Published in final edited form as:

J Med Virol. 2013 January ; 85(1): 179–186. doi:10.1002/jmv.23340.

Inhibition of Tulane Virus Replication *in vitro* with RNA Interference

Qiang Fan¹, Chao Wei¹, Ming Xia¹, and Xi Jiang^{1,2}

¹Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

²Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

Abstract

RNA interference (RNAi), a conserved mechanism triggered by small interfering RNA (siRNA), has been used for suppressing gene expression through RNA degradation. The replication of caliciviruses (CVs) with RNAi was studied using the Tulane virus (TV) as a model. Five siRNAs targeting the non-structural, the major (VP1) and minor (VP2) structural genes of the TV were developed and the viruses were quantified using qPCR and TCID₅₀ assay. Treatment of the cells with siRNA 4 hours before viral inoculation significantly reduced viral titer by up to 2.6 logs and dramatically decreased viral RNA copy numbers and viral titers 48 hours post infection in four of the five siRNAs studied. The results were confirmed by Western blot, in which the major structural protein VP1 was markedly reduced in both the cells and the culture medium. Two small protein bands of the S and P domains of the viral capsid protein were also detected in the cell lysates, although their role in viral replication remains unknown. Since the TV shares many biological properties with human noroviruses (NoVs), the successful demonstration of RNAi in TV replication would provide valuable information in control of acute gastroenteritis caused by human NoVs.

Introduction

Noroviruses (NoVs), belonging to the calicivirus (CV) family, are small, non-enveloped, single stranded, positive-sense RNA viruses. NoVs are a leading cause of epidemics of non-bacterial acute gastroenteritis, affecting millions of people worldwide. The viruses are spread by fecal-oral pathways, through person-to-person contact, or by contaminations of environmental surfaces, water or food. They are highly contagious which usually result in large outbreaks in crowded communities or institutions such as schools, restaurants, hospitals, child care centers, nursing homes for the elderly, cruise-ships, and military settings. According to a recent estimation, NoVs cause 64,000 episodes of diarrhea requiring hospitalization and 900,000 clinic visits among children in industrialized countries, and 1,000,000 inpatient hospitalizations and 200,000 deaths of children under the age of five in developing countries (Patel et al. 2008). NoVs are also the leading cause of foodborne illnesses and attributable to ~ 21 million cases in the United States annually (Hall et al. 2011).

Human NoVs are difficult to study due to the lack of a cell culture or a proper animal model for NoVs. Recently a cultivable monkey enteric calicivirus, the Tulane virus (TV) has been isolated, which could be a useful surrogate for human NoVs. TV is genetically more closely related to NoVs than to any other calicivirus genera (Farkas et al. 2008, Wei et al. 2008). TV

also recognizes the histo-blood group antigens (HBGAs) like human NoVs (Farkas et al. 2010). The complete genome of TV is organized into three open reading frames (ORFs) similar to that of NoVs (Wei et al. 2008). ORF1 is composed of two-thirds of the genome, encoding the non-structural (NS) polyprotein, which is autocleaved to at least six functional units, including the N-terminal protein, NTPase, a small protein with yet unknown function, VPg, viral protease, and RNA-dependent RNA polymerase (RdRp) (Wei et al. 2008). Both NTPase and RdRp play an essential role in viral RNA replication (Tao and Ye 2010). ORF2 encodes the major structural protein VP1, while ORF3 encodes a minor structural protein VP2 (Hardy 2005).

RNA interference (RNAi) is a molecular mechanism in which fragments of double-stranded, small interfering RNA (siRNA) interfere with the expression of a particular gene by mediating the degradation of mRNA (Elbashir et al. 2001, Gitlin et al. 2002, Li et al. 2002). Since the discovery of siRNA, a new field of drug discovery has emerged for a wide variety of human diseases, such as cancer (Haasnoot et al. 2003, Urban-Klein et al. 2005, Grzelinski et al. 2006, Haasnoot and Berkhout 2006, Ahn et al. 2010, Palumberi et al. 2010), metabolic diseases and viral infections (Leonard and Schaffer 2006, Rossi 2006, Ding 2010, Haasnoot and Berkhout 2011). A number of studies have successfully demonstrated that siRNA is able to effectively inhibit viral replication and infection, including SARS (Li et al. 2005, Wu et al. 2005), respiratory syncytial virus (Zhang and Tripp 2008, Alvarez et al. 2009), rotavirus (Dector et al. 2002, Arias et al. 2004), ebola virus (Geisbert et al. 2010), HIV (Morris and Rossi 2006), influenza virus (Ge et al. 2003, Ge et al. 2004), dengue virus (Travanty et al. 2004, Wu et al. 2010), poliovirus (Saleh et al. 2004) and hepatitis B and C viruses (Saulnier et al. 2006, Panjaworayan et al. 2010, Pelletier et al. 2010).

In this study, siRNA was designed and evaluated to inhibit CV replication in cell cultures using the TV as a model. Five siRNA duplexes were designed to target different genes of the TV genome and significant reduction of TV replication was observed following treatment of four of the five siRNAs. Future study for optimal delivery of siRNA is required.

Materials and methods

Virus, cells and antibodies

TV used in the experiments has been maintained in our laboratory for ~50 passages since its original isolation from rhesus macaques stool samples. The monkey kidney LLC-MK2 cells (ATCC) were used to cultivate the TV. Two hyperimmune antibodies against the shell (S) and the protruding (P) domains of the TV capsid protein VP1, respectively, were developed using recombinant S and P domain proteins described below.

Development of recombinant TV P and S domain proteins and antibodies against the proteins

The P domain of TV capsid protein VP1 (TVPD) coding cDNA was cloned into pQE-30 vector (Qiagen, Maryland, MD) by RT-PCR with a primer pair containing BamH I and Hind III sites, CGCGGATCCAATGCACTGCCAATTTGGTCC/CCCAAGCTTTTATCTAAAGACAAGTGCCTGCAG. Recombinant TVPD protein with a His-tag was expressed in *E. coli* (BL21, DE3) with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at room temperature (~22°C) overnight. Due to a low solubility of the TVPD proteins, the purification of the TVPD protein was performed under a denaturing condition using TALON metal affinity resins (BD, Franklin Lakes, NJ, USA). Briefly, *E. coli* cells were suspended in 1X equilibration buffer containing 50 mM sodium phosphate, 6M guanidine-HCl and 300 mM NaCl. After sonication (12 min, 0°C) and centrifugation (15,600 $\times$ g, 30 min), soluble TVPD His-tag fusion proteins were purified by

TALON resin and eluted by imidazole elution buffer containing 5.4 M guanidine. The concentration of guanidine was reduced to 3M through dialysis for antibody induction.

The coding region of TV VP1 S domain was amplified with a primer pair containing EcoR I and Xho I sites, AATGAATTCATGGAAAACAGCAAAACTG/CTGCTCGAGTTATCTAAAGACAACTGC, and was cloned into pGEX-4T1 vector (GE Healthscience, Pittsburgh, PA). The GST-TVSD fusion proteins were then expressed in *E. coli* plysS (NoVagen, Darmstadt, Germany) with 0.4 mM IPTG overnight at room temperature. The cells were harvested by centrifugation at $5000 \times g$ for 15 min and re-suspended in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspensions were then subjected to sonication and further clarification by centrifugation at $12,000 \times g$ for 60 min. The GST-fusion proteins in the supernatant were then purified by affinity chromatography using Glutathione Sepharose 4B resins (GE Healthcare, Pittsburgh, PA). The GST-tag was then removed by digestion of the fusion proteins with thrombin, and further purified with gel filtration chromatography using the Superdex 200 column (GE Healthcare, Pittsburgh, PA). A mouse anti-TVPD antibody and a rabbit anti-TVSD polyclonal antibody were generated following animal immunization with these two proteins using a procedure described previously (Tan et al. 2004b).

Design and synthesis of siRNA

The TV genome sequence (EU391643) was used as a template for designing siRNA duplexes. Five siRNAs targeting at different structural and non-structural genes of the TV genome were designed using the web-based siDESIGN program from Dharmacon Inc. (Thermo Scientific, Rockford, IL). All 5 siRNAs contained 19 nts double-stranded RNA plus UU overhang at the 3' ends of both strands of the RNAs (Figure 1). The siRNAs were synthesized by Dharmacon. SiGENOME Non-Targeting siRNA #1 was also purchased from Dharmacon as a negative control.

Cell culture, siRNA transfection and viral infection

LLC-MK2 cells were cultured with medium 199 (M199, Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum, 100 unit/ml penicillin, 100 μ g/ml streptomycin, and maintained at 37°C with 5% CO₂. Transfection of siRNA was performed on 6 well plates or 60 mm dishes at 60–70% cell confluence as described previously (Fan et al. 2009). Briefly, siRNA duplexes at 120 pmol were mixed with 6 μ l of lipofectamine 2000 (Invitrogen, Carlsbad, CA) and the mixtures were applied to cells. Four hours post transfection, the cells were infected with TV at multiple of infection (MOI) of 1 for one hour at 37°C with slight rocking to ensure every cell to be infected. After infection, the cells were washed four times with PBS and once with M199 medium to remove any virus left in the media before adding new culture media (M199 and 10% FBS). The infected cell cultures were maintained at 37°C for 48 hours to score the antiviral effects by CPE, TCID₅₀, qRT-PCR and Western blot assays.

Assays of viral titer by TCID₅₀

The viral titer in both supernatant and cells was determined by titration using CPE-based 50% tissue culture infective dose (TCID₅₀) assay. Collected culture media was spun at $860 \times g$ at 4°C for 5 min and serially diluted 10-fold with M199. The virus suspension was added to LLC-MK2 cells in 96 well plates with 7 replicating wells prepared for each dilution. Cells were checked daily for CPE and the results were scored at day 5 dpi (Ge et al., 2004).

Determination of viral titer by quantitative real time PCR (qPCR)

QPCR was performed using the iCycle IQ (Bio-Rad) real time PCR detection system. Total viral RNA of TV in both cells and culture media were extracted using QIAamp Viral RNA Mini Kit (Qiagen, Maryland, MD). The RNA yield was determined with NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE). Reverse transcription (RT) was carried out using the GoScript Reverse Transcription System (Promega, San Luis Obispo, CA) in a 20 μ l reaction mixture containing 300 ng of total RNA and an Oligo(dT)₁₅ primer at 42°C for 1h according manufacturer's instruction. An aliquot of 1.5 μ l of the RT reaction mixture was then used for the qPCR reaction using the SYBR green PCR method (Bio-Rad, Hercules, CA). The qPCR was performed at 95°C for 3 min, then 95°C for another 30 sec and 60°C for 1 min for 45 cycles. A post amplification melt curve analysis was performed to confirm production specificity of the reactions. All reactions were done in duplicate and analyzed with iCycle IQ software version 3.0 (Bio-Rad). Viral RNA copy number of each sample was converted based on the Ct value of the sample compared to the standard curve of total TV RNA with known copy numbers. The $2^{-\Delta\Delta C_t}$ was used for viral replication and normalized to no TV infection control. PCR primers used for detection of the 18s rRNA (as an endogenous control) were: For. 5-GTAACCCGTTGAACCCCAT-3 and Rev. 5-CCATCCAATCGGTAGTAGCG-3. Primer pairs for detection of TV at both the 5'-end of the genome (For. 5-GACTAGAGCTATGGATACGTCC-3 and Rev. 5-GCCCCAGGGATGGGTCAGGCCATTGAGACGG-3) and the 3'-end of the genome (5-ATCTTCCTCTGTTCTTC-3 and Oligo (dT)₁₅ AGTAGCCTCGAGCGGCCGTTTTTTTTTTTTTTT) were used.

Detection of viral capsid proteins by Western blot analysis

The TV infected cells were analyzed for TV capsid S and P proteins by Western blot analysis. In brief, total proteins from the cells and the culture supernatant were harvested at 30 hpi when the majority of cells remained attached. Cells were washed with PBS, lysed with RIPA buffer (Thermo Sci. Rockford, IL) containing PMSF 1mM, aprotinin 0.5 μ g/ml and leupeptin 1 μ g/ml. The total protein in the cell culture supernatant was harvested by centrifugation at 200,000 $\times$ g (Optima L-90K ultracentrifuge, Beckman Coulter, Brea, CA) for 3 h at 4°C and the pellets were re-suspended in the RIPA buffer as mentioned above. Equivalent volumes of cell lysates and culture supernatant were prepared for each sample and analyzed by 10% SDS-PAGE. For Western blot analysis, proteins were transferred to nitrocellulose and firstly detected for the P domain protein using the mouse anti-TVPD antibody followed by a HRP-linked secondary antibody (MP Biomedicals, LLC, Solon, OH). The signals were detected by HyGLO chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Inc. Metuchen, NJ). To detect the S domain protein, the membranes were stripped with strip buffer (Thermo Sci. Rockford, IL) and incubated with the rabbit anti TVSD antibody (1:6000) before detection by a HRP-linked 2nd antibody. A potential 80 kDa cellular protein stained by the P domain specific antibody in the original gel was used as a loading control because the signal of β -actin was very faint after twice stripping.

Statistical analysis

All treatments were performed in triplicate and the results were expressed as means $\pm$ SD from three independent experiments. Data were analyzed using the paired *t*-test and statistical significance was defined as *p* < 0.05.

Results

Optimization of RNAi against TV infection in LLC-MK2 cells

In our initial experiments, four siRNAs, targeting at the non-structural genes (NTPase and RdRp), the major (VP1) and minor (VP2) structural genes of TV genome, respectively, were designed. Three (siNTPase, siRdRp and siVP2) of them revealed significant interference against TV replication but one targeting the major structural gene (siVP1-1) was much less efficient. To verify the result, another siRNA targeting at a different location of the VP1 gene (siVP1-2) was designed and a comparable interference effect was observed as those of the other three siRNAs (Figures 1 and 2).

The conditions for effective transfections of siRNA into host cells were evaluated based on the morphology of the host cell monolayer and the outcome of CPE following the siRNA transfection and viral infection. A dose of 120 pmol of siRNA was selected for all five siRNAs studied because it did not cause visual cytotoxicity but protected clearly against viral infection in the LLC-MK 2 cells. The ratio between siRNA and transfection reagents used to deliver the siRNAs into the host cells was also determined based on our previous experiences (Fan et al. 2009) and pilot results (data not shown), in which good cell morphology and high efficiency of RNAi were obtained. In addition, a 4-hour interval between transfection of siRNA and viral inoculation was selected, which resulted in clear antiviral effects within 24 hours post infection (hpi).

Furthermore, the effects of multiple deliveries of different siRNAs simultaneously or the same siRNA repeatedly on the viral replication were also evaluated but no significant enhancement on antiviral effect was found by either approaches. Thus, in the subsequent experiments, one dose of siRNA using the optimal transfection conditions at 4 hours before viral inoculation was used and the cell cultures were maintained for 48 hpi before being scored for viral replication by qRT-PCR, TCID₅₀ and Western blot analysis (30 hpi).

Finally, primer pairs of both the 5' - and 3' -ends of the TV genome were used for qPCR and no significant differences between these two primer pairs were found in detection of TV in the cell cultures (data not shown). Since the 3' -primer pair would be more likely to detect "full-length" genome compared with 5' -primer pair, the 3' -primers were selected in our subsequent experiments.

Effects of individual siRNAs against TV replication in LLC-MK2 cells

As described above, four of the five siRNAs resulted in apparent protection against TV replication in LLC-MK2 cells. Typical CPE with round and detached cells floating around was seen at ~20 hpi in wells with the control siRNA and the siVP1-1 but not in wells with the other four siRNAs (data not shown) or in wells without TV infection. The CPE were much clearer at 40 hpi when all cells transfected with siControl or siVP1-1 were rounded and detached but no change in the other wells (Figure 2).

To further evaluate the antiviral effect of the siRNAs on TV infection, the amounts of viral RNAs in the cells and the culture supernatant were measured by real time RT-PCR at 24 and 48 hpi. The viral copy numbers in both the supernatant and infected cells were dramatically reduced at both time points, while the viral titers continued increasing from 24 to 48 hpi in wells containing the siControl (Figures 3A and 3B). The antiviral effects seemed much stronger by both non-structure siRNAs (siNTPase and siRdRp) and the siVP1-2 as measured at 24 hpi ($P < 0.05$) and 48 hpi ($P < 0.01$). Low levels of viral reduction were seen at 24 hpi for both siVP1-1 and siVP1-2, but such reduction was only seen for siVP1-2 at 48 hpi.

The antiviral effects were further evaluated by CPE-based TCID₅₀ analysis and the outcomes correlated with the qRT-PCR results well, in which significant reduction of TCID₅₀ was observed for the same four siRNAs at 48 hpi but not for siVP1-1. The antiviral effects seemed to be the highest by the siNTPase in which an estimated 2.6 logs of TCID₅₀ reduction was observed in the supernatant of cells treated with siNTPase at 48 hpi (Figure 4).

Confirmation of viral reduction by Western blot analysis

To further confirm the antiviral effects of siRNA on TV, Western blot analysis of the TV structural protein VP1 was performed on viruses harvested from both the infected cells and the cell culture supernatant at 30 hpi. The results further confirmed the qRT-PCR and TCID₅₀ data, in which significant decreases of the expression of the viral capsid protein VP1 were detected in cells treated with the four siRNAs (siNTPase, siRdRp, siVP1-2 and siVP2) but not in cells treated with siVP1-1 by using both S and P domain specific antibodies (Figure 5). Most viruses remained cell associated as the signals of the full length VP1 inside the cells were much stronger than that in the supernatants (Figure 5). Interestingly, two small protein bands besides the full length VP1 were also detected by the S and P domain specific antibodies respectively. According to the molecular weights of the two proteins (around 29 kDa and 36 kDa), they are likely cleaved products from the VP1, representing the S and P domains of the viral capsid protein (Figure 5 A and B). The detection of P proteins in stool samples of NoVs infected patients has been reported (Greenberg et al. 1981, Graham et al. 1994) although the function of this protein in viral replication and pathogenesis remains unknown. To our knowledge, it is the first time of detection of S proteins for a CV, future study is needed to determine if it is a side product of the viral capsid or has some biological function in viral replication and/or pathogenesis to the hosts.

Discussion

In this study, effective RNAi in inhibition of viral replication as potential treatments against NoVs has been demonstrated using the TV cell culture as a model. The current results showed that siRNAs targeting either the non-structural or the structural genes of the TV genome were highly effective in reduction of TV replication in the LLC-MK2 cells. Effective inhibition of calicivirus replication by antisense RNA was also observed for the feline CV (Stein et al. 2001) and the murine norovirus (MNV) (Bok et al. 2008). The antiviral effects have been determined by qRT-PCR and TCID₅₀ and confirmed by Western blot analyses. One dose of siRNA delivered four hours before viral inoculation was highly effective for controlling TV replication for at least 48 hpi, with up to 2.6 logs of TCID₅₀ reduction by the most effective siRNA targeting to the viral NTPase gene.

This study also showed that the antiviral activity mediated by siRNA was genomic location-dependent and/or gene sequence-dependent (Zheng et al. 2004, McDonagh et al. 2011, Siu et al. 2011). For example, two siRNA duplexes targeting the viral non-structural proteins, NTPase and RdRp, respectively, had very potent inhibition effect on TV replication. Although the function of NTPase remains unclear for TV, studies on the flavivirus have shown that functional inhibition of the NTPase may occur by competition or blockade of the NTP binding site, by conformational changes through immobilization of the switch region, by competitive inhibition of RNA binding, or by inhibition of unwinding via steric blockade of translocation of the NTPase along the template RNA (Borowski et al. 2002b, Borowski et al. 2002a). The RdRp is involved in the synthesis of genomic and subgenomic RNA that is essential for calicivirus replication. Thus the selection of siRNA targeting the non-structural genes may be a choice of targets for highly efficient RNAi against CVs.

The reason for the lack of inhibition of TV replication by the first siRNA targeting the VP1 gene remains unknown. Although one point mutation has been found at T5379G of the TV genome, where the targeting region of siVP1-1 is, based on a recent sequencing result (data not shown), which could be the cause of failure of siVP1-1, we still cannot exclude other cause for the relative ineffectiveness of siVP1-1. However, the success of antiviral effect by the second siRNA targeting at a different region of the VP1 gene as well as by the siRNA targeting the VP2 gene suggested that the viral structural genes could also be a good target for RNAi against calicivirus, although a different finding by others was reported (Zheng et al. 2004). The relative less efficiency of the structural siRNA in comparison with the non-structural siRNA could be due to a potential high copy number of the structural genes than the non-structural genes, in which the structural genes were encoded in both genomic and subgenomic RNAs (Hardy 2005).

We also studied timing for treatment and combination of siRNAs in reduction of TV for potential clinical applications of RNAi. In general, a single dose of siRNA at four hours before viral inoculation was found to be sufficient for preventing TV replication for at least 48 hours. This result is consistent with the fact that no clear additive effect by additional dose of the same siRNA in a later time within the 48 hour period was necessary in our multiple dose treatment experiments (data not shown). However, slight increase of efficiency using a combination of more than one siRNA in compared with one single siRNA in a single dose treatment was indeed observed. The combination of both siRNAs targeting the non-structural genes (NTPase and RdRp, with half dosage for each) seemed more effective than a combination of siRNA targeting structural and non-structural genes (data not shown). While one dose seemed to control TV infection sufficiently for 48 hours, future study on extended course of disease may be necessary, because the illness of NoVs gastroenteritis usually lasts for 3–5 days and even longer for viral shedding (up to 4 weeks). This may require an alternative model, because almost all cells in siControl were infected (round and detached), which might no longer be susceptible to TV.

The detection of both the S and P domain proteins in the TV infected LLC-MK2 cells was unexpected, particularly for the S protein which was never reported by others. Since the soluble P protein was also found in stool specimens of NoVs infected patients (Greenberg et al. 1981, Graham et al. 1994, Hardy et al. 1995) it could have certain biological function in viral replication or pathogenesis. The finding of both proteins inside cells but not in the culture medium supernatant indicated that neither of these protein fragments were part of the mature virions. Suggested function of P protein included involvement of host receptor recognition and immune regulation (Tan et al. 2004a, Tan et al. 2004b, Chen et al. 2006, Tan et al. 2008). On the other hand, we still cannot exclude the possibility that these two protein fragments were random degradation products, particularly the molecular weight of the S protein seen on the Western blotting analysis was bigger than what was expected (~ 22 kDa) based on the predicted hinge region in the TV genome. The previous studies of detection of the P protein in stools used serum samples from NoVs infected patients (Greenberg et al. 1981, Graham et al. 1994) which might not be able to detect the S domain protein. In this study, we used an S domain antibody raised against recombinant S protein. Thus, both the S and P domain fragments could be natural degradation product of the VP1 without any function in viral replication.

In summary, this study has demonstrated the feasibility of siRNA in TV replication, which may be valuable for prophylactic and therapeutic treatment of calicivirus particularly NoVs. Although a single dose at four hours before viral infection has been demonstrated effective in inhibition of TV, there is still a long way to go before this approach can be used in clinical practice to treat and prevent NoVs gastroenteritis. The major challenges include the procedures to deliver the drugs to host cells and to maintain the appropriate concentration of

the siRNA to fight against the viruses. The genetic variation of NoVs is another challenge for broad protection against different NoVs. Finally, no appropriate animal model to assess the efficacy of the treatment exists, which requires continual effort in the future studies.

Acknowledgments

The work was supported by the National Institute of Health, the National Institute of Allergy and Infectious Diseases (R01 AI37093, R01 AI055649 and R01 AI089634), and the Department of Defense (PR033018) to X.J. We also thank the helpful discuss from Drs. Ming Tan, Pengwei Huang and other members of our lab.

References

- Ahn SJ, Jeon YH, Lee YJ, Lee YL, Lee SW, Ahn BC, Ha JH, Lee J. Enhanced anti-tumor effects of combined MDR1 RNA interference and human sodium/iodide symporter (NIS) radioiodine gene therapy using an adenoviral system in a colon cancer model. *Cancer Gene Ther.* 2010; 17:492–500. [PubMed: 20186172]
- Alvarez R, Elbashir S, Borland T, Toudjarska I, Hadwiger P, John M, Roehl I, Morskaya SS, Martinello R, Kahn J, Van Ranst M, Tripp RA, DeVincenzo JP, Pandey R, Maier M, Nechev L, Manoharan M, Kotlianski V, Meyers R. RNA interference-mediated silencing of the respiratory syncytial virus nucleocapsid defines a potent antiviral strategy. *Antimicrob Agents Chemother.* 2009; 53:3952–3962. [PubMed: 19506055]
- Arias CF, Dector MA, Segovia L, Lopez T, Camacho M, Isa P, Espinosa R, Lopez S. RNA silencing of rotavirus gene expression. *Virus Res.* 2004; 102:43–51. [PubMed: 15068879]
- Bok K V, Cavanaugh J, Matson DO, Gonzalez-Molleda L, Chang KO, Zintz C, Smith AW, Iversen P, Green KY, Campbell AE. Inhibition of norovirus replication by morpholino oligomers targeting the 5'-end of the genome. *Virology.* 2008; 380:328–337. [PubMed: 18783811]
- Borowski P, Schalinski S, Schmitz H. Nucleotide triphosphatase/helicase of hepatitis C virus as a target for antiviral therapy. *Antiviral Res.* 2002a; 55:397–412. [PubMed: 12206878]
- Borowski P, Niebuhr A, Schmitz H, Hosmane RS, Bretner M, Siwecka MA, Kulikowski T. NTPase/helicase of Flaviviridae: inhibitors and inhibition of the enzyme. *Acta Biochim Pol.* 2002b; 49:597–614. [PubMed: 12422230]
- Chen R, Neill JD, Estes MK, Prasad BV. X-ray structure of a native calicivirus: structural insights into antigenic diversity and host specificity. *Proc Natl Acad Sci U S A.* 2006; 103:8048–8053. [PubMed: 16702551]
- Dector MA, Romero P, Lopez S, Arias CF. Rotavirus gene silencing by small interfering RNAs. *EMBO Rep.* 2002; 3:1175–1180. [PubMed: 12446562]
- Ding SW. RNA-based antiviral immunity. *Nat Rev Immunol.* 2010; 10:632–644. [PubMed: 20706278]
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 2001; 411:494–498. [PubMed: 11373684]
- Fan Q, Zhang F, Barrett B, Ren K, Andreassen PR. A role for monoubiquitinated FANCD2 at telomeres in ALT cells. *Nucleic Acids Res.* 2009; 37:1740–1754. [PubMed: 19129235]
- Farkas T, Sestak K, Wei C, Jiang X. Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae. *J Virol.* 2008; 82:5408–5416. [PubMed: 18385231]
- Farkas T, Cross RW, Hargitt E 3rd, Lerche NW, Morrow AL, Sestak K. Genetic diversity and histoblood group antigen interactions of rhesus enteric caliciviruses. *J Virol.* 2010; 84:8617–8625. [PubMed: 20554772]
- Ge Q, Filip L, Bai A, Nguyen T, Eisen HN, Chen J. Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc Natl Acad Sci U S A.* 2004; 101:8676–8681. [PubMed: 15173599]
- Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen HN, Chen J. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc Natl Acad Sci U S A.* 2003; 100:2718–2723. [PubMed: 12594334]

- Geisbert TW, Lee AC, Robbins M, Geisbert JB, Honko AN, Sood V, Johnson JC, de Jong S, Tavakoli I, Judge A, Hensley LE, Maclachlan I. Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet*. 2010; 375:1896–1905. [PubMed: 20511019]
- Gitlin L, Karelsky S, Andino R. Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature*. 2002; 418:430–434. [PubMed: 12087357]
- Graham DY, Jiang X, Tanaka T, Opekun AR, Madore HP, Estes MK. Norwalk virus infection of volunteers: new insights based on improved assays. *J Infect Dis*. 1994; 170:34–43. [PubMed: 8014518]
- Greenberg HB, Valdesuso JR, Kalica AR, Wyatt RG, McAuliffe VJ, Kapikian AZ, Chanock RM. Proteins of Norwalk virus. *J Virol*. 1981; 37:994–999. [PubMed: 6785451]
- Gzrelinski M, Urban-Klein B, Martens T, Lamszus K, Bakowsky U, Hobel S, Czubyko F, Aigner A. RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs in vivo exerts antitumoral effects in glioblastoma xenografts. *Hum Gene Ther*. 2006; 17:751–766. [PubMed: 16839274]
- Haasnoot J, Berkhout B. RNA interference: its use as antiviral therapy. *Handb Exp Pharmacol*. 2006; 117–150. [PubMed: 16594614]
- Haasnoot J, Berkhout B. RNAi and cellular miRNAs in infections by mammalian viruses. *Methods Mol Biol*. 2011; 721:23–41. [PubMed: 21431677]
- Haasnoot PC, Cupac D, Berkhout B. Inhibition of virus replication by RNA interference. *J Biomed Sci*. 2003; 10:607–616. [PubMed: 14576463]
- Hall AJ, Vinje J, Lopman B, Park GW, Yen C, Gregoricus N, Parashar U. Updated Norovirus Outbreak Management and Disease Prevention Guidelines. *Morbidity and Mortality Weekly Report (MMWR)*. 2011; 60:1–15.
- Hardy ME. Norovirus protein structure and function. *FEMS Microbiol Lett*. 2005; 253:1–8. [PubMed: 16168575]
- Hardy ME, White LJ, Ball JM, Estes MK. Specific proteolytic cleavage of recombinant Norwalk virus capsid protein. *J Virol*. 1995; 69:1693–1698. [PubMed: 7853506]
- Leonard JN, Schaffer DV. Antiviral RNAi therapy: emerging approaches for hitting a moving target. *Gene Ther*. 2006; 13:532–540. [PubMed: 16177819]
- Li BJ, Tang Q, Cheng D, Qin C, Xie FY, Wei Q, Xu J, Liu Y, Zheng BJ, Woodle MC, Zhong N, Lu PY. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat Med*. 2005; 11:944–951. [PubMed: 16116432]
- Li H, Li WX, Ding SW. Induction and suppression of RNA silencing by an animal virus. *Science*. 2002; 296:1319–1321. [PubMed: 12016316]
- McDonagh P, Sheehy PA, Norris JM. In vitro inhibition of feline coronavirus replication by small interfering RNAs. *Vet Microbiol*. 2011; 150:220–229. [PubMed: 21367541]
- Morris KV, Rossi JJ. Lentivirus-mediated RNA interference therapy for human immunodeficiency virus type 1 infection. *Hum Gene Ther*. 2006; 17:479–486. [PubMed: 16716105]
- Palumberi D, Aldi S, Ermini L, Ziche M, Finetti F, Donnini S, Rosati F. RNA-mediated gene silencing of FUT1 and FUT2 influences expression and activities of bovine and human fucosylated nucleolin and inhibits cell adhesion and proliferation. *J Cell Biochem*. 2010; 111:229–238. [PubMed: 20506485]
- Panjaworayan N, Payungporn S, Poovorawan Y, Brown CM. Identification of an effective siRNA target site and functional regulatory elements, within the hepatitis B virus posttranscriptional regulatory element. *Virol J*. 2010; 7:216. [PubMed: 20822550]
- Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinje J, Parashar UD. Systematic literature review of role of noroviruses in sporadic gastroenteritis. *Emerg Infect Dis*. 2008; 14:1224–1231. [PubMed: 18680645]
- Pelletier I, Saulnier A, Brisac C, Jegouic S, Vabret N, Tangy F, Blondel B, Colbere-Garapin F. Enhanced gene silencing in cells cured of persistent virus infection by RNA interference. *J Virol*. 2010; 84:6880–6885. [PubMed: 20392843]
- Rossi JJ. Topical antiviral siRNA: a practical siRNA microbicide? *Gene Ther*. 2006; 13:1493–1494. [PubMed: 16554841]

- Saleh MC, Van Rij RP, Andino R. RNA silencing in viral infections: insights from poliovirus. *Virus Res.* 2004; 102:11–17. [PubMed: 15068875]
- Saulnier A, Pelletier I, Labadie K, Colbere-Garapin F. Complete cure of persistent virus infections by antiviral siRNAs. *Mol Ther.* 2006; 13:142–150. [PubMed: 16157509]
- Siu RW, Fragkoudis R, Simmonds P, Donald CL, Chase-Topping ME, Barry G, Attarzadeh-Yazdi G, Rodriguez-Andres J, Nash AA, Merits A, Fazakerley JK, Kohl A. Antiviral RNA interference responses induced by Semliki Forest virus infection of mosquito cells: characterization, origin, and frequency-dependent functions of virus-derived small interfering RNAs. *J Virol.* 2011; 85:2907–2917. [PubMed: 21191029]
- Stein DA, Skilling DE, Iversen PL, Smith AW. Inhibition of Vesivirus infections in mammalian tissue culture with antisense morpholino oligomers. *Antisense Nucleic Acid Drug Dev.* 2001; 11:317–325. [PubMed: 11763348]
- Tan M, Hegde RS, Jiang X. The P domain of norovirus capsid protein forms dimer and binds to histo-blood group antigen receptors. *J Virol.* 2004a; 78:6233–6242. [PubMed: 15163716]
- Tan M, Zhong W, Song D, Thornton S, Jiang X. E. coli-expressed recombinant norovirus capsid proteins maintain authentic antigenicity and receptor binding capability. *J Med Virol.* 2004b; 74:641–649. [PubMed: 15484274]
- Tan M, Fang P, Chachiyo T, Xia M, Huang P, Fang Z, Jiang W, Jiang X. Noroviral P particle: structure, function and applications in virus-host interaction. *Virology.* 2008; 382:115–123. [PubMed: 18926552]
- Tao YJ, Ye Q. RNA virus replication complexes. *PLoS Pathog.* 2010; 6:e1000943. [PubMed: 20661480]
- Travanty EA, Adelman ZN, Franz AW, Keene KM, Beaty BJ, Blair CD, James AA, Olson KE. Using RNA interference to develop dengue virus resistance in genetically modified *Aedes aegypti*. *Insect Biochem Mol Biol.* 2004; 34:607–613. [PubMed: 15242701]
- Urban-Klein B, Werth S, Abuharbeid S, Czubayko F, Aigner A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther.* 2005; 12:461–466. [PubMed: 15616603]
- Wei C, Farkas T, Sestak K, Jiang X. Recovery of infectious virus by transfection of in vitro-generated RNA from tulane calicivirus cDNA. *J Virol.* 2008; 82:11429–11436. [PubMed: 18787011]
- Wu CJ, Huang HW, Liu CY, Hong CF, Chan YL. Inhibition of SARS-CoV replication by siRNA. *Antiviral Res.* 2005; 65:45–48. [PubMed: 15652970]
- Wu X, Hong H, Yue J, Wu Y, Li X, Jiang L, Li L, Li Q, Gao G, Yang X. Inhibitory effect of small interfering RNA on dengue virus replication in mosquito cells. *Virol J.* 2010; 7:270. [PubMed: 20946645]
- Zhang W, Tripp RA. RNA interference inhibits respiratory syncytial virus replication and disease pathogenesis without inhibiting priming of the memory immune response. *J Virol.* 2008; 82:12221–12231. [PubMed: 18818323]
- Zheng BJ, Guan Y, Tang Q, Du C, Xie FY, He ML, Chan KW, Wong KL, Lader E, Woodle MC, Lu PY, Li B, Zhong N. Prophylactic and therapeutic effects of small interfering RNA targeting SARS-coronavirus. *Antivir Ther.* 2004; 9:365–374. [PubMed: 15259899]

siNTPase (1453 nts): CCACAAACUUGGAGGACAUUU

siRdRp (3603 nts): GAUUAUAGUAGAUGGGAUUU

siVP1-1 (5375 nts): GAAAUUUGCAGGAGGGUUUUU

siVP1-2 (4860 nts): GGUGAUAAAUUGGGUAAAUUU

siVP2 (6352 nts): GCAAAUUUCAAGUGGGAUAAU

Fig. 1.

Genomic organization of TV and locations of siRNAs. The TV genome is about 6714 bps and encodes a large non-structural polypeptide, the major (VP1) and minor (VP2) structural proteins in three open reading frames (ORFs). The locations of siRNAs targeting the non-structural (siNTPase, siRdRp), VP1 (siVP1-1, siVP1-2) and VP2 (siVP2) genes are indicated.

Fig. 2.

Morphological change of LLC-MK2 cells following TV infection with or without treatment of siRNAs. The cell cultures were imaged at 40 hpi and typical CPE appearance with round, detached and floating cells were seen in wells of transfected with siControl and siVP1-1. Cells treated with siNTPase, siRdRp, siVP1-2 and siVP2 revealed a few round cells or did not have CPE similar to uninfected cells with or without transfection reagents.

Fig. 3.

Titration of TV in cells treated with siRNAs 4 hours before virus inoculation in LLC-MK2 cells or cell culture supernatant by qPCR at 24 and 48 hpi. (A) Relative viral copy number in cell culture supernatant. Viral copy number was determined based on a comparison of Ct value with a standard curve of TV RNA. (B) Relative viral replication ($2^{-\Delta\Delta C_t}$) in the cells. Ct values of siRNA treatments and an uninfected treatment (normalizer) were used to calculate $2^{-\Delta\Delta C_t}$. Significant difference between individual treatments and siControl was indicated (*, $p < 0.05$, and **, $p < 0.01$).

Fig. 4.

Relative virus yield (TCID₅₀) in the media at 24 and 48 hpi. Viral titer of treatments with siRNAs was measured at day 5 post infection. Significant difference between individual treatments and siControl was indicated (*, $p < 0.05$ and **, $p < 0.01$).

Fig. 5.

Western blot analysis of TV capsid protein (VP1) in LLC-MK2 cells following treatment of siRNAs. The cells were harvested at 30 hpi and total proteins in both cell lysates (A and B) and the culture supernatant (C and D) were analyzed by electrophoresis followed by detection of TV P domain (A and C) and S domain (B and D) antibodies. The blots were detected with the P domain antibody first and then with the S domain antibody after stripping. The location of the VP1 and the truncated P and S domain proteins are indicated. A non-specific band at around 80 kDa from the original gels was used as loading control for the assay. Lane 1, No TV; Lane 2, siControl; Lane 3, siNTPase; Lane 4, siRdRp; Lane 5, siVP1-1; Lane 6, siVP1-2; Lane 7, siVP2.