Short hairpin RNA-mediated silencing of bovine rotavirus NSP4 gene prevents diarrhoea in suckling mice

Fangyuan Chen, Hongmei Wang, Hongbin He, Lingling Song, Jianming Wu, Yundong Gao, Xiao Liu, Chengqiang He, Hongjun Yang, Lili Chen, Liqun Wang, Guangpeng Li, Yonghai Li, David E. Kaplan and Jifeng Zhong

While RNA interference (RNAi) has been widely used to study rotavirus gene function in vitro, the potential therapeutic role for RNAi in vivo has not been explored. To this end, we constructed two recombinant lentiviral vectors containing short hairpin RNA (shRNA) against non-structural protein-4 (NSP4) of bovine rotavirus (BRV), RNAi-351 and RNAi-492. RNAi-351 and RNAi-492 strongly suppressed the transient expression of a FLAG-tagged NSP4 fusion protein in 293T cells. In BRV-susceptible MA104 cells, RNAi-492 more potently silenced NSP4 mRNA than RNAi-351 and combination of the two shRNAs almost completely silenced viral NSP4 gene expression. While 100% of suckling mice exposed to BRV and control shRNA developed severe diarrhoea, no suckling mice exposed to BRV in the presence of RNAi-492 or a combination of RNAi-492/RNAi-351 developed severe diarrhoea, and only 20 and 3.3% developed mild diarrhoea, respectively. In addition, RNAi-492 and RNAi-351 markedly abrogated rotaviral replication in MA104 cells and significantly inhibited BRV replication in mouse pups. These results indicated that shRNAs silencing NSP4 gene had substantial antiviral properties and inhibited replication of BRV in a sequence-specific manner that may have clinical application.

INTRODUCTION

Rotavirus diarrhoea is a leading cause of infant mortality in the developing world as well as a major killer of livestock (Dhama et al., 2009). Bovine rotavirus (BRV) infects neonatal calves, resulting in acute, severe diarrhoea, dehydration, anorexia and often death. The mortality rate due to rotavirus diarrhoea approximates 5–20% (Chauhan & Singh, 1996), and thus development of effective prevention and therapeutic strategies is critical.

RNA interference (RNAi) using short hairpin RNA (shRNA) is an antiviral approach that can be utilized to protect either plants (Waterhouse et al., 2001) or animal species (Bitko & Barik, 2001; Gitlin et al., 2002) from viruses. shRNA, which hybridize with target viral mRNA and direct these mRNA for degradation are highly specific tools to downregulate gene expression (Leonard & Schaffer, 2006) and have been extensively utilized to study rotavirus gene function in vitro (Ayala-Breton et al., 2009; Campagna et al., 2005; Cuadras et al., 2006; Décot et al., 2002; Díaz et al., 2008; López et al., 2005a, b; Montero et al., 2006, Silvestri et al., 2004, 2005, Zambrano et al., 2008). Despite the fact that shRNA are easy to design, simple to construct, and inexpensive to produce, little has been published regarding the use of rotavirus gene-specific shRNA for the therapy of rotavirus infection in vivo.

Rotaviruses contain 11 segments of dsRNA that encode structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) as well as several non-structural proteins (NSP). Among the NSPs, NSP4 has been implicated in a number of biological functions during the rotavirus replication cycle and pathogenesis (Campagna et al., 2005). In this study, we demonstrate that shRNA targeting NSP4 inhibit BRV replication in a sequence-specific manner. We first
identified NSP4-specific shRNAs that suppressed transient expression of NSP4 in 293T cells, and then confirmed that these shRNA silence viral NSP4 in BRV-susceptible MA104 cells. Finally, we showed that shRNAs silencing NSP4 prevented suckling mice from developing BRV diarrhea by inhibiting BRV replication and reducing viral shedding. In summary, this study suggests that rotavirus NSP4-specific shRNA may have therapeutic potential in vivo.

RESULTS

shRNA of NSP4 recombinant lentivirus vectors inhibited transient expression of NSP4 in 293T cells

Two lentiviral shRNAs targeting NSP4, RNAi-351 and RNAi-492, were constructed corresponding to amino acids from 103 to 109 or from 151 to 157 of NSP4 (GenBank accession no. FJ972713.1), respectively. pcDNA3-FLAG-NSP4 was co-transfected into 293T cells, along with each shRNA recombinant lentiviral vector alone or in combination. FLAG–NSP4 fusion protein expression was determined by Western blotting. As shown in Fig. 1, shRNAs against NSP4 significantly inhibited transient expression of FLAG–NSP4 relative to LacZ-control shRNA. RNAi-492 and the combination of RNAi-492 plus RNAi-351 shRNAs almost completely eliminated expression of NSP4. These results strongly suggest a direct role of shRNAs in specifically blocking transient expression of NSP4 protein in 293T cells.

shRNA recombinant lentivirus silenced viral NSP4 gene in MA104 cells infected by BRV CHLY strain

We next determined whether shRNAs could block natural expression of viral NSP4 gene during BRV replication. The shRNA recombinant lentivirus was packaged and titrated (transduction unit, TU ml⁻¹) by fluorescence-activated cell sorter (FACS) analysis based on enhanced (e) GFP expression. As shown in Fig. 2(a), 96% of BRV-susceptible MA104 cells when transduced with 1 × 10⁶ TU RNAi-492 and/or RNAi-351 shRNA vectors expressed eGFP after 24 h. As shown in Fig. 2(b), after inoculation of shRNA-transduced MA104 cells with 100 TCID₅₀ of the BRV CHLY strain, NSP4 mRNA expression was strongly inhibited by either RNAi-492, RNAi-351, or the combination thereof as compared with control shRNA (LacZ). VP7 mRNA expression by contrast was not altered by either shRNA. By ELISA, RNAi-351 inhibited over 70% of BRV replication as compared with LacZ control, while RNAi-492 showed more than 90% inhibition of BRV replication (Fig. 2c). Furthermore, the combination of RNAi-492 and RNAi-351 inhibited over 95% of BRV replication. Thus, lentivirally encoded NSP4-specific shRNA resulted in specific NSP4 mRNA silencing and reduced viral replication in vitro.

Recombinant lentiviruses encoding shRNAs against NSP4s prevented suckling mice from diarrhea induced by BRV

We then determined whether NSP4-directed shRNAs could inhibit BRV infection in suckling mice. Four groups of ten suckling mice each were inoculated with recombinant lentiviruses encoding NSP4-directed shRNAs and then 24 h later were infected with BRV. As shown in Fig. 3(a), all control suckling mice, which were pre-treated with shRNA against LacZ developed diarrhea, whereas only 60 (18/30), 20 (12/30) and 3.3% (1/30) of suckling mice pre-treated with RNAi-351, RNAi-492 or RNAi-351/492 developed diarrhea, respectively. All control suckling mice manifested severe diarrhea, while RNAi-492 or RNAi-351/492 pre-treated suckling mice developed only mild diarrhea when diarrhea occurred (Fig. 3b). While control mice developed diarrhea within 48 h of infection, diarrhea was significantly delayed after pre-treatment with RNAi-492 or RNAi-351/492 by an additional 24–48 h (Fig. 3c). Statistical analysis was based on data from three independent experiments. Thus, shRNA targeting NSP4 prevented, ameliorated or markedly delayed BRV diarrhea in suckling mice.

shRNA targeting NSP4 inhibited replication of BRV in vivo

In order to determine whether shRNA against NSP4 prevents suckling mice from diarrhea via direct viral inhibition of BRV, we quantified the amount of shedding BRV in mouse faeces after BRV challenge. As shown in Fig. 4(a), we first determined the faecal BRV shedding profile with five normal mouse pups infected with the BRV CHLY strain. Faecal BRV shedding was not detectable on the day of infection (day 1), but the virus titre reached over 2600 ng ml⁻¹ on day 2 post-infection, further increased and/or stabilized until day 5, then decreased significantly on days 6 and 7. BRV shedding became undetectable by day 8. This faecal BRV shedding profile was highly reproducible. We next determined the faecal BRV shedding...
profile from mouse pups pre-transduced with NSP4-directed shRNA. As shown in Fig. 4(b), cumulative BRV viral shedding was over 14,000 ng over the 8 days post-challenge in control suckling mice transduced with shRNA against LacZ. Among the pups pre-transduced with RNAi-351, RNAi-492 or RNAi-351/492, of whom 60, 20 and 3.3% developed slight diarrhoea, cumulative BRV shedding ranged from 150 to 430 ng. In non-diarrhoeal pups, there was no more than 10 ng of BRV antigen detected. Thus, lentivirally encoded NSP4-directed shRNA potently inhibit rotaviral replication in vivo.

DISCUSSION

The rotavirus NSP4, a 28 kDa glycoprotein encoded by rotavirus genome segment 10, is the first recognized rotavirus-encoded enterotoxin and has been implicated in a number of biological functions in rotavirus infection and pathogenesis. For example, NSP4 changes Ca\(^{2+}\) homeostasis (Zambrano et al., 2008), resulting in the maldigestion and malabsorption seen in rotavirus diarrhoea (Dhama et al., 2009). NSP4 influences the development of viroplasms (López et al., 2005b; Silvestri et al., 2005), linking genome packaging with particle assembly, and acts as a modulator of viral transcription (Silvestri et al., 2005). Although RNAi techniques were used to study these functions of NSP4 (Cuadras et al., 2006; López et al., 2005b; Silvestri et al., 2005; Zambrano et al., 2008), no further studies of rotavirus RNAi in experimental animals have been published.

In this study, shRNAs delivered by pseudotyped lentivirus under the U6 promoter were used to silence the NSP4 gene. The two shRNAs used in this study, which targeted sequences of CHLY strain NSP4, differed from shRNAs used in previous studies (López et al., 2005b; Silvestri et al.,...
These shRNA were screened for inhibition of transient expression of an NSP4–FLAG fusion protein in 293T cells then tested in BRV-susceptible MA104 cells. RNAi-351 partially blocked expression of NSP4 fusion protein, while RNAi-492 completely silenced NSP4 in both systems. While effective silencing of a single viral gene does not always translate into antiviral effect due to genetic compensation or redundancy (Alkhalil et al., 2009; Hummler et al., 1994; Sebat et al., 2004), shRNAs targeting BRV NSP4 nearly completely abrogated BRV replication. The more potent shRNA (RNAi-492) blocked BRV replication in vivo that markedly abrogated symptoms, which could reduce epidemic transmission.

Rotaviruses are known to exhibit extreme genetic diversity and rapidly escape disinfection procedures (Dhama et al., 2009). Due to their high degree of sequence specificity, shRNAs become ineffective in the presence of escape mutations within and outside the targeted regions (Westerhout et al., 2005). Thus, it may be important either to multiplex shRNAs in a single vector, or to combine different RNA-based inhibitors (Li et al., 2005) if RNAi is to be developed for therapeutic use.

Biotechnology and pharmaceutical companies have focused on the development of RNAi therapeutics for viral infections, cancer, hypercholesterolemia, cardiovascular disease, macular degeneration, and neurodegenerative diseases (Perrimon et al., 2010). Pivotal issues of RNAi therapeutics are delivery, specificity and stability of the RNAi reagents. Of these, delivery is currently considered the biggest hurdle (Perrimon et al., 2010). Lentiviral vectors are one of most useful tools currently available for delivering and stable expression of shRNAs in target cells (Poeschla et al., 1996). Unlike retroviruses, lentiviruses appear to have limited potential for oncogenicity (Wu et al., 2003). Conditionally replicating lentiviral vectors with limited

---

![Graph](image_url)

**Fig. 3.** shRNA targeting NSP4 prevented suckling mice from diarrhoea induced by BRV. Suckling mice, divided into four groups, ten mice in each group, were infected with shRNAs recombinant lentiviruses, and then inoculated with BRV 24 h later. Diarrhoea, dehydration and inappetance are checked and recorded at different times ranging from 24 to 96 h. (a) The results from three independent experiments are presented as a percentage of mean number of all morbid suckling mice to that in LacZ shRNA recombinant lentivirus infected control mice ± one SD, all control suckling mice presented severe diarrhoea, whereas, only a few of shRNA of NSP4 targeting mice presented slight diarrhoea. (b) Frequency of different grades of diarrhoea symptoms. (c) All control suckling mice were ill within 48 h post-incubation of BRV; however, only a little of shRNA of NSP4 targeting mice were morbid after 48 h. The results from three independent experiments were presented as a percentage of mean number of morbid suckling mice to that in LacZ shRNA recombinant lentivirus infected control mice at different time ± one SD. Duncan’s multiple range test by SAS 8.0 system was used for (a), means with the same letter are not significantly different (P>0.05), whereas means with a different letter (A, B or C) are significantly different (P<0.05).
tissue tropism can be specifically pseudotyped (Kobinger et al., 2001; Sandrin et al., 2003), which could prove an effective tool for delivering shRNAs into experimental animals. Based on our results, we believe that lentivirally encoded shRNA RNAi-492 and/or the combination of RNAi-492/351 could be further evaluated for the prevention of BRV in BRV-sensitive calves. The safety, duration of the BRV shedding profile of five mice experimentally infected with BRV CHLY. Stool samples were collected each day after infection during an 8-day-period. BRV antigen was quantified in murine faeces with ELISA. A sample was considered positive if the optical density at 450 nm (OD450) was >0.1 plus the mean of the OD values of the negative control wells. The amount of BRV antigen shed in each stool sample was inferred from the standard curve and expressed as nanograms of BRV antigen per millilitre of sample. (b) Faecal virus shedding in mouse pups transduced with shRNA recombinant lentivirus after BRV challenge. The amount of faecal virus shedding by each mouse was summed over the 8-day-period of faeces collection. Formulation of shRNA are indicated along the axis. For groups including shedding mice, bars show the median faecal virus shedding values and the SD.

Fig. 4. Faecal virus shedding after BRV infection. (a) Normal faecal BRV shedding profile of five mice experimentally infected with BRV CHLY. Stool samples were collected each day after infection during an 8-day-period. BRV antigen was quantified in murine faeces with ELISA. A sample was considered positive if the optical density at 450 nm (OD450) was >0.1 plus the mean of the OD values of the negative control wells. The amount of BRV antigen shed in each stool sample was inferred from the standard curve and expressed as nanograms of BRV antigen per millilitre of sample. (b) Faecal virus shedding in mouse pups transduced with shRNA recombinant lentivirus after BRV challenge. The amount of faecal virus shedding by each mouse was summed over the 8-day-period of faeces collection. Formulation of shRNA are indicated along the axis. For groups including shedding mice, bars show the median faecal virus shedding values and the SD.

In conclusion, we identified two NSP4-directed shRNAs that when transduced in recombinant lentiviruses prevent BRV replication in suckling mice, which results in a marked reduction of diarrhoeal symptoms. This approach merits further investigation as a potential therapeutic to prevent morbidity and mortality of rotavirus infections in livestock and human populations.

**METHODS**

**Cell lines, animals and viruses.** 293T cells and MA104 cells used in this study were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. BALB/c mice were purchased from Experimental Animal Center of Shandong in China. BRV CHLY strain was isolated and identified in our laboratory. The CHLY NSP4 gene sequence has been accepted by GenBank (accession no. FJ972713.1).

**Titration of virus.** MA104 cells, in 96-well plates, were infected with 10-fold dilutions of the CHLY strain virus in four replicates per dilution. After 48 h, viral cytopathic effect (CPE) was assessed and TCID₅₀ of virus was calculated using the Reed & Muench (1938) method.

Groups of five 4-day-old BALB/c mouse pups received 100 μl aliquots of solutions containing varying doses (TCID₅₀) of BRV CHLY via intragastric gavage. A control group of five mice received 100 μl saline. BRV-induced diarrhoea was clinically evaluated by direct palpation of the abdomen over the subsequent 48 h by well-trained personnel blinded to treatment group assignment. A 50% diarrhoeagenic dose (DD₅₀) was estimated by the Reed & Muench method.

Five suckling BALB/c mouse pups were then intragastrically gavaged with 30 DD₅₀ of BRV. Stool samples were harvested on days 1–8 post-infection, BRV was quantified in stool samples by ELISA.

**Construction of shRNA recombinant lentiviruses and NSP4 recombinant pcDNA3.** Lentivirus-based shRNA vectors were constructed as described previously (He & Sun, 2007). The sequences of these of NSP4 shRNA oligonucleotides are RNAi-492-P1: 5’-AGAGATGAGACGTCAGCTATATTCAAGAGAGTCGTTCTCATCTGTATTATTGT-3’ and RNAi-351-P2: 5’-CTAGACAAAGAAAGAGATGAGACGTCAGCTATATTCAAGAGAGTCGTTCTCATCTGTATTATTGT-3’; RNAi-351-P1: 5’-GAGACCGTGCTAGCTATATTCAAGAGAGTCGTTCTCATCTGTATTATTGT-3’ and RNAi-492-P2: 5’-CTAGACAAAGAAAGAGATGAGACGTCAGCTATATTCAAGAGAGTCGTTCTCATCTGTATTATTGT-3’.

The control shRNA sequences are LacZ-P1: 5’-CAGATTGCGAGCCTATTGTTAACAGACATCTGCCGCGCAACTGTTTTTGT-3’ and LacZ-P2: 5’-CTAGACAAAGAAAGAGATGAGACGTCAGCTATATTCAAGAGAGTCGTTCTCATCTGTATTATTGT-3’. The control shRNA sequences were annealed to each other and ligated into H1 lentivirus vector, followed by DNA sequence confirmation. The sequence of LacZ shRNA was not homologous to those of viral and cellular genes, so shRNA of LacZ recombinant lentivirus was the negative shRNA control. NSP4 recombinant pcDNA3 plasmid was constructed. The PCR primer pair used for pcDNA3-FLAG-NSP4 was NSP4-P1: 5’-GGGTACCGCGCCACCATGCTAGCTACACAGGACGAGTCGAAAGAAAAGCTTACCGACCT-3’ and NSP4-P2: 5’-GCTCTAGATATCCACGCGCTGCAGTCACT-3’. PCR fragments were digested with KpnI and XbaI, and subcloned into previously digested pcDNA3, followed by DNA sequence confirmation.

**Co-transduction of lentiviral shRNAs and FLAG-tagged NSP4 gene in 293T cells.** pcDNA3-FLAG-NSP4 (4 μg) was co-transfected into 293T cells, along with either 2 or 4 μg of recombinant shRNA lentivirus vectors, using Lipofectamine 2000, according to manufacturer’s instruction (Invitrogen). After 48 h, transient expression of the FLAG–NSP4 fusion protein was determined by Western blot analysis.
performed as described previously (He et al., 2007). In brief, 293T cells were lysed using lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 % Triton, 5 mM EGTA, 5 mM EDTA, 1 mM NaF, 1 mM Na3VO4, freshly added proteinase inhibitor tablet) and supernatants were collected by centrifugation. Proteins were separated on polyacrylamide gels in the presence of SDS and electrophoretically transferred onto nitrocellulose membrane. The membranes were blocked with 5 % Blotto in TBS-T (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween 20) for 1 h at room temperature and probed with various antibodies against FLAG and β-actin (Sigma). Specific proteins were visualized by enhanced chemiluminescence (Amersham Biosciences) detection. NSP4 expression was quantified relative to the LacZ shRNA control.

**Packaging of recombinant shRNA lentivirus.** The recombinant shRNA lentivirus vectors were co-transfected into 293T cells, along with gag- and env-expressing plasmids as described previously (He & Sun, 2007). Supernatants were collected 48 h post-transfection. Transduction of MA104 cells was then carried out in 12-well plates with 10-fold serial dilutions of different recombinant H1 lentivirus supernatants. After 4 h of incubation, the medium was refreshed. Two days later, cells were removed and fixed in 2 % paraformaldehyde. Transduction efficiency of shRNA recombinant H1 lentivirus encoding eGFP was determined by FACS analysis, and transduction titers (TU ml-1) were calculated with the Reed & Muench method.

**Transduction of lentivirus-based shRNA in BRV-susceptible MA104 cells.** MA104 cells in 12-well plates were transduced with 1 × 106 TU of shRNA of NSP4 recombinant lentivirus (single or combination) or LacZ shRNA in eight replicates at each dilution. After 24 h, the cells of four replicate wells were removed for FACS-based assessment of shRNA transduction. The medium of the remaining four wells was changed and each well inoculated with 100 TCID50 of the BRV CHLY strain. After 48 h, the CPE of each well was measured. Cells and supernatant were collected for assessment of shRNA gene silencing efficiency and BRV titre, respectively. The mRNA expression of viral genes in cells transfected with recombinant shRNA was detected by RT-PCR. Total RNA was isolated from the cells with TRIzol reagent (Promega). The cDNA was synthesized using NSP4-P4 (5'-GGTCACATTAAGACCGTTCCT-3') or VP7-P2 (5'-CGTATGCTGTGTCCATTGAACCTGTAATTGGCAA-3') as primers and moloney murine leukemia virus reverse transcriptase (Promega) in the reverse transcription reaction. Amplification was performed using the following primer pairs: NSP4-P3: 5'-GGCTTTTTAAAGGTCTTGTGCTCTC-3' and NSP4-P4; VP7-P1: 5'-GGCAGTTAAAGAGAGATCTTGGC-3' and VP7-P2. Amplified cDNA was analysed with 1 % agarose gel electrophoresis.

**NSP4 shRNA introduction into suckling mice.** Four groups of ten suckling BALB/c mouse pups were intragastrically administered 5 × 106 TU of shRNA recombinant lentivirus encoding shRNA for one of the following: RNAi-351, RNAi-492, RNAi-351 plus RNAi-492 and LacZ control. Twenty-four hours later, pups were challenged with 30 DD50 of BRV using an intragastric gavage. Faecal samples were collected on the day of challenge (D1) and on days 3, 4, 5 (D2–D5) and 8 (D8) post-challenge. Each stool collection was assessed at the time of collection for diarrhoeal disease as described previously (Wolber et al., 2005). In brief, faeces with brown colour (normal colour) and normal consistency, with green or yellow/green colour (abnormal colour) and normal consistency, with normal colour and watery (abnormal consistency), or with abnormal colour and abnormal consistency, were scored as 'normal', 'mild', 'moderate' or 'severe'. Faecal staining of skin was usually seen in conjunction with severe symptoms, as was abdominal bloating. BRV titres were quantified in each stool sample by ELISA. The statistical analysis was based on data from three independent experiments.

**Detection and quantification of BRV shedding by ELISA.** BRV shedding was quantified as described previously (Parez et al., 2006). In brief, all stool samples were stored at −20 °C until testing. Before being tested, stool samples were weighed, diluted in buffer to obtain a 6 % (w/v) suspension and clarified by centrifugation. BRV antigen in mouse faeces was determined with a commercial ELISA (IDEIA RV; Dako) according to the manufacturer’s recommendations. To quantify BRV antigen shedding, fourfold serial dilutions of CaCl2-purified CHLY (ranging from 4 to 62.5 ng ml−1) were assayed to establish a standard curve. The amount of BRV antigen shed in each stool sample was inferred from the standard curve and expressed as nanograms of BRV antigen per millilitre of sample. A value of zero was assigned when no virus shedding was detected. For each group of mice, these integrated values were used to calculate the median values of faecal virus shedding and for statistical comparison between groups.

**ACKNOWLEDGEMENTS**

This work was partially supported by grants from State Major Project of Transgenic (2009ZX008007–006B to J.Z.; 2011ZX008007–002 to G.L.), National Natural Science Fund of China (31072160 to H.H.), Natural Science Fund of Shandong Province (Y2008D20 to H.H.), Science and Technology Research Fund of Shandong Province (2009GG2002032 to H.H.), A Major Application of Technological Innovations of Agriculture of Shandong Province (H.H.), State Scientist in Industrial Technology System of Dairy Cattle (H.H.), and Taishan Scholar and Distinguished Experts from overseas (H.H.).

**REFERENCES**


**shRNA prevents rotavirus diarrhoea in suckling mice**


