Effect of small interfering RNAs on in vitro replication and gene expression of feline coronavirus

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Objective—To evaluate the ability of small interfering RNAs (siRNAs) to inhibit in vitro viral replication and gene expression of feline coronavirus (FCoV).

Sample—Cell cultures of Crandell-Rees feline kidney cells.

Procedures—5 synthetic siRNAs that each targeted a different region of the FCoV genome were tested individually and in various combinations for their antiviral effects against 2 strains of FCoV (feline infectious peritonitis virus WSU 79-1146 and feline enteric coronavirus WSU 79-1683) in cell cultures. Tested combinations targeted the FCoV leader and 3′ untranslated region, FCoV leader region and nucleocapsid gene, and FCoV leader region, 3′ untranslated region, and nucleocapsid gene. For each test condition, assessments included relative quantification of the inhibition of intracellular viral genomic RNA synthesis by means of real-time, reverse-transcription PCR analysis; flow cytometric evaluation of the reduction of viral protein expression in infected cells; and assessment of virus replication inhibition via titration of extracellular virus with a $TCID_{50}$ infectivity assay.

Results—The 5 siRNAs had variable inhibitory effects on FCoV when used singly. Combinations of siRNAs that targeted different regions of the viral genome resulted in more effective viral inhibition than did individual siRNAs that targeted a single gene. The tested siRNA combinations resulted in approximately 95% reduction in viral replication (based on virus titration results), compared with findings in negative control, nontargeting siRNA–treated, FCoV-infected cells.

Conclusions and Clinical Relevance—In vitro replication of FCoV was specifically inhibited by siRNAs that targeted coding and noncoding regions of the viral genome, suggesting a potential therapeutic application of RNA interference in treatment of feline infectious peritonitis. (*Am J Vet Res* 2014;75:828–834)

Feline coronavirus is a single-stranded, positive-sense RNA virus with a membrane envelope. The FCoV genome contains 11 putative ORFs. These include 2 large ORFs that encode viral nonstructural replicase proteins; 4 structural ORFs that encode spike, envelope, membrane, and nucleocapsid proteins; and 5 accessory ORFs of unknown function. Similar to other coronaviruses, FCoV uses a discontinuous transcription mechanism to synthesize both full-length and subgenomic negative-strand RNAs, which then function as templates for synthesis of full-length genomic mRNAs and sgRNAs.[1] Both viral genome and sgRNAs share a common 5′ leader sequence and a common 3′ UTR.[2]

Abbreviations

CRFK	Crandell-Rees feline kidney
DMEM	Dulbecco modified Eagle medium
FCoV	Feline coronavirus
FECV	Feline enteric coronavirus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus
MOI	Multiplicity of infection
ORF	Open reading frame
RT	Reverse transcription
sgRNA	Subgenomic mRNA
siRNA	Small interfering RNA
UTR	Untranslated region

Feline coronavirus is a virus that commonly infects domestic cats, typically resulting in subclinical infection or mild enteritis. However, some FCoV-infected cats develop FIP, a progressive and fatal disease that accounts for most infectious disease–related deaths in pet cats.[3] Feline infectious peritonitis may have an acute onset with peritoneal or pleural effusion (or both) or may have a more protracted course with pyogranulomatous infiltration of multiple tissues. Both clinical scenarios invariably end in death. The pathogenesis of FIP

involves mutant FCoV that can replicate efficiently in monocytes and macrophages, which results in dysregulation of host cell-mediated immunity and allows the virus to replicate unchecked to a high titer. Contributing to the pathogenesis of FIP are cytokines and inflammatory mediators released from infected macrophages and other inflammatory cells that infiltrate the tissue as well as antigen-antibody complexes and complement activation.[4] Thus, efficient systemic viral replication appears to have a critical role in FIP pathogenesis.

To date, there is no specific treatment for this fatal disease. The use of siRNAs is a novel antiviral strategy that specifically targets viral mRNA and genomic RNA for degradation by endogenous cellular enzymes.[5] This technology has been used successfully for viral diseases such as viral hepatitis and severe acute respiratory syndrome both in vitro and in ex vivo studies.[6–8] Recently, 2 individual siRNAs that target the FCoV leader region and nucleocapsid gene were shown to inhibit FCoV replication in vitro.[9] The purpose of the study reported here was to evaluate the ability of siRNAs (singly and in combination) to inhibit in vitro replication and gene expression of FCoV. To this end, FCoV-specific siRNAs that hybridize to viral coding or noncoding regulatory regions of the FCoV genome were designed and evaluated in various single or combination siRNA test conditions. Assessments included relative quantification of the inhibition of intracellular viral genomic RNA synthesis by means of real-time, RT-PCR analysis; flow cytometric evaluation of the reduction of viral protein expression in infected cells; and assessment of virus replication inhibition via titration of extracellular virus with a $TCID_{50}$ infectivity assay.

Materials and Methods

siRNAs—Five siRNAs[a] (**Appendix**) were designed on the basis of published sequence data for FCoVs with an RNA interference designer[b] as follows: siRNA-L (targets the common 5′ leader region), siRNA-U (targets the common 3′ UTR), siRNA-R (targets the replicase gene), siRNA-M (targets the membrane gene), and siRNA-N (targets the nucleocapsid gene). The sequences of these siRNAs were confirmed for specificity by comparing them with FCoV sequences in the GenBank database. The designed stealth siRNAs used in the study were chemically modified to reduce off-target and nonspecific effects, eliminate the induction of the interferon response pathway, minimize nonspecific cellular stress, and enhance specificity and stability in serum.[10]

Cell culture, transfection, and virus infection—Crandell-Rees feline kidney cells[c] were propagated in a DMEM-based nutrient solution[d] supplemented with 10% heat-inactivated fetal bovine serum[e] and maintained at 37°C and 5% CO_2 in an incubator. The cells were plated in 12-well plates; at approximately 80% confluency, transfection was performed with siRNA (100nM/well) and transfection reagent[f] (1.5 µL/well). In experiments that included combinations of siRNAs, 2 or 3 siRNAs were combined to provide a total concentration of 100nM/well. Tested siRNA combinations targeted the FCoV leader and 3′ UTR (ie, siRNA-L and siRNA-U), the leader region and nucleocapsid gene (ie, siRNA-L and siRNA-N), and the leader region, 3′ UTR, and nucleocapsid gene (siRNA-L, siRNA-U, and siRNA-N).

Twenty-four hours following transfection, cells were either infected with FIPV WSU 79-1146[g] or FECV WSU 79-1683[h] virus at an MOI of 0.1. One hour after incubation, the cells were washed with DMEM-based nutrient solution, and fresh DMEM-based nutrient solution with supplemental 10% heat-inactivated fetal bovine serum was added to each well. After 48 hours, samples of cell culture medium as well as cultured cells were collected for viral titration, relative quantification of intracellular FCoV genomic RNA, and assessment of viral protein expression. Control samples used in each experiment included FCoV-infected cells and untreated CRFK cells; FCoV-infected cells treated with a negative control nontargeting siRNA[a] were included as controls to test for potential nonspecific effects. Fluorescein-labeled siRNA[i] was used to evaluate transfection efficiency. The CRFK cells underwent mock transfection with the transfection agent only to evaluate for potential toxic effects. Each siRNA and siRNA combination was tested in duplicate, and each experiment was performed twice.

Flow cytometry analysis—Forty-eight hours after viral infection, siRNA-treated and control CRFK cells were treated with a permeabilization reagent[j] (according to manufacturer's protocol) to assess virus protein expression. The cells were stained with 200 µL of fluorescein isothiocyanate–labeled anti–FCoV polyclonal antibody[k] for 30 minutes on ice in the dark. The cells were washed with PBS solution, pelleted by centrifugation (1,000 $\times$ g), and resuspended in 500 L of PBS solution. Finally, FCoV protein expression was measured as the intensity of fluorescence detected by flow cytometry.[l] Experimental controls included cells transfected with negative control, nontargeting siRNA–treated, FCoV-infected cells or uninfected cells.

To assess the transfection efficiency, the transfection control cells were evaluated with flow cytometry. Cells were trypsinized and pelleted 24 hours following transfection and resuspended in 1 mL of PBS solution/sample. The intensity of intracellular fluorescein was measured via flow cytometry to evaluate the efficiency of the transfection reagent.

Quantitative real-time RT-PCR assay—For cellular viral RNA quantification, cells were harvested 48 hours following virus infection, and nucleic acid was extracted with an RNA purification kit[m] according to manufacturer recommendations. Reverse transcription and quantitative PCR assays were performed with primers and a carboxyfluorescein-labeled probe that targets the 7b coding region,[11] a region that is highly conserved in FCoVs. Reactions were done with a quantitative RT-PCR kit[n] in a thermal cycler.[o] To standardize the total amount of RNA in each reaction, mRNA expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase was used to allow relative quantitation of the viral RNA by the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.[12]

$TCID_{50}$ assay—Forty-eight hours after infection with FCoV, 500 µL of tissue culture medium was col-

lected from each well and stored at –80°C prior to viral titration with the TCID$_{50}$ assay. In brief, samples of culture medium were serially diluted 10-fold with DMEM. Diluted virus suspensions were added to monolayer CRFK cells cultured in 96-well plates (6 wells/dilution). Wells were monitored for cytopathic effects, such as cells rounding, clumping, and detachment, at 72 hours after infection by means of an inverted phase-contrast microscope. The TCID$_{50}$ endpoint values were calculated according to the method of Reed and Muench.[13]

Cell viability assay—A cell proliferation assay[p] was performed to determine any potential cellular toxic effects resulting from transfection of the CRFK cells. The CRFK cells were propagated to 80% confluency in a 96-well tissue culture plate. The CRFK cells were transfected with 1 of the 3 siRNA combinations or underwent mock transfection. After 24 hours, cell viability reagent was added to each well. A colored product produced by metabolically active cells was detected by a microplate reader[q] that measured absorbance at 490 nm. The quantity of this colored product was directly proportional to the number of living cells in the sample. The samples were evaluated and compared with findings for untreated CRFK cell control wells. Each sample was tested in triplicate, and the experiment was done twice.

Statistical analysis—An ANOVA[r] was used to identify significant differences between the mean values for treated samples and the negative control, nontargeting siRNA–treated, FCoV-infected samples. The ANOVA assumption of normality was tested with the Shapiro-Wilk statistic. When this assumption was not met, a logarithmic transformation or ANOVA on rank transformation of the data was done, and the Levene test was used to test the ANOVA equality of variance assumption. Bonferroni adjustment was used to minimize the type I error rate. The Fisher least significant difference method of mean separation was used to determine which means differed significantly. A value of $P \leq 0.05$ was considered significant.

Results

Transfection efficiency—Cell transfection efficiency was evaluated by means of fluorescein-labeled siRNA,[i] which was detected in CRFK cells by use of flow cytometry. The CRFK cells were 95% effectively transfected with the transfection reagent.[d]

Effect of individual synthetic siRNAs on FCoV protein expression in CRFK cells—The expression of FCoV protein was measured by means of flow cytometry to assess the effectiveness of individual siRNAs to inhibit FCoV gene expression, and therefore viral replication, in cultured cells. The 5 siRNAs (siRNA-L, siRNA-U, siRNA-R, siRNA-M, and siRNA-N) used in the study had variable inhibitory effects on viral replication in vitro; siRNA-L, siRNA-U, siRNA-R, siRNA-M, and siRNA-N resulted in mean ± SD decreases in the protein expression of FIPV WSU 79-1146 of 80 ± 11%, 43 ± 23%, 13 ± 11%, 33 ± 7%, and 50 ± 17%, respectively, compared with findings for the negative control, nontargeting siRNA–treated, FCoV-infected sample. In contrast, siRNA-R had no effect on the protein expression of FECV WSU 79-1683, whereas siRNA-L, siRNA-U, siRNA-M, and siRNA-N resulted in decreases in viral protein expression of 75 ± 6%, 30 ± 11%, 14 ± 5%, and 13 ± 0.9%, respectively, compared with the negative control, nontargeting siRNA–treated, FCoV-infected sample.

Effect of siRNA combinations on the expression of FCoV proteins—Flow cytometric evaluation of viral protein expression in cells transfected with any of the 3 siRNA combinations revealed a significant ($P = 0.005$) decrease in protein expression following infection with FIPV WSU 79-1146 ($P < 0.001$) or FECV WSU 79-1683, compared with findings for the negative control, nontargeting siRNA–treated, FCoV-infected sample. Protein expression of the FIPV WSU 79-1146 strain was decreased (compared with the negative control sample findings) by 91 ± 6%, 91 ± 2%, and 90 ± 5% after CRFK cells were treated with siRNAs that targeted the FCoV leader and 3′ UTR (ie, siRNA-L and siRNA-
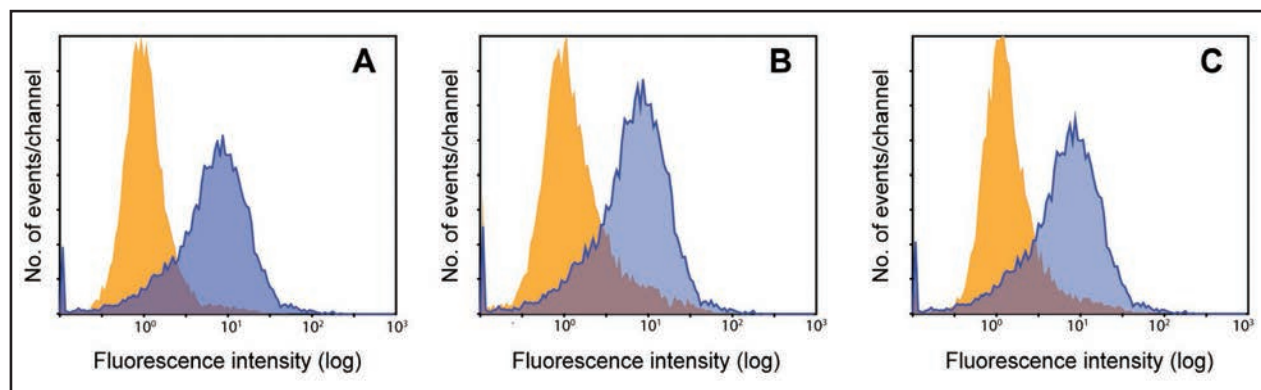

Figure 1—Results of flow cytometric analysis (No. of events/channel vs fluorescence intensity [logarithm base 10]) illustrating the effect of siRNA combinations on the expression of FIPV WSU 79-1146 protein in virus-infected CRFK cells. The CRFK cells were transfected with 1 of 3 of siRNA combinations (concentration, 100nM [orange]) or a negative control siRNA (purple) 24 hours prior to viral infection (MOI, 0.1). At 48 hours after viral infection, CRFK cells were stained with labeled anti–FCoV polyclonal antibody following a standard protocol, and cell fluorescence was assessed by flow cytometry. The tested siRNA combinations included siRNA-L (targeting the FCoV leader region) and siRNA-U (targeting the 3′ UTR [A]), siRNA-L and siRNA-N (targeting the nucleocapsid gene [B]), and siRNA-L, siRNA-U, and siRNA-N (C). In each panel, the data represent the result of 1 of 2 independent experiments that yielded similar results; each sample was assessed in duplicate.
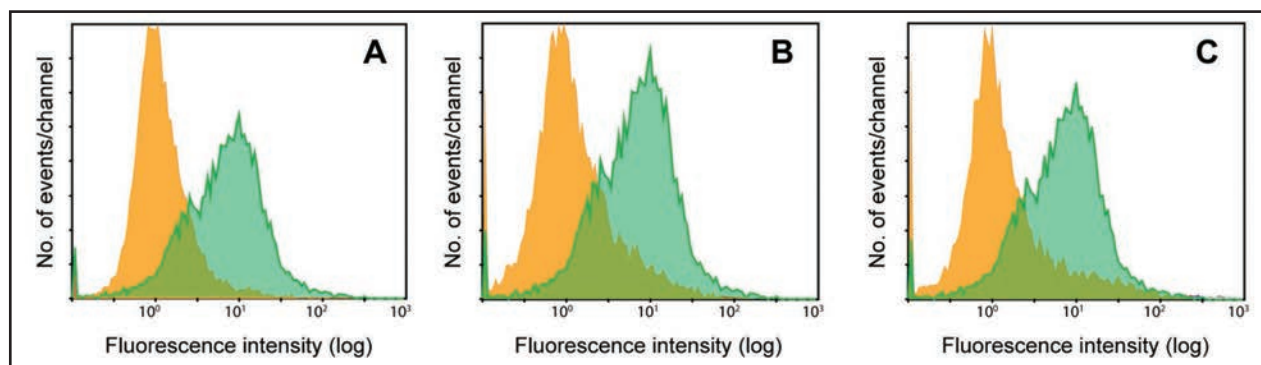
Figure 2—Results of flow cytometric analysis (No. of events/channel vs fluorescence intensity [logarithm base 10]) to illustrate the effect of siRNA combinations on the expression of FECV WSU 79-1683 protein in virus-infected CRFK cells. The CRFK cells were transfected with 1 of 3 of siRNA combinations (concentration, 100nM [orange]) or a negative control nontargeting siRNA (green) 24 hours prior to viral infection (MOI, 0.1). At 48 hours after viral infection, CRFK cells were stained with labeled anti–FCoV polyclonal antibody following a standard protocol, and cell fluorescence was assessed by flow cytometry. The tested siRNA combinations included siRNA-L (targeting the FCoV leader region) and siRNA-U (targeting the 3′ UTR [A]), siRNA-L and siRNA-N (targeting the nucleocapsid gene [B]), and siRNA-L, siRNA-U, and siRNA-N (C). In each panel, the data represent the result of 1 of 2 independent experiments that yielded similar results; each sample was assessed in duplicate.

U), the leader region and nucleocapsid gene (ie, siRNA-L and siRNA-N), and the leader region, 3′ UTR, and nucleocapsid gene (siRNA-L, siRNA-U, and siRNA-N), respectively (**Figure 1**). The protein expression of the FECV WSU 79-1683 strain was decreased (compared with the negative control sample findings) by $95 \pm 1\%$, $94 \pm 1\%$, and $95 \pm 1\%$ after CRFK cells were treated with the siRNA-L and siRNA-U combination, siRNA-L and siRNA-N combination, and the siRNA-L, siRNA-U, and siRNA-N combination, respectively (**Figure 2**). On the basis of the results obtained after treatment of cells with combinations of siRNAs, compared with findings following treatment with individual siRNAs, further experiments were performed for the siRNAs used in combination only.

Inhibition of viral genomic RNA by siRNA combinations—A quantitative real-time RT-PCR assay was used to evaluate the reduction of the intracellular viral genomic RNA following cell treatment. The 3 siRNA combinations significantly ($P < 0.001$) inhibited genomic RNA production in both strains of FCoV. With regard to FIPV WSU 79-1146 genomic RNA, each of the 3 combinations resulted in a reduction of approximately $97 \pm 1.4\%$, compared with findings for the negative control, nontargeting siRNA–treated, FCoV-infected sample. With regard to FECV WSU 79-1683 genomic RNA, reductions of $96 \pm 4\%$, $92 \pm 9\%$, and $93 \pm 7\%$ (compared with findings for the negative control sample) were evident in CRFK cells treated with the siRNA-L and siRNA-U combination, siRNA-L and siRNA-N combination, and the siRNA-L, siRNA-U, and siRNA-N combination, respectively.

Effect of siRNA combinations on the yield of progeny virus (extracellular virus titer)—To deter-
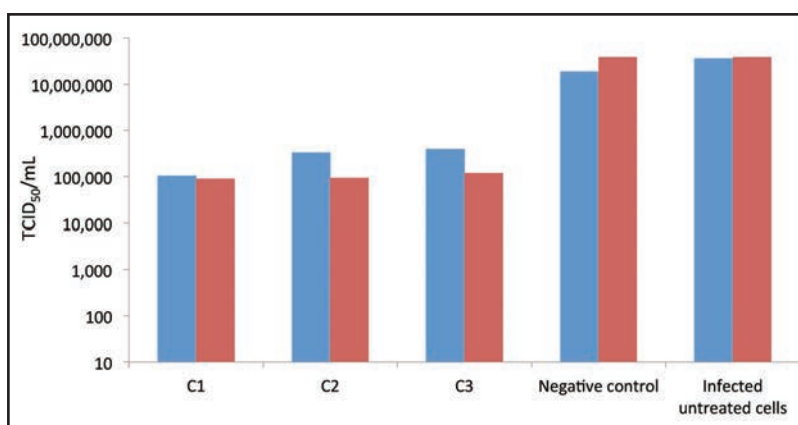

Figure 3—Results of a TCID$_{50}$ assay to illustrate the effect of siRNA combinations on the yield of FIPV WSU 79-1146 (blue bars) and FECV WSU 79-1683 (red bars) progeny virus in CRFK cells. The CRFK cells were transfected with 1 of 3 siRNA combinations (concentration, 100nM) or a negative control nontargeting siRNA 24 hours prior to viral infection (MOI, 0.1) or remained untreated. The tested siRNA combinations included siRNA-L (targeting the FCoV leader region) and siRNA-U (targeting the 3′ UTR [C1]), siRNA-L and siRNA-N (targeting the nucleocapsid gene C2]), and siRNA-L, siRNA-U, and siRNA-N (C3). Forty-eight hours after viral infection, a sample of cell culture supernatant was collected from each well for titration. Each combination was tested in duplicate, and the data represent the mean ± SD of 2 independent experiments. For cells treated with any of the 3 siRNA combinations, viral replication was significantly different from that in infected cells treated with the negative control nontargeting siRNA. There was no significant difference in extracellular virus titers for untreated cells infected with either FIPV WSU 79-1146 or FECV WSU 79-1683, compared with the infected cells treated with the negative control, nontargeting siRNA.

mine whether reductions in viral protein expressions by the siRNA combinations had any effect on the yield of progeny virus, TCID$_{50}$ assays were done. Each of the 3 siRNA combinations resulted in approximately a 100-fold reduction ($P < 0.001$) in the extracellular virus titer of both viral strains, compared with findings for the negative control, nontargeting siRNA–treated, FCoV-infected sample, which represented a reduction in virus replication of approximately 95% (**Figure 3**).

To confirm that siRNAs were designed to specifically target FCoV, the titer of the extracellular virus from the FCoV-infected cells and the negative control, nontargeting siRNA–treated, FCoV-infected cells were assessed with TCID$_{50}$ assays. There was no significant difference in the titers of extracellular virus for cells

infected with FECV WSU 79-1683 or FIPV WSU 79-1146, compared with the negative control, nontargeting siRNA–treated, FCoV- infected cells ($P = 0.211$ and $P = 0.5055$, respectively). This indicated that the transfection or nontargeting siRNA did not have a nonspecific inhibitory effect on FCoV and confirmed the specificity of the designed siRNAs.

Effect of siRNA transfection on cell viability— Microscopic examination (by means of an inverted phase-contrast microscope) revealed that the number of viable cells among CRFK cells transfected with each of the 3 siRNA combinations was comparable to the number of viable cells among untreated CRFK cells as well as the numbers of viable cells among cells transfected with negative nontargeting siRNA and cells that underwent mock transfection. Furthermore, a cell proliferation assay was used to investigate any potential cytotoxic effect of the siRNA combinations. Assay results indicated that the 3 siRNA combinations and the transfection reagent had no significant effect on cell viability, compared with viability of untreated CRFK cells. The mean ± SD optical density of the untreated cells was 0.9920 ± 0.017 absorbance units, which was not significantly ($P = 0.1$) different from the mean optical density of the mock-transfected cells (0.9353 ± 0.2 absorbance units) or from the cells transfected with the siRNA-L and siRNA-U combination (0.9576 ± 0.02 absorbance units), the siRNA-L and siRNA-N combination (1.0433 ± 0.049 absorbance units), or the siRNA-L, siRNA-U, and siRNA-N combination (1.0636 ± 0.018 absorbance units).

Discussion

Feline infectious peritonitis is a fatal progressive disease of cats that is characterized by a poor cell-mediated immune response and continued systemic virus replication as well as exaggerated humoral response to the virus. To date, treatment of FIP has been ineffective, with affected cats progressing inevitably to death. Because of the immune-mediated component of the disease, treatments directed at enhancing the cell-mediated response (eg, interferon) or suppressing the humoral response (eg, corticosteroids and cyclophosphamide) have been used with little or no success.[14] Recently, polyprenyl, an agent that is postulated to enhance T-lymphocyte activity, has been used with some success in cats with dry forms of FIP but not in cats with the common effusive (wet) form of the disease. These results reflect the importance of limiting the viral load (replication); viral spread and replication are greater with the wet form than with the dry form.[15] It seems that the high viral burden associated with the wet form of the disease interferes with the ability of the host immune response to restore its normal physiologic function. Reducing virus replication in infected cats as well as enhancing the host immune response could improve the ability of an infected animal to control development of disease.

In the present study, we found that individual siRNAs and siRNA combinations that target highly conserved regions of FCoV coding and noncoding genes are able to inhibit virus gene expression and thereafter inhibit replication of the virus. Feline coronavirus is considered a good target for siRNA treatment because the viral full-length genome contains nearly all the sequences found in any of the sgRNAs. Thus, an siRNA that targets the sgRNAs will target the full-length genomic RNA too. The siRNAs used in the present study (siRNA-L, siRNA-U, siRNA-N, siRNA-M, and siRNA-R) each targeted a single gene and had a variable inhibitory effect on FCoV replication in vitro. Each of the 3 siRNA combinations targeted a different combination of regions of the viral genome, and inhibition of the viral replication of both FCoV strains by each combination was more effective than that achieved by the individual siRNAs, compared with findings for the negative control, nontargeting siRNA–treated, FCoV-infected sample. Combinations of siRNAs targeting different regions have been known to lead to a significant synergistic effect on viral inhibition, compared with results of treatment with individual siRNAs.[16] Additionally, in the present study, the cell proliferation assay revealed that the siRNA combinations produced no cytotoxic effects in the transfected cells.

Because FCoV has a high mutation rate, the probability that the virus may evade siRNA targeting through viral mutations is considered a challenge to the design of siRNAs. To overcome this limitation, siRNAs that target highly conserved regions of essential viral genes as well as combinations of these siRNAs were used in the present study to prevent or reduce development of viral escape mutants. The siRNA-L, which targets the FCoV leader sequence, was included in each of the 3 combinations used in the present study because this region is highly conserved in FCoV and has a pivotal role in virus gene expression and replication.[2] Another siRNA used was siRNA-U, which targets the 3′ UTR. The 3′ UTR is a noncoding region that is highly conserved in FCoV and has an essential role in regulating the viral transcription and replication cycle.[16] The earliest steps of both genome replication and sgRNA transcription are initiated at the 3′ end of the genome, which makes it an important siRNA target. Furthermore, during virus replication, a 5′ common leader sequence and 3′ coterminal nested set of sgRNAs are made.[1] Hence, siRNA-L and siRNA-U target genomic RNAs and all the viral nested sgRNAs. The other siRNA used in 2 of the 3 tested combinations was siRNA-N, which targets the viral nucleocapsid gene. Mutation is less likely to occur in the highly conserved regions that have an important role during the viral replication cycle because such mutations might have a deleterious effect on the virus. Furthermore, in the present study, the siRNA targets chosen had 100% homology with FCoV sequences deposited in GenBank. This level of homology reveals a selective potential against mutation during the evolution of the virus. An additional benefit of targeting highly conserved regions of the viral genome is to overcome the genetic diversity problem among FCoVs, which is considered an additional challenge to designing specific siRNAs. Thus, siRNAs can be designed to recognize and target various strains of FCoVs that circulate in the field. Given the poor host or cell-mediated immune response associated with enhanced viral replication in cats with FIP, the effective reduction of vi-

ral replication by the siRNA combinations used in the present in vitro study may be useful in vivo; treatment of FIP-affected cats with an siRNA combination may, in part, restore the host immune response and thereafter improve viral clearance. Reduction of viral replication with siRNA treatment could also reduce the likelihood of mutation and reduce the expansion of any virus population containing the mutation of importance in development of FIP.

Interestingly, each siRNA (25 mer) used in the present study was manufactured with an RNA interference designer that provides siRNAs that have higher specificity for the intended target gene and increased stability in serum and cell culture than standard siRNAs (21 mer). This method of manufacture eliminates unwanted off-target effects. Therefore, the siRNA combinations used in the present study can be directly applied in an in vivo study without the need for any chemical modification that may interfere with their targeting effect.[10]

Unlike a previous study[9] that investigated the inhibitory effect of individual siRNAs that each targeted a single gene, the present study investigated the effectiveness of siRNA combinations that targeted 2 or 3 regions of the viral genome on in vitro inhibition of FCoV replication. The siRNA combinations markedly inhibited FCoV genomic RNA, FCoV protein, and FCoV replication. These results indicated that FCoV replication can be specifically and significantly inhibited with siRNA combinations that target different highly conserved coding and regulatory noncoding regions of the viral genome or mRNA, suggesting a potential therapeutic application of RNA interference in the treatment of cats with FIP. Development of agents such as siRNAs for treatment of FIP is a potential means to combat this highly fatal viral disease that is now considered to be the primary infectious cause of death in young cats. With improvements in siRNA design and delivery methods, RNA interference might be an effective treatment option for such a life-limiting viral infection.

a. Stealth siRNA, Invitrogen, Carlsbad, Calif.
b. Block-iT RNAi designer, Invitrogen, Carlsbad, Calif.
c. Crandell-Rees feline kidney cell line, American Type Culture Collection, Manassas, Va.
d. DMEM:F-12, Lonza, Walkersville, Md.
e. Fetal Bovine serum, Atlanta Biologicals, Lawrenceville, Ga.
f. Lipofectamine 2000, Invitrogen, Carlsbad, Calif.
g. FIPV WSU 79-1146, American Type Culture Collection, Manassas, Va.
h. FECV WSU 79-1683, American Type Culture Collection, Manassas, Va.
i. Fluorescently labeled siRNA, Invitrogen, Carlsbad, Calif.
j. IntraPrep permeabilization reagent, Immunotech, Marseille, France.
k. Polyclonal Ab, VMRD, Pullman, Wash.
l. Flow cytometr, Epics XL, Beckman Coulter Inc, Fullerton, Calif.
m. RNeasy Plus Mini Kit, Qiagen, Valencia, Calif.
n. Superscript III Platinum One-Step qRT-PCR Kit, Invitrogen, Carlsbad, Calif.
o. SmartCycler II, Cepheid, Sunnyvale, Calif.
p. CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, Wis.
q. ELx800 Universal Microplate Reader, Bio-Tek Inc, Winooski, Vt.
r. SAS, version 9.3, SAS Institute Inc, Cary, NC.

References

1. Sawicki SG, Sawicki DL, Siddell SG. A contemporary view of coronavirus transcription. *J Virol* 2007;81:20–29.
2. Masters PS. The molecular biology of coronaviruses. *Adv Virus Res* 2006;66:193–292.
3. Hartmann K. Feline infectious peritonitis. *Vet Clin North Am Small Anim Pract* 2005;35:39–79.
4. de Groot-Mijnes JDF, Van Dun JM, van der Most RG, et al. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. *J Virol* 2005;79:1036–1044.
5. Spurgers KB, Sharkey CM, Warfield KL, et al. Oligonucleotide antiviral therapeutics: antisense and RNA interference for highly pathogenic RNA viruses. *Antiviral Res* 2008;78:26–36.
6. Chandra PK, Kundu AK, Hazari S, et al. Inhibition of hepatitis C virus replication by intracellular delivery of multiple siRNAs by nanosomes. *Mol Ther* 2012;20:1724–1736.
7. Li T, Zhang Y, Fu L, et al. siRNA targeting the leader sequence of SARS-CoV inhibits virus replication. *Gene Ther* 2005;12:751–761.
8. Chang Z, Babiuk LA, Hu J. Therapeutic and prophylactic potential of small interfering RNAs against severe acute respiratory syndrome. *BioDrugs* 2007;21:9–15.
9. McDonagh P, Sheehy PA, Norris JM. In vitro inhibition of feline coronavirus replication by small interfering RNAs. *Vet Microbiol* 2011;150:220–229.
10. Li S, Crothers J, Haqq CM, et al. Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. *J Biol Chem* 2005;280:23709–23717.
11. Gut M, Leutenegger CM, Huder JB, et al. One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses. *J Virol Methods* 1999;77:37–46.
12. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 2008;3:1101–1108.
13. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938;27:493–497.
14. Hartmann K, Ritz S. Treatment of cats with feline infectious peritonitis. *Vet Immunol Immunopathol* 2008;123:172–175.
15. Legendre AM, Bartges JW. Effect of polyprenyl immunostimulant on the survival times of three cats with the dry form of feline infectious peritonitis. *J Feline Med Surg* 2009;11:624–626.
16. He ML, Zheng BJ, Chen Y, et al. Kinetics and synergistic effects of siRNAs targeting structural and replicase genes of SARS-associated coronavirus. *FEBS Lett* 2006;580:2414–2420.

Appendix appears on the next page

Appendix

Sequences of siRNAs (and their position within the FIPV WSU 79-1146 genome) used in experiments to evaluate the ability of siRNAs to inhibit in vitro viral replication and gene expression of FIPV WSU 79-1146 and FECV WSU 79-1683.

siRNA	Nucleotide sequence*	Position in the genome (bp range)
siRNA-L		
Sense	UCUUCGGACACCAACUCGAACUAAA	74–98
Antisense	UUUAGUUCGAGUUGGUGUCCGAAGA	
siRNA-M		
Sense	GACCACAAUUUAGCUGGCUCGUUUA	26,198–26,222
Antisense	UAAACGAGCCAGCUAAAUUGUGGUC	
siRNA-R		
Sense	CAUUGCAGCUUUGAGUACUGGUGUU	13,330–13,354
Antisense	AACACCAGUACUCAAAGCUGCAAUG	
siRNA-N		
Sense	GACAACUUUCUAUGGUGCUAGAAGU	27,507–27,531
Antisense	ACUUCUAGCACCAUAGAAAGUUGUC	
siRNA-U		
Sense	GAGGUACAAGCAACCCUAUUGCAUA	28,941–28,965
Antisense	UAUGCAAUAGGGUUGCUUGUACCUC	

*Nucleotide sequences are based on GenBank accession No. DQ010921.