nucleotides 10280 and 10281 abutting the TAA stop codon. Each inserted target consisted of the exact complementary sequence of its corresponding cellular miRNA, which should direct cleavage of the modified TBEV/DEN4 RNA genome mediated by the cellular RISC (4, 7, 9).

Mosquito C6/36 and simian Vero cells were selected for virus recovery since both cell lines maintained an efficient replication of parental TBEV/DEN4 virus. The latter cell line is certified as a cell substrate for production of vaccines for use in humans. Genomic RNA transcripts were generated by SP6 polymerase from the engineered full-length cDNAs and transfected into cells as described previously (6, 30, 33). We found that mosquito C6/36 cells, which do not express the above-mentioned miRNAs as measured by TaqMan microRNA assay, were able to support the generation of each TBEV/DEN4 mutant virus (designated let-7cT, mir-9T, mir-124aT, mir-128aT, and mir-218T) containing the insertion of corresponding miRNA target. The miRNA target insertions did not significantly affect virus replication in C6/36 cells since the virus yield of each miRNA target virus did not differ statistically from that of unmodified parental TBEV/DEN4 virus (Fig. 1A). Viruses bearing targets for brain tissue-expressed miRNAs (mir-9, mir-124a, mir-128a, and mir-218) were also recovered in Vero cells, and their levels of replication were not significantly different from the replication level of the parental TBEV/DEN4 virus (Fig. 1A). In contrast, recovery of the let-7cT virus in Vero cells failed despite several attempts. Using the TaqMan miRNA assay, we did not detect the corresponding let-7c miRNA in C6/36 cells, but in Vero cells this miRNA was expressed up to 463 copies/ng of total cellular RNA. Furthermore, despite the efficient replication of the C6/36 cell-derived let-7cT virus in mosquito C6/36 cells, its replication was significantly restricted in simian Vero cells (Fig. 1A) ($P < 0.001$) compared to that of other miRNA target viruses. Two escape mutant viruses (let-7cTΔ and let-7cT*) emerged in Vero cells that were isolated after a long-term incubation (on days 12 and 15 postinfection) in two separate experiments. Sequence analysis of the let-7cTΔ virus genome revealed a deletion of 14 nt in the 3′ end of the miRNA target sequence (Fig. 1B), which is required for efficient base pairing with the "seed" sequence of the miRNA let-7c and subsequent repression of viral RNA translation and replication. A second Vero cell escape mutant (let-7cT*) contained a single A-to-G mutation at a position 13 nt from the 3′ end of the target sequence (Fig. 1B) that resulted in a U · G mismatch between the let-7c miRNA and its target in the viral genome. This mutation in the let-7cT* genome completely restored the ability of the virus to efficiently replicate in Vero cells (Fig. 1A). These findings support the miRNA-mediated inhibition of let-7cT virus rep-

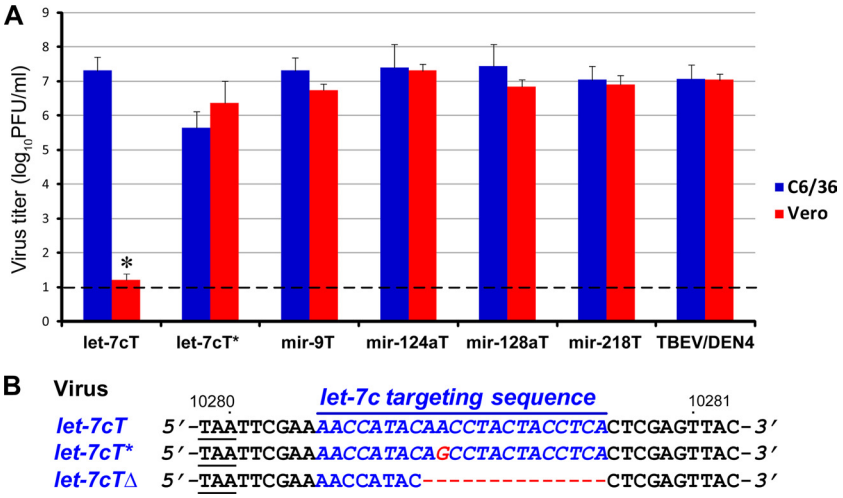

FIG. 1. Effect of miRNA target insertions on the TBEV/DEN4 replication in C6/36 and Vero cells. (A) Replication of indicated viruses was assessed in mosquito C6/36 or simian Vero cells following inoculation at an MOI of 0.01. Virus titers were determined on day 4 postinoculation by PFA on C6/36 or Vero cells. Mean virus titers and standard errors are shown. The dashed line indicates the limit of virus detection (1.0 $\log_{10}$ PFU/ml). Mean virus titers did not differ statistically unless noted. The asterisk indicates that replication of let-7cT virus was significantly different from that of all other miRNA target viruses and from parental TBEV/DEN4 virus in Vero cells (one-way ANOVA followed by a Tukey posthoc test; $P < 0.001$). (B) Mutations identified in the let-7c targeting sequence of two escape mutant viruses (let-7cT* and let-7cTΔ) that were isolated from Vero cells infected with C6/36 cell-recovered let-7cT virus. Deletion of 14 nt in the let-7cTΔ genome and a single A-to-G mutation in the let-7cT* genome are shown in red.

lication in Vero cells, which can easily be eliminated by imperfect base pairing between the target and let-7c miRNA.

Replication of miRNA target TBEV/DEN4 viruses in primary neuronal cells. To provide further evidence of miRNA-mediated inhibition of TBEV/DEN4 mutant viruses *in vitro*, growth analysis of the C6/36 cell-recovered let-7cT virus and the Vero cell-recovered mir-9T, mir-124aT, mir-128aT, and mir-218T viruses was performed in primary rat neuronal cells derived from the cortex of embryonic rat brains. During prenatal brain development, rat neurons express increased levels of miRNAs mir-9 and mir-124a but not mir-128a (18). Parental TBEV/DEN4 and engineered mir-218T virus replicated efficiently with nearly identical kinetics (Fig. 2A), reaching virus titers of 7.0 or 7.5 $\log_{10}$ PFU/ml by day 3 or 4 postinfection, respectively. At that time, the vast majority of cells infected with these two viruses expressed the TBEV-specific antigen in their cytoplasms, as detected by immunofluorescence assay (Fig. 2B to E). Immunostaining of the TBEV antigen in the neurons infected with mir-128aT virus (Fig. 2F and G) was diminished compared to that observed for TBEV/DEN4-infected cells (Fig. 2B and C). Also, the mir-128T virus titer in cell culture medium was 50-fold lower than that attained by the TBEV/DEN4 virus (Fig. 2A). The growth of let-7cT, mir-9T, and mir-124aT viruses was significantly impaired in the neurons (Fig. 2A) ($P < 0.0001$) since these viruses exhibited a 1,000-fold or higher reduction in titer compared to the parent virus and displayed a markedly reduced fluorescence signal (Fig. 2H to M). Thus, these findings clearly indicate that the presence in the viral genome of a target sequence for the miRNAs highly expressed in the brain (let-7c, mir-9, and mir-124a) restricted or attenuated viral replication in developing primary neurons to a greater extent than the insertion of a target for the miRNAs (mir-128a and mir-218) which are not as highly expressed in the brain (1, 18).

miRNA target insertions altered the neurovirulent phenotype of TBEV/DEN4 *in vivo*. Since TBEV/DEN4 retains the high neurovirulence of its TBEV parent when inoculated directly into the brain of mice (31), we next sought to investigate the effect of miRNA target insertions on viral neuropathogenesis by determining the 50% lethal dose (LD_{50}) of viruses in immunocompetent mice. Adult mice were used because they are a highly sensitive animal model for assessment of TBEV neurovirulence and because the expression patterns of brain tissue-specific and brain tissue-enriched miRNAs are conserved in adult mice, monkeys, and humans (1, 25, 38). Six-week-old Swiss mice were inoculated by the intracerebral route with 10-fold serial dilutions ranging from 1 to 10^3 PFU of TBEV/DEN4 or from 10 to 10^5 PFU of miRNA target viruses. The morbidity/mortality rate of TBEV/DEN4-inoculated mice was dose dependent, and all mice which received the highest doses (10^2 or 10^3 PFU) of TBEV/DEN4 developed paralysis or died while 40% or 60% of the mice which received a dose of 1 or 10 PFU survived, respectively (Fig. 3). Thus, in adult mice, parental TBEV/DEN4 virus was highly neurovirulent, with a calculated i.c. LD_{50} of 6 PFU, and replicated efficiently in the mouse brain, attaining a mean peak virus titer of 7.1 $\log_{10}$ PFU/g of brain tissue. In contrast, mice inoculated with an even higher dose of 10^5 PFU (16,600-fold higher than the i.c. LD_{50} of the parent virus) of virus carrying a let-7c, mir-9, mir-124a, mir-128a, or mir-218 miRNA target remained healthy without showing any neurological signs during the 21-day observation. On day 22 of the study, brains of five surviving animals from each group of mice infected with the highest dose (10^5 PFU) of the above-mentioned miRNA target viruses were harvested, and the virus titer of each individual brain suspension was determined on Vero or C6/36 cells. All mouse brain suspensions tested were found to be free from inoculated virus as determined by PFA, and viral RNA was not detected using

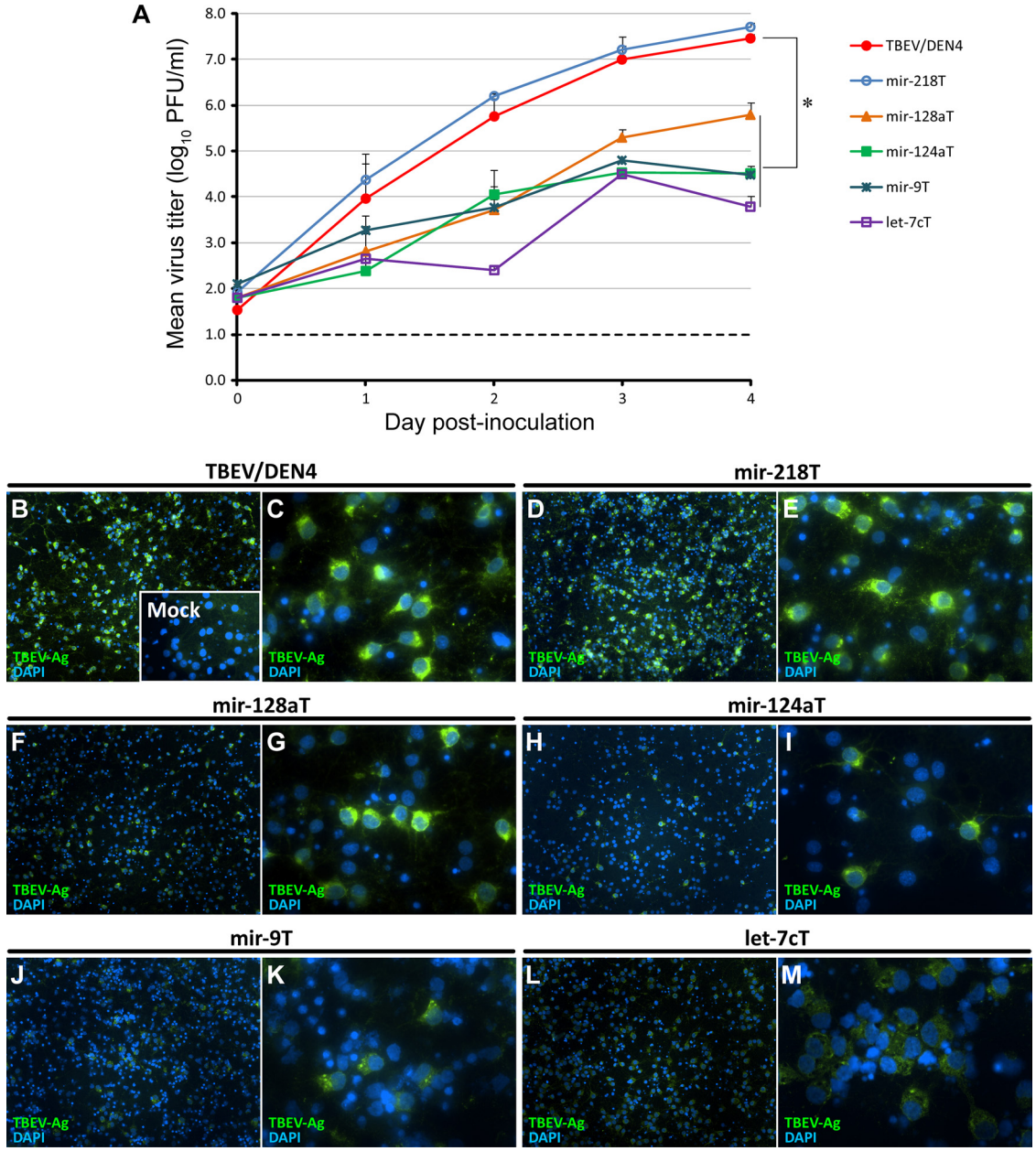

FIG. 2. Effect of miRNA target insertions on the TBEV/DEN4 replication in primary neuronal cells. (A) Growth kinetics of TBEV/DEN4, let-7cT, mir-9T, mir-124aT, mir-128aT, and mir-218T viruses in primary neuronal cells infected at an MOI of 0.5. Virus titers in the daily harvested culture medium were determined by PFA on C6/36 cells for let-7cT and on Vero cells for mir-9T, mir-124aT, mir-128aT, and mir-218T. Mean virus titers and standard errors are shown. The dashed line indicates the limit of virus detection (1.0 $\log_{10}$ PFU/ml). The asterisk indicates that replication of let-7cT, mir-9T, mir-124aT, and mir-128aT virus was significantly different from replication of TBEV/DEN4 and mir-218T viruses in primary neuronal cells on days 3 and 4 postinfection (one-way ANOVA followed by a Tukey posthoc test; $P \leq 0.0004$). (B to M) TBEV antigen (TBEV-Ag) expression in primary neuronal cells infected with indicated virus or mock infected (inset in B) on day 3 postinfection. Representative merged images show the expression of TBEV-Ag (green) and DAPI (blue). Original magnifications: ×100 (B, D, F, H, J, and L) and ×400 (C, E, G, I, K, M, and inset in B).

the RT-PCR analysis. Based on these data, we conclude that the neurovirulence of TBEV/DEN4 in mice was greatly reduced or abolished by the introduction of the let-7c, mir-9, mir-124a, mir-128a, or mir-218 miRNA target sequence.

Next, the engineered viruses were assessed in immunodeficient SCID mice following peripheral inoculation to determine the ability of virus to replicate, possibly accumulate mutations, invade the CNS, and cause fatal encephalitis by potentially escaping the miRNA-mediated inhibition. Adult immunodeficient mice were used since they are deficient for immune functions mediated by B and T lymphocytes and represent a considerably more sensitive animal model for detection of flavivirus neuroinvasiveness (32, 37). Unlike TBEV, which is highly pathogenic for immunocompetent adult mice by intra-

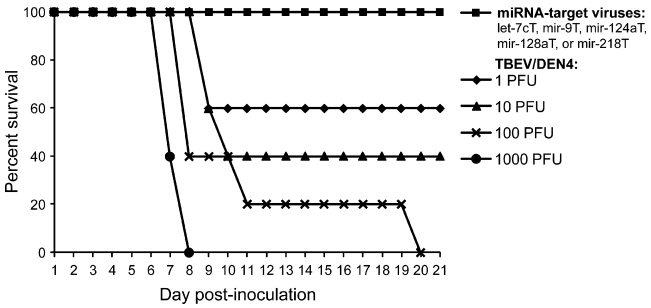

FIG. 3. Neurovirulence of TBEV/DEN4 in the highly permissive Swiss mice was greatly attenuated by insertion of either let-7c, mir-9, mir-124a, mir-128a, or mir-218 target sequence into the virus genome. Mice inoculated i.c. with 10, 10^2, 10^3, 10^4, or 10^5 PFU of either let-7cT, mir-9T, mir-124aT, mir-128aT, or mir-218T virus survived without showing any neurological signs during the 21-day observation (squares). Survival curves of mice infected with TBEV/DEN4 at doses from 1 to 10^3 PFU are shown.

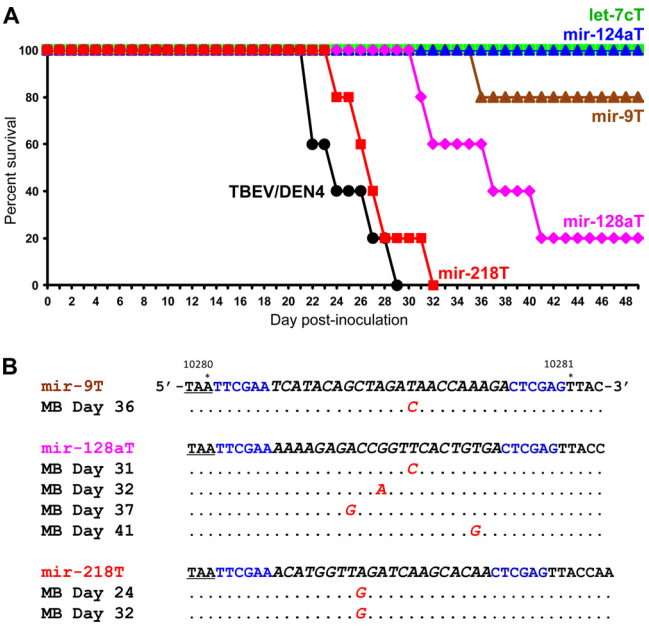

FIG. 4. Insertion of miRNA-target sequence for let-7c, mir-9, mir-124a, or mir-128a, but not for mir-218, into the TBEV/DEN4 genome increased survival of SCID mice infected with a lethal virus dose. (A) Survival curves of SCID mice infected i.p. with a dose of 10^5 PFU of the indicated virus. (B) Mutations that accumulated in the miRNA target sequences of viruses isolated from the mouse brain (MB) on the indicated day postinoculation. Nucleotides that differ from the inoculated virus sequence are shown in red. The sequences of miRNA targets (shown in italics) are flanked with BstBI and XhoI sites (blue) inserted between positions 10280 and 10281 of the TBEV/DEN4 genome. The ORF stop codon is underlined.

peritoneal (i.p.) inoculation, chimeric TBEV/DEN4 virus, even at a high dose of 10^7 PFU, failed to produce CNS disease during the 28-day observation period (31). However, TBEV/DEN4 is a neuroinvasive virus in immunodeficient SCID mice, with an estimated i.p. LD_{50} of approximately 25,000 PFU (37). In SCID mice, we found that the mir-218T virus exhibited a pathogenicity similar to that of the parental TBEV/DEN4 virus when mice were infected at a dose of 10^5 PFU: all mice succumbed to TBEV/DEN4 or mir-218T virus infection between day 23 and 32 postinoculation (Fig. 4A). A decrease in neuroinvasiveness was observed for mir-9T and mir-128aT viruses, as demonstrated by the reduction in morbidity/mortality of mice and by a significant delay in the onset of encephalitis compared to results with the parental virus. Each of the seven individual viruses that were present in brains of mice which succumbed to mir-9T, mir-128aT, or mir-218T infection contained single nucleotide mutations within the miRNA target sequence (Fig. 4B). The identified mutations were located at the central part or the 3′ end of the target sequence and resulted in the disruption of the complementary pairing between the miRNA sequence and its target. These findings suggest that the acquired mutations in the miRNA target region permit the virus to escape from the miRNA-mediated inhibition of virus replication. Interestingly, immunodeficient mice were completely resistant to the let-7cT and mir-124aT infections. Thus, introduction of a target sequence for brain tissue-expressed let-7c, mir-9, mir-124a, or mir-128a miRNA, but not for mir-218 miRNA, decreased the neuroinvasive potential of TBEV/DEN4 in immunodeficient mice.

A single nucleotide mutation in the miRNA target sequence restored virus neurovirulence. To address the question of whether the mutations in the miRNA target sequences attenuate the miRNA-mediated inhibition of virus replication and pathogenicity, we used a let-7cT* virus as one representative of viruses containing a mutation in the miRNA target for the additional study of neuropathogenesis in newborn mice, a highly sensitive animal model for evaluation of flavivirus neurovirulence (32, 37). In the comparative study, 3-day-old Swiss mice, in litters of 10 animals, were inoculated i.c. with 10-fold dilutions ranging from 1 to 100 PFU of let-7cT, let-7cT*, or TBEV/DEN4 (as determined previously [37], the i.c. LD_{50} of TBEV/DEN4 for this age of mice was 1.0 PFU). All mice which received a dose of 10 or 100 PFU of TBEV/DEN4 or let-7cT* developed paralysis or died between days 7 and 10 p.i. (Fig. 5B and C). At the lowest dose (1 PFU), 60% or 10% of the mice succumbed to the TBEV/DEN4 or let-7cT* infection, respectively (Fig. 5A). However, all mice inoculated with let-7cT survived during a 21-day observation. These data clearly indicate that a single nucleotide mutation in the let-7c target sequence restored the neurovirulent phenotype of the virus.

Viremia and immune response of rhesus monkeys following infection with TBEV/DEN4 carrying the target complementary to mir-9 or mir-124a miRNA. Since the TBEV/DEN4 virus is poorly infectious in immunocompetent mice when the peripheral route of inoculation is used, presumably due to the DEN4 genetic background, we sought to evaluate the level of attenuation and immunogenicity of two engineered viruses (mir-9T and mir-124aT) in a more relevant rhesus monkey model. TBEV and its chimeric viruses usually cause an asymptomatic infection in nonhuman primates following peripheral inoculation (27, 37). Presence, duration, and magnitude of viremia serve as reliable criteria of virus virulence while immunogenicity is often assessed by measuring the level of virus-induced neutralizing antibodies. Although clinical illness was not seen in any monkey tested, each monkey inoculated subcutaneously with the TBEV/DEN4 virus developed viremia lasting 3 to 4 days, with a mean peak virus titer of 1.8 $\log_{10}$ PFU/ml (Table

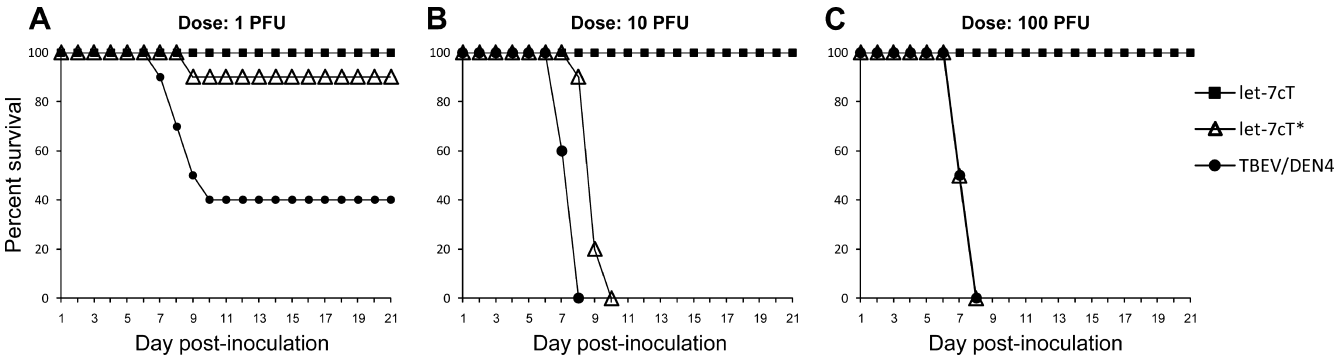

FIG. 5. The relative neurovirulence levels of let-7cT, let-7cT*, and TBEV/DEN4 viruses. Litters of 10 3-day-old Swiss Webster mice were inoculated i.c. with serial 10-fold dilutions ranging from 1 to 100 PFU of the indicated viruses and then monitored daily for signs of encephalitis and morbidity for 21 days.

1). The level of viremia and its duration were lower and shorter than those observed in macaques infected with TBEV (27). The replication of mir-124aT virus was not reduced compared to that of its TBEV/DEN4 progenitor. In addition, the mir-124aT viruses isolated from monkey sera were shown to retain the authentic miRNA target sequence. In contrast, incorporation of a mir-9 target into the TBEV/DEN4 genome attenuated the virus as none of the four monkeys infected with 10^5 PFU of mir-9T developed detectable viremia (<0.7 $\log_{10}$ PFU/ml). However, despite the relatively low level of replication of parental TBEV/DEN4 and its two derivatives in monkeys, all viruses induced a high level of serum TBEV-specific neutralizing antibodies in each immunized animal as measured on day 28 following inoculation. The humoral immune response to a single dose of either virus was comparable (for mir-9T) or higher (for TBEV/DEN4 and mir-124aT) than that induced by three doses of a licensed inactivated TBEV vaccine (Table 1). Also, these titers of TBEV-specific neutralizing antibodies were higher than those generally considered protective levels in humans (antibody titer of $\geq$1:10) (36).

TABLE 1. TBEV/DEN4 bearing mir-9 or mir-124a target sequence is highly immunogenic in rhesus monkeys

Virus or vaccine	No. of viremic monkeys/no. of monkeys tested	Mean no. of viremic days per monkey	Mean peak virus titer ($\log_{10}$ PFU/ml)	Geometric mean serum TBEV-specific neutralizing antibody titer[b]	
				Day 0	Day 28
Viruses[a]					
TBEV/DEN4	4/4	3.5	1.8	<5	1,692
mir-9T	0/4	0	<0.7	<5	252
mir-124aT	4/4	4.0	1.4	<5	625
Vaccine					
Encepur[c]	0/4	0	<0.7	<5	281

[a] Groups of rhesus monkeys were inoculated subcutaneously with 10^5 PFU of indicated virus on day 0. Serum used to measure viremia was collected daily for 7 days, and virus titers were determined by PFA on Vero cells. The lower limit of detection was 0.7 $\log_{10}$ PFU/ml. Viremia was not detected in any monkey after day 4.

[b] Plaque reduction (60%) neutralizing antibody titers were determined against TBEV/DEN4.

[c] Serum samples of four monkeys inoculated subcutaneously with the formalin-inactivated TBEV vaccine Encepur in three human doses (3×0.5 ml) on days 0, 7, and 21 were from our previous studies (37) and were collected for neutralization assay 21 days after the third dose.

DISCUSSION

We demonstrate that the incorporation of a target sequence for microRNAs expressed in the central nervous system into the viral genome enables alteration of the neurotropic flavivirus phenotype and control of neuropathogenesis. A similar approach was also successfully used to control virus tropism and pathogenesis of adenovirus, coxsackievirus, influenza virus, poliovirus, and vesicular stomatitis virus (3, 15–17, 20). From the five selected miRNA target sequences that were introduced into the flavivirus genome, let-7c and mir-124a miRNA targeting was most effective in terms of both restriction of TBEV/DEN4 virus replication *in vitro* and reduction of virus neurovirulence and neuroinvasiveness *in vivo*.

Chimeric TBEV/DEN4 virus, like many other flaviviruses, is neurotropic and efficiently replicates in human cells of neuronal origin and in the brains of mice and monkeys inoculated i.c., in which virus antigens were detected exclusively in neurons (6, 24, 37). MicroRNA mir-124a is CNS specific, broadly distributed throughout many regions of brain, expressed only in neurons, and regulates neuronal differentiation (1, 22, 38). Replication of the virus bearing the mir-124a target was 1,000-fold reduced in primary rat neuronal cells compared to that of parental TBEV/DEN4 virus. The mir-124aT virus was also highly attenuated *in vivo* and did not induce any clinical signs of CNS involvement in immunocompetent mice infected directly into the brain with a large dose of virus. In addition, the introduction of mir-124a target sequence into the TBEV/DEN4 genome completely abolished the neuroinvasive potential of the virus in immunodeficient mice. Nevertheless, the mir-124aT virus retained the ability of its parent to replicate in peripheral tissues of monkeys and induced a strong neutralizing antibody response against TBEV, which was higher than that induced by a licensed inactivated TBEV vaccine given at three doses of immunization. However, the level of TBEV-specific antibodies induced by the inactivated TBEV vaccine was shown to be sufficient to completely protect the immunized

monkeys against challenge with tick-borne Langat virus, as observed previously (37).

Although the inclusion of target sequences for three other miRNAs expressed in the CNS (mir-9, mir-128a, and mir-218) completely abolished the neurovirulence of the TBEV/DEN4 virus in immunocompetent mice, the effect of these miRNA target insertions on the viral neuroinvasive phenotype in immunodeficient mice was less evident than that of a let-7c or mir-124a target insertion. The absence of potent B and T cell responses in SCID mice allows for prolonged replication of virus, possibly leading to the emergence of mutations which restore the ability of virus to replicate in the CNS and cause neurological disease. Indeed, all of the mir-9T, mir-128aT, and mir-218T viruses that were isolated from the brains of SCID mice with signs of the CNS disease contained single nucleotide mutations located within the miRNA target sequences, suggesting that these mutations allowed viruses to overcome miRNA-mediated inhibition. The emergence of escape mutations could have occurred in the peripheral tissues before the invasion into the CNS of SCID mice or after invasion, when virus was replicating under miRNA pressure in the CNS. Additional studies are needed to analyze the genetic stability of miRNA target viruses in the CNS of mice with a compromised immune system after direct inoculation into the brain. Also, future work will need to evaluate the effect of the miRNA targeting on flavivirus replication and pathogenesis in the immature CNS of newborn mice since miRNA expression patterns during the CNS development are different from those in adult animals (1, 14, 39).

In this study, the observed virulence of miRNA target viruses in SCID mice was inversely proportional to the level of expression of the corresponding miRNAs in the mouse brain (1). For example, the virus bearing the target for the most abundant CNS-expressed mir-124a or let-7c miRNA was less pathogenic, suggesting the important role of miRNA expression level in controlling viral replication within the CNS. The expression of mir-128a and mir-218 miRNAs is limited in many regions of the brain, and it did not surprise us that among the miRNA target sequences tested, mir-128a and mir-218 targets were less effective in restricting virus replication in primary neuronal cells and in preventing the development of lethal encephalitis in SCID mice. Inclusion of a target for miRNA mir-9, which is highly expressed in many regions of the CNS (1, 18), significantly restricted replication of TBEV/DEN4 in primary neuronal cells, adult mice, and monkeys. The greatly restricted viremia in mir-9T-infected monkeys suggests that it might be a result of virus inhibition by the mir-9 miRNA expressed in non-CNS tissues. In support of this notion, findings in recent studies indicate that mir-9 is also expressed in peripheral tissues (29, 40).

The inclusion of the let-7c target into the TBEV/DEN4 genome did not allow us to rescue virus in Vero cells in which the let-7c miRNA is expressed. Thus, the let-7cT virus was generated in mosquito cells where let-7c miRNA was not detected. Following infection of Vero cells with let-7cT virus recovered from mosquito cells, we showed that a single nucleotide mutation within the target sequence of let-7cT* virus resulted in a mismatched base paring with the corresponding miRNA and restored the ability of the mutant virus to efficiently replicate in Vero cells. Furthermore, comparative studies of let-7cT and let-7cT* viruses in suckling mice indicate that this single nucleotide mutation in the let-7c target sequence restored the ability of the let-7cT* virus to cause paralysis and death in mice following i.c. inoculation. These observations further support the role of miRNA-mediated regulation in controlling viral infection. let-7c is a member of a large let-7 family of miRNAs which are ubiquitously expressed in a multitude of tissues and cells and thus can potentially restrict virus replication in these tissues. The mosquito cell-derived let-7cT virus was restricted in replication in simian Vero or primary rat neuronal cells and was not able to cause neurologic disease in mice inoculated via the i.c. or i.p. route. Based on our experience with mir-9T virus, we would expect that the high level and broad expression of let-7c in peripheral tissues would also significantly impair virus replication, resulting in a reduced level of immunogenicity. Therefore, we suggest that the targeting of the flavivirus genome for miRNA let-7c would not be beneficial for the development of effective live attenuated vaccines.

In conclusion, we demonstrate that the modification of a highly neurovirulent flavivirus by the inclusion of a single copy of the target for miRNAs expressed in the CNS alters or completely abolishes the neurovirulent phenotype of the virus *in vivo*. These findings support the rationale of the miRNA targeting approach to control flavivirus neurotropism and to develop live attenuated virus vaccines against various neurotropic viruses. From the perspective of the safety and stability of new vaccine candidates carrying miRNA targets, the potential benefit of multiple target copies for a single designated CNS-specific miRNA (such as mir-124a) alone or in combination with other miRNA targets inserted into the virus genome remains to be investigated.

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