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Effect of Small Interfering RNAs on in Vitro Replication and Gene Expression of Feline Coronavirus

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Effect of Small Interfering RNAs on in Vitro Replication and Gene Expression of Feline Coronavirus

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Abstract

Feline corononavirus (FCoV) infection is ubiquitous in domestic cat populations worldwide and is usually associated with subclinical or mild enteritis. However, in some cats infection may result in the development of a fatal progressive disease called feline infectious peritonitis (FIP). FIP is considered to be the major cause of infectious-related death in pet cats. Currently, there is no protective vaccine or curative treatment to this highly fatal disease. In this study, we evaluated the ability of small interfering RNAs (siRNAs) to inhibit the in vitro viral replication and gene expression of FCoV as a potential treatment for FIP.

Five synthetic siRNAs were designed to target different regions of the FCoV genome. The siRNAs were tested individually and in various combinations in vitro for their antiviral effects against 2 strains of FCoV (feline infectious peritonitis virus WSU 79-1146 and feline enteric coronavirus WSU 79-1683). Tested combinations targeted the FCoV leader and 3′ untranslated region; FCoV leader region and nucleocapsid gene; and FCoV leader, 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 Polymerase chain reaction (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 50% tissue culture infective dose ($TCID_{50}$) assay.

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 non-targeting siRNA-treated FCoV-infected cells. This study shows that FCoV replication can be specifically inhibited by siRNAs that target coding and noncoding regions of the viral genome, suggesting a potential therapeutic application of RNA interference in treatment of feline infectious peritonitis.

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List of Abbreviations

AGO	Argonaute
CCoV	Canine coronavirus
CMI	Cell-mediated immune response
CPPs	Cell-penterating peptides
CRFK	Crandell-Rees feline kidney
DMEM	Dulbecco modified Eagle medium
dsRNA	Double stranded RNA
E protein	Envelope protein
FCoV	Feline coronavirus
FECV	Feline enteric coronavirus
FIPV	Feline infectious peritonitis virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IFN	Interferon
M protein	Membrane protein
miRNA	microRNA
MOI	Multiplicity of infection
N protein	Nucleocapsid protein
NK	Natural killer

nt	Nucleotide
ORF	Open reading frame
PKR	Protein kinase R
RIG-1	Retinoic acid-inducible gene I
RISC	RNA-induced silencing complex
RNAi	RNA interference
RSV	Respiratory syncytial virus
RT	Reverse transcription
S protein	Spike protein
SARS	Sever acute respiratory syndrome
sg	Subgenomic
sh	Short hairpin
siRNA	Short interfering RNA
TGEV	porcine transmissible gastroenteritis virus
TC	Cytotoxic T lymphocytes
TH	T helper cell
TLR	Toll-like receptor
Treg	regulatory T
UTR	Untranslated region
VSRs	viral suppressors RNAi
VA	viral associated

Chapter 1

General Introduction

Introduction

Feline coronavirus (FCoV) is a common virus in domestic cats usually associated with subclinical infection or mild enteritis. However, some FCoV-infected cats develop feline infectious peritonitis (FIP), a progressive and fatal disease that accounts for the majority of infectious disease-related deaths in pet cats [1].   FIP may manifest acutely with peritoneal and/or pleural effusion or may have a more protracted course with pyogranulomatous infiltration of multiple tissues. Both manifestations invariably end in death. The pathogenesis of FIP involves a mutant FCoV that can replicate efficiently in monocytes and macrophages leading to dysregulation of host cell-mediated immunity, allowing the virus to replicate unchecked to a high titer [2]. Systemic viral replication appears to play a critical role in FIP pathogenesis. Further, Contributing to the pathogenesis of FIP are cytokines and inflammatory mediators released from infected macrophages, other inflammatory cells that infiltrate tissue, as well as antigen-antibody complexes and complement activation [3].

To date there is no specific treatment for this fatal disease. In this work, we describe the use of individual siRNAs and/or siRNA combinations as a means of inhibiting FCoV replication. Using siRNAs is a novel antiviral strategy that specifically targets viral mRNA and genomic RNA for degradation by endogenous cellular enzymes.[4] This technology has been employed successfully for viral diseases such as viral hepatitis and severe acute respiratory syndrome (SARS) both in vitro and in ex-vivo [5].

Study hypothesis:

Inhibition of FCoV translation and replication using siRNAs (singly and in combinations) that specifically target coding and noncoding regions of the viral genome can help in treating FIP disease.

Study objectives:

1. Design feline coronavirus-specific siRNAs that can hybridize to viral coding or noncoding regulatory regions of the genome.
2. Optimize transfection condition.
3. Screen synthesized siRNAs individually and in different combinations for their effectiveness in inhibiting FCoV replication in cell culture as assessed by: a) relative quantification of the inhibition of intracellular viral genomic RNA synthesis by means of real-time, reverse-transcription PCR (RT-PCR) analysis; b) flow cytometric evaluation of the reduction of viral protein expression in infected cells; and c) assessment of virus replication inhibition via titration of extracellular virus with a $TCID_{50}$ infectivity assay.

Feline Coronavirus Review of Literature

Feline coronavirus

Feline coronavirus infection (FCoV) is ubiquitous in domestic cats and particularly common in multi-cat settings, where cats are shedding the virus in feces and sharing litter boxes. FCoV infection may be asymptomatic or may result in mild enteritis. In this case, the causative agent is referred as feline enteric coronavirus (FECV). In a relatively small

percentage of cats, a fatal, multisystemic, immune-mediated disease develops and is known as feline infectious peritonitis (FIP). In this case, the causative agent is referred as feline infectious peritonitis virus (FIPV) [6]. It is believed that cats most likely acquire FIPV by mutation of an endogenous FECV [7, 8] or rarely through excreted virus from other FIPV infected cat [9].

Classification and virion properties

FCoV is a member of the family Coronaviridae, order Nidovirales. The family Coronaviridae comprises two subfamilies, Coronavirinae and Torovirinae. Based on the serological and the genetic properties, the Coronavirinae subfamily has been divided into three new genera, Alphacoronavirus, Betacoronavirus, and Gammacoronavirus formerly named group 1, 2 and 3, respectively [10]. Further, each genus is divided into different species according to the identity of the sequence of the replicase domains of the polyprotein 1ab.The FCoV, porcine transmissible gastroenteritis virus (TGEV) and canine coronavirus (CCoV) show more than 96% homology within these domains and have been grouped in the same species, *alphacoronavirus 1*, within the *Alphacoronavirus* genus [11].

FCoV is an enveloped virus with a large single-stranded, positive sense capped and polyadenylated RNA genome of about 30 kilobases (Kb). The FCoV genome contains 11 putative open reading frames (ORFs). These ORFs encode viral non-structural replicase proteins; structural proteins (spike, envelope, membrane, and nucleocapsid proteins); and 5 accessory ORFs of unknown function. Based on sequence analysis of strain FIPV WSU-79/1146, the genome 5' untranslated region (UTR) consists

of about 310 nucleotides (nt) including the leader sequence (nt 1 to 92) that play an important role in the viral mRNA synthesis. The viral 3' UTR contains a putative bulged stem loop and pseudoknot followed by the poly (A) tail and is known to also play an important role in regulating the virus replication [12].

Viral proteins

Viral non-structural proteins

Approximately two-thirds of the viral genome encodes the replicase gene comprising ORFs 1a and 1b. Translation of the coronavirus replicase gene gives rise to the primary translation products polyprotein 1a (pp1a) and polyprotein 1 ab (pp1ab), which are processed by virus-encoded protease to generate 16 functional subunits of the replication/transcription machinery. Among these subunits are the RNA-dependent RNA polymerase and the helicase. ORF1 also encodes the viral proteases needed for this proteolytic process. The replicase products assemble into replication-transcription complexes (RTC) which are embedded in cellular membranes to make double-membrane vesicles that serve as viral RNA synthesis sites [12].

Viral structural proteins

There are four ORFs that encods the viral structural proteins; spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. Trimers of the (S) protein form the characteristic peplomers that protrude from the virion membrane. The S protein is responsible for viral attachment to specific host cell receptors and for cell-to-cell fusion [11]. The E protein has ion channel activity and plays an important role in viral envelope

assembly [13]. The M protein, the most abundant structural component, is a type III glycoprotein consisting of a short amino-terminal ectodomain, a triple spanning transmembrane domain, and a carboxyl-terminal inner domain [14]. The interaction between the M and S proteins facilitates the encapsulation of the S protein into new virion. The N protein encloses the viral RNA genome and forms the ribonucleocapsid. Also the N protein plays an important role in viral replication and is part of the RTC. The N protein has also been shown to localize to the nucleoli to exert an impact on the cell cycle progression to ensure maximal translation of viral mRNAs. In addition, the N protein plays an important role in circumventing the innate immune response because it is reported to be an interferon (IFN) antagonist [15].

Viral accessory proteins

The ORFs 3 and 7 encode the 5 accessory proteins; 3abc and 7ab. The function of these proteins is still unknown. They are not required for infection because a mutated virus in which ORF 3 and 7 are deleted can still replicate in vitro as well as the wild type strain. ORF7b encodes a secretory, hydrophilic protein of 22 kDa that has been suggested to serves as an immune modulator. The ORF 7a encoded protein is reported to be an IFN antagonist and protects the virus from the antiviral state induced by IFN, but it needs the presence of ORF3 encoded proteins to exert its antagonistic function [16].

Virus replication

Like other coronaviruses, FCoV uses a discontinuous transcription mechanism to synthesize both full-length and subgenomic (sg) negative-strand RNAs, which then function as templates for synthesis of full-length genomic RNA and positive-strand

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sgRNAs, respectively. Both viral genome and sgRNAs share a common 5' leader sequence and a common 3' UTR [17].

The replication cycle of the coronavirus starts by attachment of the S protein to the host cell receptor, followed by cell pentration via endocytosis. The low pH in the cytoplasm enables the release of the viral genome into the cytoplasm. In the infected cell, coronavirus gene expression starts with the translation of the replicase gene. The synthesized 16 replicase-transcriptase proteins assemble into the RTC together with other viral proteins (such as the N protein) and cellular proteins to mediate the replication of the genomic RNA and transcription of a nested set of (sg) mRNAs [18]. The RNA dependent RNA polymerase transcribes the genome to its complementary full length negative-stranded RNA, which serves as a template for the viral progeny genomes.

For viral genome transcription, the viral polymerases transcribe a negative sense sgRNA for each gene. The elongation of the synthesized negative-stranded sgRNA continues until the first transcription regulatory sequence (TRS) is encountered. TRS is a short motif (about 10 nucleotides) that can be found in the genome at the 3' end of the leader sequence and in front of each ORF and is very important to identify each gene boundary. Then the RTC will either disregard the TRS motif and continue the elongation or will stop synthesis of the minus strand and relocate to the 5' end of the genome in order to complete its synthesis and to copy the leader sequence located at the 5' most end of the viral genome. The leader sequence is then fused with the body of the synthesized sgRNA. This completed sg minus-strand RNA will then serve as a template for the positive stranded mRNA synthesis. The incorporation of the leader sequence in each sgRNA is important for signaling for the ribosome to translate the encoded protein [18]. After the

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synthesis of the viral proteins and progeny viral genome, the N proteins bind to the newly replicated genomic RNA strand to form the helical ribonucleocapsid. Then the M protein and the E proteins are integrated into the membrane of the endoplasmic reticulum and guide the virus assembly [14]. Subsequently the virus buds and gains its envelope from the endoplasmic reticulum membrane. Finally these viral progeny are transported by Golgi vesicles to the cell membrane and released via exocytosis.

Viral serotypes and biotypes

FCoV is classified into 2 main serotypes based on the sequence of the S protein; FCoV serotype I and FCoV serotype II. FCoV serotype I is highly prevalent in the field causing approximately 80% of all infections. FCoV serotype II is less prevalent and arose from a recombination event between a type I FCoV and CCoV that resulted in the replacement of the FCoV spike gene with the CCoV spike gene [19]. The majority of the research on FCoV has been conducted on serotype II, because it can propagate easily in cell culture and is, therefore, easy to work with in the laboratory.

Each serotype is further subdivided according to its pathogenicity into two biotypes: the avirulent FECV and the virulent FIPV. FECV is ubiquitous among cats and causes mild to often subclinical enteritis. In contrast, FIPV leads to a devastating, fatal, systemic disease that is marked by severe pyogranulomatous inflammation. Neither serotype is more commonly associated with FIP than the other, nor is the virus of FIP antigenically distinct from the avirulent FECV [6]. Furthermore, there is no consistent genetic difference that defines the two biotypes, though mutation of an avirulent FECV is thought to give rise to FIP in an individual. The differences among isolates from the same

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group of cats are small, rarely more than 1-2%, but can be up to 20% among isolates from different geographic regions [5]. Currently the marked difference between the two biotypes is the replication site. The primary replication site of FECV is localized in the lower portion of the intestinal tract, whereas the FIPV main replication site is the macrophages and monocytes. Thus FIPV and FECV can only be distinguished by the clinical disease they cause in cats.

Feline infectious peritonitis (FIP)

Feline infectious peritonitis (FIP) is a fatal, progressive disease of cats that responds poorly to treatment. This disease was first documented in the late 1950s by necropsy records at the Angell Memorial Animal Hospital, Boston MA [20]. Since then, there has been a steady increase in the incidence of the disease and this disease now accounts for the majority of infectious disease-related deaths in pet cats. In 1963 FIP was officially described as an important cats' disease, but the causative agent of FIP was not known to be viral until 1966 [21]. Then in 1972, it was known that the virus can cause two different forms of the disease identified as wet and dry forms [22]. In 1978 the antigenic relationship of FIPV to canine, porcine, and human Coronaviruses was determined and the notion that FIPV is a mutant form of FECV was first verified in 1998 [8].

Clinical and pathological features

Early in the disease, the FIPV infected cat develops nonspecific symptoms such as loss of appetite, weight loss, depression, rough hair coat, fluctuating antibiotic resistant fever and increased susceptibility to secondary infections. Once symptoms develop, usually there is an increase in the severity of the disease over the course of several

weeks, ending in death. Whereas the more specific signs of the disease depend on the form of the disease. There are two major forms of FIP, an effusive or wet form, and a non-effusive, parenchymatous, or dry form [6].

The effusive form of FIP is the most common form that can occur within 4-6 weeks of infection and is characterized by effusive inflammation of the serosal membrane and accumulation of fluid in the peritoneal cavity, or less commonly in the pleural cavity [23]. In some cases excessive pleural effusion can lead to respiratory distress and dyspnea. The peritoneal and pleural fluid is secondary to vasculitis and leakage of fluid from the vasculature. The fluid is usually a yellow-tinged, slight to moderately cloudy pyogranulomatous inflammatory exudate with diffuse granular fibrinous exudation covering serosal surfaces or floating in the effusion. Microscopically the pyogranulomas are made up of central aggregates of viral infected macrophages around small venules and surrounded by a rich inflammatory exudate containing mainly neutrophils and macrophages with a scattering of plasma cells and T-lymphocytes. Occasionally the wet form is associated with eye and/or nervous tissue lesions [6].

The dry, parenchymatous, or non-effusive FIP form is the chronic form of the disease that can incubate for months to years and is characterized by pyogranulomatous lesions of parenchymatous organs such as the kidneys, mesenteric lymph nodes, bowel wall, liver, central nervous system (CNS) and the eyes. The pyogranulomas of the dry FIP consist of foci of macrophages around vessels surrounded by dense infiltrates of lymphocytes (mainly B-cells) and plasma cells that extend into surrounding tissues, as seen in classical granulomas [6]. The clinical signs associated with the dry form depend on which organ is affected. If the kidneys are affected, they become enlarged due to

pyogranulomatous lesions in the cortex. The infected cat may exhibit polyuria, polydypsia, vomiting, proteinuria, and azotemia. The liver may have focal necrotizing pyogranulomatous inflammation involving hepatic capsule and adjacent hepatic parenchyma and affected animals may have increased liver enzymes, including ALT, GGT, alkaline phosphatase, or hyperbilirubinemia [24]. The brain and spinal cord may have meningoencephalitis and myelitis, with vasculitis. Thus the cats may exhibit seizures, abnormal behavior, central vestibular signs, ataxia, hyperesthesia, and abnormal postural reactions [25]. The eyes may have bilateral anterior uveitis due to lymphatic and plasmacytic infiltration that is manifested by swelling and discoloration of the iris, Keratic precipitates on the caudal aspect of the cornea may occur due to accumulations of fibrin, macrophages, and other inflammatory cells that have exuded from the inflamed uveal tract [26]. Lungs may have granulomatous pleuritis and pneumonia.

Lymphoid lesions are common in effusive and non-effusive FIP [24,6]. Splenic enlargement may be due to histiocytic and plasmacytic infiltration of the red pulp, hyperplasia of lymphoid elements in the white pulp, necrotizing splenitis with fibrin deposition and polymorphonuclear cell infiltrates (more common in wet FIP), or by more organized pyogranulomatous reactions(more common in dry FIP). Lymph node enlargement is usually limited to thoracic and abdominal nodes with lesions resembling those described for the spleen.

FIP pathogenesis

FCoV like other RNA viruses has a high mutation rate because viral polymerases lack proof-reading ability. Thus, several genetic forms of the virus may co-exist in the same animal at the same time. Most of these mutations have very little effect on the behavior of the virus. However, certain mutations that affects the function of certain genes may have a marked effect on the biological behavior of the virus and may lead to change in the viral tropism and/or virulence. Thus, the pathogenesis of FIP is believed to be caused by mutant FECV that gain the ability to replicate efficiently in monocytes/macrophages and lead to dysregulation of host cell-mediated immunity, allowing the virus to replicate systemically unchecked to a high titer. Speculation on the genomic locale of this mutation has involved the genes encoding the viral structural and nonstructural proteins. The spike gene was one of the viral structural genes that was implicated in the mutation of the FECV to FIP [27, 28]. Recently, one study looked at mutations in the S protein domain referred to as the S1/S2 domain that is cleaved by the viral protease to produce a functional S protein. This study found mutations in the S1/S2 motif in FIPV, whereas, many but not all of the FECV have a conserved motif. Though, these mutations influence the efficiency of the protease to cleave the precursor S protein, their exact role in the viral pathogenesis is not established. Some mutations in the motif were discovered to increased protease cleavage efficiency, other mutations decreased the efficiency, and in some cases the mutation had no effect on the cleavage efficiency [29]. Other researchers have proposed that mutation of viral accessory genes might be associated with variation in virulence observed in FCoV biotypes. Studies showed that the ORF 7 gene is often mutated in FECV, while conserved in FIPV [8]. Thus, the viral ORF 7 accessory gene was proposed

to be a virulence marker. However, mutation of this gene was identified later in the genomes of both FECV and FIPV, implying that the mutation/deletion in this gene is not correlated with FIP pathogenicity [30]. Further, several FIPVs that have been shown to display decreased virulence with tissue culture passaging have maintained intact, non-mutated 7b genes, confirming that mutation in ORF 7 gene is not associated with FIPV virulence.

Another accessory gene that was considered to be involved in FIPV pathogenesis was the 3c gene [31]. ORF3c is intact in all strains of FECV. Thus, it was proposed that the 3c is essential for the replication of FECV in the gut and mutation in this gene may lead to change in the viral tropism and enhance the viral replication in the monocytes and macrophages. However, as some FIPV strains (about 30%) seem to have intact ORF3c [6, 31], it is likely that 3c mutations are not the only cause of FIP pathogenesis and combinations of mutations might be required for FIP to develop. Despite intensive research in this area, there is no consistent genetic difference identified that defines virulent versus avirulent biotypes. At least one study found 100% homology between the structural and accessory genes of enteric and non-enteric (liver) viral genomes from a cat with FIP [9]. A distinguishing factor appears to be quantitative differences in viral RNA levels in the blood of cats with and without FIP [32]. Rising amounts of viral RNA in the blood seen in the end-stage of FIP may indicate that loss of immune control leads to enhanced viral replication and disease progression. Thus, the increased viral replicative capacity seems to be an important element of FIP pathogenesis.

Another crucial element of FIP development is the lack of an effective immune response. Specifically cell-mediated immune response (CMI) is essential for protection

against FIP. Cats with a strong CMI will survive, while cats with poor CMI will develop FIP. Lymphocyte depletion has been observed in affected cats, despite lack of viral infection of these cells. The cause of the lymphocyte depletion is unknown, but the release of inflammatory mediators and cytokines from viral infected cell has been suggested to play a critical role in this depletion [2]. Cytotoxic T lymphocytes (TC), T helper (TH), regulatory T (Treg) as well as natural killer (NK) cells are markedly depleted in the blood of cats with FIP[2, 33]. As a consequence the capacity of the innate CMI mediated by the NK as well as the adaptive CMI mediated by the TC to destroy the infected macrophages and to clear the virus is reduced. Further the depletion of the Treg cells enhances the immune mediated pathology associated with the FIP infection. While cell-mediated immunity is compromised, the humoral response is exaggerated. The virus-specific antibodies bind to the virus in circulation, or in tissue sites, precipitating cellular damage. As a consequence, the lesions associated with FIP are due primarily to immune-mediated destruction. Contributing to the pathogenesis of FIP are cytokines and inflammatory mediators released from infected monocytes/macrophages, other inflammatory cells that infiltrate tissues, as well as antigen-antibody complexes and complement activation [3]. Also leukocytes from FIP cats were found to have increased expression of adhesion molecules that enhance their endothelial adherence ability and subsequent migration to the surrounding tissue to add to the pyogranulomatus lesion associated with FIP. Further, vascular endothelial growth factor (VEGF), produced by FIPV infected monocytes and macrophages, induces vascular permeability and may be an important contributor to abdominal and thoracic effusions in cats with wet FIP [34].

In addition to virus specific factors, genetic and environmental factors may have a role in FIP. The ability of an animal to produce an effective immune response depends in part on its genetic composition. Studies have shown a genetic predisposition to disease occurrence. Certain breeds, including Bengals, Birmans, and Himalayans, are more likely to develop FIP. However, the exact host genetic factors that may be involved remain to be identified. Studies have shown that the monocytes from different cats do not have the same susceptibility to FCoV infection, suggesting a genetic role in the susceptibility of monocytes to FCoV infection. Environmental factors including; stressors, such as crowded housing, trauma, pregnancy, surgery or other infections have been assumed to be associated with FIP development. Stress can depress the host immune system increasing the likelihood that FIPV will establish itself in the body or may allow an FIPV that is being successfully contained to become active. Disease caused by feline herpesvirus and other common upper respiratory pathogens are good indicators of cattery or shelter stresses. If a cattery or shelter is having a lot of problems with these upper respiratory infections, it is likely that they will also have problems with FIP. Therefore, it is important to minimize the exposure of cats to stress especially FCoV seropositive ones.

FIP treatment and control

To date, treatment of FIP has been ineffective, with affected cats progressing inevitably to death. Because of the immune-mediated component of the disease, therapies directed at enhancing the CMI response and/or suppressing the humoral response have been tried with little or no success. For the enhancement of the host immune response, human and feline recombinant IFNs have been tried. Although IFN showed inhibition of FCoV replication in vitro, in vivo studies have shown no effect on

survival time or quality of life [35]. Recently, polyprenyl, a new therapeutic that is postulated to enhance T lymphocyte activity, has been used with some success in dry forms of FIP but not with the common effusive form of the disease, which involves wider viral spread and replication than the dry form [36]. Drugs used to suppress the host humoral response including; glucocorticoids and cyclophosphamide could not alleviate the FIP signs [37].

Another approach of treatment is to target the virus. Ribavirin a nucleoside analog antiviral drug that has been tried experimentally to treat FIP but it was toxic in cats and resulted in severe side effects [38]. Pyridine N-oxide derivatives have been tried and shown to inhibit FCoV replication in vitro, however, the exact anti-coronavirus molecular target is not known [39]. Recently inhibitors of the viral protease have been tried to inhibit FIPV replication in cell culture. Protease is an enzyme encoded by the viral protease gene and responsible for cutting viral polyproteins into their final form, which is essential for the assembly of the progeny virus. Clinical trials to show the effect of protease inhibitor in FIP infected cats are currently lacking [40].

To overcome the lack of an effective treatment of this disease, various vaccination trials have been investigated. Unfortunately, there is only one commercially available vaccine for FCoV on the market. It is an intranasal vaccine containing a temperature-sensitive mutant of FCoV that replicates in the upper respiratory tract to induce a strong mucosal IgA response and stimulate cellular immunity [41]. This vaccine provided protection in about 70% of seronegative cats, while in seropositive cats the vaccine showed no protection. Thus the efficiency of the vaccine is not 100% [42]. Furthermore, the vaccine should not be given until 16 weeks of age whereas at-risk cats often get

infected by 4-6 weeks of age after the maternal antibody has waned. Other vaccination trials using a recombinant poxvirus that expressed the viral M protein or using live attenuated vaccine consisting of FIPV strains in which the 3abc or the 7ab accessory proteins were deleted showed either partial protection or appeared to be ineffective in controlling FIP [43, 44, 45].

 In general, prevention of FIP is dependent on preventing FECV infection because FIPV arises from mutant FECV. Therefore, FECV should be eradicated from catteries and certain steps must be followed to achieve this goal. The chronic shedders should be removed, the seropositive cats should be kept in quarantine until they have cleared the virus, and all cats that enter the cattery should be coronavirus free as tested by real-time, reverse-transcription PCR (RT-PCR) that can detect the viral genome in the feces and/or serology to determine the antibody titer. Also high hygiene standards should be maintained to prevent the fecal –oral transmission of the virus. Nonetheless, these procedures are labor intensive and not easy because of the widespread nature of the disease and the ease transmission of the virus [6].

RNA Interference Review of Literature

History RNA interference

RNA interference (RNAi) is a natural cellular response to the presence of double stranded RNA (dsRNA) which results in sequence-specific silencing of gene expression in eukaryotes, and is considered to be a natural defense mechanism against viruses and transposons in some organisms. RNAi is also thought to play a role in regulating cellular gene expression. Since its discovery, RNAi has been developed into a widely used

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technique for generating genetic knock-outs and for studying gene function by reverse genetics. The RNAi phenomenon was first discovered in 1990 in plants [46]. In an attempt to make the color of the flower brighter, researchers created transgenic plants in which the pigment making gene was over-expressed. The extra copies of this gene resulted in suppression of the plant endogenous gene expression as well as the transgenes. This phenomenon was then known as "posttranscriptional gene silencing" and "quelling". In 1992, a similar phenomenon was observed in *Neurospora crassa*, where the introduction of homologous RNA sequences caused quelling of the endogenous gene [47]. Later, the RNAi phenomenon was also documented in animals [48].

It was thought that introduction of sense or antisense strand RNAs of certain endogenous genes resulted in gene suppression, but in 1998, studies showed that dsRNA was the source of sequence-specific inhibition of protein expression, The phenomenon was renamed RNAi [48]. At that time, the use of RNAi as a tool was limited to lower organisms because delivering long dsRNA for RNAi was nonspecifically inhibitory in mammalian cells as described later. Studies later showed that the actual molecules that led to RNAi were short dsRNA oligonucleotides, 21 nucleotides in length, processed internally by an enzyme called Dicer. These short dsRNA oligonucleotides were termed short interfering RNAs (siRNAs), and in 2001, it was demonstrated that these siRNA could directly trigger RNAi in mammalian cells without evoking nonspecific effects. . This has resulted in the use of siRNAs in biomedical research to selectively knockdown genes of interest to identify and assess their functionality. Also, siRNAs have been considered to be a new therapeutic means to combat genetic and/or viral disease [49].

Sources and processing of small regulatory RNAs in the RNAi Pathways

The sequence specificity of RNAi is assured by a group of small regulatory RNAs. These small RNAs are classified according to their cellular origin and biogenesis pathways into: microRNAs (miRNAs) and siRNAs. miRNAs originate from endogenous genes and are involved in the regulation of development and physiological processes. In human about 5% of the genome encodes more than 1,000 miRNAs that regulate at least 30% of our genes. Whereas, siRNAs are naturally generated in some eukaryotes from exogenous and endogenous long dsRNAs such as infecting viruses and transposable elements [50]. Although siRNA and miRNA have different origins, both types of small RNAs are closely related in their biogenesis, as each small RNA associates with an Argonaute (AGO) family protein to form a sequence-specific, gene-silencing ribonucleoprotein with specificity conferred by base-pairing between the small RNA and its target mRNA .

Typically, the biogenesis of a miRNA occurs in the nucleus with a transcript known as a primary miRNA (pri-miRNA); such transcripts are at least 1,000 nt long, containing single or clustered double-stranded hairpins that have single-stranded 5′ and 3′ terminal overhangs and ~10-nt distal loops. These stem-loops are recognized by a microprocessor complex, comprising Drosha, an RNase III family enzyme, and its cofactor DiGeorge syndrome critical region gene 8 (DGCR8), a protein containing two dsRNA-binding domains (dsRBDs). DGCR8 recognizes the pri-miRNA's junction of stem and single-stranded RNA, which likely helps position Drosha for the endonucleolytic cleavage it performs on the stem ~11 base pairs (bp) from the junction to yield a 60-nt precursor miRNA (pre-miRNA) containing 2- nt 3'overhangs [51, 52]. Exportin 5

transports this pre-miRNA to the cytoplasm. In the cytoplasm, the biogenesis pathways of both endogenous miRNAs and exogenous siRNAs converge. Both miRNA and siRNA precursors are trimmed down to a dsRNA duplex by Dicer, a second RNase III enzyme, acting in association with Tar RNA binding protein (TRBP) [53, 54]. The resulting dsRNA is a duplex of 21- to 25-nt strands, bearing a 2-nt overhang at each 3' terminus and a phosphate at the 5' end. Subsequently, one strand of the small RNA duplex is incorporated into RISC to function as a mature strand and guide RISC to target mRNAs [55], while the passenger strand is discarded (cleavage) [56]. Strand selection depends on the degree of base pairing at the duplex 5' end; the strand less stably base paired at its 5' end (has the weakest binding energy) is preferentially incorporated into RISC. The RISC mainly consists of three proteins; Dicer, AGO, and a dsRNA binding protein (dsRBP). Once the dsRNA helix is presented to AGO, the 3' end and 5' phosphate of the guide strand are bound by the AGO's PAZ and MID domains, respectively, generating the RISC. Then the loaded RISC performs cellular surveillance, binding ssRNA such as mRNA with complementarity to the AGO-bound guide strand. Guide strand nucleotides 2–8 from the 5'end constitute the seed sequence and initialize binding to the target [57]. The fate of a targeted mRNA is dependent on the degree of complementarity. Perfect complementarity, often observed in plants and exogenous siRNA, generally results in endonucleolytic cleavage of the mRNA. Imperfect complementarity, observed for most mammalian and viral miRNA targets, results in translational repression that can then lead to mRNA destabilization. RISC-bound mRNAs often localize to cytoplasmic processing bodies (Pbodies),which exclude the translational machinery and contain proteins involved in mRNA remodeling, decapping, and deadenylation, as well as exonucleases. P bodies

themselves, however, are not necessary for translational silencing. Recently, data showed that there are several other pathways for the biogenesis of mature miRNA that depend mainly on AGO for cleavage of pre-miRNA to mature miRNA.

Viral infections and the RNAi response in mammalian cells

Since the discovery of the RNAi phenomenon, there was an important question raised "Does RNAi contribute to the innate antiviral defense mechanism in mammalian cells?" [58] To address this question, initially, an understanding of the mammalian response to viral infection is needed, so a brief discussion is included. Mammals have evolved highly sophisticated and effective systems of innate and adaptive immune responses to infections. The adaptive immune system employs receptor systems which have hundreds to thousands of genes that recombine and mutate to evolve highly specific humoral (antibody) and cell mediate immunity (T-cell) responses. The innate immune response involves IFNs, NK cells and macrophages. IFNs are cytokines that function as the host's first line of defense against viral infection. Activation of this innate immune response is triggered partly by dsRNA, a common viral replication intermediate. This long dsRNA (>30 bases) interacts directly with cellular proteins, such as protein kinase R (PKR), retinoic acid-inducible gene I (RIG-1) or Toll-like receptor (TLR) which triggers signaling pathways that lead to the expression of type I IFNs (IFNα and IFNβ) and the activation of non-specific RNases. Binding of these IFNs with their receptor leads to the expression of a large number of genes which leads to a generalized antiviral response at multiple cell levels. Expressed genes include those like 2,5 oligoadenylate synthetase that activates RNase L to catalyze the degradation of the viral RNA genome in non-sequence specific manner [59]. Thus, it appears that the mammalian cell does not require

the simple RNAi based antiviral mechanism to defend against viral infection. Therefore, RNAi machinery is mainly conserved in mammalian cells to regulate gene expression. In contrast, in plants the RNAi mechanism functions as an adaptive antiviral immune response [58]. In plants, RNA virus infection can stimulate production of viral dsRNA-derived siRNAs to specifically target viral genomes and mRNAs for degradation. The difference of function of RNAi machinery in mammalian cell and plant cell may be related to the difference in the composition of the RNAi machinery in both cells. The plant cell has up to four dicers as compared with the mammalian cell that only has one dicer [60]. Also in plant cell the siRNA can spread systemically to distribute its immune response function [61]. This systemic spread does not occur in mammalian cell due to lack of an RNA dependent RNA polymerase to produce additional siRNA molecules. In spite of the lack of natural antiviral function, studies in mammalian cell suggest that certain viruses can interact with cellular RNAi machinery to generate viral miRNAs [62]. These virally encoded miRNAs can regulate both cellular and viral genes, to lead to a successful viral infection. Several of the virally encoded microRNAs function to down-regulate the expression of factors of the innate immune system, including proteins involved in promoting apoptosis and recruiting effector cells of the immune system. Viruses have also evolved the ability to downregulate or upregulate the expression of specific cellular miRNAs in the favor of their replication [63].

RNAi based antiviral therapy

Harnessing RNAi machinery to silence disease in mammalian cells was initially challenging because introduction of foreign dsRNA into mammalian cell activates a nonspecific IFN response, as mentioned above. Nevertheless, this limitation was shortly

solved by using chemically modified synthetic siRNA duplexes that are too short to induce this non-specific inhibition (< 30 bases) [64]. Currently, siRNA can be either applied directly to cells or to be expressed in cells (such as short hairpin RNA (shRNA)) for successful RNAi in cell culture and in vivo. shRNA is short hairpin dsRNA which is created in the cell from a DNA construct encoding a sequence of single stranded RNA and its complement, which ultimately is processed to siRNA. Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors.

The specificity, efficiency and the ability to design siRNA (or vector encoding them) to target any gene of interest shows that siRNA is a promising therapeutic tool. Using siRNAs that specifically target viral mRNA and/or genomic RNA for degradation by endogenous cellular enzymes is considered to be a powerful antiviral therapeutic strategy. Various viruses including; Semliki forest virus, poliovirus, dengue virus, influenza virus, hepatitis C virus (HCV), and SARS have been successfully targeted by siRNAs in vitro and ex-vivo [5, 65-68]. Despite the great success of the RNAi study in vitro, the therapeutic application of this technology clinically has not progressed as well, because of several practical obstacles including; the potential of inducing off-target effects, triggering innate immune responses and most importantly delivering siRNA into the target cell. These obstacles are addressed in greater detail below. Despite these challenges, several RNAi based therapeutic approaches that target respiratory syncytial virus, Ebola virus, and human immunodeficiency virus (HIV), have progressed to clinical trials. Topical administration(via intranasal aspiration) of unmodified siRNA directed against the mRNA of the respiratory syncytial virus (RSV) nucleocapsid (N) protein showed a promising antiviral effect in phase II clinical trials [69, 70] . An anti-Ebola siRNA

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targeting the Ebola virus RNA polymerase L protein formulated in stable nucleic acid-lipid particles is currently in phase I clinical trials. [71, 72]. RNAi based therapy against HIV is being evaluated in phase I and II clinical trials in which autologous hematopoietic cells are transduced ex vivo, followed by infusion back into the patient [73].

Obstacles of RNAi based therapy

Efficiency and specificity

siRNA and target mRNA accessibility play an important role in achieving potent gene knockdown. The gene-silencing efficiency of RNAi is strongly affected by the local structure of mRNA at the targeted region [74]. Nucleotides in the mRNA can often form hydrogen bonds (i.e., becoming double-stranded) with other nucleotides in the same mRNA molecule, and form secondary structure (hairpin or stem) that may affect the accessibility of the siRNA to its target region. Therefore, computer software has been used to predict possible secondary structures for a given mRNA [75]. Also, it is recommended to stay away from target sites at either the 5' or 3' the mRNA, since proteins involved in translational regulation or mRNA processing may bind to these terminal regions and could interfere with the siRNA–mRNA interaction [76].

One of the advantages of RNAi is its ability to specifically target the gene of interest. However, this specificity can be masked by a nonspecific effect or in another words the unanticipated off-target effects that may occur due to; 1) unintended incorporation of sense strand (passenger strand) into RISC and subsequently silencing of unintended targets or 2) siRNA recognition of unintended mRNAs having partial homology, especially in the seed region of the antisense strand (guide strand) with

sequences in the 3'UTR of the off-targeted gene. [77, 78]. The imperfect pairing of the siRNAs with unintended mRNA enables the siRNA to function as miRNA and lead to translational repression (miRNA like off-targeting) [77]. Although the magnitude of off-target transcript silencing is generally lower than that of the on-target gene, small changes in the expression levels of host proteins, such as essential transcription factors, might translate to serious unintended side-effect.

In general, because siRNA off-targeting is concentration dependent, it is recommended to use siRNA at its lowest effective concentration to reduce or eliminate siRNA off-target effects while preserving on-target effect.[79, 80]. However, in some experiments, use of a low dose of siRNA did result in reduction of off-target effects, but it was at the expense of target gene knockdown, which was also reduced. [81]. To resolve this problem, siRNA combinations that include two or more potent individual siRNA that target the same gene have been used. Use of siRNA combinations has shown strong knockdown of the target gene while also significantly minimizing the off-targeting effects [81]. Further to avoid off-target silencing, various chemical as well as backbone modifications have been introduced to the siRNA structure (both the sense and antisense strand). On the sense strand, modifications have been made to prevent strand entry into RISC, thus eliminating off-target effects by this strand. Studies showed that internally destabilized duplexes and 5' O- methylation of the sense strand itself impaired its silencing effect [82, 83]. Another strategy that has been used for strand selection and to abrogate gene silencing of the modified strand is locked and unlocked nucleic acid modified siRNAs. Locked nucleic acids are a family of conformational locked nucleotide analogues, where the 2′-position of the ribose is connected to the 4′-position via a

methylene bridge to lock the sugar backbone in the 3′-endo conformation, whereas unlocked nucleic acid monomers are acyclic derivatives of RNA lacking the C2–C3-bond of the ribose ring of RNA that lower the stability of the duplex and force the correct strand to into RISC [84].

To address off-targets generated by the antisense strand, key nucleotides that are essential for off-targeting were modified to impair the majority of antisense strand off-target effects, while preserving on-target knockdown. In this regard, a study showed that siRNAs with the seed arm replaced with a cognate DNA sequence exert very few off-target silencing [85]. Other studies showed that a modified siRNA backbone structure with a single nucleotide bulge placed in the antisense strand were reported to be able to discriminate better between perfectly matched and mismatched targets and significantly reduced off-target silencing, with no loss in silencing of the intended target [86].

Side-effects

The RNAi phenomenon is a fundamentally important regulatory mechanism in the cell, and harnessing this phenomenon in the interests of therapeutic application could result in serious side effects and cell toxicity. Exogenously introduced siRNA/shRNA sequences utilize the components that make up the cellular RNAi machinery involved in gene regulatory mechanism, thereby reducing the accessibility of the machinery to the cellular miRNAs that are essential for regulating the cellular gene expression [87, 88].

In addition to the off-target effects, mentioned earlier, studies have shown that siRNAs can be recognized by the host innate immune response and stimulate the production of pro-inflammatory cytokines and type I IFN, which may predispose to a

pathological inflammatory response [89]. Studies have shown that some siRNAs can be recognized by TLR3 that recognize dsRNA in a sequence-independent pattern or recognized by TLR7 and 8 that recognize ssRNA in sequence dependent pattern. Due to the primary localization of TLR in the endosomal compartment, these receptors most readily recognize and bind ligands during internalization of siRNAs delivered by cationic lipids and polymers [90]. Other delivery system such as electroporation, hydrodynamic delivery, or peptide transduction can bypass transit through this compartment and can evade recognition by these receptors. The other receptors that can recognize foreign RNA including PKR and RIG-I are localized in the cytoplasm and mainly recognize dsRNA which are longer than traditional siRNAs (>30 nt) or have different end structures than standard siRNAs. For example, RIG-I recognizes a 5′-triphosphate end, which is generated in vivo during viral replication but is not triggered by the 5′-cap structure present on mammalian RNA. Long dsRNAs naturally exist within mammalian cells and these usually do not elicit an immune response; this is achieved by exclusion of these RNAs from endosomal TLRs and endogenous chemical modification [90]. Sugar modifications, such as 2′O Methylated RNA, and some base modifications, such as pseudouridine, are common in mammalian tRNAs and rRNAs to help these RNAs to evade initiating an autoimmune response. Likewise, there are various chemical modification strategies (mentioned earlier) that can enable the synthetic siRNAs to evade the immune system detection [91]. Indeed, better understanding of the mechanisms that drive the immunostimulatory properties of the siRNA as well as the recognition pathway is very important to overcome this side effect.

Viral escape mutants

Viruses have a unique ability to develop resistance to antiviral drugs. Viruses, especially RNA viruses replicate with high mutation rates that result in genetic diversity and evolution of resistance to antiviral therapy. Therefore, it is not surprising to know that viruses have great ability to escape from RNAi inhibition as well. Single mismatches within the targeted region, or even its entire deletion, resulted in escape from RNAi inhibition effect by poliovirus, HCV and HIV-1 [92-94]. The location of the point mutation in the target sequence determines the level of resistance. 100% homology in the seed region nt 2 - 8 in the guide strand are important for target recognition so any mutation in this region may affect the siRNA inhibition efficiency. However, a few mutations within the 3' and 5' ends of the target sequence can be tolerated. Therefore, differences observed in the level of resistance conferred by point mutations are associated with the location of the mutation in the target sequence. The basis for this positional resistance difference is related to the mechanism of incorporation of the siRNA into the RISC and its AGO protein induced cleavage. To overcome this limitation, combinations of different siRNAs have been used to delay viral escape of poliovirus and HCV [92, 93]. Further, targeting multiple genes has been shown to be more effective than targeting a single gene. However, with use of multiple siRNAs, the RNAi machinery may become saturated, with no boosted effect.

A second approach to overcome viral escape mutants is to target highly conserved genes that are essential for viral replication, as mutation in these regions could lead to a deleterious effect on the virus [95]. Thus, targeting these regions may prevent or delay viral escape mutant development. Nevertheless, silent mutations that lead to changes in

nucleotides but not in amino acids may occur in these regions without compromising the viral replication ability and allow the virus to escape the RNAi inhibition effect. The UTRs of RNA viruses are considered to be a good example of a highly conserved region. However, it is important to determine the susceptibility of viral untranslated regions because interaction in the UTRs with proteins or RNAs might shield them from RISC mediated recognition and cleavage. A third approach to prevent virus escape is to target the host factors required for virus replication, such as the virus receptor [96]. Indeed, it seems that combination of these three approaches will be very effective in preventing virus escape mutants.

Viral suppressors of RNAi

Across eukaryotes including; plants, *Drosophila*, mosquitoes, nematode worms and fungi, antiviral RNAi directed by virus-derived siRNAs represents a major antiviral defense that the invading viruses have to overcome in order to establish infection. As a counter defense mechanism, viruses of these hosts express proteins termed viral suppressors of RNAi (VSRs) that can block the biogenesis and/or function of viral siRNAs. VSRs may inhibit the viral RNAi pathway at various stages and in multiple ways. Some bind dsRNA and sequester siRNAs away from the RNAi pathway such as P10 of vitiviruses. In some viruses the VSRs either bind to or degrade AGO, to prevent the RISC from cleaving target RNA such as 2b protein of cucumoviruses and P0 of poleroviruses respectively. Others VSRs inhibit cell-to-cell signalling of immunity, such as the P30 of tobamoviruses [97].

In mammalian cells, though siRNA is not a natural defense mechanism, some research suggests mammalian viruses have evolved mechanisms that suppress RNAi. This RNAi suppression mechanism may be raised to subvert the effect of the cellular miRNAs that may interfere with virus replication. Since cellular miRNAs are highly conserved during evolution it seems unlikely that viruses would fail to evolve mechanisms to prevent their inhibitory effect. In mammalian cells, good example is adenovirus. Adenovirus has developed several strategies to target the RNAi pathways during infection through its viral associated RNAs; VA RNAI and VA RNAII. VA RNAs are short RNA polymerase III transcripts which have highly folded structure with imperfect stem that resemble cellular pre-miRNAs. Therefore, the VA RNA is able to interfere with the RNAi/miRNA pathways both at the level of pre-miRNA processing and RISC assembly. The VA RNAs are produced in a high abundance, enabling them to act as a competitive substrate for dicer, and as a consequence, they become a preferred substrate for RISC assembly. Further, it has been shown that the terminal stem of VA RNAi binds to the Exportin-5 receptor and therefore, reduces the efficacy of cellular pre-miRNA binding to Exportin-5 and its transportation from the nucleus to the cytoplasm [62].

Influenza virus has been shown to suppress RNAi in plants and in Drosophila but not in mammalian cells [98]. Although various virus-encoded RNAi suppressors have been identified, recently, some of these suppressors have been questioned and the ability of mammalian viruses to suppress RNAi pathway in natural host cells has remained controversial.

Delivery

Safe and effective delivery of siRNA to the target sites and then into cells is one of biggest challenges preventing siRNA clinical applications. In this regard, viruses differ greatly with respect to cell and tissue tropism, which will pose further hurdles to the delivery challenge. For example, chronic systemic viral infections require systemic, prolonged expression or repeated delivery of siRNAs. However, local delivery of siRNAs is feasible for the treatment of acute viruses with a relatively restricted tissue tropism, such as RSV, parainfluenza virus and influenza [67, 99]. Indeed, local regional delivery of siRNAs has fewer barriers compared to systemic delivery. siRNA is short double-stranded RNA with a net negative charge, which makes it difficult to transfect into cells. Additionally, siRNA is rapidly degraded by nuclease activity in plasma and cells, and siRNA is also rapidly excreted by the kidneys. Therefore, as previously described, several types of chemically modified siRNAs have been developed to not only minimize off-target effects and inhibit immune system stimulation, but these modifications also improve the siRNAs stability to nuclease and its pharmacokinetic properties [100]. Several studies have indicated that chemically modified siRNAs can maintain potency and show increased serum stability beyond 72 hours whereas the same sequence in unmodified form is completely degraded in just 5 hours in mouse serum.

The delivery system used for uptake of the negatively charged siRNAs into the cells can also provide stability against nucleases in the serum. In addition an ideal delivery system should be able to provide: evasion of the immune system, avoidance of non-specific interactions with serum proteins and non-targeted cells, prevention of renal clearance, and exit from blood vessels to reach target tissues. Once internalized into the

cells, the siRNA must be able to release from the delivery system in a reversible manner to conserve its activity in the cytosol, incorporate into the RISC and induce gene silencing.

To achieve effective siRNA delivery, several delivery systems have been developed and examined. These delivery systems can be classified into two main groups; viral and non-viral delivery systems. Viral delivery systems are mostly used to deliver DNA-based expression cassettes that express shRNA. There are five groups of viral delivery systems used for RNAi (Retrovirus, Lentivirus, Adenovirus, Adeno-Associated-Virus (AAV), and Baculovirus). Although viral delivery systems have high transduction efficacy, due to the inherent ability of viruses to transport genetic material into cells, the potential of mutagenicity or oncogenesis, inflammation and host immune responses, limited viral loading capacities and high cost hinder their application [101]. For these reasons, different non-viral siRNA delivery systems have been developed including; cationic carrier, nanoparticles and cell-penetrating peptides (CPPs) [102, 103].

Positively charged cationic liposomes and polymers have been shown to be able to deliver siRNAs through the cellular membrane and achieve a high level of RNAi [104]. However, the highly positive surface charges of these molecules tend to interact with the erythrocytes and/or serum proteins, leading to aggregation with erythrocytes and bad pharmacokinetics, mainly due to the rapid uptake of these aggregates by the mononuclear phagocytic system [90]. In some cases this interaction has been used to enhance the delivery to certain tissue and cell types. For example, many liposomal delivery systems, as well as siRNA conjugated to lipophilic molecules, interact with serum lipoproteins and subsequently gain entry into hepatocytes that take up those lipoproteins [105]. Means to improve the cationic delivery vehicles have been explored, including use

of hydrophilic polymerases such as polyethylene glycol (PEG), which was used to shield the cationic vehicles to increase circulation time and minimize non-specific interactions of particles with serum proteins, cells of the innate immune system and other non-targeted tissues [106]. Despite the improvements, the use of cationic carriers as delivery vehicle is limited in vivo because of their toxicity. Use of cationic liposomes in vivo was shown to elicit dose-dependent toxicity and pulmonary inflammation by promoting release of reactive oxygen intermediates [107].

Another widely used cationic delivery vehicles is polyethyleneimine (PEI). PEI is a synthetic polymer that has strong buffering capacity and can increase the endosomal escape of the siRNA, consequently preventing enzymatic degradation and TLR mediated immunological response in endosomes/lysosomes. However the toxic effect caused by the non-degradable nature of this synthetic polymer limits its wide application in vivo. Recently, various synthetic cationic liposomes and polymers-based nanoparticles have been developed that offer enhanced transfection efficiency combined with reduced cytotoxicity, as compared to traditional ones. The incorporation of distinct layers composed of lipid molecules with varying physical and chemical characteristics into the polymer nanoparticle formulation resulted in improved RNAi efficiency through better fusion with cell membrane and entry into the cell, enhanced release of RNAi molecules inside the cell, and reduced intracellular degradation of RNAi-nanoparticle complexes [108].

In general, various delivery systems improved the rate of cellular uptake by incorporating targeting ligands that bind specifically to receptors on target cells to induce receptor-mediated endocytosis [109]. Other systems use cationic CPPs, such as

transporten (a chimeric peptide composed of galanin and mastoparan), penetratin (from Drosophila) or TAT (Peptides derived from the transactivating regulatory protein of HIV), the VP22 protein from herpes simplex virus and polyarginine [110-112]. The strong positive charge on these peptides promotes binding and condensation of negatively charged siRNA, allowing CPPs to be used for the delivery of siRNA into a variety of cultured cells through endocytosis or non-endocytic mechanism with minimal toxicity [113]. Most siRNA delivery systems undergo cellular internalization through endocytosis. Endocytosed materials are taken up into membrane-bound endocytic vesicles, which fuse with early endosomes and become increasingly acidic as they mature into late endosomes. Some delivery systems incorporate materials that are designed to respond to this low-pH environment by becoming membrane-disruptive in order to trigger the release of siRNA from endosomes into the cytoplasm.

Development of an efficient delivery system is one of the most challenging obstacles to turn siRNAs into clinical therapeutic applications. Many different siRNA delivery systems were synthesized, characterized and their gene silencing efficiency were tested in vitro and in vivo, and some of them have showed a promising results. Indeed, the improvement in siRNAs designs rules and the continuous progress in the development of new delivery system will lead to promising clinical application of RNAi as antiviral therapy.

Chapter 2

Effectiveness of Small Interfering RNA (siRNA) to Inhibit Feline Coronavirus Replication in Vitro

Abstract

Feline coronavirus (FCoV) is an enveloped, single-stranded RNA virus which is ubiquitous in domestic cat populations worldwide. Mutation of FCoV to a biotype that has the ability to replicate in the macrophages and monocytes results in the development of feline infectious peritonitis (FIP). FIP is an invariably fatal disease that is considered to be the major cause of infectious death in young cats. To date there is no effective treatment for FIP affected cats.

In this study, we evaluated the ability of siRNA to inhibit the in vitro viral replication and gene expression of FCoV. Five synthetic siRNAs targeting five different regions of the FCoV genome were screened for their antiviral effects against two different strains of FCoV; FIPV WSU 79-1146 and FECV WSU 79-1683. Efficacy was assessed by flow cytometric evaluation of the reduction of viral protein expression in infected cells and relative quantification of the inhibition of intracellular viral genomic RNA synthesis by means of real-time, reverse-transcription PCR analysis.

The 5 examined siRNAs targeted the leader sequence, untranslated region, replicase gene, membrane gene, and nucleocapsid gene. These siRNAs exhibited a variable inhibitory effect on viral replication in vitro and resulted in decreases in the protein expression of FIPV WSU 79-1146 and FECV WSU 79-1683. These preliminary findings shows that FCoV translation and replication can be specifically inhibited using siRNA targeting coding and noncoding region of viral genome, suggesting a potential therapeutic application of RNAi in treating FIP.

Introduction

Feline coronavirus (FCoV) infection is common in domestic cats and usually causes subclinical or mild enteritis. Primarily, FCoV targets intestinal epithelial cells and is shed in feces of infected cats, often continuously or intermittently for months or years [6]. In certain cats, virus mutation may occur that allows the virus to replicate efficiently in monocytes and macrophages, resulting in the development of FIP [8]. FIP is a fatal progressive disease that is manifested in two lethal forms; the effusive form and non-effusive form [6]. To date, therapeutic possibilities for cats with FIP are extremely limited and do not specifically target the virus. Therefore, development of a new therapeutic for FIP would be valuable. Recently, a novel antiviral strategy has been developed that utilizes siRNAs to specifically target viral mRNA and genomic RNA (gRNA) for degradation. siRNA utilize the cellular machinery for gene silencing in a sequence dependent manner [4]. This technology has been employed successfully for viral diseases such as human immunodeficiency virus (HIV), viral hepatitis and severe acute respiratory syndrome (SARS) both in vitro and in vivo [114-116].

Given the important role of increased FCoV replication during the pathogenesis of FIP, it is likely that the development of siRNAs that specifically target the virus and limit its replication will help in treating this fatal disease. The objectives of our study were to design a panel of feline coronavirus-specific siRNAs that can hybridize to viral coding or noncoding regulatory regions of the genome and screen synthesized siRNAs for their effectiveness in inhibiting FCoV replication in cell culture. Inhibition of viral growth was measured by quantitative real-time RT-PCR for relative quantification of the inhibition of

intracellular viral genomic RNA synthesis and flow cytometry for evaluating the reduction of viral protein expression in infected cells.

Materials and Methods

Cell culture and viruses

Crandell-Rees feline kidney cells (CRFK)[a] were propagated in DMEM/F-12[b]supplemented with 10% heat inactivated fetal bovine serum[c] and maintained at 37°C and 5% CO_2 in an incubator.

Two different strains of FCoV; FIPV WSU 79-1146[d] and FECV WSU 79-1683[e] were used for the study. Working stocks viruses were produced in CRFK cells[a] and quantified by plaque assay. Plaque assays were performed by inoculating monolayers of CRFK cells[a] in 96-well plates with 100 µl of ten-fold virus dilutions. After adsorption for 1 hour at 37 °C the cells were overlaid with 100 µl of 0.75% carboxymethylcellulose. After 96 hours incubation, plaques were counted. Virus stocks were stored at -80 °C until use and each aliquot was used only once for each experiment.

siRNAs design

Five siRNAs[f] (Table 2.1) were designed based on published sequence data for FCoV using an RNAi designer[g]. siRNA-L that targets the common 5′ leader region, siRNA-U that targets the common 3′ UTR, siRNA-R that targets the replicase gene, siRNA-M that targets the membrane gene, and siRNA-N that targets the nucleocapsid gene. The sequences of these siRNAs were confirmed for specificity by comparing them with FCoV sequences in GenBank database. Also the sequences of the siRNAs were

selected to have a minimal homology to cat genome available in GenBank. The stealth siRNAs[f] used in this study were chemically modified to reduce off-target and nonspecific effects, eliminate the induction of the interferon response pathway, minimize nonspecific cellular stress, and to be highly specific and stable in serum [117].

Optimization of transfection conditions

To optimize transfection condition and obtain the highest transfection efficiency, 80% confluent CRFK cells in 12-well plates were transfected with 30 nM fluorescein labeled siRNA[h]/well. Transfection was performed with increasing concentrations of the transfection agent[i] (1.5 µL, 3 µL and 5 µL/well of transfection reagent) according to the manufacturer's protocol [i]. Then, 24 hours following transfection, cells were trypsinized and pelleted (1000 x g). Finally, the cells were resuspended in 500µl phosphate-buffered saline (PBS) per sample and the intracellular fluorescein was measured using flow cytometry.

Optimization of siRNA concentration

The quantity of siRNA used for transfection is very important to obtain the highest transfection efficiency. To optimize the concentration of siRNA, 80% confluent CRFK cells[a] in 12-well plates were transfected with increasing concentration of either siRNA-L or siRNA-M (30 nM, 50nM and 100nM siRNA/well) and 1.5µL/well of transfection reagent. After 24 hours, cells were either infected with FIPV WSU 79-1146[d] or FECV WSU 79-1683[e] virus at MOI 0.1. One hour after incubation the cells were washed with DMEM/F-12[b] and fresh DMEM/F-12[b] with supplemental 10% heat inactivated fetal bovine serum[c] was added to each well. After 48 hours cultured cells were harvested and FCoV protein

Table 2.1— *Sequences of siRNAs (and their position within the FIPV WSU 79-1146 genome based on GenBank accession No. DQ010921) 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	26198–26222
Antisense	UAAACGAGCCAGCUAAAUUGUGGUC	
siRNA-R		
Sense	CAUUGCAGCUUUGAGUACUGGUGUU	13330–13354
Antisense	AACACCAGUACUCAAAGCUGCAAUG	
siRNA-N		
Sense	GACAACUUUCUAUGGUGCUAGAAGU	27507–27531
Antisense	ACUUCUAGCACCAUAGAAAGUUGUC	
siRNA-U		
Sense	GAGGUACAAGCAACCCUAUUGCAUA	28941–28965
Antisense	UAUGCAAUAGGGUUGCUUGUACCUC	

expression was measured using flow cytometry[l] as described below.

siRNA transfection and virus infection

80% confluent CRFK cells in 12-well plates were transfected with 100 nM siRNA/well and 1.5µL/well of transfection reagent. Cells were either infected with FIPV WSU 79-1146[g] or FECV WSU 79-1683[h] virus at MOI 0.1, 24 hours after transfection. One hour after incubation the cells were washed with DMEM/F-12[b] and fresh DMEM/F-12 [b] with supplemental 10% heat inactivated fetal bovine serum[c] was added to each well. After 48 hours cultured cells were harvested for relative quantification of intracellular FCoV genomic RNA and viral protein expression. Control samples used in each experiment included FCoV-infected cells and untreated CRFK cells. Also, FCoV-infected cells treated with a negative control siRNA[a] that is non-targeting were included as controls to test for potential nonspecific effects. CRFK cells were also mock transfected, using the transfection agent only, to evaluate for potential toxic effects. Each siRNA[a] was tested in duplicate, and each experiment was performed twice.

Flow cytometry analysis

To assess virus protein expression in infected cells, 48 hours after viral infection, CRFK cells in each test and control well were trypsinized, washed with PBS, and resuspended in 1 mL of PBS per sample. 100µL of each suspension was removed and placed on ice for later RNA extraction and the rest of each sample was processed for flow cytometry.

The flow cytometry samples were first treated with a permeabilization reagent [j] according to manufacturer's protocol. Then the cells were stained with 200 µl of FITC-labeled FCoV polyclonal antibody[k] for 30 minutes on ice in the dark. Then the cells were washed with PBS, pelleted (1000 x g), and resuspended in 500µl PBS. Finally, FCoV protein expression was measured as the intensity of fluorescence using flow cytometry[l].

Quantitative real-time RT-PCR

For cellular viral RNA quantification, cells were harvested 48-hours following virus infection and nucleic acid was extracted using an RNA purification kit [m] according to manufacturer recommendations. Briefly, samples were first lysed and homogenized using a homogenizer[n]. Then the lysate was passed through a gDNA eliminator spin column provided with the kit that selectively and efficiently removes genomic DNA. Purified RNA samples were stored at -80°C until tested by real-time RT-PCR.

Reverse transcription and quantitative PCR was done using previously published primers and a FAM-labeled probe targeting the 7b coding region, as this region is highly conserved in FCoV [118]. Reactions were done using a qRT-PCR Kit[o] in a thermal cycler[p] as follows; 5 µL of extracted RNA was used in 25 µL total volume reactions, which contained 200nM of each probe and 300nM of each primer, and 40U of recombinant ribonuclease inhibitor[q]. Cycling parameters were as follows: cDNA production at 42°C for 30 min, hot start Taq polymerase activation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 40 seconds, and extension at 72°C for 30 seconds.

To standardize the total amount of RNA in each reaction, mRNA expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to allow relative quantitation of the viral RNA by the $2^{-\Delta\Delta Ct}$ method [119].

Statistical analysis

All statistical tests[r] were done using ANOVA to compare and test for significant mean differences between treated samples and the negative control non-targeting siRNA, FCoV-infected samples. The ANOVA assumption of normality was tested using the Shapiro-Wilk statistic. When this assumption was not met, a log transformation of the data and/or ANOVA on rank transformed data was done, and the Levene's test was used to test the ANOVA equality of variance assumption. Bonferroni adjustment was used to minimize the Type I error rate. Fisher's Least Significant Difference (LSD) method of mean separation was used to determine which means differed significantly.

Results

Optimization of transfection condition

CRFK cells were 95% effectively transfected with 1.5 µL of the transfection reagent. Further, to optimize the concentration of the siRNAs that will be used throughout the study, cells were transfected with increasing concentration of either siRNA-L or siRNA-M. The transfection was performed with 1.5 µL of the transfection reagent. Both tested siRNAs demonstrated concentration dependent inhibition of viral protein expression as evaluated by the flow cytometery. Reductions in viral protein expression were increased at 100nM of the siRNA. To this end, transfection of CRFK cells was

optimized at 100nM siRNA in combination with 1.5 µL of the transfection reagent to obtain optimal target gene reduction.

Effect of individual synthetic siRNAs on FCoV protein expression in CRFK cells

The 5 siRNAs–siRNA-L, siRNA-U, siRNA-R, siRNA-M, and siRNA-N–used in this study exhibited a variable inhibitory effect on viral replication in vitro, resulting in decreases in the protein expression of FIPV WSU 79-1146 by 80 ± 11%, 43 ± 23%, 13 ± 11%, 33 ± 7%, and 50 ± 17% respectively (Fig. 2.1). siRNA-R had no effect on the protein expression of FECV WSU 79-1683 while siRNA-L, siRNA-U, siRNA-M, and siRNA-N resulted in 75 ± 6%, 30 ± 11%, 14 ± 5%, and 13± 0.9% decreases in viral protein expression, respectively, compared with the negative control non-targeting siRNA, FCoV-infected sample (Fig. 2.2).

Inhibition of viral genomic RNA by individual synthetic siRNAs

siRNA-L, siRNA-U, siRNA-N showed 99%, 75% and 47% reduction in FIPV WSU 79-1146 genomic RNA and 96%,41% and 35 % reduction in FECV WSU 79-1683, respectively. While, siRNA-R and siRNA-M had no inhibitory on the replication of viral genomic RNA, (This data is the result of one experiment in which each siRNA treatment was assessed in duplicate).
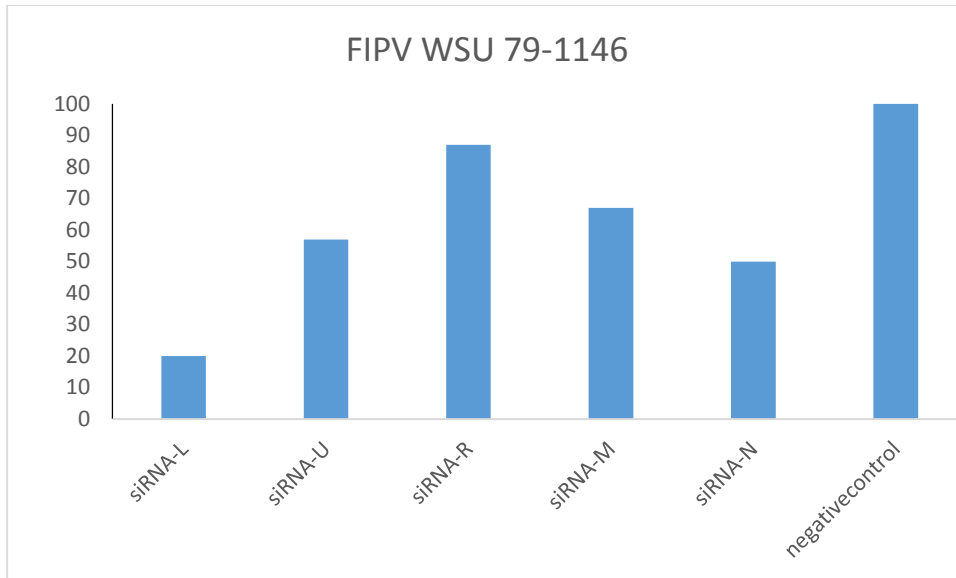
Figure 2.1— *Results of flow cytometric analysis to illustrate the effect of individual siRNAs on the expression of FIPV WSU 79-1964 protein in virus-infected CRFK cells (the Y axis represent the relative viral protein expression (%)). The CRFK cells were transfected with 1 of 5 screened siRNAs (concentration, 100nM) or a negative control non-targeting siRNA 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 data represent the result of 2 independent experiments, each sample was assessed in duplicate.*
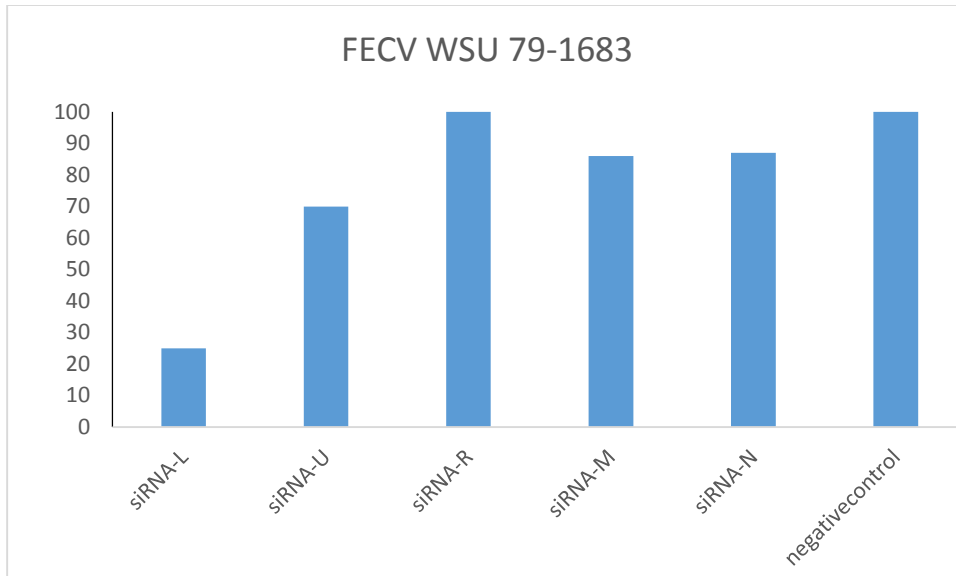
Figure 2.2—*Results of flow cytometric analysis (% viral protein expression on the Y axis) to illustrate the effect of individual siRNAs on the expression of FECV WSU 79-1683 protein in virus-infected CRFK cells (the Y axis represent the relative viral protein expression (%)). The CRFK cells were transfected with 1 of 5 screened siRNAs (concentration, 100nM) or a negative control nontargeting siRNA 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 data represent the result of 2 independent experiments, each sample was assessed in duplicate*

Discussion

Inhibition of FCoV replication using the RNAi phenomenon provides a promising therapeutic application against the fatal FIP disease which is caused by a mutant FCoV biotype. FCoV is considered a good target for siRNA treatment, as the viral full-length genome contains nearly all the sequences found in any of the sgRNAs [18]. Thus the siRNA that targets the sgRNAs will target the full-length genomic RNA too. In spite of that, the development of siRNA-based FCoV strategy faced several challenges. An obvious challenge is the selection of an appropriate target within the viral RNA genome that can be used for all viral strains. Coronavirus has the largest animal RNA genome and is prone to a high mutation rate [120]. Therefore, there are various FCoV strains circulating in the field. To overcome this limitation we designed and screened the ability of five siRNAs that target various highly conserved viral non-coding and coding regions. siRNA-L targets the leader sequence, which is highly conserved in FCoV and plays a pivotal role in virus gene expression and replication [121]. siRNA-U, targets the 3′ UTR. This non-coding region is highly conserved in FCoV and plays an essential role in regulating the viral transcription and replication cycle. The earliest steps of both genome replication and sgRNA transcription initiate at the 3′ end of the genome, which makes it an important siRNA target [18]. siRNAs that target viral coding regions include; siRNA-R, siRNA-M and siRNA-N. siRNA-R targets the viral replicase gene, which encodes the viral nonstructural replicase that plays a critical role during the virus replication. siRNA-N and siRNA-M, target the viral structural nucleocapsid gene and membrane gene. These five siRNAs exhibited a variable inhibitory effect on FCoV replication in vitro as compared with the negative control non-targeting siRNA sample. siRNA-L exhibited the highest inhibitory effect on

FCoV protein expression, followed by siRNA-U and siRNA-N. The leader sequence is present in the 5'end of each viral mRNA as well as the viral genome [18]. Therefore siRNAs directed against the regulatory leader sequence can target genomic RNA as well as all viral mRNA species and has been reported as an effective method for inhibiting coronavirus replication using an in vitro SARS-CoV model [122]. All these screened siRNAs except siRNA-R and siRNA-M resulted in inhibition of the viral protein expression as well as the viral genomic RNA. siRNA-R and siRNA-M showed low inhibitory effect on the viral protein expression(siRNA-M) and no effect on the viral genome replication. Using M –fold software that predict the secondary structure of single stranded nucleic acid showed that the target sites of the replicase and membrane genes have the ability to self-anneal and to develop secondary structure that is known to interfere with and/or inhibit the siRNA effect. Further, the low inhibitory effect of siRNA-M on the viral protein expression may be attributed to the degradation of viral sg (mRNA) that encodes the viral membrane protein and not because of the degradation of the viral full length genome. The difference in RNA secondary structure between the sg (mRNA) and the viral genomic RNA seems to play role in limiting target accessibility.

Development of viral escape mutants is another important challenge that faces siRNA-based antiviral therapy. Selecting and targeting highly conserved regions can minimize this challenge. Highly conserved regions are critical for the viral life cycle and therefore do not tolerate nucleotide changes and is highly conserved among the different virus strains. However, any silent point mutation in these critical conserved regions might affect the efficiency of the siRNA. Thus, further strategies are required to minimize the development of escape mutants. The use of multiple siRNA targets in combinations has

been demonstrated to dramatically decrease the ability of the virus to develop escape mutants and resist the siRNA inhibitory effect [93].

These preliminary findings shows that FCoV replication can be specifically inhibited using siRNA targeting coding and noncoding region of viral genome. Identification of siRNAs that specifically targets FCoV is necessary to develop siRNA technology into an effective anti-FCoV therapy and treat FIP disease for which there is no specific treatment.

Chapter 3
siRNA Combinations Inhibit Feline Coronavirus Replication and Expression in Cell Culture

Abstract

Feline infectious peritonitis (FIP) continues to be a significant cause of mortality in cats. Feline coronavirus (FCoV), the agent of FIP, primarily targets intestinal epithelial cells, but in certain cats, virus mutation may occur that allows the virus to replicate efficiently in monocytes and macrophages, resulting in FIP development to which currently, there is no specific effective treatment.

In this study, we evaluated the ability of siRNA combinations to inhibit FCOV replication and expression in vitro. Three combinations of previously screened individual siRNAs were tested for their antiviral effects against two different strains of FCoV. Efficacy of the siRNAs combinations was determined by 1) quantification of the inhibition of intracellular viral genomic RNA using real time RT-PCR, 2) evaluation of the reduction of viral protein expression in infected cells using flow cytometry and 3) assessment of virus replication inhibition in cell culture via titration of extracellular virus using $TCID_{50}$ assay.

Combinations of siRNAs tested included; siRNA-L (targeting the FCoV leader region) and siRNA-U (targeting the 3′ untranslated region); siRNA-L and siRNA-N (targeting the nucleocapsid gene); and siRNA-L, siRNA-U, and siRNA-N. These combinations resulted in more than 99.5%, 98.7%, and 98.4% reduction in viral replication, respectively, in comparison to siRNA negative control cells, based on virus titration results. These preliminary findings show that FCoV replication can be specifically inhibited using siRNAs combinations targeting various regions of the viral genome, suggesting a promising therapeutic application of siRNA in treating FIP.

Introduction

Given the central role played by the increased virus replication in the development of FIP, antiviral therapy that specifically targets the virus and reduces its replication could effectively help treat this highly fatal disease. In the previous chapter, we showed the ability of individual siRNAs that each target a single region to inhibit the replication of FCoV. Improvement of the inhibitory effect of these siRNAs can be achieved by combining the most effective siRNAs into combinations. The use of siRNA combinations that target various regions has been reported to have a synergistic effect, help to reduce off-target effects of the siRNA and prevent the development of escape mutants that may resist the effect of the designed siRNA [123]. To achieve this objective, three combinations of previously screened siRNAs (siRNA-L, siRNA-U and siRNA-N) have been examined and their efficiency were assessed via 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$_{50}$ assay.

Material and methods

Selection of siRNA combinations

The selection of the siRNA combinations was based on the percentage of in vitro FCoV inhibition. The three siRNAs, siRNA-L, siRNA-U and siRNA-N, which gave better viral inhibition as compared with the other examined siRNAs were included in the screened siRNA

combinations. The tested siRNA combinations included siRNA-L (targeting the FCoV leader region) and siRNA-U (targeting the 3′ untranslated region [C1]); siRNA-L and siRNA-N (targeting the nucleocapsid gene C2]); and siRNA-L, siRNA-U, and siRNA-N (C3).

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, then transfection was performed at about 80% confluency with siRNA combinations and transfection reagent[f] (1.5 µL/well). In each well 2 or 3 siRNAs were combined to provide a total concentration of 100nM/well. 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 non-targeting siRNA[a] were also 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 X g), and resuspended in 500 µL 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.

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. Briefly, samples were first lysed and homogenized using homogenizer[n]. Then the lysate was passed through a gDNA eliminator spin column provided with the kit that selectively and efficiently removes genomic DNA. Purified RNA samples were stored at -80°C until tested by real-time RT-PCR.

Reverse transcription and quantitative PCR assays were performed with primers and a FAM-labeled probe that targets the 7b coding region, a region that is highly conserved in FCoVs [118]. Reactions were done with a quantitative RT-PCR kit[n] in a thermal cycler.[o] as follow; 5 µL of extracted RNA was used in 25 µL total volume reactions, which contained 200nM of each probe and 300nM of each primer, and 40U of

recombinant ribonuclease inhibitor[q]. Cycling parameters were as follows: cDNA production at 42ºC for 30 min, hot start Taq polymerase activation at 95ºC for 2 minutes, followed by 45 cycles of denaturation at 95ºC for 10 seconds, annealing at 60ºC for 40 seconds, and extension at 72ºC for 30 seconds.

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 [119].

TCID$_{50}$ assay

Forty-eight hours after infection with FCoV, 500 µL of tissue culture medium was collected 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 monolayers 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 [124].

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

each of the 3 siRNAs 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.

Resistance of FCoV to siRNA combination treatment

To investigate the ability of the treated FCoV to tolerate siRNA treatment without developing drug resistance via mutation in the target region, extracellular progeny virus from siRNA combination 3 (siRNA-L, siRNA-U, and siRNA-N combination) transfected, FIPV WSU 79-1146-infected cells was harvested. This harvested virus was used to infect CRFK cells 24 hours after being transfected with siRNA combination 3. Forty-eight hours after viral infection, *siRNA treated* as well as control CRFK cells *w*ere treated with a permeabilization reagent [j] (according to manufacturer's protocol) to assess virus protein expression by flow cytometry[l] as described earlier. Experimental controls included 1) cells transfected with siRNA combination 3, FIPV WSU 79-1146 (stock virus) infected cells 2) cells transfected with negative control non-targeting siRNA-treated, FCoV-infected cells and 3) uninfected cells. Inhibition of protein expression of siRNA combination 3 treated progeny virus was compared to that of siRNA treated stock virus FIPV WSU 79-1146.

Further, to confirm the sequence identity between the designed siRNA and the progeny virus nucleic acid as well as the stock virus nucleic acid, the viral nucleic acid

was extracted from the harvested extracellular virus and the stock virus as mentioned previously. Three Primer sets were designed (using Primer3 software) using the published sequence of FIPV WSU 79-1146 to amplify ~150 to 220 nucleotides each of the viral leader sequence, 3′ untranslated region and nucleocapsid gene (Table 3.1). Each of these amplicons includes the siRNA complementary region and was used to compare the sequence identity using BlastN on the NCBI website.

Statistical analysis

All statistical tests[r] were done with an ANOVA to compare any significant differences between the mean values of 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 $P = 0.05$ was considered significant.

Table 3.1— Design of the primers that were used to amplify ~150-220 nucleotides each of the viral leader sequence, 3' UTR region and nucleocapsid gene. Nucleotide sequences and locations are based on GenBank accession No. DQ010921.

primer	Oligonucleotide sequence	Amplimer size (nucleotides)	Nucleotide sequence location
Leader region			
Forward	5'– AAAGTGAGTGTAGCGTGGCTAT –3'	209	12-33
Reverse	5'– TTACCAAACAGGGACGAAC –3'		221-206
UTR region			
Forward	5'– CGCGCTGYCTACTCTTGTA –3'	156	28898-28916
Reverse	5'– CTCTTCCATTGTTGGCTCGT –3'		29054-29035
Nucleocapsid			
Forward	5'– GCCGTGCTTSAAAAATTAGG –3'	221	27364-27383
Reverse	5'– GTTTGGCAGCGTTACCATTG –3'		27585-27567

Results

Effect of siRNA combinations on the expression of FCoV proteins

Flow cytometric evaluation of viral protein expression in cells transfected with siRNA combinations revealed a significant decrease in protein expression following infection with FIPV WSU 79-1146 ($p < 0.001$) and FECV WSU 79-1683 ($p = 0.0046$) as compared with the negative control sample. Protein expression of the FIPV WSU 79-1146 strain was decreased by $91 \pm 6\%$, $91 \pm 2\%$, and $90 \pm 5\%$ after treating CRFK cells with siRNA combination1 (UTR + L), combination 2 (UTR + NC), and combination 3 (UTR + L + NC), respectively, as compared with the negative control sample (Fig. 3.1). The protein expression of the FECV WSU 79-1683 strain was decreased by $95 \pm 1\%$, $94 \pm 1\%$, and $95 \pm 1\%$ after treating CRFK cells with siRNA combinations (1), (2), and (3), respectively, as compared with the negative control sample (Fig.3.2).

Inhibition of viral genomic RNA by siRNA combinations

The 3 siRNA combinations showed significant inhibition of both strains of FCoV genomic RNA ($p < 0.001$). The 3 combinations resulted in ~ $97 \pm 1.4\%$ reduction in FIPV WSU 79-1146 genomic RNA (Fig.3.3) and $96 \pm 4\%$, $92 \pm 9\%$, and $93 \pm 7\%$ reductions in FECV WSU 79-1683 genomic RNA after treating CRFK cells with siRNA combinations (1), (2), and (3), respectively, as compared with the negative control sample (Fig. 3.4).
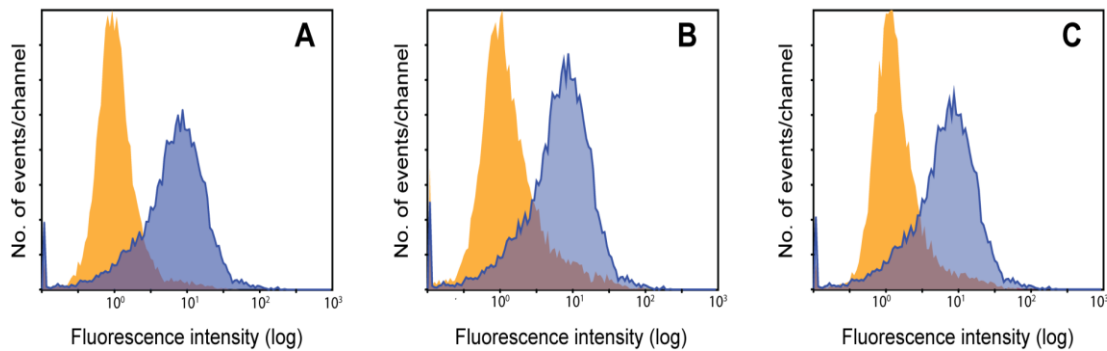
Figure 3.1—*Results of flow cytometric analysis (no. of events/channel vs fluorescence intensity [logarithm base 10]) illustrating the effect of siRNA combinations on the expression of FIPV WSU 79-1146 protein in virus-infected CRFK cells. The CRFK cells were transfected with 1 of 3 of siRNA combinations (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' untranslated region [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 results of 1 of 2 independent experiments that yielded similar results; each sample was assessed in duplicate.*
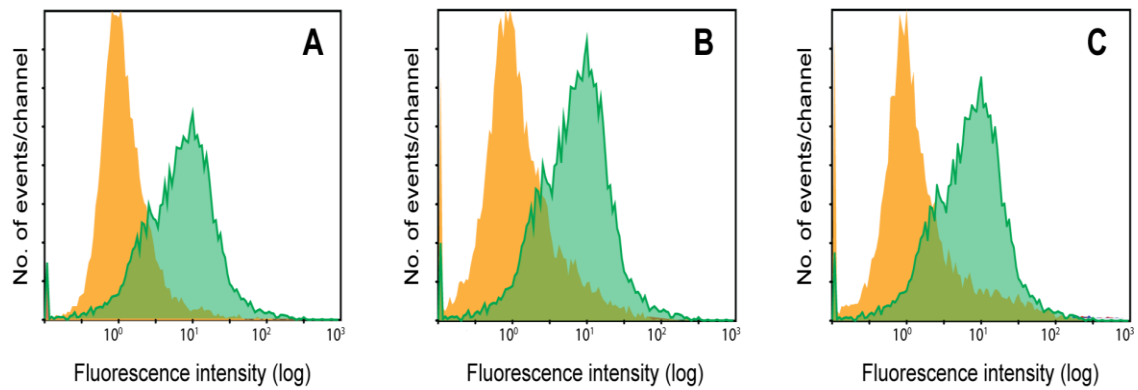
Figure 3.2—_Results of flow cytometric analysis (no. of events/channel vs fluorescence intensity [logarithm base 10]) to illustrate the effect of siRNA combinations on the expression of FECV WSU 79-1683 protein in virus-infected CRFK cells. The CRFK cells were transfected with 1 of 3 of siRNA combinations (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' untranslated region [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 results of 1 of 2 independent experiments that yielded similar results; each sample was assessed in duplicate._

Figure 3.3— *Results of* quantitative real-time RT-PCR *to illustrate the effect of siRNA combinations on FIPV WSU 79-1146 genomic RNA replication in CRFK cells (the Y axis represents the relative viral genomic production %). 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 siRNAs\ combinations included siRNA-L (targeting the FCoV leader region) and siRNA-U (targeting the 3' untranslated region [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, CRFK cells were collected and the nucleic acid was extracted to perform real-time RT-PCR. Each combination was tested in duplicate, and the data represent the mean ± SD of 2 independent experiments.*
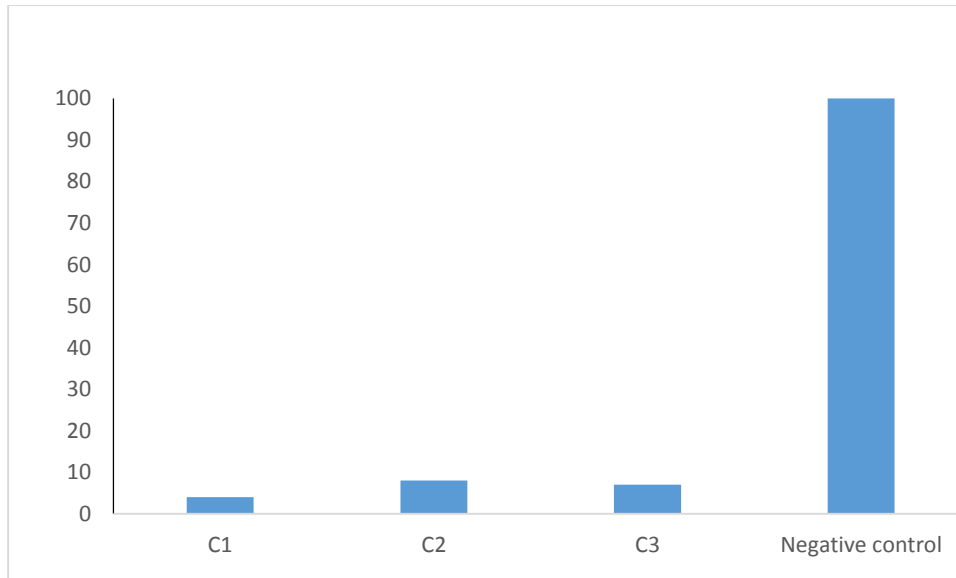
Figure 3.4— *Results of quantitative real-time RT-PCR to illustrate the effect of siRNA combinations on FIPV WSU 79-1146 genomic RNA replication in CRFK cells (the Y axis represents the relative viral genomic production %). The CRFK cells were transfected with 1 of 3 siRNA combinations (concentration, 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' untranslated region [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, CRFK cells were collected and the nucleic acid was extracted to perform real-time RT-PCR. Each combination was tested in duplicate, and the data represent the mean ± SD of 2 independent experiments.*
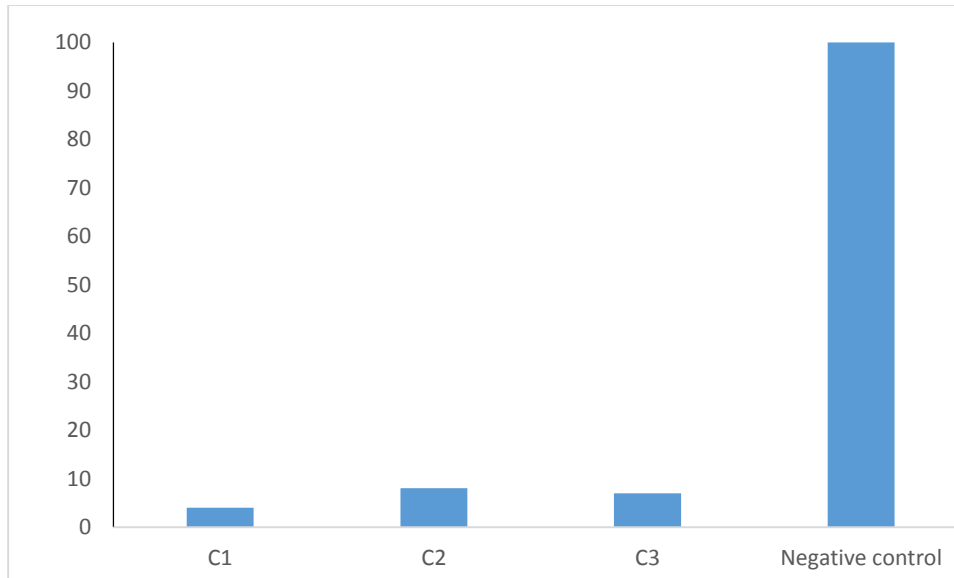
Effect of siRNA combinations on the yield of progeny virus (extracellular virus titer)

The 3 siRNA combinations resulted in about a 100 fold reduction in the extracellular virus titer of both viral strains ($p < 0.001$), as compared with the negative control siRNA sample, which represented ~ 95% reduction in virus replication (Fig. 3.5 and Fig. 3.6). 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 non-targeting siRNA, FCoV-infected cells were assessed with TCID$_{50}$ assay. There was no significant difference in the titers of the extracellular virus from FECV WSU 79-1683 nor FIPV WSU 79-1146 infected cells, as compared with the negative control non-targeting siRNA, FCoV- infected cells ($p = 0.211$ and $p = 0.5055$, respectively). This showed that the transfection and/or non-targeting siRNA does not have a nonspecific inhibitory effect on FCoV and confirms 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 non-targeting 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, which was not significantly ($P = 0.1$) different from the mean optical density

Figure 3.5— *Results of a $TCID_{50}$ assay to illustrate the effect of siRNA combinations on the yield of FIPV WSU 79-1146 progeny virus in CRFK cells. The CRFK cells were transfected with 1 of 3 siRNA combinations (concentration, 100nM), or a negative control non-targeting 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' untranslated region [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 non-targeting siRNA. There was no significant difference in extracellular virus titers for untreated cells infected with FIPV WSU 79-1146, compared with the infected cells treated with the negative control non-targeting siRNA.*
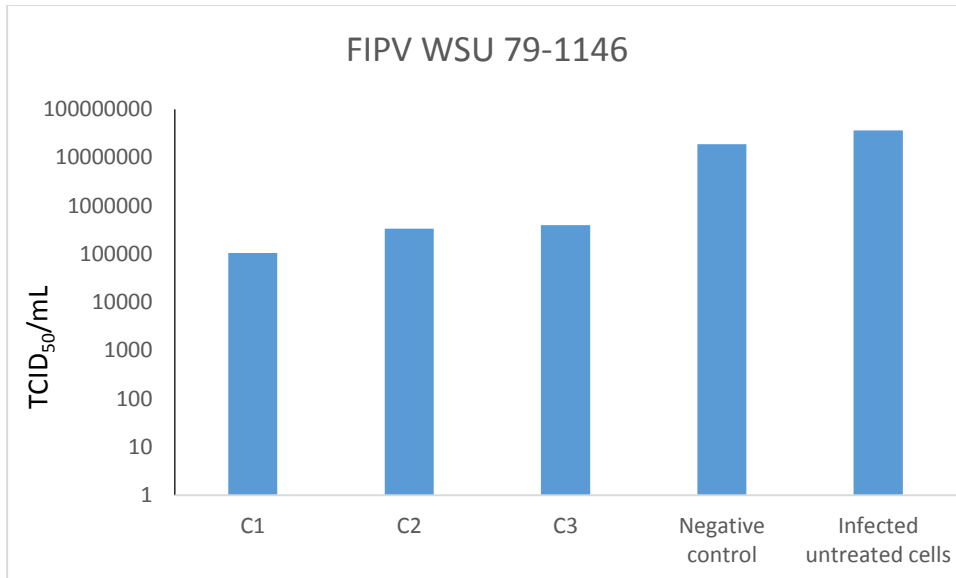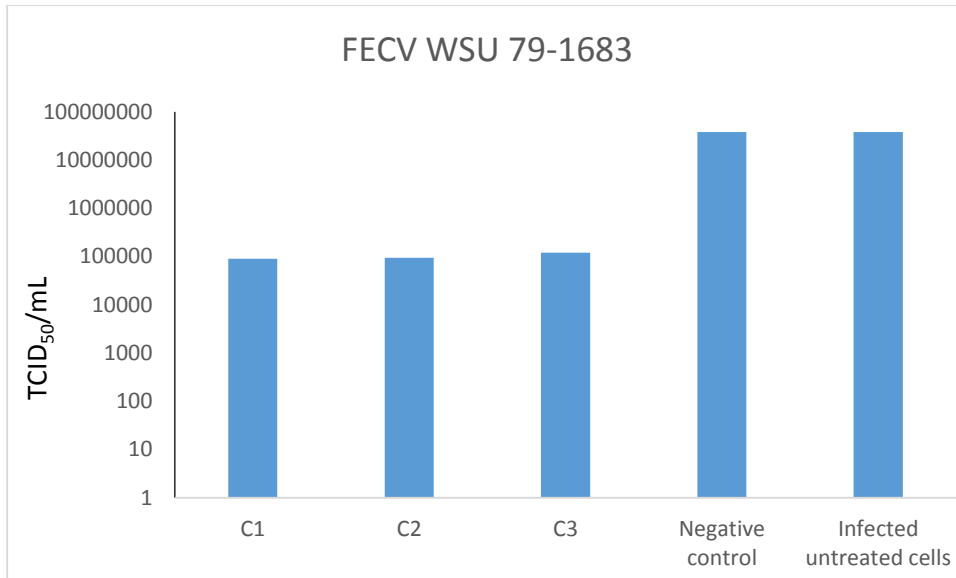
Figure 3.6—*Results of a $TCID_{50}$ assay to illustrate the effect of siRNA combinations on the yield of FECV WSU 79-1683 progeny virus in CRFK cells. The CRFK cells were transfected with 1 of 3 siRNA combinations (concentration, 100nM), or a negative control non-targeting siRNA 24 hours prior to viral 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' untranslated region [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 non-targeting siRNA. There was no significant difference in extracellular virus titers for untreated cells infected and FECV WSU 79-1683, compared with the infected cells treated with the negative control non-targeting siRNA.*

of the mock-transfected cells (0.9353 ± 0.2 absorbance) or from the cells transfected with the siRNA-L and siRNA-U combination (0.9576 ± 0.02 absorbance), the siRNA-L and siRNA-N combination (1.0433 ± 0.049 absorbance), or the siRNA-L, siRNA-U, and siRNA-N combination (1.0636 ± 0.018 absorbance).

Resistance of FCoV to siRNA combination treatment

To determine the ability of treated FCoV strain to tolerate the siRNA treatment without developing mutation in the siRNA target region, we compared siRNA combination 3 (siRNA-L, siRNA-U, and siRNA-N combination) inhibitory effect on the viral protein expression of; extracellular progeny virus (harvested from siRNA treated, FCoV infected cells) and FIPV WSU 79-1146 (stock virus) using flow cytometery. Flow cytometric evaluation of the viral protein expression showed 60.5± 5% and 65 ± 5%, inhibition in the viral protein expression of the extracellular progeny virus and FIPV WSU 79-1146 respectively as compared with the negative control non-targeting siRNA, FIPV WSU 79-1146- infected cells. This result revealed no significant difference in protein expression following infection of siRNA treated cells with either the extracellular progeny virus or FIPV WSU 79-1146 (p= 0.05) based on paired t-test analysis. Further, sequencing of the siRNAs target regions of both the progeny virus and FIPV WSU 79-1146 genome showed 100% homology.

Discussion

Feline infectious peritonitis (FIP) is a progressive, fatal disease of cats that is characterized by a poor cell-mediated immune response and continued systemic virus replication, as well as an exaggerated humoral response to the virus. Currently, there is

no effective treatment for FIP. Our previous results (chapter 2) showed that individual siRNAs each targeting a single gene were able to inhibit FCoV replication in vitro. The inhibitory effect of siRNA is stated to be increased by combining multiple siRNAs targeting either the same or different gene.

In the present study, siRNA combinations that target highly conserved regions of FCoV coding and noncoding genes were able to inhibit virus gene expression and thereafter inhibit replication of the virus. The 3 combinations of siRNAs that target different regions of the viral genome showed more effective inhibition on the viral replication of both FCoV strains than individual siRNAs as compared with the negative control non-targeting siRNA sample. Combinations of siRNAs targeting different regions led to a significant synergistic effect on viral inhibition compared to individual siRNA treatment [123]. Moreover, using combinations of siRNAs that target highly conserved regions has been reported to be able to reduce or prevent the development of escape mutants that could resist the inhibitory effect of the introduced siRNA. Nucleotide substitution occurs frequently in the FCoV genome, thus, reducing the probability of drug resistance development via mutation is very important to achieve an effective antiviral effect.

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 plays an important role in virus gene expression and replication [121]. Another siRNA used in combination was siRNA-U, which targets the 3′ UTR noncoding region, which is highly conserved in FCoV and has an essential role in regulating the viral transcription and replication cycle.[93] Moreover, during virus replication, a 5′ common leader

sequence and 3′ co-terminal nested set of sgRNAs are made [18]. Therefore, siRNA-L and siRNA-U target genomic RNAs and all the viral nested sgRNAs. The other siRNA used in tested combinations was siRNA-N, which targets the viral nucleocapsid gene. Viral nucleocapsid protein is a virion structural protein that protein plays an important role in the viral replication and proposed to be an interferon antagonist that likely plays a role in circumventing the innate immune response.

Given the poor host or cell-mediated immune response associated with enhanced viral replication in cats with FIP, the effective reduction of viral 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 RNAi 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 [117]. 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. In vivo delivery of these siRNA combinations to FIPV target cells (Monocytes and macrophages) could successfully inhibit the viral replication and limit the disease. Unfortunately, systemic delivery of siRNA is considered to be a big challenge that face the clinical application of this technology. However, recently there are various delivery

systems, successfully delivered siRNA to the monocytes and macrophages, suggesting a promising clinical application of RNAi in treating FIP [125, 126].

To our knowledge, this is the first study to investigate the effectiveness of siRNA combinations to inhibit FCoV replication. Our siRNAs combinations markedly inhibited FCoV genomic RNA, FCoV protein and FCoV replication in vitro. These results indicate that FCoV replication can be specifically and significantly inhibited with siRNAs combinations targeting different highly conserved coding and regulatory noncoding regions of the viral genome/mRNA. Since the exact genetic changes that led to the mutation of the non-virulent FECV to the virulent FIPV is still controversial, it was very important to designed siRNAs that target FCoVs of different genetic background and therefore, can inhibit the replication of any viral strains circulating in the field. These siRNA combinations seemed to be a promising tool to prevent and/or treat FIP. Inhibition of FECV replication in seropositive cats and chronic shedders using siRNA technology can help these cats to clear the virus and prevent the possibility of the mutation of FECV to FIPV and the development of this fatal disease.

Chapter 4
General Summary

Feline coronavirus (FCoV) is enveloped virus with a large, capped, polyadenylated RNA genome of about 30 Kb nucleotides and belongs to Genus alphacoronavirus, in the family Coronaviridae. FCoV genome includes 11 putative ORFs; two large ORFs encode viral non-structural replicase proteins; structural ORFs that encode spike, envelope, membrane, and nucleocapsid proteins and five ORFs encode the nonstructural proteins 3a, 3b, 3c, 7a, and 7b [9].

FCoV causes a mild or often subclinical enteric infection, especially in kittens. However, in some cats it can cause fatal systemic disease known as feline infectious peritonitis (FIP). FIP can manifest in two forms; 1) the wet form, which is characterized by fibrinous, granulomatous serositis, with protein-rich effusions in the body cavities of affected cats and 2) the dry form, which is characterized by granulomatous inflammatory lesions of several organs especially, liver, kidney, spleen, CNS, and eyes [6]. It has been proposed that a mutant FCoV which is capable of infecting and replicating in the monocytes and macrophages is responsible for the development of FIP[8].

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 [37]. Recently, polyprenyl, an agent that is postulated to enhance T lymphocyte activity, has been used with some success in cats with the dry form of FIP but not in cats with the common effusive form of the disease. These results reflect the importance of limiting the viral load (replication); viral spread and replication is greater with the wet form than with the dry form [36]. 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 physiological 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.

Recently, RNAi mechanism has been studied as a therapeutic tool for various mammalian infections including viral infection [5, 72, 127]. RNAi is a highly conserved biological process found in plants and animal cells. This biological process can be induced artificially via the introduction of synthetic, short interfering RNA (siRNA). Subsequently, this siRNA is incorporated into RNA induced silencing complex (RISC), an enzyme complex with RNAase and helicase activity [4]. The RISC guides the introduced siRNA to the target complementary sequences and results in sequence specific silencing of the gene of interest. In the present study, we found that individual siRNAs and siRNAs 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. FCoV 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, 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. Further, multiple combinations of the most effective individual siRNAs were examined. Inhibition of the viral replication by each combination was more effective than that achieved by the individual siRNAs, compared with findings for the negative control non-targeting siRNA FCoV-infected sample. Combinations of siRNAs targeting different regions showed a

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significant synergistic effect on viral inhibition, compared to results of treatment with individual siRNAs. Additionally, in our 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 to prevent or reduce development of viral escape mutants. 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 [120, 128]. 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 viral 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.

Unlike a previous study [129] 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 [1]. With improvements in siRNA design and delivery methods, RNA interference might be an effective treatment option for such a life-limiting viral infection.

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a. Crandell-Rees feline kidney cell line, American Type Culture Collection, Manassas, Va.

b. DMEM:F-12, Lonza, Walkersville, MD.

c. Fetal Bovine serum, Atlanta Biologicals, Lawrenceville, Ga.

d. FIPV WSU 79-1146, American Type Culture Collection, Manassas, Va.

e. FECV WSU 79-1683, American Type Culture Collection, Manassas, Va.

f. Stealth siRNA, Invitrogen, Carlsbad, Calif.

g. Block-iT RNAi designer, Invitrogen, Carlsbad, Calif.

h. Lipofectamine 2000, Invitrogen, Carlsbad, Calif.

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, Fullerton, Calif.

m. RNeasy Plus Mini Kit, Qiagen, Valencia, Calif.

n. QIAshredder, Qiagen, Valencia, Calif.

o. Superscript III Platinum One-Step qRT-PCR Kit, Invitrogen, Carlsbad, Calif.

p. SmartCycler II, Cepheid, Sunnyvale, Calif.

q. Qia shredder, Invitrogen, Carlsbad, Calif.

r. CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, Wis.

s.   ELx800 Universal Microplate Reader, Bio-Tek Inc, Winooski, Vermont.

t. SAS, version 9.3, SAS Institute, Cary, NC.

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Vita

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