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Originally published as:

Achazi, K., Patel, P., Paliwal, R., Radonić, A., Niedrig, M., Donoso-Mantke, O.
RNA interference inhibits replication of tick-borne encephalitis virus in vitro
(2012) Antiviral Research, 93 (1), pp. 94-100.

DOI: 10.1016/j.antiviral.2011.10.023

This is an author manuscript.

The definitive version is available at: <http://www.sciencedirect.com/>

RNA interference inhibits replication of tick-borne encephalitis virus *in vitro*

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Abstract

Each year, up to 10,000 cases of infections with the flavivirus tick-borne encephalitis (TBE) virus that affect the central nervous system are reported in Europe and Asia. Due to the potentially severe adverse effects of post-exposure prophylaxis with TBE virus hyperimmunoglobulin, TBE can currently only be treated symptomatically. An RNA interference (RNAi) approach to inhibiting TBE virus replication was therefore developed. In this study we demonstrate for the first time that small interfering RNAs (siRNA) targeted to the TBE virus genome reduce the quantity of infectious TBE virus particles, TBE virus genome, and TBE virus protein *in vitro* by up to 85%. The 50% inhibitory dose (DI50) of the shRNA plasmid was only 0.05 µg/ml. As RNAi-based therapeutics for other diseases are already being evaluated in phases II and III clinical trials, it is possible that RNAi could become valuable tool for controlling TBE virus infection.

Keywords

Flavivirus, tick-borne encephalitis virus, tick-borne encephalitis, RNA interference, small interfering RNA, antiviral drug

Abbreviations

DAPI; 4',6-diamidino-2-phenylindole

DI50; 50% inhibitory dose

DMEM; Dulbecco's modified Eagle medium

dsRNA; double stranded RNA

EGFP; enhanced green fluorescent protein

GE; genome equivalent

HIV; human immunodeficiency virus

MOI; multiplicity of infection

PFU; plaque forming units

RISC; RNA-induced silencing complex

RNAi; RNA interference

RT-qPCR; quantitative real-time RT-PCR

shRNA; short hairpin RNA

siRNA; small interfering RNA

TBE; tick-borne encephalitis

1. Introduction

Viruses of the genus *Flavivirus* are small enveloped viruses with a single positive-stranded RNA genome of about 11 kilobases. The genome codes for three structural (E, M/preM, and C) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) (Mansfield et al., 2009). Flaviviruses, transmitted mainly by mosquito or tick vectors and a cause of serious health issues throughout the world, are divided into three groups: mosquito-borne flaviviruses, tick-borne flaviviruses, and flaviviruses with unknown vector (Grard et al., 2007).

The prototype of tick-borne flaviviruses, tick-borne encephalitis (TBE) virus, is the causative agent of TBE that, with an annual average of 10,000 cases, is the most important viral encephalitis in Europe and Asia (Süss, 2008). Three mainly human-pathogenic TBE virus subtypes are known: the European, the Siberian, and the Far Eastern subtypes, each associated with varying degrees of disease severity (Grard et al., 2007; Gritsun et al., 2003). To date, there is no specific therapy to treat TBE virus infection although a vaccine is available (Lindquist and Vapalahti, 2008). Post-exposure prophylaxis with TBE virus hyperimmunoglobulin is no longer

recommended because it can cause adverse events in children and adults and there is no evidence for efficacy in adults (Aebi and Schaad, 1994; Noack, 1997; Valdueza et al., 1996; Waldvogel et al., 1996).

The use of RNA interference (RNAi) to regulate gene expression and defend against bacterial and viral pathogens is common to all eukaryotic species (Katiyar-Agarwal et al., 2006; Navarro et al., 2006). RNAi is induced by double-stranded RNA (dsRNA) that in the eukaryotic cell is recognized by an enzyme complex (Dicer) able to cut it into pieces of 21 to 23 nucleotides in length (Fire et al., 1998). These fragments, known as small interfering RNA (siRNAs), are incorporated into a multi-protein complex termed the RNA-induced silencing complex (RISC) that degrades or inactivates complementary mRNAs and viral RNAs.

Although siRNAs can be synthesized chemically and transfected into cells it is also possible to clone a synthetic shRNA gene cassette into a plasmid or viral vector for subsequent transfection. Such a vector encodes the shRNA that is processed by Dicer in the cell into siRNA (Bernstein et al., 2001; Guil and Esteller, 2009; Hajeri and Singh, 2009; Hammond et al., 2001; MATHONNET et al., 2007; Stevenson, 2004; Valencia-Sanchez et al., 2006).

Many studies have shown RNAi to be a useful tool for inhibiting replication of viruses such as the human immunodeficiency virus (HIV) *in vitro* or viral hepatitis *in vitro* and *in vivo* (Coburn and Cullen, 2002; Eekels et al., 2011; Hannon and Rossi, 2004; Huang et al., 2010; Jacque et al., 2002; Song et al., 2003; Weinberg and Arbuthnot, 2010; Zhang et al., 2010). Reduction of virus replication by RNAi *in vitro* and *in vivo* has also been described for many flaviviruses such as Dengue virus, West Nile virus, Japanese encephalitis virus and Yellow fever virus (Bai et al., 2005; Haasnoot and Berkhout, 2006; Haasnoot et al., 2007; Haasnoot et al., 2003; Hajeri and Singh, 2009; Kumar et al., 2006; Murakami et al., 2005; Pacca et al., 2009; Singh and

Hajeri, 2009; Stein and Shi, 2008). However, so far no studies describing the effects of RNAi on TBE virus infection have been reported.

We therefore designed and tested *in vitro* a number of siRNAs targeted to the TBE virus genome. The siRNAs were found to reduce the quantity of infectious virus particles, viral genomes and virus proteins in cultures of infected cells.

2. Material and Methods

2.1. Virus propagation

Three cell culture-derived TBE virus strains, one strain for each subtype, were used to test the efficacy of the siRNAs: European subtype K23 (GenBank accession no. **AM600965**), Siberian subtype Aina (GenBank accession no. **AF091006**) and Far Eastern subtype Sofjin (GenBank accession no. **X03870**). Viruses were propagated in Vero E6 cells (ATCC CRL-1586) cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% glutamine, 1% penicillin and 1% streptomycin after infection with TBE virus-containing cell culture supernatants at a multiplicity of infection (MOI) of 1. Supernatants were harvested after 3 to 5 days and the TBE virus titer determined by plaque assay as described below. Unless otherwise stated, the TBE virus strain K23 was used as a prototype of all TBE virus strains.

2.2. SiRNA target sequence selection and cloning

Suitable siRNA target sequences in the TBE virus E-protein-coding region (prototype strain K23) were identified using the siRNA Target Designer (Promega, Madison, WI, USA) and analyzed using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for homology with human and mouse genes. Only sequences that showed a

difference to the human or murine genome of more than three base pairs were considered. siRNA-targeted sequences were also compared to those of TBE virus strains described in the NCBI database (<http://blast.ncbi.nlm.nih.gov>). SiRNA sequences targeting the most conserved viral sequences (no more than two mismatches compared to the siRNA target sequences) were selected (Table 1) and used to design the corresponding shRNA gene cassettes.

The shRNA plasmids were produced using the Promega siStrike U6 hairpin cloning system kit according to the manufacturer's instructions. Briefly, the shRNA gene cassettes obtained from Invitrogen (Karlsruhe, Germany) were cloned into the psiStrike vector using the Promega protocol. Incorporation of the shRNA gene cassette into the plasmid was confirmed by a PstI digestion.

ShRNA plasmids were checked for errors by sequencing. The plasmid SC#1 containing an shRNA gene cassette based on a randomized siRNA sequence (SC#1) was used as negative control.

2.3. Transfection of cells

5×10^5 HEK293T cells in 200 μ l medium without antibiotics were seeded into each well of poly-L-lysine coated (Sigma, Steinheim, Germany) 24-well culture plates one day before transfection. Cells were approximately 90% confluent after 24 h.

Transfection of shRNA plasmids was performed using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Briefly, for each well, 1 μ g shRNA plasmid and 1 μ l Lipofectamine 2000 were diluted in 200 μ l Opti-MEM (Invitrogen, Karlsruhe, Germany), added to the pre-seeded HEK293T cells and incubated for 48 h at 37°C and 5% CO₂. The transfection efficiency was determined by counting the total cell number and the number of cells expressing enhanced green fluorescent protein (EGFP).

2.4. Infection of cells

To analyze the effect of the siRNAs on TBE virus infection, HEK293T cells transiently transfected with shRNA plasmids, and therefore transiently expressing shRNAs that are processed to siRNAs targeted against the TBE virus genome, were infected with TBE virus-containing cell culture supernatant. After removing the transfection media, 200 µl of virus-containing medium were added to each well to give an MOI of 0.1 for the K23 and Aina strains and 0.01 for the Sofjin strain. Cells were incubated for 30 min at 37°C and 5% CO₂ before removing the virus-containing medium, washing the cells twice with 200 µl of PBS (140 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) and adding 200 µl fresh medium. After 24 h, 48 h, and/or 72 h of incubation, cells were analyzed by immunofluorescence microscopy or harvested to be further analyzed by RT-qPCR, Western blot and plaque assay. As a negative control, cells were transfected with an shRNA plasmid containing an shRNA gene cassette based on a nonspecific randomized siRNA sequence (SC#1).

2.5. Isolation of viral nucleic acids

Total RNA was prepared according to the manufacturer's instructions from cells and cell culture supernatants using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) or the Qiagen viral extraction kit (Qiagen, Hilden, Germany), respectively. To avoid DNA contamination, RNA was also treated with the Turbo DNase Kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions.

2.6. Virus detection by quantitative real-time RT-PCR

cDNA was reverse-transcribed from 5 µl RNA in a final reaction volume of 20 µl using the Superscript II kit (Invitrogen, Karlsruhe, Germany). The RNA was first incubated

for 10 min at 65°C and reverse transcription performed at 37°C for 60 min and at 93°C for 10 min using a Biometra thermoblock cycler (Biometra, Göttingen, Germany). The presence of viral genomes and tubulin genes (as reference) in the cDNA was tested by TBE virus-specific quantitative real-time RT-PCR (RT-qPCR) or by a tubulin-specific in-house RT-qPCR as described previously (Achazi et al., 2011; Radonic et al., 2004).

2.7. Detection of infectious virus particles by plaque assay

Titers of virus in culture supernatants and cells were determined by plaque assay as described by Bae et al. (2003). Culture supernatants were used directly whereas cells were first resuspended in 250 µl PBS, frozen and thawed twice (30 min at -80°C, 30 min at 37°C) and centrifuged for 5 min at 5,000×g to yield supernatants to be tested in the plaque assay.

2.8. Isolation of membrane proteins, SDS page, and Western blot

To isolate membrane proteins from HEK293T cells, culture medium was removed and the cells washed twice with 200 µl ice-cold PBS before lysing in 100 µl RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8, 1 mM PMSF, 1% protease inhibitor cocktail III, 25 units / ml Benzonase, 1 mM DTT) and centrifugation at 4°C for 10 min at 15,000× g. The protein-containing supernatant was then transferred to a new reaction tube for storage at -80°C. Protein concentrations were determined using the Invitrogen Quant-iT Protein Assay Kit and a Qubit fluorometer (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

Protein extracts were subjected to SDS-PAGE and Western blotting as described by Muller et al. (2007). Detection of the TBE virus E-protein was used to confirm the

presence of virus proteins in the infected cells with β -actin serving as a positive control. The murine monoclonal anti-TBE virus antibody MAB 1367 described by Niedrig et al. (1994) diluted 1:1,000 was used as first antibody and an HRP-labeled anti-mouse immunoglobulin G antibody (Pierce, Rockford, USA, order no. 31450) diluted 1:5,000 served as the secondary antibody. The SuperSignal West Femto Chemiluminescent system was used for detection (Pierce Biotechnology, Rockford, IL, USA) with 1 min exposure of the membrane to the X-ray film.

2.9. Immunofluorescence staining and microscopy

Immunofluorescence staining was performed as described by Niedrig et al. (1999) using MAB 1367 (see above) diluted 1:40 as first antibody and an Alexa 594-labeled anti-mouse immunoglobulin G antibody (Invitrogen, Karlsruhe, Germany, order no. A11005) diluted 1:500 as secondary antibody.

Cells were observed using a fluorescence microscope Axioskop 20 (Zeiss, Jena, Germany) or a confocal laser scanning microscope cLSM 510 Meta (Zeiss, Jena, Germany).

2.10. Statistical analysis

Laboratory data were handled and analyzed using PASW statistics 17 (version 17.0.3; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Identification of siRNA target sequences

A thorough analysis of all available TBE virus sequences was performed to allow development of siRNA molecules binding to the RNA of all three human-pathogenic TBE virus subtypes. However, the 15% to 20% diversity between the genomes precluded the identification of an siRNA target sequence perfectly fitting all TBE virus subtypes. Three siRNA-targeted sequences (Table 1) with no more than one or two mismatches to the genome sequences of all TBE virus strains were therefore selected.

Because most sequences available on NCBI are for the E-protein-coding region, the selected target sequences are complementary to this region. Furthermore, as the E-protein is the most important flaviviral structural protein, being responsible for virus adherence and absorption, it has been previously targeted by RNAi in studies of other flaviviruses (Kumar et al., 2006; Murakami et al., 2005; Pacca et al., 2009).

3.2. Exclusion of aspecific effects on TBE virus infection and transfection efficiency

To exclude potential aspecific effects of shRNA plasmids, the degree of virus replication in non-transfected cells was compared to that in cells transfected with the control shRNA plasmid SC#1. The quantity of virus was standardized to the amount of tubulin expression in the cells.

The SC#1 control shRNA plasmid had no statistically significant effect (t-test >0.05) on the virus genome content of infected cells (data not shown) and was therefore used as a negative control in subsequent experiments.

The transfection efficiency with 5 $\mu\text{g/ml}$ of shRNA plasmid was found to be 47%, dropping to 34% with 0.5 $\mu\text{g/ml}$ and 2.5% with 0.05 $\mu\text{g/ml}$. There was no significant difference between experiments using the same amount of shRNA plasmid (t-test $p < 0.05$).

3.3. Effects of the siRNAs on a TBE virus infection in cells

First, cells infected with the TBE virus strain K23 (MOI=0.1) and transfected with the shRNA plasmid (5 µg/ml) were sampled every day for 72 h to determine the optimal sampling time. siRNA TBEV#2 and TBEV#3 were most effective after one day, reducing the virus genome yield by up to 85%. In contrast, siRNA TBEV#1 showed the best effect after 72 h with a 65% reduction (Figure 1), although already at 24 h inhibition was as high as 58%. In subsequent experiments, the cells were therefore harvested 24 h after transfection.

Different concentrations of shRNA plasmid used to transfect cells (5, 0.5 and 0.05 µg/ml) were tested to find the optimum and to demonstrate the dose-dependent effects of the siRNAs. All three siRNAs significantly reduced the quantity of TBE virus genome generated in the cells (t-test <0.05) compared to the control SC#1 (Figure 1). SiRNA TBEV#2 was most effective (Figure 2) with 0.05 µg/ml being sufficient to inhibit the quantity of virus genome by more than 50%, compared to TBEV#3 and TBEV#1 that required at least 0.5 µg/ml and 5 µg/ml respectively to achieve the same degree of inhibition.

A plaque assay was used to verify that the RNAi had reduced not only the quantity of virus genome but also the number of infectious particles produced (Figure 2). All three siRNA tested (TBEV#1, #2, and #3) inhibited the production of infectious particles by up to approximately 80%. Although inhibition by the different siRNAs varied by about 5%, the siRNAs TBEV#2 and TBEV#3 were again most effective. Whether or not shRNA-expressing cells were susceptible to infection was investigated by immunofluorescence staining of transfected, TBE virus-infected cells. Transfected cells fluoresce green (due to expression of GFP in the shRNA plasmid) and an Alexa 594-labeled anti-mouse immunoglobulin G antibody (red) was used to

visualise the monoclonal anti-TBE virus antibody bound to virus. Cells that were both infected and transfected therefore appeared orange. In addition, cell nuclei were stained blue using 4',6-diamidino-2-phenylindole (DAPI).

TBE virus was present in a proportion of the cells transfected with the control shRNA plasmid SC#1. Virus was also observed in cells expressing the siRNA TBEV#1, albeit at a lower frequency compared to the control SC#1. In contrast, virus could not be detected in any of the cells expressing the shRNA plasmids TBEV#2 or TBEV#3 (Figure 3A-D).

Western blot analysis was used to give a semi-quantitative measure of the TBE virus E-protein content in shRNA plasmid-treated cells (Figure 4). ShRNA plasmids TBEV#2 and TBEV#3 were used as they have been the most effective against the TBE virus strain K23 in previous experiments and β -actin was used as a reference protein.

Although 43 kDa bands for the β -actin protein were present in lysates of non-infected and infected cells, the TBE virus E-protein could only be detected in infected cells. The intensity of the band from infected cells treated with the control SC#1 was stronger than those from shRNA plasmid TBEV#2- and TBEV#3-treated cells, indicating an inhibition of E-protein production by the siRNAs.

3.4. Effects of the siRNAs on different TBE virus subtypes

Finally, we infected cells with three different TBE virus strains, one for each subtype: K23 (European), Aina (Siberian), and Sofjin (Far Eastern) to determine the specificity of the antiviral effect of the siRNAs. The efficacy of the siRNAs varied according to the TBE virus subtype (Figure 5). SiRNA TBEV#1 was most effective for all strains, reducing virus content by up to 85%. SiRNA TBEV#3 also reduced virus production for all strains observed, although the inhibition was not statistically significant (t-test

>0.05) for the Siberian subtype (strain Aina). Due to mismatches between the Siberian and Far Eastern subtypes, siRNA TBEV#2 only inhibited replication of the European subtype (strain K23).

In summary, the results demonstrate that siRNAs targeted against the TBE virus genome efficiently reduced the TBE virus load in cell culture with regard to quantity of infectious virus particles, viral genome and virus protein.

4. Discussion

The results of this study demonstrate that siRNAs can inhibit the replication of TBE virus in infected cell culture. The efficacy of the three different siRNAs varied according to each subtype, which is partly due to mismatches between the siRNA and TBE virus strain sequences and possibly also to secondary structures in the virus genome that are known to influence the effects (Westerhout and Berkhout, 2007). SiRNA TBEV#1 exactly matches the TBE virus strains Sofjin (Far Eastern subtype) and Aina (Siberian subtype) and most efficiently reduced the yield of virus genome for these two strains compared to siRNA TBEV#2 and siRNA TBEV#3. For example, siRNA TBEV#1 has two mismatches with TBE virus strain K23 (European subtype) and is therefore less inhibitory for this strain. However, some mismatches can be tolerated (Amarzguioui and Prydz, 2004; Lin et al., 2003), and indeed the siRNA TBEV#3 inhibited the TBE virus strains Aina and Sofjin despite having up to two mismatches.

Complete inhibition of TBE virus by the siRNAs was not achieved with the method used here, possibly because cells were only transiently transfected and with an average efficiency of only 47% (when using the highest concentration of shRNA plasmid). This efficiency is, however, comparable to that reported for other RNAi-based experiments described in the literature (Wu et al., 2010).

siRNAs reduced the yield of virus genome by up to 85% and the production of infectious virus particles by 80%. These values are similar to those achieved by Pacca et al. (2009) with yellow fever virus and Murakami et al. (2005) with Japanese encephalitis virus, who showed a decrease in virus production of between 12% and 97% depending on the siRNA used. This reduction in virus production in our study was also confirmed at the protein level by Western blot analysis.

Furthermore, similar to the study by Murakami et al. (2005) with Japanese encephalitis virus, we showed siRNA to act on TBE virus in a dose-dependent manner. The dose of the most efficient siRNA (TBEV#2) required to achieve 50% virus inhibition (inhibitory dose [DI50]) of the prototype strain (K23) was 0.05 µg/ml of the shRNA plasmid. For the other two shRNA plasmids TBEV#1 and TBEV#3 the DI50s were 5 µg/ml and 0.5 µg/ml, respectively. Since high concentrations of siRNAs are toxic and could induce non-specific immune responses *in vivo*, from a medical point of view it is important to minimize the amount of siRNA used (Hornung et al., 2005; Sioud, 2004). SiRNA TBEV# 2 would therefore seem to be the most appropriate candidate to serve as a basis for developing antiviral therapies against the European subtype, whereas TBEV#1 would be better for all three subtypes. In future experiments, all three siRNA could be combined to improve reduction of viral replication and to reduce the possibility of TBE virus escape mutants developing. The next step will be to test whether the shRNA plasmids are effective when administered after the infection of the cells. If successful, the molecules could then be tested *in vivo* (mouse model) to evaluate their efficacy and toxicity.

The main challenge facing the use of RNAi in living organisms is delivery of the siRNAs and plasmid or viral shRNA vectors to their site of action. For the treatment of tick-borne encephalitis, the siRNAs or shRNA vectors need to be introduced into the brain and different approaches have been developed. In mice, siRNA molecules or

even plasmid and viral shRNA vectors can be injected directly into the brain or with high pressure into the tail vein (Bai et al., 2005; Harper et al., 2005; Kumar et al., 2006; Van den Haute et al., 2003) and delivery strategies more appropriate for humans have been described in the literature. For example, siRNA molecules can be targeted by coupling them to aptamers that bind specifically to certain cell types (Chu et al., 2006; McNamara et al., 2006). In addition, modified lentiviral vectors expressing specific envelope glycoproteins can be used to transduce cells in the CNS, or cell specific promoters can be used to allow cell specific expression. The use and safety of the different approaches involving modified lentivirus vectors have recently been summarized by Manfredsson and Mandel (2011). A further option to introduce siRNA molecules into neural cells is to couple them to a rabies virus glycoprotein that passes the blood-brain barrier and binds specifically to neuronal cells (Kumar et al., 2007).

The successful inhibition of virus replication *in vivo* (mice) has been shown for other flaviviruses. Indeed, therapeutic approaches have been developed against many other viruses such as hepatitis C virus or HIV and against other diseases such as cancer or Alzheimer's disease (Bai et al., 2005; Kumar et al., 2006; Murakami et al., 2005; Pacca et al., 2009; Ray and Shi, 2006). Finally, RNAi-based drugs against human respiratory syncytial virus and age-related macular degeneration have been tested in phase II and III clinical trials (Federici et al., 2007; Kim and Rossi, 2007).

In conclusion, RNAi is a new approach that may develop into a suitable tool for controlling TBE virus infection and a therapeutic agent for post-exposure prophylaxis.

5. Acknowledgements

We thank M. Böthe, N. Litzba, N. Stock, A. Teichmann, R. Schädler, U. Erikli, and S. Norley for excellent support. The work was supported by a grant from the German

National Academic Foundation and by the grant 01 KI 0714 of the Federal Ministry of Education and Research (BMBF).

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Table 1: SiRNA target sequences compared to the sequences of the three different TBE virus strains and control sequence.

siRNA/Virus strain	sequence (5' → 3')	nt in TBE virus genome	protein-coding region
SC#1^a	gCggTAgCgCCCATATTAT		
TBEV#1	gggACTggTTCAATgATCT		
K23 (AM600965)	gggACTggTT <u>TA</u> ATgA <u>C</u> CT	1599-1617	E-protein
Aina (AF091006)	gggACTggTTCAATgATCT	650-668	
Sofjin (X03870)	gggACTggTTCAATgATCT	1540-1558	
TBEV#2	ggTCTTACgTACACAATgT		
K23 (AM600965)	ggTCTTACgTACACAATgT	1850-1868	E-protein
Aina (AF091006)	ggTCT <u>CAC</u> A <u>T</u> ACACAATgT	901-919	
Sofjin (X03870)	ggTCTTAC <u>A</u> TACACAATgT	1791-1809	
TBEV#3	gAggTggCTTCATAgAgAT		
K23 (AM600965)	gAggTggCTTCATAgAgAT	2052-2070	E-protein
Aina (AF091006)	gAgg <u>A</u> ggCTTCATAgAgAT	1103-1121	
Sofjin (X03870)	g <u>C</u> ggTggCTTCATAg <u>AA</u> AT	1993-2011	

^a SC#1, a negative control shRNA plasmid containing a gene cassette based on a randomized, scrambled siRNA sequence. Base mismatches in the TBE virus genome sequences compared to the siRNA target sequences are underlined.

Figure 1: Time course of TBE virus genome equivalent (GE) copy number in infected (strain K23) HEK293T cells transfected with shRNA plasmids TBEV#1, #2, or #3 compared to HEK293T cells transfected with the control shRNA plasmid SC#1.

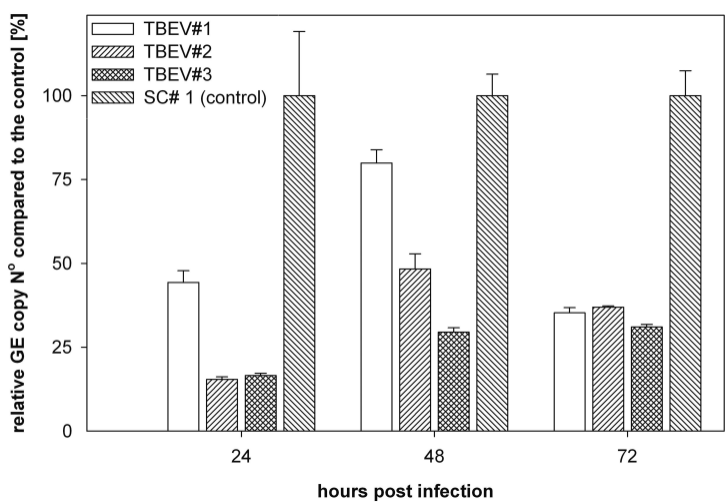
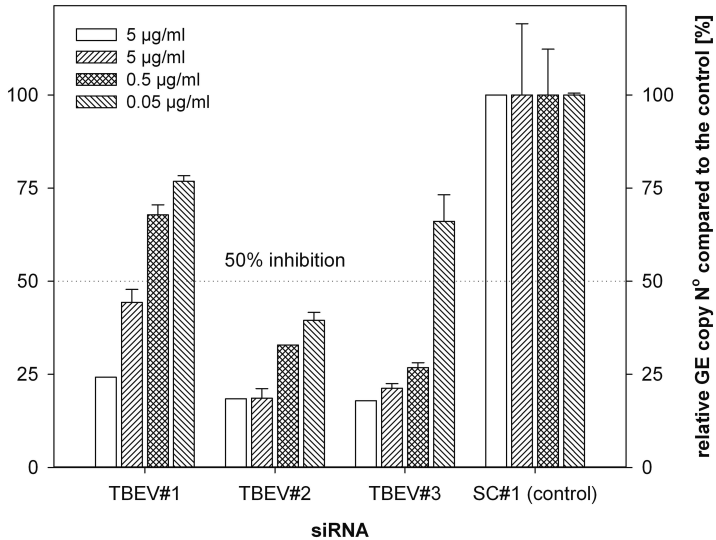


Figure 2: Effects of different shRNA plasmid concentrations on TBE virus plaque-forming units (PFU, white bars) and TBE virus genome equivalent copy number (GE, hatched bars) in HEK293T cells infected with TBE virus (strain K23) at 24h post infection.

All three siRNAs (TBEV#1, #2, and #3) significantly reduced (t-test <0.05) the quantity of TBE virus genome compared to the control SC#1.

relative PFU compared to the control [%]

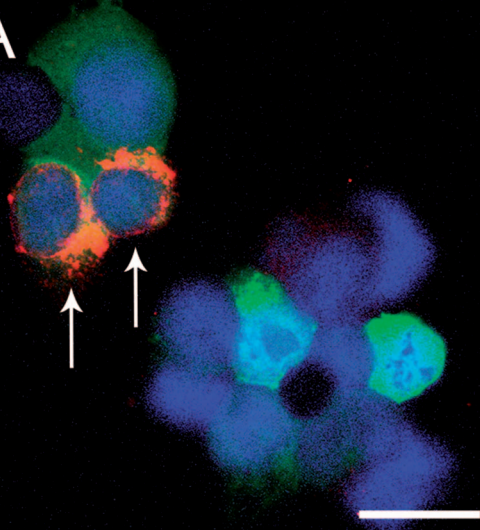


relative GE copy N° compared to the control [%]

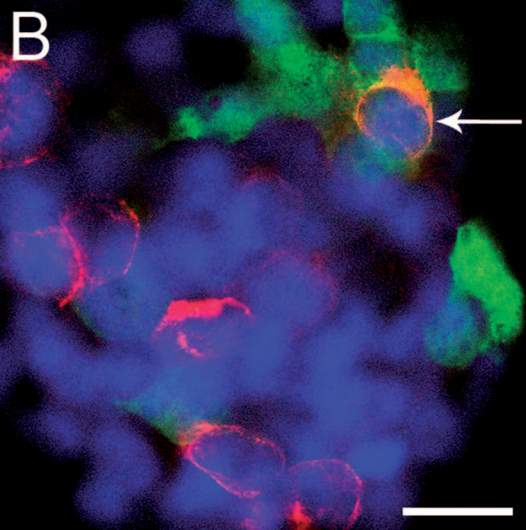
Figure 3: Immunofluorescence staining of HEK293T cells infected 24h earlier with TBE virus and transfected with shRNA plasmids TBEV#1, #2, and #3.

Cells are expressing either the control shRNA SC#1 (A), or shRNAs TBEV#1 (B), TBEV#2 (C), or TBEV#3 (D). Viral proteins are stained red, shRNA-expressing cells fluoresce green, and the nuclei are stained blue (DAPI). Cells expressing shRNAs and containing TBE virus appear orange and are indicated by white arrows. The bars represent a length of 20 μm .

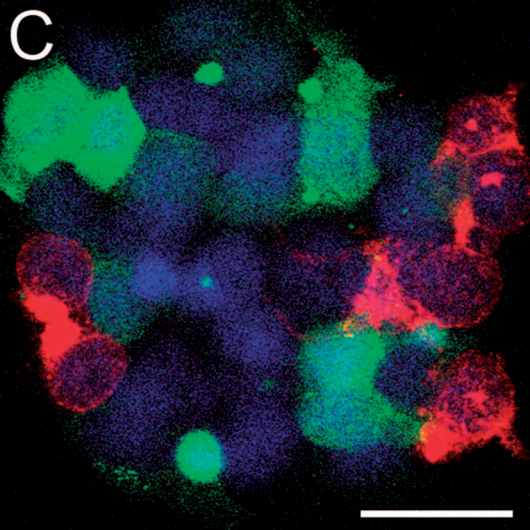
A



B



C



D

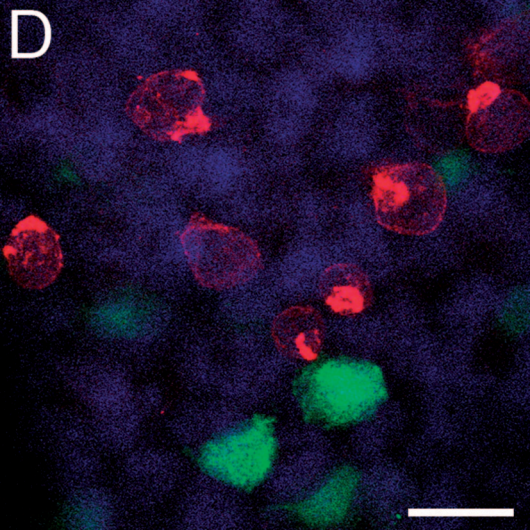


Figure 4: Effect of siRNAs on the quantity of E-protein in TBE virus (strain K23)-infected HEK293T cells at 24h post infection.

Western Blot analysis was performed using lysates from cells transfected with the control shRNA plasmid SC#1 or with the most efficient inhibitory plasmids TBEV#2, and TBEV#3.

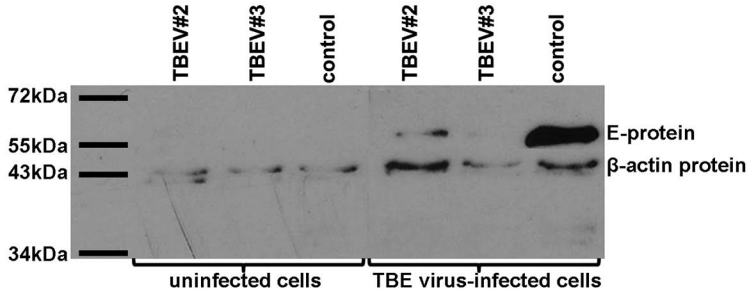
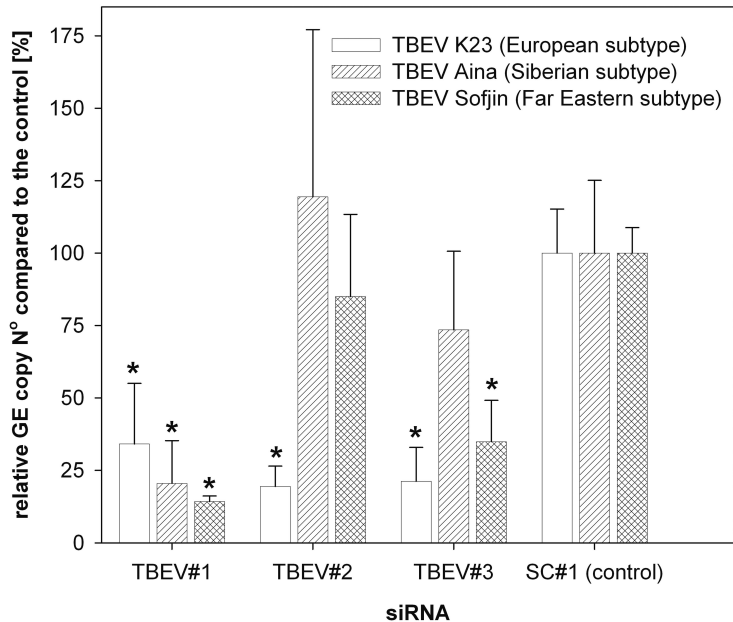


Figure 5: Effects of siRNAs TBEV#1, #2, and #3 on different TBE virus subtypes.

Experiments with every siRNA and strain were performed at least three times in duplicate (5 times for TBEV strain K23). Standard deviations were calculated for all the experiments and replicates. The TBE virus genome equivalent (GE) copy number was normalized to the reference gene tubulin at 24h post infection. Statistically significant differences (t-test $p < 0.05$) are marked with an asterisk.



- >We developed small interfering RNAs (siRNA) targeted to the TBE virus genome.
- >siRNAs reduce the quantity of TBE virus particles, genome, and protein up to 85%.
- >The 50% inhibitory dose of the shRNA plasmid was 0.05 µg/ml. >RNA interference could become valuable tool for controlling TBE virus infections.