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Inhibition of porcine circovirus type 2 replication in mice by RNA interference

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Abstract

Porcine circovirus type 2 (PCV2) is the primary causative agent of an emerging swine disease, postweaning multisystemic wasting syndrome (PMWS) for which no antiviral treatment is available. To exploit the possibility of using RNA interference (RNAi) as a therapeutic approach against the disease, plasmid-borne short hairpin RNAs (shRNAs) were generated to target the PCV2 genome. Transfection of these shRNAs into cultured PK15 cells caused a significant reduction in viral RNA production that was accompanied by inhibiting viral DNA replication and protein synthesis in infected cells. The effect was further tested in vivo in a mouse model that has been developed for PCV2 infection. Mice injected with shRNA before PCV2 infection showed substantially decreased microscopic lesions in inguinal lymph nodes compared to controls. In situ hybridization and immunohistochemical analyses showed that shRNA caused a significant inhibition in the level of viral DNA and protein synthesis detected in the lymph nodes of the treated mice relative to the controls. Taken together, these results indicate that shRNAs are capable of inhibiting PCV2 infection in vitro as well as in vivo and thus may constitute an effective therapeutic strategy for PCV2 infection.

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Keywords: Porcine circovirus type 2 (PCV2); RNA interference (RNAi); shRNA; DNA replication; Protein synthesis; Mouse

Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a new emerging disease affecting pigs primarily at 5 to 18 weeks of age. This disease was first identified within high-health herds in Western Canada in 1991 (Clark, 1997) and now is considered to be endemic in many swine-producing countries of the world (Allan and Ellis, 2000). Pigs affected with PMWS show weight loss, dyspnea, tachypnea, anemia, diarrhea, and jaundice. Characteristic macroscopic findings are enlargement of lymph nodes and noncollapsed lungs with tan mottling. Microscopic lesions can be detected in a number of tissues, the most characteristic being those of lymphoid tissues. These lesions consist of lymphocyte depletion of follicular and interfollicular areas together with macrophage infiltration of lymphoid tissues. Other common lesions described for PMWS include interstitial pneumonia, periporal to diffuse hepatitis, and interstitial nephritis (Clark, 1997; Rosell et al., 1999).

Isolation of virus from tissues of affected pigs led to the identification of a porcine circovirus type 2 (PCV2), considered to be of etiological importance in PMWS (Allan et al., 1999; Ellis et al., 1998; Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). Porcine circovirus type 1 (PCV1) was originally identified as a contaminant of porcine kidney cell cultures (PK15 ATCC CCL-33) (Tischer et al., 1982) and is nonpathogenic to pigs (Allan et al., 1995; Tischer et al., 1982).

Porcine circovirus (PCV) is classified in the genus Circovirus of the Circoviridae family (Pringle, 1999). The PCV virion is icosahedral, non-enveloped, and 17 nm in diameter. The genome of PCV is a single-stranded circular DNA of about 1.76 kb. The overall DNA sequence homology within the PCV1 or PCV2 isolates is greater than 90%, while the homology between PCV1 and PCV2 isolates is 68–76%. Two major open reading frames (ORFs) have been recognized for PCV, ORF1, called rep gene, which encodes a protein of 35.7 kDa involved in virus replication (Mankertz et al., 1998), and ORF2, called cap gene, which encodes the major immunogenic capsid protein of 27.8 kDa (Cheung, 2003a; Nawagitgul et al., 2000). In addition to the replicase ORF1 and
the capsid protein ORF2, a novel protein, ORF3, has been detected in PCV2 productive infection and is not essential for PCV2 replication in cultured cells but is involved in virus-induced apoptosis (Liu et al., 2005). ORF2 protein has been demonstrated to stimulate protective responses to injected pigs with baculovirus-expressed product or DNA prepared from the gene (Blanchard et al., 2003; Kamstrup et al., 2004). Furthermore, other studies have shown that chimeric PCV1–2 virus with the immunogenic ORF2 capsid gene of pathogenic PCV2 cloned into the nonpathogenic PCV1 genomic backbone as well as an attenuated PCV2 after 120 passages could induce specific antibody responses (Fenaux et al., 2004a, 2004b). However, there is a great need for the development of a new therapy for PCV2 infection.

RNA interference (RNAi) is a natural process by which double-stranded RNA directs sequence-specific post-transcriptional gene silencing (Fire, 1999; Hammond et al., 2001a,b; Sharp, 2001). Specific inhibition of endogenous or pathogen mRNA by RNAi can be triggered by the introduction of 21–23 nucleotide (nt) duplexes of RNA (siRNA) or by transcription of DNA precursor into short hairpin RNAs (shRNA) homologous to target sequences (Brummelkamp et al., 2002; Elbashir et al., 2001a; Paddison et al., 2002), opening up possibilities for controlling replicative processes of pathogens. This discovery promoted the use of RNAi for specifically inhibiting gene expression and replication of infectious viruses. Over the past years, RNAi approaches have shown to be effective against a variety of viruses in cell culture, among these are prominent viruses such as human immunodeficiency virus 1 (Capodici et al., 2002), influenza virus (Ge et al., 2003), poliovirus (Gitlin et al., 2002), severe acute respiratory syndrome virus (Wang et al., 2004), herpes viruses (Jia and Sun, 2003), human papillomavirus (Hamel et al., 1998), dengue virus (Caplen et al., 2002), and West Nile virus (McCown et al., 2003). Synthetic siRNAs as well as shRNAs transcribed in vivo from DNA templates have been shown to be potent inhibitors of gene expression in adult mice (McCaffrey et al., 2003). Studies in mice injected with shRNAs or siRNAs directed against hepatitis B virus have raised expectations about the use of RNAi as an antiviral strategy (Giladi et al., 2003; McCaffrey et al., 2003). Furthermore, siRNAs or DNA vectors produced shRNAs have also been shown to reduce the influenza viral load in murine lungs and increase survival (Ge et al., 2004; Tompkins et al., 2004). Recently, there is a report that administration of siRNAs to mice before West Nile virus infection has reduced the viral load and offered partial protection from lethal infection (Bai et al., 2005).

Inhibition of PCV2 viral transcription and DNA replication by shRNA in cell culture

To test whether RNAi could inhibit PCV2 in cell culture, we generated six shRNA-expressing plasmids targeting the ORF1 and ORF2 regions of the PCV2 genome (Fig. 1A) for their ability to suppress expression of PCV2 viral proteins. The PK15 cells were transfected with the shRNA-expressing plasmids and then infected with PCV2, and the levels of PCV2 viral protein production were determined by IFA at 48 h after infection. The time points for measuring the effects of shRNA in cells were chosen following preliminary optimization experiments (data not shown). To confirm the specificity of the inhibition, pVP2, an shRNA-expressing plasmid directed against porcine parvovirus viral sequence, was used as a negative control in the experiments. The results showed that all shRNA-expressing plasmids targeting the PCV2 genome caused a decrease in PCV2 antigen levels to varying degrees (Figs. 1B and C). Among these specific shRNAs, pSIR3 and pSIR6 exhibited the highest level of inhibition (Figs. 1B and C). Therefore, we chose these two shRNA-expressing plasmids for the following experiments. In contrast to the PCV2-specific shRNAs, the control pVP2 had no effect on PCV2 antigen level in the treated cells, indicating that inhibition by the PCV2-targeted shRNAs was specific and due to RNAi.

A dose–response analysis (Fig. 2A) was conducted by transfecting PK15 cells with increasing amounts of pSIR3, pSIR6, or negative control pVP2-expressing plasmid followed by measuring the numbers of PCV2 antigen cells by IFA 72 h post-transfection. The results showed that, at concentrations of 1500 to 2500 ng shRNA-expressing plasmid per 5 × 10^4 cells, the highest level of inhibition (fivefold) of viral antigen was obtained with pSIR3 and pSIR6 plasmids, whereas pVP2 had no effect (Fig. 2A). At higher doses of shRNA, we observed a slight nonspecific inhibitory effect of pVP2 on viral protein production (Fig. 2A). In addition, kinetic study results (Fig. 2B) revealed a continuous trend in the specific inhibition of PCV2 viral production by pSIR3 and pSIR6 from 48 to 120 h after transfection. The inhibition of PCV2 viral production was maximal at 72 h but thereafter started to decline by 96 h after transfection.
ORF2 mRNAs was significantly reduced in PCV2-infected cells transfected with pSIR3- or pSIR6-expressing plasmids (Fig. 3A). shRNAs are known to be incorporated into an RNA-induced silencing complex and to direct RNA-induced silencing complex-mediated sequence-specific mRNA degradation (Hammond et al., 2000). ORF1 and ORF2 of PCV2 have been demonstrated to contain different mRNA, but their mRNA levels were reduced in the PCV2-infected cells regardless of ORF1 or ORF2 shRNA transfection. One possible explanation for the effects of this coordination is that the target mRNA bound with a shRNA might affect the other non-target mRNA of the virus through a downstream effect on all the viral transcripts because viral replication is inhibited, but the detailed mechanism of this process remains unclear.

To further study the inhibitory effect of pSIR3- or pSIR6-expressing plasmids on PCV2 DNA replication, the supernatants of infected PK15 cells after being transfected with the shRNA plasmids at 72 h post-transfection were directly assayed by PCR amplification with primers 1446F and 420R. The results showed that reduced level of newly synthesized viral replicative form (RF) DNA was observed in the treated cells with pSIR3- or pSIR6-expressing plasmid manifested a marked depletion of viral proteins compared with cells transfected with pVP2-expressing plasmid (Fig. 3B). In contrast, the viral RF DNA was detected in the treated cells with the control pVP2-expressing plasmid as seen in that of PCV2-alone-infected cells (Fig. 3B).

**Effects of mutations on antiviral activity of shRNA**

To further examine the ability of ORF1 shRNA to suppress viral gene expression in PCV2-infected PK15 cells, single nucleotide mutations were introduced into pSIR3 sequence as shown in Fig. 4A. Cells were infected with PCV2 after being transfected with these shRNA-expressing plasmids, and total proteins were extracted and examined by an immunoblot analysis with mouse polyclonal antibody against ORF1 or ORF2 protein at 48 h post-infection. The cells transfected with pSIR3- or pSIR6-expressing plasmid manifested a marked suppression of ORF2 protein expression and noticeable decreases in the level of the ORF1 protein were detected after the pSIR6 treatment. In addition, drastic suppression or not was detected in the mutant shRNA-expressing plasmids dependent on the location of mutation in the RNAi sequence. As shown in Fig. 4B, a mutant with one nucleotide mutation in the 5′-end of the sense strand (pSIR3-m1) still had an effective reduction in the synthesis of ORF1 and ORF2 proteins, whereas with a nucleotide substitution at the center (pSIR3-m2) or 3′-end (pSIR3-m3) of the sense sequence, the resultant shRNAs failed to significantly suppress expression of PCV2 ORF1 and ORF2 in the transfected cells, further verifying the specificity of RNAi as demonstrated (Amarzguioui et al., 2003; Harborth et al., 2003). Neither viral infection nor treatment with shRNA alone influenced the level of GAPDH production (Fig. 4B).

We also examined the effects of pSIR3 and pSIR6 as well as mutated shRNAs by FACS with porcine antibody raised against PCV2 72 h after shRNA-expressing plasmids transfection. As shown in Fig. 4C, PCV2 viral production was...
significantly reduced in PK15 cells that were pretreated with pSIR3- or pSIR6-expressing plasmids. In addition, the pSIR3-m1 significantly suppressed PCV2 protein synthesis, whereas the two other shRNA mutants (pSIR3-m2 and pSIR3-m3) had no effect (Fig. 4C), indicating that the effects of PCV2 shRNAs are indeed highly specific. As expected, the pVP2 shRNA also did not inhibit PCV2 viral expression (Fig. 4C).

**Attenuation of PCV2-induced pathology in mice by shRNA**

To determine whether shRNAs exert an antiviral response in vivo in BALB/c mice, we administered the pSIR3 or pSIR6 plasmids 1 day before viral inoculation. No clinical signs or gross lesions were observed in shRNA-expressing plasmids transfected or mock-transfected mice that received PCV2 at any time during the 12-day observation period. Microscopic alterations in PCV2 alone mice were first noticed at day 5 post-infection, slight germinal centers developed in the parenchyma of the inguinal lymph nodes (data not shown). At 11 days post-infection, the mice developed prominent germinal centers that consisted of large lymphoblastic cells and histiocytic cells, and the paracortex had loss of lymphocytes and was infiltrated by large numbers of histiocytes (Fig. 5A). In contrast, the lymph nodes showed normal microscopic morphology in treated mice with pSIR3 or pSIR6 at 5 days post-infection as seen in that of mock-infected mice (data not shown). At 11 days post-infection, mice treated with pSIR3 or pSIR6 showed significantly lower ($P < 0.05$) lesion scores than that of untreated mice and showed a considerable reduction in inguinal lymph...
Fig. 5. shRNAs mediate attenuation of PCV2 infection in mice. Histology (staining with hematoxylin and eosin) of inguinal lymph node sections of mice with pSIR3, pSIR6, or control pVP2, at day 11 after infection. (A) Prominent germinal centers occupy most of the parenchyma in PCV2-infected control. (B and C) Showed normal morphology of inguinal lymph nodes after transfection with pSIR3 or pSIR6, respectively. No germinal centers were noticeable. (D) No germinal centers are noticeable in negative control. Scale bar, 200 µm.

Fig. 4. Effects of mutated pSIR3 on PCV2 protein synthesis in cell culture. (A) Sequences of the position of each mutation in the pSIR3 region. The targeting sequence for pSIR3 is listed at the top. The sequences of wild-type and mutated shRNAs are shown, with mutated nucleotides underlined. (B) Whole-cell extracts prepared from transfected PK15 cells 72 h after treatment with the indicated shRNA-expressing plasmids were assayed by Western blotting for the presence of ORF1 and ORF2 expression in PCV2-infected cells. GAPDH was used as the loading control. (C) PK15 cells transfected with the indicated shRNAs were infected with PCV2 24 h later. PCV2 protein expression was measured by flow cytometry with porcine antibody against PCV2 72 h after transfection. Graphic key: black (filled), mock-infected control; gray (line), shRNA transfection; black (line), PCV2 infection without shRNA infection. Representative FACS plots from one of three experiments are shown.
node lesions, as evidenced by decreases in the lymphocytes and in the number of infiltrating histiocytes compared to controls (Figs. 5B and C). In addition, the nonspecific pVP2 shRNA did not attenuate the lymph node lesions induced by PCV2 infection at 5 and 11 days post-infection (data not shown). No microscopic lesions were observed in mock-infected mice (Fig. 5D). The results suggest that PCV2 shRNA can significantly attenuate PCV2-induced lymph node pathology in mice.

Inhibition of PCV2 DNA replication in mice by shRNA

To further study the effect of pSIR3 or pSIR6 on viral DNA replication in vivo, we performed an in situ hybridization analysis with inguinal lymph nodes of mice treated with shRNAs at 6 and 12 days after injection. Replicated PCV2 genomic DNA was present in 2.0 ± 0.5% of infected cells in inguinal lymph node sections of mice that received PCV2 alone at 6 days after injection, with slight inhibitory effect observed in the pSIR3 or pSIR6-treated mice (data not shown). At 12 day post-injection, PCV2 nucleic acid was detected in 8.2 ± 1.5% of infected cells such as macrophages, histiocytes, and occasional lymphocytes in inguinal lymph node tissue sections from mice that were inoculated with PCV2 alone. Positive cells typically exhibited a dark purple to black reaction product in the cytoplasm as well as in the nucleus (Figs. 6A and B). Lymph node sections from mice receiving pSIR3 had significantly reduced numbers of positive cells (P < 0.05), with 1.2 ± 0.5% of stained cells (Fig. 6C). The number of stained cells in sections from pSIR6-treated mice was also significantly reduced (P < 0.05, 1.6 ± 1.2% of infected cells) (Fig. 6D). In contrast, the number of positive cells in sections of mice treated with pVP2 RNAi did not show any reduction (P > 0.05, 8.5 ± 2.3%) (Fig. 6E). No staining was seen in sections from mock-infected mice (Fig. 6F). These results demonstrate that PCV2 shRNA can inhibit the PCV2 DNA replication in vivo.

Fig. 6. The effects of shRNA on PCV2 viral replication in mouse inguinal lymph node sections. In situ hybridization staining of inguinal lymph node sections of mice with pSIR3, pSIR6, or control pVP2, at day 11 after infection. Hybridization signals for PCV2 nucleic acids are seen as purple color (arrow). Treatment with pSIR3 or pSIR6 reduces the number of cells that stain for PCV2 nucleic acids. Representative sections are shown. (A) Mock-transfected group, many hybridization signals are seen in most fields. (B) Partial enlargement of panel A. (C) pSIR3, no hybridization signals were observed in most fields. (D) pSIR6, few signals were indicated. (E) Control pVP2, hybridization signals can be seen as mock-transfected group. (F) Lymph node section from a normal mouse, no positive signals were observed. Scale bar, 80 μm.
Inhibition of PCV2 protein synthesis in mice by shRNA

We also assessed the effect of shRNA on PCV2 protein synthesis by immunohistochemical staining for PCV2 in inguinal lymph node sections from mice treated in the experiment described above. At 6 days after injection of the shRNAs followed by inoculation of PCV2, the numbers of cells stained positively for PCV2 in PCV2-alone-infected group were 2.5 ± 0.6%, with slight inhibitory effect observed in the pSIR3- or pSIR6-treated mice (data not shown). As shown in Figs. 7A and B, the numbers of PCV2 antigen-positive cells increased significantly in the inguinal lymph nodes of the virus-inoculated cells at 12 days after injection (P < 0.05, 8.9 ± 2.1%), but mice treated with pSIR3 or pSIR6 exhibited small increase in PCV2 antigen-positive cells (1.5 ± 0.4% for pSIR3 or 1.8 ± 0.7% for pSIR6 treatment) (Figs. 7C and D) and, thus, overall had a significant inhibition (P < 0.05) compared to the virus-inoculated alone mice. PCV2 staining was detected mainly in the cytoplasm but for some in the nucleus as well. As a control, mice treated with pVP2 showed similar PCV2-positive cells (8.7 ± 2.2%) to the virus control at 12 days after injection (Fig. 7E). No staining was seen in sections from mice that were not inoculated with PCV2 (Fig. 7F). The results emphasized the significant antiviral activity of the pSIR3 and pSIR6 and their ability to suppress PCV2 protein synthesis in vivo.

Discussion

RNAi operates at the post-transcription level to suppress gene expression and has developed into a powerful method to down-regulate the expression of endogenous as well as exogenous sequences of the cell (McManus and Sharp, 2002; Morris et al., 2004). The ability to silence mammalian gene expression using siRNA opens new and exciting routes to the understanding of mammalian cell biology and its pathology. There are many precedents for RNAi being effective against a wide range of viral pathogens in vitro. In addition, RNAi has been demonstrated to be functional in mice and can inhibit viral replication and

![Fig. 7. The effects of shRNA on PCV2 protein synthesis in inguinal lymph node sections. Immunohistochemical staining of inguinal lymph node sections of mice with pSIR3, pSIR6, or control pVP2, at day 11 after infection. PCV2 antigen-positive cells are seen as brown color (arrow). Treatment with pSIR3 or pSIR6 reduces the number of cells that stain for PCV2 antigen. Representative sections are shown. (A) Mock-transfected group, many positive cells for PCV2 antigen are seen. (B) Partial enlargement of panel A. (C) pSIR3, no stained cells were observed in most fields. (D) pSIR6, faintly stained cells were indicated. (E) Control pVP2, stained cells can be seen as mock-transfected group. (F) Lymph node section from a normal mouse, no staining was observed. Scale bar, 80 μm.](image-url)
expression when injected with specific siRNAs against hepatitis B and influenza viruses (Ge et al., 2004; Giladi et al., 2003; McCaffrey et al., 2003; Uprichard et al., 2005). These studies have shown that RNAi can potentially be used as a therapeutic or prophylactic mechanism against viruses. Here, we have provided evidence that shRNAs targeting the ORF1 and ORF2 regions of PCV2 are capable of inhibiting all the steps of PCV2 infection in cultured cells, which support viral production, and in vivo in a mouse model.

In PCV2, three ORF regions have been recognized. In this study, to test which region of the PCV2 genome is the most effective site for siRNA targeting, six different anti-PCV2 shRNAs were selected at the ORF1 and ORF2 genes that have important functions in viral replication and production, respectively. After testing the effect of these vector-based siRNAs on the inhibition of PCV2 replication in cultured PK15 cells, we found that all six shRNAs could cause a decrease in PCV2 replication to varying degrees, with two of them being more effective. These results may be due to different positional accessibility caused by steric hindrance by a secondary or tertiary structure and/or protein binding (Kamstrup et al., 2004). We also noticed that the nonspecific pVP2-expressing plasmid has some inhibitory effect on PCV2 replication after being transfected at higher concentration. This could be due to the nongene-specific suppression of siRNA. Higher concentrations of siRNA have been demonstrated to exhibit more measurable nonspecific gene suppression activity (Persengiev et al., 2004). Therefore, identifying highly active siRNAs and decreasing siRNA concentrations can alleviate some nonspecific effects while maintaining efficient silencing.

It has been thought that siRNAs target mRNAs containing the same sequences and induce their cleavage. In this study, we used real-time RT-PCR to detect ORF1 or ORF2 mRNA level after being transfected with pSIR3- or pSIR6-expressing plasmids and found that not only can shRNA significantly reduce its corresponding mRNA level but inhibit other viral gene transcription as well (Fig. 3A). This could be related to the life cycle of the virus. It can be assumed that the PCV2-specific shRNA first recognizes the corresponding sequence of the viral mRNA and initiates the degradation of this mRNA. As a consequence, the degradation of the mRNA results in blockade of the corresponding protein synthesis, i.e., silencing of viral gene expression. Thus, the amount of newly synthesized mature virions might have been reduced in the subsequent virus infection. Therefore, significant inhibition in other mRNA level could be due to reduction of newly synthesized mature virions induced by shRNA when it does not directly target this mRNA. This hypothesis has been further confirmed by the data that viral DNA was reduced in supernatants of transfected cells (Fig. 3B) and PCV2 protein synthesis was inhibited in transfected cells (Fig. 4C).

The roles of siRNA target recognition have been examined employing diverse siRNAs with nucleotide substitutions (Amarzguioui et al., 2003; Chiu and Rana, 2003; Elbashir et al., 2001b; Harborth et al., 2003; Pusch et al., 2003). Introducing a single nucleotide change to the siRNA sequence could abrogate siRNA-mediated silencing. To clarify this question, we performed evaluations using a series of pSIR3 mutants containing point mutations at different locations. The data (Figs. 4B and C) suggest that point mutations in the middle and 3′-end sequence of pSIR3 sense strand could eliminate the anti-PCV2 activity probably due to a decrease in the affinity of the siRNA for the target mRNA (Schwarz et al., 2003), whereas the point mutation of the 5′-end sequence did not interfere with the antiviral activity. Sequence specificity of siRNA is very stringent as single base pair mismatches between the siRNA and its target sequence can dramatically reduce the silencing capability (Elbashir et al., 2001a, 2001b; Yuan et al., 2005). However, the molecular mechanism of this phenomenon needs to be further studied. In addition, it has been demonstrated that escaping mutants were generated following siRNA or shRNA treatment (Boden et al., 2003; Gitlin et al., 2002, 2005), this can be prevented by treatment with multiplex specific siRNAs or targeting long portions of the viral genome. In this study, we also performed three passages of PCV2 on newly pSIR3-expressing plasmid-transfected cells to investigate whether escaping PCV2 mutants were generated, but did not detect any mutants (data not shown), suggesting that the pSIR3 targeting sequence is a critical site for the fitness of the virus. Therefore, this kind of targeting sequence as pSIR3 can be used as targets for designing siRNA in PCV2 therapy.

A few studies have addressed the potential of siRNA-based therapeutics in vivo using model animal systems. Interference with transgene and endogenous gene expression in mice following administration of siRNA duplexes has been reported (McCaffrey et al., 2002; Song et al., 2003). To prolong the expression of siRNA, a plasmid or an integrating viral vector expressing endogenous siRNA can be used to achieve this purpose (McCaffrey et al., 2003; Uprichard et al., 2005). Such vector-based strategies with generation of siRNA may be used to test inhibition or suppression of viral gene expression and replication in animal models and permit greater understanding of the role and activity of viral genes in vivo. By contrast to that PCV2 was capable of replicating in BALB/c mice and caused microscopic lesions (Kuipel et al., 2001), Quintana et al., (2002) reported that no microscopic lesions compatible with PCV2 infections in pigs were detected in inoculated mice, but it might be due to the inoculum of the dosage and administration route (Quintana et al., 2002). In the present study, we observed enlargement of germinal centers by infiltration with large numbers of lymphoblastic and histiocytic cells, apoptosis of histiocytes, and lymphocytic depletion in the paracortex of lymph nodes in PCV2-inoculated BALB/c mice (Fig. 5A and data not shown) which resembled early lesions in lymph nodes in PCV2-inoculated gnotobiotic pigs. Furthermore, PCV2 replication was confirmed by the amounts of viral DNA and protein synthesis in the lymph nodes (Figs. 6A and 7A) as well as other tissues (data not shown) in the PCV2-inoculated mice. Therefore, we used BALB/c mice as an animal model for inhibiting PCV2 infection by RNAi in vivo. The study described herein shows that RNAi induced by plasmid-borne small interfering RNAs can be used to inhibit...
replication of a porcine pathogen in a mouse model system for 12 days after injection. The antiviral treatment decreased viral expression in the inguinal lymph node and attenuated lymph node lesions in PCV2-infected mice and thus may be applicable as a therapeutic strategy for naturally occurring PCV2 infection.

In conclusion, we show that shRNA can be designed to induce an antiviral effect on PCV2 DNA replication and protein synthesis both in cultured cells and in animal model. These data demonstrate that RNAi is a potential prophylaxis and therapy for PCV2 infection.

Materials and methods

Virus and cells

The permanent PK15 cell line, which was free of PCV, was maintained in minimal essential medium (MEM, Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 5% L-glutamine, 100 U/ml of penicillin G, and 100 μl/ml streptomycin at 37 °C in a humidified 5% CO₂ incubator. The PCV2 virus strain BJW (Liu et al., 2005) was used in the study.

siRNA target sequences and vector construction

The mammalian expression vector RNAi-Ready pSIREN (pSIR) (Clontech, BD) was used for the expression of siRNA. Six specific anti-PCV2 shRNA expression plasmids were constructed to target ORF1 and ORF2 genes of the PCV2 strain BJW (GenBank accession no. AY847748) genome: pSIR1 (targeting sequence, nt F527 to F545), 5'-AACGAAATGGGCTGCTAAT-3'; pSIR2 (nt F590 to F608), 5'-AAATGGCATCTTCAACC-3'; pSIR3 (nt F624 to F642), 5'-GCCATCTTGCAAACCAC-3'; pSIR4 (nt R1612 to R1594), 5'-AAATGGCATCTTCAACCAC-3'; pSIR5 (nt R1429 to R1411), 5'-GGTTGAATTCTGGCCCTGC-3'; and pSIR6 (nt R1307 to R1289), 5'-ACTACTCTCCCCGCCCATTC-3' (Fig. 1A). The suffix (F or R) of the oligonucleotide indicates the orientation of the targeting sequence. F indicates forward direction from nt 0 to 1767, while R indicates reverse direction from nt 1767 to 0. A nonspecific shRNA expression vector, pVP2 (5'-CCTACCTGAGCTGGCCTAA-3'), targeting capsid protein VP2 gene of porcine parvovirus, was constructed as a negative control. These sequences were all analyzed by a BLAST search of the GenBank database to avoid similar sequences found in any other genes but share 100% homology within the published PCV2 strains as well. Oligonucleotides were synthesized and inserted into the pSIR vector in the BamHI and EcoRI sites according to the manufacturer’s instructions.

Transfection and infection

The transfection of shRNAs was performed under optimal conditions. Briefly, PK15 cells were seeded and grown at 37 °C overnight. When cells reached 60 to 70% confluency, they were transfected with shRNA expression plasmids with Lipofectamine reagent (Invitrogen). Following 24 h of transfection, cells were washed and infected with PCV2 strain BJW at the indicated multiplicity of infection (MOI) for 1 h. The cells were then overlaid with complete medium and were incubated at 37 °C in 5% CO₂. Cells were additionally treated with 300 mM D-glucosamine at 24 h after infection as described previously (Tischer et al., 1987). At 48 h post-infection, they were analyzed by IFA, and supernatants and cell lysates were collected for PCR analysis and Western blotting, respectively.

Indirect fluorescence assay (IFA)

At 24 h of post-transfection with shRNA expression plasmids, PK15 cells grown on 24-well plate were incubated with the PCV2 for 60 min at 37 °C at an MOI of 1 TCID₅₀ and added MEM for incubation. Following the incubation at 37 °C, cells at 48 h post-infection were washed with PBS and fixed for 30 min at room temperature (RT) with 4% paraformaldehyde (PFA) in PBS. After fixation, the cells were blocked by PBS with 3% BSA at RT for 1 h. Primary antibody, porcine anti-PCV2 antibody, was diluted in PBS with 3% BSA and incubated with the cells for 1 h at 37 °C. After washing with PBS, the cells were incubated with rabbit anti-porcine fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma) diluted in PBS with 3% BSA for 1 h at 37 °C. The cells were washed three times with PBS and examined using a fluorescence microscope. Cells positive for PCV2 viral antigens were counted in six fields of view.

Flow cytometry

PK15 cells were harvested and fixed for 30 min in a 2% PFA solution. Nonspecific binding was reduced by exposure to 10% equine serum for 30 min and three washes with fluorescence-activated cell sorter (FACS) wash buffer (phosphate-buffered saline with 1% fetal calf serum and 0.1% sodium azide) prior to incubation with a 1:200 dilution of porcine anti-PCV2 antibody for 30 min at RT. The cells were then washed three times with FACS buffer and incubated at RT with a 1:100 dilution of FITC-conjugated anti-swine IgG (Sigma) for 30 min. After incubation, the cells were washed three times with FACS buffer and resuspended in 400 μl of FACS buffer before acquisition and analysis on a FACScalibur instrument (Becton Dickinson) using WinMDI 2.8 software (Purdue University Cytometry Laboratories).

Western blotting

Western blot analysis was performed after the cells were harvested by centrifugation at 500 × g for 8 min. The pellets were lysed for 10 min on ice in 150 μl lysis buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 5 mM DTT, and 20 μg/ml of the proteinase inhibitor cocktail (Novagen)]. Lysates were then collected by centrifugation at 14,000 × g for 10 min.
at 4 °C and the concentration was measured. Twenty micrograms of total cellular protein from each sample was resolved on 10 or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose (NC) membranes (Stratagene) with a semidytransfer cell (Bio-Rad Trans-Blot SD). The membranes were blocked for 2 h at RT in blocking buffer TBST (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Tween-20) containing 5% skim milk powder to prevent nonspecific binding and then incubated with mouse anti-ORF1, anti-ORF2 antibody (Liu et al., 2005), or monoclonal antibody against human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon International, Inc., CA, USA) at RT for 2 h. Then, the membranes were washed three times with TBST and incubated for 1 h at RT with horseradish-peroxidase-conjugated anti-mouse secondary antibody (DAKO) diluted in blocking buffer (1:2000). After washing, the membrane was reacted with 3,3′-diaminobenzidine tetrahydrochloride (Pierce, Rockford, Ill., USA) substrate and then stopped with distilled water.

PCR

Newly synthesized viral DNA in cell culture was assayed by PCR as elsewhere described (Cheung, 2003b). Briefly, the supernatants of infected PK15 cells after being transfected with shRNA-expressing plasmids 72 h post-transfection were used as templates for PCR amplification. The sense primer 1446F: 5′-GTCATAGCCAAACCAGTCAAAAC-3′, and antisense primer 420R: 5′-TCACAGCAGTAGACGGTCATCCCG-3′ were used for amplifying PCV2 DNA. The PCR consisted of an initial enzyme activation step at 94°C for 15 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was electrophoresed in 1% agarose gel and photographed.

Real-time RT-PCR

Total cell RNAs were prepared from virus-infected PK15 cells 72 h after being transfected with shRNA-expressing plasmids by using Trizol RNA Extract reagent (Invitrogen) for reverse transcription (RT)-PCR. The RNA samples were incubated with DNase I for 60 min at 37°C to remove any contaminating viral RNA. The following primers were used: F223, 5′-TGTAGATTCAAGGGTGATTAGA-3′ and R412, 5′-GGTCACTGGTGAGGCTGAGG-3′, for amplifying PCV2 RNA. For real-time PCR, the cDNA was amplified with the specific oligonucleotide primers: 5′-ATCAAGCGCACCCAGTCAAAAC-3′ and 5′-GGTCACTAGTGAGGCTGAGG-3′, for PCV2-ORF2; and sense, 5′-CATCAGCAGTAGCAGTAGTCC-3′, and antisense, 5′-GCTGTAGCCAAATTCCGGT-3′, for GAPDH. cDNAs were reverse transcribed from total RNAs by the use of antisense primers and the first-strand synthesis system (AMV Reverse Transcriptase kit, Roche). Quantitative real-time PCRs were performed on a LightCycler (Roche). The amplification protocol followed the instructions of a TaqMan Gold PCR kit. Each sample was run in triplicates. The relative amount of target gene mRNA was normalized to that of GAPDH mRNA in the same sample.

Viral challenge assay in BALB/c mice

Fifty BALB/c mice of 8 weeks of age were randomly assigned into five rooms of 10 animals each. Mice in group 1 were uninoculated and served as negative controls. pSIR3, pSIR6, and pVP2 expression plasmids (10 µg per mouse) were injected via both tail vein and intramuscular routes to mice in groups 2 to 4, respectively. Mice in group 5 was served as positive controls. After 24 h, all mice in groups 2 to 5 were each inoculated intranasally and interperitoneally with about 105 TCID50 of the PCV2 strain BJW. The animals were monitored daily for clinical signs of disease. At 5 and 11 days post-infection, five mice were randomly selected from each group and necropsied. Inguinal lymph nodes were collected during necropsy and processed for histological examination and in situ hybridization (ISH) as well as immunohistochemical (IHC) staining.

For histological examination, inguinal lymph nodes were collected and fixed by immersion in 4% PFA. Fixed samples were dehydrated, embedded in paraffin wax, and sectioned at 5 µm then stained with hematoxylin and eosin (HE). Lymph node scores were assessed for depletion of lymphoid cells, infiltration by histocytic cells, and/or the presence of multinucleated cells.

For ISH, a PCV2-specific oligoprobe was used for demonstration of PCV2 nucleic acid. Briefly, a 253 bp DNA fragment from ORF2 of PCV2 was amplified using a pair of primers: 5′-ATCAAGCGCACCCAGTCAAAAC-3′ and 5′-GGTCACTAGTGAGGCTGAGG-3′. The specific oligoprobe was labeled with digoxigenin (DIG) (Roche). After deparaffinization and rehydration, the lymph node sections were proteolytically digested with 10 µg/ml of proteinase K in PBS buffer for 15 min at 37°C and then post-fixed with 0.4% PFA in PBS for 30 min at 4°C. After acetylation, the slides were prehybridized in hybridization buffer (50% formamide, 5× SSC, 50 µg/ml denatured salmon sperm DNA) for 120 min at 37°C followed by overnight hybridization at 42°C with the DIG-labeled DNA probe at a concentration of 300 ng/ml. After post-hybridization washes, the slides were incubated with antidi-DIG (Roche) (150 µl of 1:5000 diluted in buffer [10% fetal calf serum; 100 mM Tris–HCl, 150 mM NaCl, pH 7.5]) overnight at 4°C. The slides were washed, incubated with Nitro Blue Tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) mixture for color development, and then mounted for examination under the microscope.

For IHC, a polyclonal anti-PCV2 antibody was used to detect the presence of PCV2 viral antigen. Briefly, tissue sections were deparaffinized and rehydrated through graded alcohol. Endogenous peroxidase activity was inhibited by flooding the slides with 3% H2O2 in 1% Triton X-PBS for 10 min. After being blocked in 5% normal mouse serum for 1 h at RT, the slides were incubated with purified PCV2 antibody (1:100) overnight at RT followed by incubation with a secondary biotinylated anti-swine IgG (1:200) for 1 h at RT. An avidin–biotin peroxidase (ABC) method (Pierce, IL, USA) diluted in 1:100 was applied for 1 h at RT. The sections were finally incubated in diaminobenzidine (DAB)–hydrogen per-
oxide solution for 5 min and counterstained with 1% Methyl Green, dehydrated and mounted with Permount (Fisher Scientific Inc.), and examined microscopically.

Statistical analysis

Results are presented as averages ± standard deviations or standard errors of the means, as indicated. Statistical comparisons are made by using Student’s t test, and differences between groups were considered significant if the P value was <0.05.

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References

Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. 12, 3–14.

Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A., Adair, B., Ellis, W.A., McNulty, M.S., 1995. Pathogenesis of porcine circovirus: experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. 44, 49–64.

Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. 121, 1–11.

Amarguoiu, M., Holen, T., Babaie, E., Prydz, H., 2003. Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res. 31, 589–595.

Bai, F.W., Wang, T., Pal, U., Bao, F.K., Gould, L.H., Fikrig, E., 2005. Use of RNA interference to prevent viral spread of the marburg virus in adult mice. J. Infect. Dis. 191, 1148–1154.

Blanchard, P., Mahe, D., Cariolet, R., Keranflec’h, A., Baudouard, M.A., Cordioli, P., Albina, E., Jestin, A., 2003. Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 proteins. Vaccine 21, 4565–4575.

Boden, D., Fuchs, O., Lee, F., Tucker, L., Ramratnam, B., 2003. Human immunodeficiency virus type 1 escape from RNA interference. J. Virol. 77, 11531–11535.

Brummelkamp, T.R., Bernards, R., Agami, R., 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.

Caplen, N.J., Zhang, Z., Falgout, B., Morgan, R.A., 2002. Inhibition of viral gene expression and replication in mosquito cells by dsRNA-triggered RNA interference. Mol. Ther. 6, 243–251.

Capodici, J., Kariko, K., Weissman, D., 2002. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J. Immunol. 169, 5196–5201.

Cheung, A.K., 2003a. Transcriptional analysis of porcine circovirus type 2. Virology 305, 168–180.

Cheung, A.K., 2003b. The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology 313, 452–459.

Chiu, Y.L., Rana, T.M., 2003. SiRNA function in RNAi: a chemical modification analysis. RNA 9, 1034–1048.

Clark, E.G., 1997. Post-weaning multisystemic wasting syndrome. Proc. Am. Assoc. Swine Pract. 28, 499–501.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001a. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., Tuschl, T., 2001b. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. 20, 6877–6888.

Ellis, J.A., Hassard, L., Clark, E.G., Harding, J., Allan, G.M., Willson, P., Strokapp, J., Martin, K., McNeilly, F., Meehan, B.M., Todd, D., Haines, D.M., 1998. Isolation of circovirus from lesions of pigs with post-weaning multisystemic wasting syndrome. Can. Vet. J. 39, 44–51.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004a. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV2 cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J. Virol. 78, 6297–6303.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004b. Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. J. Virol. 78, 13440–13446.

Fire, A., 1999. RNA-triggered gene silencing. Trends Genet. 15, 358–363.

Ge, Q., McManus, M.T., Nguyen, T., Chen, H.H., Sharp, P.A., Eisen, H.N., 2003. 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. 100, 2718–2723.

Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N., Chen, J., 2004. Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc. Natl. Acad. Sci. U.S.A. 101, 8676–8681.

Giladi, H., Ketzinel-Gilad, M., Rivkin, L., Fellig, Y., Galun, E., 2003. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol. Ther. 8, 769–776.

Gillin, L., Karelsky, S., Andino, R., 2002. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 418, 430–434.

Gillin, L., Stone, J.K., Andino, R., 2005. Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. J. Virol. 79, 1027–1035.

Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. 72, 5262–5267.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2001. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404, 293–296.

Hammond, S.M., Caudy, A.A., Hannon, G.J., 2001. Post transcriptional gene silencing by double-stranded RNA. Nat. Rev. Genet. 2, 1110–1119.

Harborth, J., Elbashir, S.M., Vandenburgh, K., Manninga, H., Scaringe, S.A., Weber, K., Tuschl, T., 2003. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev. 13, 83–105.

Jia, Q.M., Sun, R., 2003. Inhibition of gammaherpesvirus replication by RNA interference. J. Virol. 77, 3301–3306.

Kamstrup, S., Barfoed, A.M., Fritman, T.H., Ladekjær-Mikkelsen, A.-S., Botner, A., 2004. Immunisation against PCV2 structural protein by DNA vaccination of mice. Vaccine 22, 1358–1361.

Kielp, M., Stevenson, G.W., Choi, J., Latimer, K.S., Kanitz, H.C., Mittal, S.K., 2001. Viral replication and lesions in BALB/c mice experimentally inoculated with porcine circovirus isolated from a pig with post weaning multisystemic wasting disease. Vet. Pathol. 38, 74–82.

Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein of porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J. Virol. 79, 8262–8274.

Mankertz, A., Mankertz, J., Wolf, K., Buhk, H.J., 1999. Identification of a protein essential for replication of porcine circovirus. J. Gen. Virol. 79, 381–383.

McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J., Kay, M.A., 2002. RNA interference in adult mice. Nature 418, 38–39.

McCaffrey, A.P., Nakai, H., Pandey, K., Huang, Z., Salazar, F.H., Xu, H., Wieland, S.F., Marion, P.L., Kay, M.A., 2003. Inhibition of hepatitis B virus in mice by RNA interference. Nat. Biotechnol. 21, 639–644.
McCown, M., Diamond, M.S., Pekosz, A., 2003. The utility of siRNA transcripts produced by RNA polymerase in down regulating viral gene expression and repression of negative- and positive-strand RNA viruses. Virology 313, 514–524.

McManus, M.T., Sharp, P.A., 2002. Gene silencing in mammals by small interfering RNAs. Nat. Rev., Genet. 3, 737–747.

Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. 79, 2171–2179.

Morozov, L., Sirinarumitr, T., Sorden, S.D., Halbur, P.G., Morgan, M.K., Yoon, K.J., Paul, P.S., 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. 36, 2535–2541.

Morris, K.V., Chan, S.W.L., Jacobsen, S.E., Looney, D.J., 2004. Small interfering RNA-induced transcriptional gene silencing in human cells. Science 27, 1289–1292.

Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. 81, 2281–2287.

Paddison, P.J., Caudy, A.A., Berstein, E., Hannon, G.J., Conklin, D.S., 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. 16, 948–958.

Persengiev, S.P., Zhu, X., Green, M.R., 2004. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA 10, 12–18.

Pringle, C.R., 1999. Virus taxonomy at the XIth international congress of virology, Sydney, Australia. Arch. Virol. 144, 2065–2070.

Push, O., Boden, D., Silbermann, R., Lee, F., Tucker, L., Ramratnam, B., 2003. Nucleotide sequence homology requirements of HIV-1-specific short hairpin RNA. Nucleic Acids Res. 31, 6444–6449.

Quintana, J., Balasch, M., Segalés, J., Calsamiglia, M., Rodríguez-Arrioja, G.M., Plana-Durán, J., Domingo, M., 2002. Experimental inoculation of porcine circoviruses type 1 (PCV1) and type 2 (PCV2) in rabbits and mice. Vet. Res. 33, 229–237.

Rosell, C., Segalés, J., Plana-Durán, J., Balasch, M., Rodríguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999. Pathological, immunohistochemical and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J. Comp. Pathol. 120, 59–78.

Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., Zamore, P.D., 2003. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115, 199–208.

Sharp, P.A., 2001. RNA interference. Genes Dev. 15, 485–490.

Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., Lieberman, J., 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347–351.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. Nature 295, 64–66.

Tischer, I., Peters, D., Rasch, R., Pociuli, S., 1987. Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. Arch. Virol. 96, 39–57.

Tompkins, S.M., Lo, C.Y., Tumpey, T.M., Epstein, S.L., 2004. Protection against lethal influenza virus challenge by RNA interference in vivo. Proc. Natl. Acad. Sci. U.S.A. 101, 8682–8686.

Uprichard, S.L., Boyd, B., Althage, A., Chisari, F.V., 2005. Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. Proc. Natl. Acad. Sci. U.S.A. 102, 773–778.

Wang, Z., Ren, L.L., Zhao, X.G., Hung, T., Meng, A.M., Wang, J.W., Chen, Y.G., 2004. Inhibition of severe acute respiratory syndrome virus replication by small interfering RNAs in mammalian cells. J. Virol. 78, 7523–7527.

Yuan, J., Cheung, P.K.M., Zhang, H.M., Chau, D., Yang, D., 2005. Inhibition of coxsackievirus B3 replication by small interfering RNAs requires perfect sequence match in the central region of the viral positive strand. J. Virol. 79, 2151–2159.