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RNA interference protects horse cells *in vitro* from infection with Equine Arteritis Virus

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ABSTRACT

Equine Arteritis Virus (EAV) belongs to the *Arteriviridae* and causes viral arteritis in horses. In an attempt to develop novel and save therapies against the infection it was tested whether EAV is susceptible to RNA interference (RNAi) in an equine *in vitro* system. Horse cells were transfected with chemically synthesized small interfering RNA oligonucleotides (siRNAs) and challenged with EAV. Application of these siRNAs led to a significant protection of the cells, and virus titers decreased drastically. siRNAs derived from DNA plasmids expressing small hairpin RNAs (shRNAs) were also effective. The protection was most pronounced with two siRNAs targeting the open reading frame 1 (coding for non-structural proteins), whereas siRNAs targeting sequences for several structural proteins had less or no effect. In addition, it was investigated whether RNAi could be used to treat cells with an already established viral infection. Only application of the siRNAs shortly after viral challenge led to significant survival rates of the cells, whereas transfection at later time points caused much less benefit for the cells. These findings are discussed in a perspective of using RNAi as a therapeutic approach to combat EAV.

1. Introduction

Equine Arteritis Virus (EAV) belongs to the family *Arteriviridae* and is the causative agent of equine viral arteritis, a serious worldwide infectious disease of horses. Clinical signs during the acute phase of infection vary widely and include depression, fever, severe conjunctivitis, respiratory problems, edema, etc. In addition, EAV can cause abortion at high rates in pregnant mares (Bryans et al., 1957). The virus persists in the genital tract of about half of the stallions infected and is shed continuously in the semen. Current vaccines against EAV do not completely block virus transmission and safe vaccine candidates are not yet available (Cardwell et al., 2002; Snijder and Meulenberg, 2001; Giese et al., 2002). Hence, there is a high demand for the development of novel and safe antiviral therapies.

Arteriviruses are small, enveloped RNA viruses with a single-stranded positive-sense genome of 12–15 kb in length. The bulk part of the EAV genome codes for the non-structural proteins of the virus, whereas most of the 3′ quarter carries the seven open reading frames (ORFs) for the structural proteins (Snijder and Meulenberg, 2001). Arteriviruses express their structural proteins from small, subgenomic RNAs (sgRNAs) (de Vries et al., 1990; Kuo et al., 1991), a feature they have in common with *Coronaviridae* and *Roniviridae*, which unites them to the order *Nidovirales*. The sgRNA coding for the capsid protein (N-protein, expressed from ORF7), is the most abundant viral RNA in an EAV-infected cell (Pasternak et al., 2006). In contrast to the structural proteins, the large ORF1 is translated as a single polypeptide which is then enzymatically processed into several peptides, mainly building the replication complex (Snijder and Meulenberg, 1998).

Since its first description in nematodes (Fire et al., 1998), RNA interference (RNAi) has been studied as a potential nucleic acid-based therapy for many diseases, including infections (de Fougerolles et al., 2007). The principle of RNAi lies in the ability of a cell to degrade double stranded RNA, which reflects an evolutionary conserved defence mechanism against viruses and transposable elements in plants, lower metazoa and possibly mammals (Voinnet, 2001; Cullen, 2006; Wilkins et al., 2005). RNAi has been used in several strategies against viral infections, with varying success (Haasnoot et al., 2007). By using RNA oligonucleotides (small interfering RNA, siRNA) complementary to viral RNA, one can achieve the degradation of the target RNA, i.e. the viral genome. SiRNAs can be delivered directly as chemically synthesized RNA (Elbashir et al., 2001) or indirectly as DNA-plasmids expressing small hairpin RNAs (shRNAs) which are then processed to siRNAs inside the

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cell (Brummelkamp et al., 2002). Several RNA viruses have been targeted by RNAi, and first antiviral effects *in vivo* have been demonstrated (Li et al., 2005; Bai et al., 2005; Kumar et al., 2006). There is also evidence that the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), an Arterivirus that infects pigs, is vulnerable to shRNA derived from a DNA-plasmid in an heterologous *in vitro* model (Huang et al., 2006; He et al., 2007; Li et al., 2007).

In this study it was tested whether RNAi can be used to protect horse cells from EAV. It was found that siRNAs significantly interfere with EAV replication both when delivered directly as chemically synthesized siRNA or indirectly as DNA-plasmid based shRNAs. Two siRNAs targeting sequences in the ORF1 region were more effective than siRNAs targeting ORFs coding for several structural proteins.

2. Materials and methods

2.1. Cell culture

The equine skin-derived cell line APH-R (Collection of Cell Lines of the Friedrich-Löffler-Institute, Riems Island, Germany) was cultured in DMEM, supplemented with 10% fetal calf serum, 1% Glutamax, 15 mM HEPES, 1% non-essential amino acids and antibiotics. Baby hamster kidney (BHK-21) cells were grown in GMEM, supplemented with 10% FCS, 1% Glutamax and 5% Tryptose Phosphate Broth and antibiotics.

2.2. SiRNAs, transfection and virus infection

SiRNAs were designed using the full genome of EAV strain Bucyrus (GeneBank accession Y07862, van Dinten et al., 1997) according to Reynolds et al. (2004). Those sequences with the highest scores at the desired locations were chosen and chemically synthesized (Eurofins MWG, Ebersberg, Germany). All siRNA-sequences were present in the EAV strain used for our experiments, as confirmed by amplifying and DNA-sequencing of the corresponding parts of the viral genome. The siRNA-sequences were absent from the horse genome, as confirmed by BLAST searches (data not shown).

For siRNA transfection, APH-R cells were seeded in 24-well plates (8×10^4 cells per well) and incubated for 21 h with siRNA, complexed to 4.5 μl transfection reagent (HiPerfect, Qiagen) at 40 nM final concentration. The Alexa-488 labelled siRNA duplex was obtained from Qiagen and used in a fivefold higher concentration to allow visualization.

Plasmids expressing shRNA constructs were constructed using the pSUPER-vector (Oligoengine). The targeting sequence of pSUPGFPb was 5′-GAACGGCATCAAGGTGAAC (kind gift of A. Kretzschmar, Leipzig).

The oligonucleotides were annealed to their complementary strands and cloned via BglII and HindIII restriction sites into pSUPER. The EGFP-encoding plasmid was based on the vector pVAX1 (Invitrogen). The correct sequences were verified by DNA-sequencing and the plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) for BHK-21 cells and ExGen 500 (Fermentas) for APH-R cells.

APH-R cells were challenged at 24 h after siRNA-transfection (or as indicated in the time-course experiment) with EAV, Bucyrus strain VR796 (ATCC), for 3 h (100 $TCID_{50}$ per well). After challenge, the cells were washed and incubated in normal growth medium. This infection resulted in a complete death of the whole culture after 6 days in the untransfected controls. BHK21 cells were challenged as described above but 48 h after transfection of the plasmids. Virus titers were determined as a $TCID_{50}$ on BHK21 cells according to Reed and Muench (1938).

2.3. Immunological procedures

Four or six days after viral challenge, the cultures were fixed with 3.7% formaldehyde and incubated with the monoclonal antibody 17D3 (VMRD) against the EAV N-protein and a secondary FITC-conjugated anti-mouse antibody (DakoCytomation). Samples were analyzed with a Leica DMI4000 inverted fluorescence microscope. The same antibody was used to detect the EAV N-protein in a Western Blot. The EGFP antibody was from Cell Signaling Technology. A polyclonal EAV-GP2b-antiserum was raised in rabbits against a C-terminal peptide of the protein. For Western Blots, cultures were lyzed 96 h after challenge. Protein content was determined (BCA protein kit, Pierce) and equal amounts of protein were loaded onto each lane of a SDS protein gel.

2.4. Statistical analysis

The Student's *t*-test was used to assess the significance of differences of viral yield or cell numbers between the groups of siRNA treated cells and the control groups. Two-tailed *P*-values were calculated.

2.5. Quantitative RT-PCR

RNA was isolated with the GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich). 100 ng total RNA was used in an one-step quantitative RT-PCR assay (Invitrogen) performed on the LightCycler 480 II (Roche). The TaqMan PCR reaction mix contained 2× Reaction Mix, 375 nM each primer and probe, 5 μl RNA and DEPC-H_2O to a final volume of 20 μl. PCR cycling conditions were: 48 °C for 15 min and 95 °C for 2 min; 50 cycles of 95 °C for 15 s, 55 °C for 30 s and 60 °C for 30 s. Data were analyzed by the relative quantification method using the instrument specific software. As house keeping gene equine β-actin was used to monitor the quantity and quality of RNA. Primer and probe sequences (5′–3′): equine INF-β forward: ATGAGATGCTCCAGCACAC; INF-β reverse: TCCTCCTCCATTA TTTCCTCC; INF-β probe: ACCATCGTTAAGAACCTCCTTGTGGAAGTC; equine β-actin forward: GACCCAGATCATGTTTGAGACC; β-actin reverse: AGTCCATCACGATGCCAGTG β-actin probe: AACACCCCGGCCATGTACGTGGCCA. As a positive control for IFN-β activation, 1 μg of Poly I:C (Sigma–Aldrich) was transfected via Lipofectamine 2000. The lower β-actin mRNA content of this sample (Table 2) reflects the cytotoxicity of the transfection reagent.

3. Results

3.1. RNAi works in horse cells

To evaluate an inhibitory effect of siRNAs on EAV replication it was first tested whether generally RNAi can be induced in horse cells. The equine cell line APH-R was transfected with DNA plasmids expressing shRNAs against the enhanced green fluorescent protein (pSUPGFP and pSUPGFPb) and, as negative control, against an EAV-sequence (pSUPs8, see below). After 24 h, the cells were transfected with a plasmid expressing EGFP. One day later, cells were lyzed and analyzed for EGFP expression. Fig. 1A shows that the EGFP signal was markedly decreased in cells transfected with two different shRNA-expressing plasmids targeting EGFP, whereas the EAV targeting construct had no effect. Hence, it can be concluded that RNAi efficiently works in the APH-R cell line. As the aim of the present study was the use of chemically synthesized siRNAs, it was next tested whether siRNAs can be delivered to APHR cells. The cells were transfected with fluorescently labelled siRNA using lipid-based transfection. After 24 h the cells were observed with a

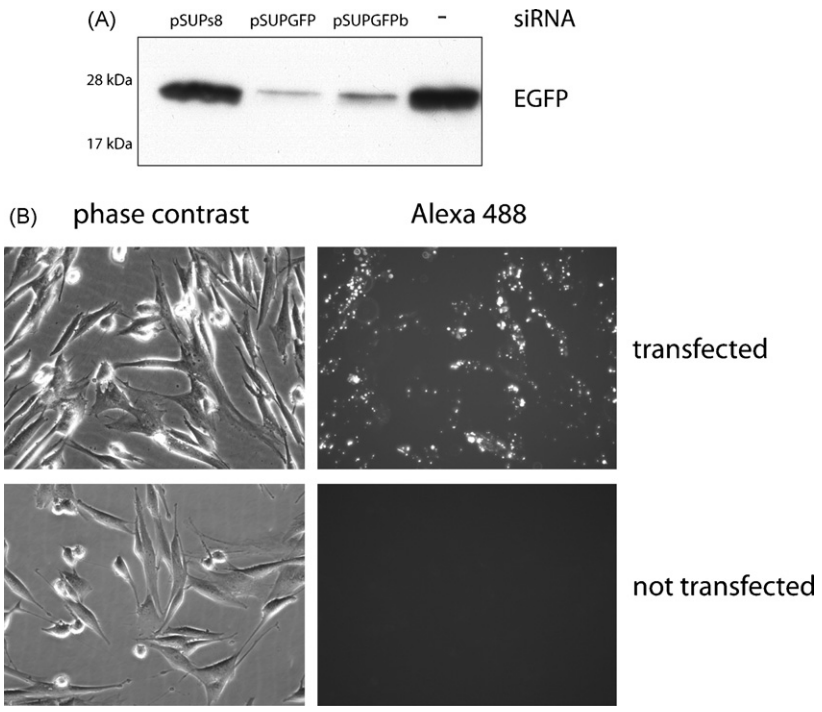

Fig. 1. RNAi in equine APH-R cells. (A) EGFP expression of cells transfected with the indicated shRNA-generating plasmids and cotransfected with a plasmid encoding EGFP. (B) Fluorescently labelled siRNA-oligonucleotides were delivered to the cells via lipid-based transfection (top row). The cells were analyzed 24 h later. The phase-contrast picture is shown on the left side, the fluorescent channel (Alexa 488) on the right side. Untransfected controls are in the bottom row. Pictures were taken at 400× magnification.

Table 1
Position of siRNAs used in this study. SiRNAs were designed using the EAV genome, Bucyrus strain (accession number Y07862). Mutated residues in Si1mut and Si2mut are shown in italics. Si1mut is based on a naturally occurring variation (strains S1512 and H6).

siRNA	Sequence (5′–3′)	Position (first nt)	ORF
Si1	GCAAUGUCCUGGCUUGUUC	380	1a
Si2	GGAGAGUCUCACUGCAACA	4577	1a
Si3	GUGGCGCAGUAUCGUAACA	4701	1a
Si4	GUUAUGCCUUCCAAUAUCA	10082	2b
Si5	AUUGCUUGCUGUGGUUGCA	10237	2b
Si6	CCGAUUGUGAUGACACCUA	11378	5
Si7	GCUACAAUGACCUACUGCG	12380	7
Si8	CGUACAACGGUUCUUCAUG	12519	7
SiGFP	GGCUACGUCCAGGAGCGCA		
Si1mut	GCA***G***UGUCCUGGC***C***UGUUC		
Si3mut	GUG***C***CGCAGUA***G***CGUAACA		

fluorescent microscope. There was an efficient uptake of the siRNA, almost all cells showed a signal (Fig. 1B). To our knowledge, this is the first report of RNAi in horse cells.

3.2. RNAi protects horse cells from EAV

Different siRNAs against the genome of EAV were designed (Table 1, Fig. 2). Three siRNAs (Si1, Si2, Si3) targeted the ORF1a region of the non-structural protein coding ORF1, the others targeted ORF2b (Si4 and Si5), ORF5 (Si6) and ORF7 (Si7 and Si8), hence structural proteins. The unrelated sequence against the GFP gene from the plasmid pSUPGFP (see above) was used as a control oligonucleotide (SiGFP).

APH-R cells were transfected with siRNAs and challenged with virus 24 h later. To monitor viral replication and protein synthesis, the cultures were fixed after 96 h and stained with an antibody against the EAV N-protein.

The infected control-cultures (not transfected with siRNA) showed clear signs of a cytopathic effect (CPE), and there was a strong signal for the EAV N-protein (Fig. 3, pos).

The same picture was seen in the infected cultures transfected with the control siRNA (Fig. 3, SiGFP). However, different results were obtained in the cells transfected with siRNAs against EAV. Two SiRNAs targeting ORF1 (Si1 and Si3) led to a clear reduction of infection with much fewer EAV-positive cells and no detectable CPE (Fig. 3, Si1 and Si3). Many cells showed an EAV-signal after transfection of Si4 and Si5, targeting ORF2b, although the CPE was less than in the control (Fig. 3, Si4 and Si5). Si8 (against ORF7) and all other siRNAs tested (not shown) did not lead to a significant decrease in viral infection, a clear CPE was observed and the cells showed strong signals for EAV (Fig. 3, Si8).

In order to quantify the protective effect of siRNAs against EAV infection, the cells were transfected and challenged as described above, but after 6 days post-infection the viable cells in the culture were counted (Fig. 4A) and the virus concentration was measured by determining the $TCID_{50}$ (Fig. 4B). In accord with the immunofluorescence data, cultures transfected with Si1 and Si3 had the highest cell numbers, approaching non-infected control cultures. Cell numbers obtained after transfection of Si1 and viral challenge

Fig. 2. Schematic map of the EAV genome in 5′ → 3′ orientation (total length is 12.7 kb). The positions of the siRNA target sequences used in this study are indicated by black bars. The ORFs are labelled with the names of the encoded polypeptides. Modified from (Pasternak et al., 2006).

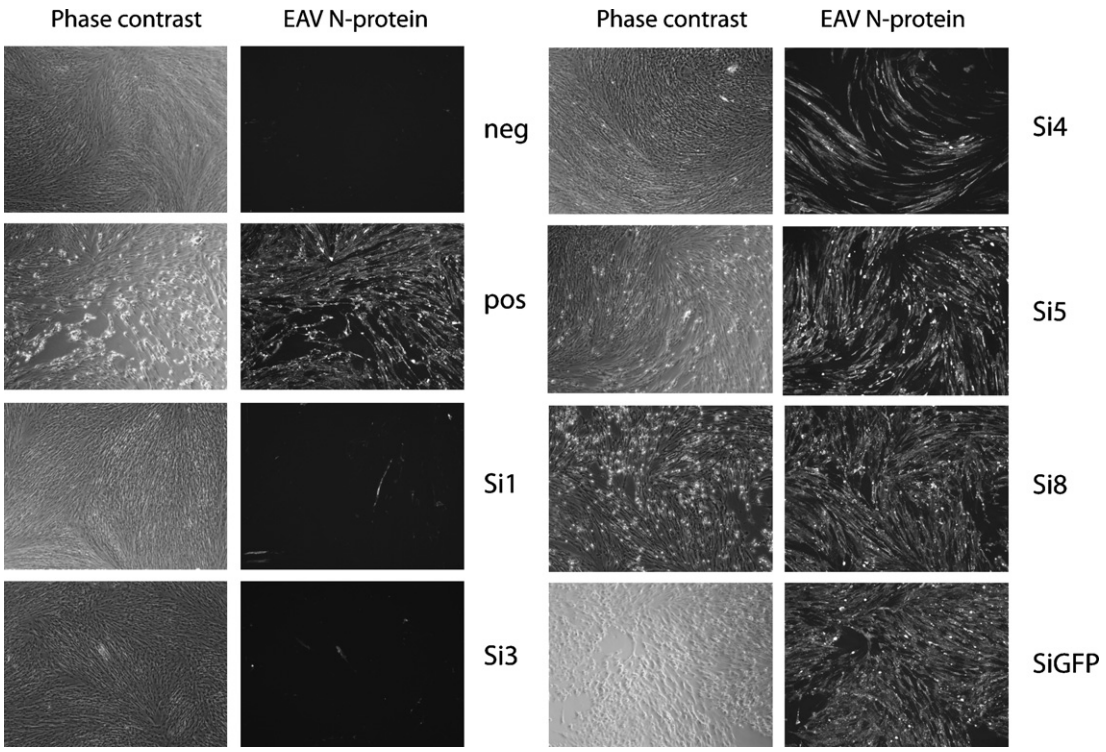

Fig. 3. Investigation of the cytopathic effect and viral replication in cells transfected with different siRNAs. Equine APH-R cells were transfected with the siRNA indicated and challenged with EAV. Cells were analyzed 96 h later. The phase contrast pictures are in the left columns, the staining for the viral N-protein is on the right side. Neg are cells transfected with siRNA but not infected, pos are cells not transfected but infected. Pictures were taken at 200× magnification.

were somewhat more variable, but Si3 repeatedly led to a significant protection of the cells against EAV. Transfection of Si2 also led to more surviving cells, although the effect was not statistically significant. Interestingly, when the sequences of Si1 and Si3 were mutated at two positions (Si1mut, Si3mut, Table 1), the cell numbers were as low as with SiGFP, underlining the sequence specificity of the effect. SiRNAs targeting ORF2b (Si4 and Si5) caused significantly more protection than the control siRNA, however, less than siRNAs against ORF1. Cells transfected with Si6, Si7 and Si8 were not protected and their numbers were as low as for the untransfected cultures, where the CPE led to the death of almost all cells.

Similar results were seen after determination of the $TCID_{50}$. The difference between the siRNAs targeting ORF1 and the controls reached 4 orders of magnitude (Fig. 4B). It should be noted that in several experiments no virus at all could be detected in the supernatant of cultures transfected with Si1 and Si3, hence protection was complete, which was not observed with the other siRNAs.

To analyze the direct effect of the siRNAs against structural proteins on their target gene expression, siRNA-treated and infected cells were analyzed by Western Blot for the viral N-protein and GP2b (the proteins encoded by ORF7 and ORF2b, respectively). As can be seen in Fig. 4C, Si4 and Si5 interfered with the production of their target protein GP2b early during infection (48 h). However, at 96 h the protein levels were comparable to samples not treated with siRNAs, indicating a more transient effect on viral replication of these siRNAs compared to Si1 and Si3. Si7 and Si8 were not able to reduce the amount of N-protein to a detectable level at early and later time points.

Cells could also be protected from EAV by an antiviral type 1 interferon response caused by the siRNAs, hence independently from the RNAi mechanism. Therefore, the mRNA levels of interferon-β (IFN-β) were determined after transfection of Si1, Si3, Si8 and SiGFP. However, no induction of IFN-β was detected, strongly suggesting that the antiviral activity of the siRNAs was via RNAi (Table 2). Interestingly, infection of the APH-R cells with EAV caused an increase in IFN-β mRNA, which will be discussed elsewhere (A. Heinrich, M. Giese, S. Ulbert, in preparation).

3.3. BHK-21 cells are protected from EAV by DNA-plasmid based RNAi

Next it was investigated whether siRNA derived from DNA-vectors would have a similar effect on EAV replication. Therefore, pSUPER-based constructs expressing Si3, Si8 and the control siRNA as shRNAs (pSUPs3, pSUPs8 and pSUPGFP) were designed. Si3 was chosen as it gave the most reproducible results when chemically synthesized (see above). BHK-21 cells (which are also susceptible to EAV) were used for these experiments, as they could be DNA-transfected more efficiently.

The cells were transfected with the siRNA-vectors and challenged with EAV 48 h later. As can be seen in Fig. 5, there was no CPE and no signal for the viral N-protein detectable after 5 days of infection in cells transfected with pSUPs3. No virus was detectable in the supernatants by $TCID_{50}$ measurements in three different experiments (data not shown). In contrast, pSUPs8 showed no protection of the cells (Fig. 5), similar to the control pSUPGFP and in line with the data obtained using chemically synthesized siRNA.

3.4. RNAi mediates less protection when administered after viral challenge

It was demonstrated that two siRNAs against ORF1 of EAV have a protective effect against the virus; however, only conditions were used where the siRNAs were transfected before the viral challenge. As to antiviral therapies, it is important to test whether RNAi is also able to interfere with viral replication when administered to

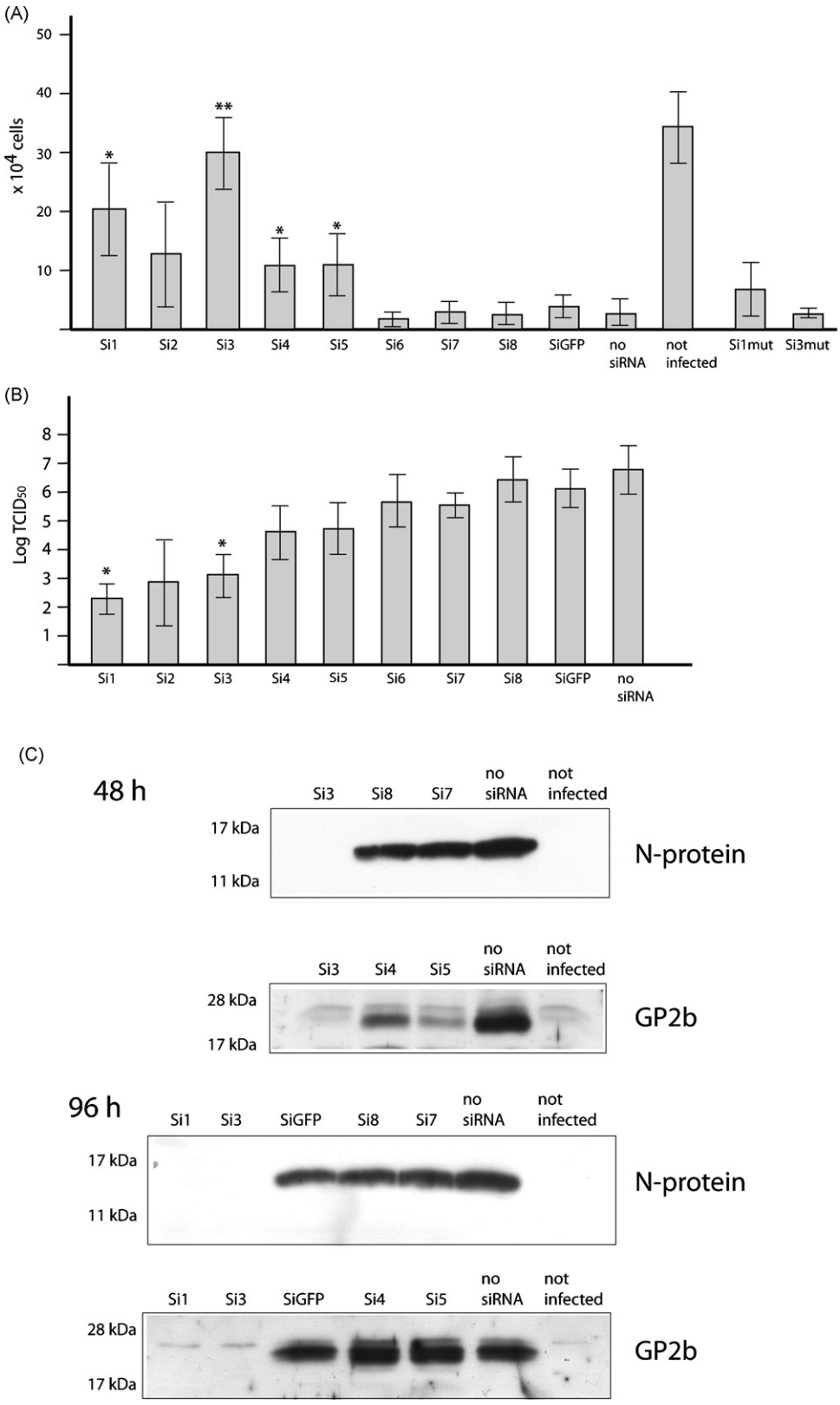

Fig. 4. (A) Quantification of cellular survival after siRNA transfection and EAV challenge. Equine APH-R cells were transfected with the siRNAs indicated and challenged with virus. At 6 days, the surviving cells were counted. (B) Consequences of siRNAs for viral titers. Supernatant from the cultures in A were spread over 96-well plates containing BHK-21 cells and the $TCID_{50}$ was determined. Values are the mean results from 7 independent experiments, error bars represent the standard deviation. Statistically significant differences between non-transfected controls ("no siRNA") and siRNA-transfected cultures are indicated by one ($P < 0.02$) or two ($P < 0.001$) asterisks. (C) Analysis of siRNA target gene expression. APH-R cells were treated as in A. At 48 h (top) and 96 h (bottom) after challenge, the cells were lyzed and analyzed for the expression of the EAV N-protein and GP2b by western blot. Equal amounts of total protein were loaded in each lane. The transfected siRNA is shown on top of each lane.

Table 2
IFN-β and β-actin mRNA levels determined by real time RT-PCR in APH-R cells treated with different reagents. Cp values are given as mean results from three measurements, the standard deviation is given in brackets. ND is not detectable.

Treatment	Cp β-actin	Cp IFN-β
Si1	19.56 (0.11)	ND
Si3	19.58 (0.11)	ND
Si8	19.55 (0.05)	ND
SiGFP	19.59 (0.07)	ND
Poly I:C	22.67 (0.11)	23.89 (0.12)
EAV	20.28 (0.24)	32.63 (0.07)
Negative control	19.53 (0.11)	ND

cells already infected. Therefore, APH-R cells were challenged with EAV and afterwards transfected with Si1, Si3 and SiGFP at different time points. After 6 days, surviving cells in the cultures were counted. Reasonable protection was only observed with Si1 and Si3 and only at the earliest time point, i.e. 30 min after infection (Fig. 6). Compared with the results obtained by transfecting siRNAs before viral challenge, the protection was less pronounced. At later time points, there were still more surviving cells in the Si1- and Si3-transfected cultures compared to the non-transfected controls (Fig. 6). As the cell numbers were also higher than in the cultures transfected with SiGFP, a low-level protective effect specific for Si1 and Si3 was observed, probably reflecting the interference of Si1 and Si3 with the spreading infection in the culture.

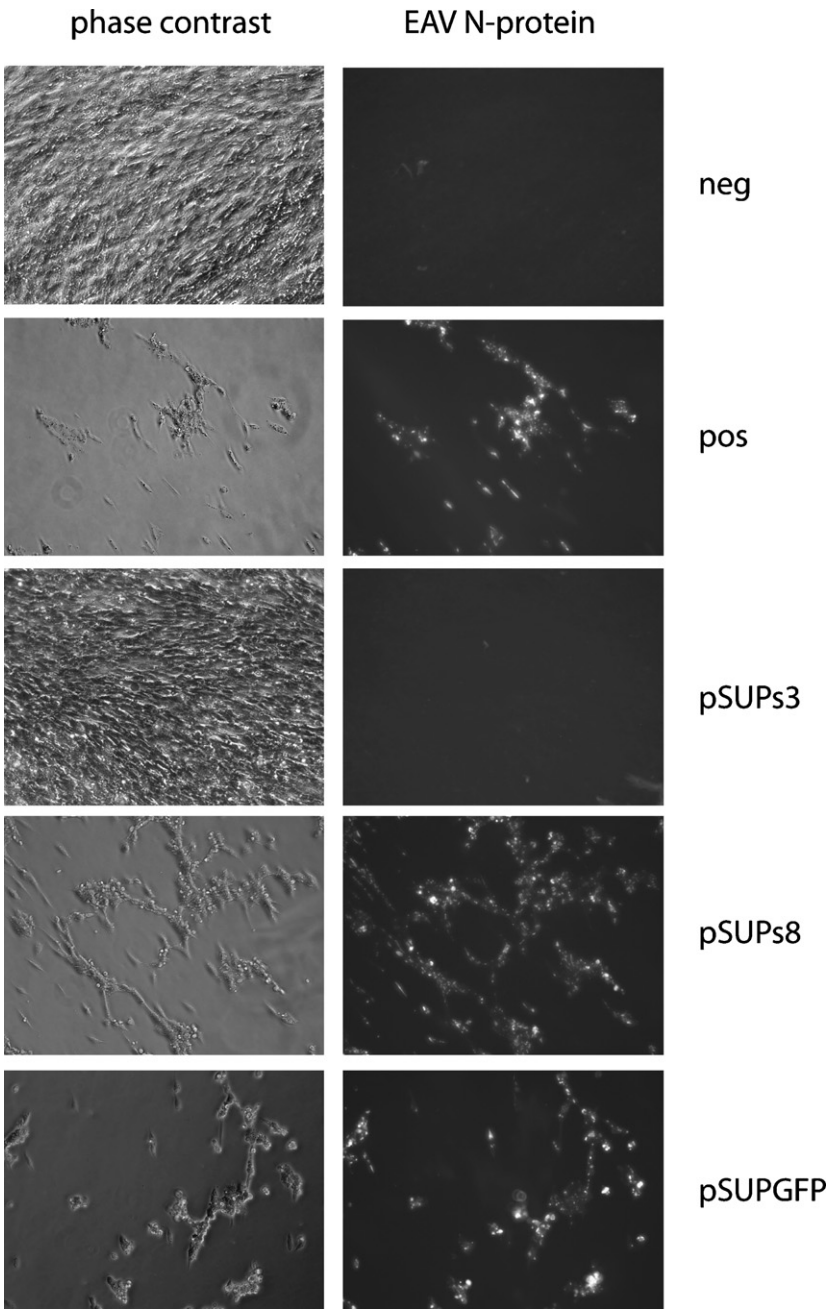

Fig. 5. Protection of BHK-21 cells from EAV by shRNAs. The cells were transfected with DNA vectors expressing the shRNAs indicated and challenged with EAV 48 h later. After 5 days the cells were analyzed microscopically for their CPE (phase contrast, left side) and the EAV N-protein (right side). Pictures were taken at 200× magnification.

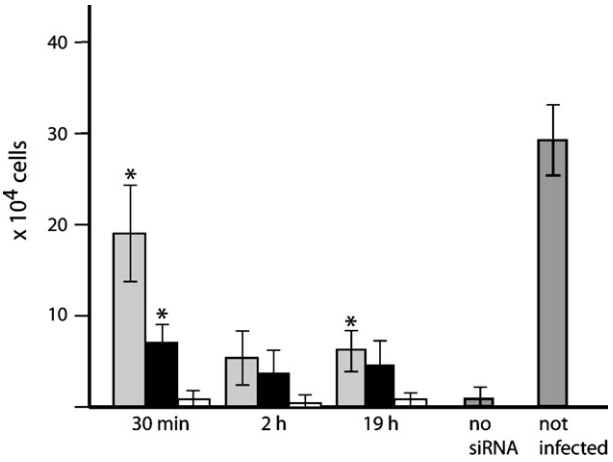

Fig. 6. Time dependence of siRNA mediated protection from EAV. Equine APH-R cells were infected with EAV and transfected with Si3 (light grey columns), Si1 (black columns) and SiGFP (white columns) at the indicated time points after infection. After 6 days the surviving cells in the cultures were counted. Controls are shown as dark grey columns. Values are the mean results from 4 independent experiments, error bars represent the standard deviation. The asterisks indicate a statistically significant difference of siRNA-treated cells to the non-transfected ("no siRNA") controls ($P < 0.02$).

4. Discussion

EAV has a serious impact on horses, it causes acute infection and can persist lifelong in stallions. Although vaccines against the virus have been developed, there is still a high demand for antiviral therapies, especially in the case of virus persistence. Here, RNAi was investigated as a potential agent to combat EAV. Due to the absence of a suitable small-animal model, *in vitro* studies were performed. Although EAV can be propagated in a variety of cell lines, horse cells were used to be as close to the original host as possible.

The results demonstrate that horse cells are protected from EAV by chemically synthesized siRNAs. From three siRNAs targeting ORF1, two led to a significant decrease in viral infection. Two siRNAs targeting ORF2b led to weakly increased survival rates of the cells. The siRNAs against the structural proteins GP5 and N failed to induce any protection.

Although it is tempting to conclude from these results that RNAi against EAV most efficiently works when ORF1 is targeted, one should also take several other explanations into account: first, the effectiveness of the particular siRNAs. Although all our siRNAs were designed following the same rules, it cannot be excluded that a given siRNA has specific physical or biological features that make it less efficient in destroying its target RNA. Second, the viral target RNA might form specific structures, like hairpins, which make it inaccessible for the siRNA (Westerhout and Berkhout, 2007). Accordingly, transfection of several siRNAs did not lead to less expression of their target proteins (Fig. 4C), and Si2, although targeting ORF1, protected the cells to a lesser extent than Si1 and Si3.

However, an alternative explanation for the ineffectiveness of siRNAs targeting structural proteins could be the molar amount of target RNA. It is well established that the sgRNA encoding ORF7 is by far the most abundant RNA in a cell infected with an Arterivirus, and the sequence is also present in all other sgRNAs (Pasternak et al., 2006). Hence, any siRNA directed against ORF7 has to be present in a substantial concentration in the cell to significantly affect the level of its target RNA, which obviously is more demanding compared to siRNAs targeting the relatively rare RNA of ORF1. This hypothesis was tested by adding more siRNA to the cells, but a significant effect of Si7 or Si8 was not seen before the concentration limits of the transfection protocol were reached (data not shown).

In order to be used as an antiviral-therapy *in vivo*, RNAi should be applied to the cells after the viral infection is established. However, it was found that the protective effect under such conditions was significantly reduced, except when the cells were transfected shortly after viral challenge. Similar to these results, it has been reported that Flaviviruses, single-stranded positive-sense RNA viruses like EAV, are resistant to RNAi once they are established in a cell (Geiss et al., 2005). This was explained by the fact that viral replication takes place in specialized membrane compartments, also found in an EAV infection (Posthuma et al., 2008). These compartments might render the viral RNA inaccessible for the RISC complex, the essential component of the RNAi machinery (Haasnoot et al., 2007). However, in a mouse model, the same Flaviviruses were susceptible to RNAi after viral challenge. In this study, the siRNA was directly injected into the brain, the organ targeted by the viruses (Kumar et al., 2006), whereas in other experiments systemic application after challenge had no effect (Bai et al., 2005). This suggests a limitation of *in vitro* studies. In mammals, there probably is an excess of uninfected target cells. RNAi is delivered to these cells and renders them resistant to the virus, although other cells have been infected before RNAi application. Hence, RNAi can well be useful to block a spreading viral infection, as long as enough potential target cells (that are not yet infected) take up the siRNA. The major challenge in RNAi-based antiviral treatment is therefore the efficient targeting of the right cells at the right time, similar to other nucleic acid-based therapeutics, such as morpholino oligomers (van den Born et al., 2005; Kinney et al., 2005).

Other concerns for using RNAi to combat viral infections are the emergence of resistant viruses due to mutations in the targeting sequences (Das et al., 2004) and the natural sequence variation among different virus strains (Stadejek et al., 1999). By doing BLAST searches, the targeting sequences in ORF1 (Si1, Si2, Si3) were found in less field isolates than those targeting structural proteins, although it should be noted that to date there are far less sequences spanning ORF1 in GeneBank compared to ORFs 2–7 (data not shown). A lack of conservation might complicate a broader use of a given siRNA in antiviral therapy. To overcome these obstacles, one should therefore use several different siRNAs together, similar to therapeutic RNAi approaches against HIV (ter Brake et al., 2008).

To date there are two principal ways of delivering RNAi to cells, either by transfecting chemically synthesized siRNAs or by transfecting DNA plasmids (or DNA viruses) encoding shRNAs which are later processed to siRNAs. Here it was found that both approaches work to interfere with EAV replication. For *in vivo* applications, synthetic siRNAs have the advantage over DNA-plasmids that they do not have to enter the nucleus to function. Therefore, application is more direct, faster and probably reaches more cells. However, RNA is less stable than DNA. It therefore has to be determined experimentally which delivery method would be best suited to eliminate EAV *in vivo*.

To sum up, in the present study it was shown that RNAi can be used to protect horse cells from EAV and to lower the impact of EAV on cells after viral challenge. As RNAi can be applied to animals, these findings have potential consequences for the development of antiviral therapies. However, the prerequisite for any RNAi-based therapy of an EAV infection is an appropriate delivery method. For systemic application, the amounts of siRNAs or shRNA-expressing plasmids may be limiting due to the horse's size. General understanding of the disease and of EAV localization in an infected animal has to be augmented before targeted delivery of RNAi becomes a reasonable option.

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