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Research Article

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Design and evaluation of small interfering RNAs for the treatment of Severe Acute Respiratory Syndrome-Coronavirus-2

Abstract

SARS-CoV-2 is the virus responsible for the COVID-19 pandemic. As the 2019 coronavirus disease continues to spread, it will be useful to have as many effective treatment options as possible. This research has the potential to create a siRNA treatment that is safe, effective, and practical in design and administration; 192 siRNAs were designed to target conserved regions of the SARS-CoV-2 genome. The first aim of this study is to confirm, via sequence analysis, that these target sites have remained highly conserved over the course of the pandemic. Multiple sequence alignments were generated for the first half of 30,312 full SARS-CoV-2 genomes, which were averaged and compared with the Wuhan-Hu-1 reference genome. Most target sites maintained a very high level of conservation, suggesting that potential repressor siRNAs could be effective in a majority of infected individuals. To evaluate the efficacy of the 192 test siRNAs, we cloned sections of the SARS-CoV-2 RNA genome into GFP fusion genes. Some of these constructs were transfected in different conditions to set up a screening assay based on GFP expression. Preliminary data on the setup of this GFP reporter assay show that the M, N, E, ORF8, and ORF10 constructs produced a good GFP signal, whereas the S, F1, F2 and F3 constructs did not produce a sufficiently strong GFP signal to detect above background. In a preliminary experiment, we evaluated siRNAs targeting the M, N, and E open reading frames and found some to be efficacious. Future directions for this project include generating alignments of the second half of the SARS-CoV-2 genome for a complete sequence conservation estimate, and cell metabolism assays for supplementing visual observations of siRNA toxicity, optimization of GFP readout, and screening of all designed siRNAs.

Introduction

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by a newly discovered strain of coronavirus: severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). SARS-CoV-2 is unlike common human coronaviruses (e.g., 229E alpha coronavirus, OC43 beta coronavirus) which cause the common cold. (1) However, SARS-CoV-2 is genetically related to SARS-CoV, the virus behind the first threat of a coronavirus pandemic in 2003. (2) Coronaviruses are transmitted when individuals are in close proximity. When people are within approximately 6 feet, respiratory droplets produced by an infected individual (e.g., through coughing, sneezing, talking) can be inhaled into the nose, mouth, airways, and lungs of an uninfected individual. This is believed to be the major way the virus spreads. (3)

Coronaviruses are enveloped, non-segmented, and positive-sensed double-stranded RNA (dsRNA) viruses. They are the largest group of viruses in the *Nidovirales* order, of the family *Coronaviridae*, and can be classified into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. (4) Coronaviruses possess large RNA genomes whose sizes range from approximately 26 to 33 kilobases (kb) and include a variable number (from 6 to 11) of open reading frames (ORFs). (5) The coronaviral genome encodes four major structural proteins: the spike surface glycoprotein (S), small envelope protein (E), matrix protein (M), and nucleocapsid protein (N). (4) The spike protein plays an essential role in binding angiotensin-converting enzyme 2 (ACE2) or CD209L receptors on the host cell and determines host tropism. (6, 7)

Our research aims to use RNA interference (RNAi) as a tool to combat the spread of COVID-19. Also known as Post-Transcriptional Gene Silencing (PTGS), RNAi is a conserved biological pathway among eukaryotes. This mechanism involves small RNAs (fewer than 30 bases long) with characteristic two-nucleotide 3' overhangs which allow them to be recognized by RNAi enzymatic machinery. (8) In some organisms, RNAi is triggered by double-stranded RNAs (dsRNA) of viral origin that lead to homology-dependent targeting of the foreign RNAs. In mammalian cells, the RNAi pathway is primarily responsible for the regulation of genes by microRNAs (miRNAs), which direct the RNAi machinery to repress the translation of several target genes. RNAi is also used to target specific genes for degradation by introducing artificially designed, 19-nucleotide short interfering RNAs (siRNAs) into mammalian cells. siRNAs are typically made to resemble the cleavage products of double stranded RNA by the RNase III endonuclease Dicer. (9) Once introduced into a cell, siRNAs are loaded into the RNA-induced silencing complex (RISC). (10) The argonaute-2 (Ago-2) protein uses its endonuclease activity to cleave and release the siRNA "passenger" strand, activating the RISC. The remaining single-stranded "guide" RNA molecule allows target specificity by intermolecular base pairing, resulting in the cleavage of the target RNA by Ago2. (11) siRNAs represent promising drugs against viral infections as they can be rapidly designed and manufactured and lyophilized for long-term storage and transport. (12) Lastly, siRNAs are attractive drug candidates against SARS-CoV-2 because their delivery into lungs by intranasal administration has been proven effective in clinical studies for other respiratory viruses, such as RSV. (13) siRNAs were also effective in monkeys infected with SARS-CoV. (14)

The threat on global health posed by SARS-CoV-2 highlights the need to develop of a multitude of treatments. Given that RNA interference could be used as a novel COVID-19 therapy, our first step was to find conserved target sites in the SARS-CoV-2 genome suitable for small interfering RNAs (siRNAs) through genetic sequence analysis. The design of siRNAs involves identifying targets in viral genomes that are conserved across all circulating strains of the virus-of-interest, as siRNA activity is dependent on accurate base pairing with the target sequence in the given virus. One way to identify conserved target sites is to generate sequence alignments of all available CoV-2 genomes and calculate the percent conservation of each possible 19-base siRNA target. This approach has been previously optimized for the design of clinically relevant ribozymes targeting HIV RNA.

(15) In the current literature, few studies focus specifically on siRNAs' efficacy against coronaviruses by targeting subgenomic RNAs (sgRNAs) as opposed to target the open reading frame 1 (ORF1). Hence, in this study, we designed and evaluated the efficiency of novel siRNAs targeting different regions of SARS-CoV-2. Specifically, our study aims to determine siRNA target sites within the SARS-CoV-2 ORF1a, ORF1b, and eight sgRNAs via sequence conservation and evolutionary conservation. We hypothesize that targeting accessible and conserved sites within the SARS-CoV-2 genome will identify efficacious siRNAs against COVID-19.

Methods

SARS-CoV-2 Sequence Conservation Estimates

The sequence alignment tool on NCBI Virus was used to generate sequence FASTA alignments containing all complete SARS-CoV-2 genome sequences (30,312 at the time of analysis). The accession number for the reference strain is NC_045512.1. FASTA files were imported into Jalview alignment editor (version 2.11.1.3), which was used to calculate the conservation percentage for each nucleotide within the selected target site (expressed as the percentage of sequences containing the consensus nucleotide at each position). A histogram representing the percent (%) of conservation for each nucleotide in the alignment with reference to the consensus nucleotide was then generated. This annotation was exported as a CSV (Spreadsheet), saved as a .txt extension, and imported as a comma delimited file into Microsoft Excel. The data was transposed from rows into columns, and the average conservation was characterized.

Cell Culture

HEK293T cells and VeroE6 (ATCC) cells were maintained in DMEM with high glucose (HyClone) supplemented with 10% fetal bovine serum (FBS) (HyClone), 50 U/mL penicillin, and 50 µg/mL streptomycin (Life Technologies).

Cell Plating and Transfections

For transfections of GFP expression plasmids, a master mix was created using 1 uL TransIT-LT1 DNA Transfection Reagent (Mirus Bio), diluted in 39 uL opti-MEM. The mix was added to DNA at different amounts, and this mixture rested for 15 minutes and was added over top of pre-plated cells at 1.40 × 105 cells/mL at a volume of 100 µL per well in a black-bottom 96-well plate. Clear-bottom plates were used for microscopy. Reverse transfection with non-targeting siRNAs and targeting siRNAs were also performed with 4 µL of 5 µm siRNA in 16 µL OptiMEM was added to 60 wells of a black-bottom plate. A master mix was made using 0.3 µL of RNAi MAX transfection reagent and 19.7 µL OPTI-MEM, and 20 µL was added to the siRNA dilutions. Cells were seeded after 15 minutes at 1.40 × 105 cells/mL 24 h prior to DNA transfection at a volume of 100 µL per well. After 24 h, GFP transfections were performed as above. The 36 border wells of the plate were seeded with 100 µL PBS. Plates were read 48 h after GFP transfection on a BioTek Instruments Synergy 4 Multi-Detection Microplate Reader. Fluorometer readings were detected at an excitation of 485 nm and an emission of 515 nm.

Results

Design of siRNAs targeting SARS-CoV-2 RNA and construction of GFP reporter constructs

In these experiments, we aimed to identify siRNAs targeting SARS-CoV-2 RNA which could potently inhibit viral replication. We designed siRNAs against conserved regions of the SARS-CoV-2 genome, and reporter constructs linking different regions of the RNA genome to GFP reporter sequences were produced. To confirm that the identified siRNA target sites remained conserved as the pandemic progresses, all available and complete SARS-CoV-2 sequences (collected between January 12, 2020 to October 23, 2020) were subject to multiple sequence alignment using NCBI Virus and analysed for nucleotide conservation with Jalview. Microsoft Excel was used to calculate sequence homology for the first half of the SARS-CoV-2 genome. The selected siRNA target sites are illustrated in Fig. 1.

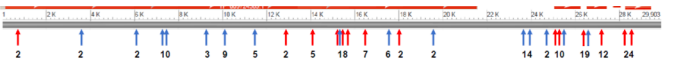

Figure 1. Target sites for siRNAs identified by sequence conservation and RNA structure analysis. The number of siRNAs identified for each target region are illustrated under the SARS-CoV-2 genome. The different open reading frames (in red) of the genome are shown, over the full-length RNA (in grey). Red arrows indicate regions that are highly conserved across SARS-CoV-2 genomes and related coronaviruses (40 siRNAs). Blue arrows indicate regions that are conserved across SARS-CoV-2 genomes and are predicted to be unstructured (56 siRNAs). 96 siRNAs were also designed by Thermo Fisher (target sites not shown).

Every site we selected is highly conserved across SARS-CoV-2 genomes, but those indicated with a red arrow in Fig. 1 are also highly conserved in various related coronaviruses infecting bats and humans. These target sites might be important for several coronaviruses and are less likely to mutate. The target sites indicated by blue arrows in Fig 1. are predicted to be unstructured, though still highly conserved across SARS-CoV-2 genomes. We chose 96 target sites (number of wells in a 96-well plate), of which 40 target areas are indicated by red arrows and 56 target regions are indicated by blue arrows (Fig. 1). These target sites were selected by eliminating siRNAs having target sites with partial homology to the human transcriptome (one of the potential off-target effects of siRNA) using GGGenome, a web-based application which helps identify targeting mismatches in the human transcriptome.(16) An i-Score (inhibitory-Score) was also used to predict which siRNAs would be highly active based on their RNA sequences (e.g., thermostability of siRNA duplex, presence of G/C nucleotides and A/T stretches on the antisense strand). The i-Score was generated from a web-based siRNA-designing algorithm created through observation of siRNAs in HEK293 cells.(17) Another 96 siRNAs were designed by Thermo Fisher to make up a total of 192 test siRNAs.

Available complete SARS-CoV-2 sequences show high percentage conservation as compared to various candidate siRNA sequences

After we determined the SARS-CoV-2 sequence homology at the nucleotide level, the average percent nucleotide identity between the first half of all available NCBI SARS-CoV-2 genomes and the SARS-CoV-2 consensus sequence was compared with the 19-nucleotide siRNA target sites identified from the first half of the genome. We did this to confirm that the siRNA target sites we selected are conserved as the pandemic progresses. So far, all siRNA target sites evaluated have remained highly conserved. As an example, the conservation at the nucleotide level of three siRNA target sites within the RNA coding for the nsp2, proteinase, and RDRP proteins located in ORF1 are shown (Fig. 2). A 19-nucleotide sequence that has not remained conserved is also shown as an example of what would be a poor siRNA target site.

Fluorescence readouts from GFP transfections show greater detection in HEK293T cells than in VeroE6 cells

Given the putative conserved regions that will be targeted for siRNA silencing, the next step was to develop a method to test if the siRNAs are effective at marking the SARS-CoV-2 gene target for degradation. We chose green fluorescent protein (GFP) to report the efficacy of the targeted degradation because of its simple tracking method. If a given siRNA is effective, it will decrease GFP fluorescence. In other words, the designed siRNAs should target the SARS-CoV-2 RNA that is also linked to the GFP RNA. As such, when the SARS-CoV-2 RNA is degraded, the GFP RNA

will also be destroyed, and the GFP will not be expressed. GFP fluorescence readouts can thus be used as a measure of siRNA efficacy in targeting chosen RNA segments of SARS-CoV-2. We divided the SARS-CoV-2 genome into seven fragment templates (using fragments obtained from Xie and colleagues), from which primers were designed to place them in frame ahead of the GFP sequence.(18) Each of the subgenomic open reading frames (S, 3a, E, M, 6, 7ab, 8, N) and the large ORF1ab (which was divided into five fragments; F1–F5) was inserted. Relative fluorescent units (RFUs) for serial dilutions of the majority sgRNAs in VeroE6 cells remained lower than those seen in HEK293T cells. Transfection into VeroE6 cells (Fig. 3) exhibited low detectable GFP fluorescence signal, with 3,611 RFU detected at 250 ng of transfected DNA, and 3,603 RFU detected at 125 ng for the empty construct. In contrast, 10,606 RFU was detected at 250 ng and 6,203 RFU was detected at 125 ng for the empty construct, in HEK293T cells (Fig. 4).

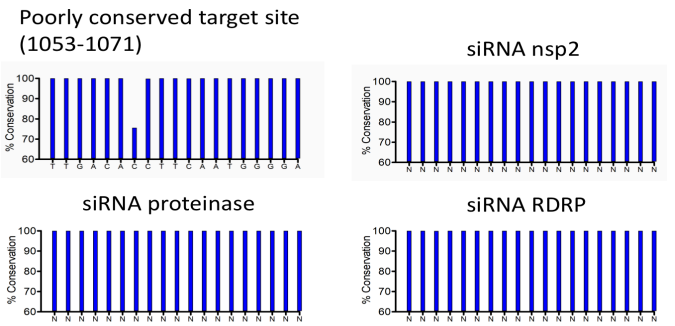

Figure 2. Sequence conservation of partial SARS-CoV-2 genome compared to candidate siRNAs. Percent conservation is shown on the vertical axis, while each nucleotide of the 19-nucleotide siRNA candidate sequence is shown on the horizontal axis. Real sequences are not shown for proprietary reasons. The upper left-hand panel provides an example of a poorly conserved target site, while the remaining three panels represent the targets site within the RNA coding for nsp2, proteinase, and RDRP, respectively.

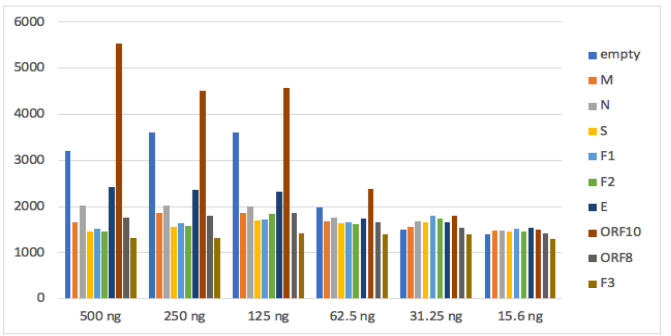

Figure 3. Graphical representation of GFP fluorescence readout in VeroE6 cells. GFP test showing RFUs in VeroE6 cells obtained with BioTek Synergy 4 plate reader (Excitation: 485 nm; Emission: 515 nm) after transfection of each GFP construct, cloned at decreasing doses.

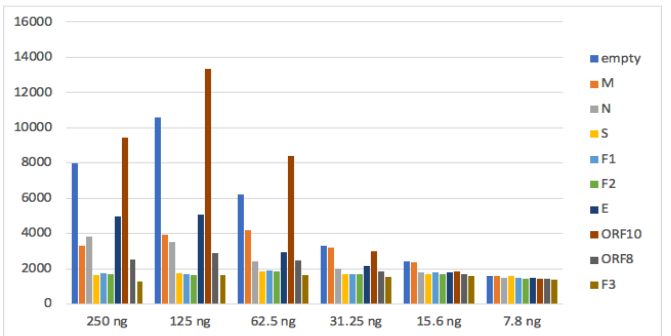

Figure 4. Graphical representation of GFP fluorescence readout in HEK293T cells. GFP test showing RFUs in HEK293T cells obtained with BioTek Synergy 4 plate reader (Excitation: 485 nm; Emission: 515 nm) after transfection of each GFP construct, cloned at decreasing doses.

Preliminary reverse transfection of HEK293T cells shows no toxicity to transfection reagent or to non-targeting siRNAs

To confirm that an siRNA did not produce toxic effects on the cells, we performed reverse transfection using negative-control siRNAs in HEK293T cells (Fig. 5). As show in Fig. 5, no conclusive differences in the viability or morphology of the cells were detected in the four transfection conditions: 1) no treatment, 2) treated with only the RNA transfection reagent, 3) treated with the RNA transfection reagent and the first non-targeting siRNA (siRNA1), and 4) treated with the RNA transfection reagent and the second non-targeting siRNA (siRNA2).

However, a few rounded cells and flat cells were found from cultures treated with RNAi max + siRNA1&2, compared to cells given no treatment. These results might indicate stress or an alternative effect on the cells.

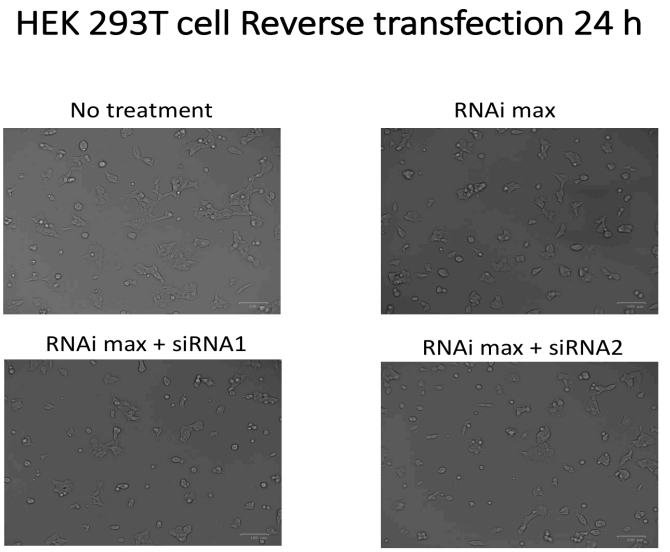

Figure 5. HEK 293T cells reverse transfected with non-targeting siRNAs after 24 h. Four reverse transfection conditions tested in HEK293T cells: no treatment, treated only with the RNAi MAX transfection reagent (RNAi max), treated with the RNA transfection reagent and scrambled siRNA1 (RNAi max + siRNA1), and treated with the RNA transfection reagent and scrambled siRNA2 (RNAi max + siRNA2).

Preliminary reverse transfection of HEK293T cells with targeting siRNAs identifies some efficacious siRNAs against the M, N, and E open reading frames

To identify effective siRNAs and confirm that our GFP assay can be used for siRNAs screening, we performed reverse transfection of siRNAs targeting the M, N, and E open reading frames and transfected the respective GFP-tagged open reading frames (Fig 6.). In subsequent experiments, all the other siRNAs will be screened once every construct expresses significant GFP signal.

Discussion

This research has the potential to create a safe and effective treatment against COVID-19. siRNAs targeting viruses have been studied preclinically for strains of Ebola and Marburg viruses in monkeys. (19-21) Additionally, three siRNAs (ARB-1740) were effective against Hepatitis B virus in human cells and are currently in clinical trials for the cure of chronic HBV infection. (22) To identify optimal siRNA target sites in coronavirus genomes, siRNAs against SARS-CoV-2 in particular, we examined the

conservation of all available sequences to evaluate homology and compared it to a limited number of candidate siRNAs. We cultured and plated two cell lines (VeroE6 andHEK293T) to test for siRNA efficacy and safety. Through this study, it was found that the target sites of all chosen siRNAs targeting the first half of the of the SARS-CoV-2 genome were conserved across all available circulating strains (examples shown in Fig 2). Confirming that the 19 nucleotides of the designed siRNAs were highly conserved within the target sequence in the SARS-CoV-2 reference genome was critical because siRNA activity relies on successful base pairing. Our study also supported that VeroE6 cells and HEK293T cells can be transfected with GFP-tagged SARS-CoV-2 RNA constructs. Measuring GFP fluorescence readouts informed the optimal concentration of GFP mRNA for screening so that, the chosen concentration produces a strong GFP fluorescence signal without saturating the potential inhibitory effects of siRNAs. In preparation for reverse transfection, HEK293T cells were reverse transfected with non-targeting siRNAs, although the results surrounding toxicity is still inconclusive. Reverse transfection with VeroE6 cells was not performed because the GFP fluorescence was not as strong. Lastly, we conducted reverse transfection of HEK293T cells with targeting siRNAs.

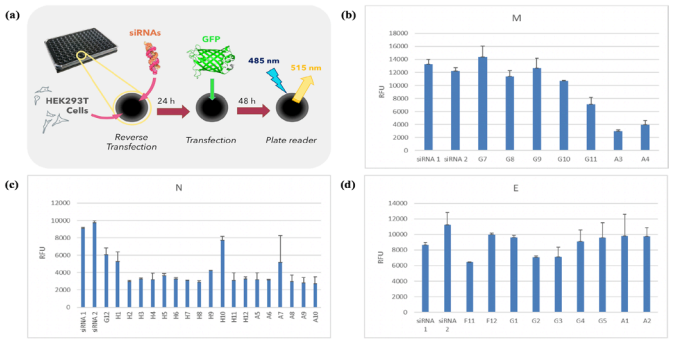

Figure 6. HEK 293T cell reverse transfected for targeting siRNAs followed by transfection of respective GFP constructs. HEK293T cells were plated onto siRNAs with RNAi Max transfection reagent (reverse transfection). Twenty-four hours later, we transfected cells with GFP constructs, and the plates on a fluorometer (excitation 485 nm; emission 515 nm) forty eight hours later. A schematic of the experimental procedures is depicted in (a). siRNAs targeting the (b) M open reading frame, (c) N open reading frame, and (d) E open reading frame are named according to their position in the 96-well plates. Two siRNA negative controls (siRNA1 and siRNA2) which do not target SARS-CoV2 are included for each GFP construct as a reference for the baseline GFP expression. Efficacious siRNAs decrease the GFP signal relative to controls. Data are expressed in relative fluorescent units (RFU) and represent the mean ± SD (standard deviation) of one experiment in duplicate (n=2).

We compared the conservation of siRNA candidate target sites using methods developed to calculate sequence conservation. As the pandemic progresses, this method can be updated with emerging sequences to verify that all siRNA target sites remain highly conserved in circulating strains of SARS-CoV-2. This data can also be used to identify new target sites, given that poorly conserved target sites can be easily excluded based on percent homology characteristics (Fig. 2). A recent study by Gilead Sciences used over 90,000 SARS-CoV-2 sequences isolated from clinics worldwide to analyze genetic diversity in the RNA replication complex in the context of potential SARS-CoV-2 genetic diversification impacting Remdesivir (RDV) efficacy as the virus spreads over time. (23) The researchers observed low genetic sequence variation; the high genetic stability of certain parts of the SARS-CoV-2 genome over time supports the prediction and hope for a minimal global risk of SARS-CoV-2 resistance to RDV. We employed the same idea to test for the entire first half of the SARS-CoV-2 genome in the context of siRNAs rather than RDV. While evidence suggests that the SARS-CoV-2 virus has remained genetically stable between January 12 and October 23 of 2020 (period for which NCBI Virus sequences were taken), there have been reports about the SARS-CoV-2 B.1.1.7 variant carrying deletions (69-70del) and mutations (N501Y, P681H) in the spike protein, resulting in increased virus infectivity and yield in human lung epithelial cells and primary airway tissue.(24) This enhanced transmissibility may imply that preventive measures against the original Wuhan-Hu-1 coronavirus reference genome may only be partly effective against new strains. Nevertheless, we have calculated that the UK B.1.1.7 lineage shared over 99.8% nucleotide identity with the original Wuhan-Hu-1 reference genome, and only three of our 192 siRNA target sites are altered in this lineage. More SARS-CoV-2 whole genome sequencing, genomic epidemiology, and collaboration for global surveillance will contribute to a better understanding of the speed and extent of mutations. (25) Our sequence conservation analysis is limited because only the first half of the SARS-CoV-2 genome was analysed due to the number of columns which can be created in Microsoft Excel. Another point worth mentioning is that two sequences did not align at the same nucleotide; coming from the same source, these two CoV-2 sequences were missing four nucleotides at the start of the sequence compared to the reference genome. We have now identified a method to analyze the second half of the genome independently and are calculating sequence conservation for the whole genome.

We performed two GFP tests, in VeroE6 cells (African Green Monkey kidney epithelial cell line) and HEK293T cells (Human embryonic kidney cell line). While the fluorescence readouts are similar in both cell lines, only HEK293T cells were used in subsequent reverse transfection experiments because there was little detectable signal obtained with the plate reader for some of the constructs in VeroE6 cells (Fig. 3). In both cell lines, the S, F1, F2 and F3 constructs did not produce a sufficiently strong GFP signal comparing to the background (Fig. 4). This weak signal can be caused by the large size of the segments (S, F1–F3). In future experiments, these constructs will be cloned into smaller fragments. The inserts (S, F1–F3) were also Sanger sequenced to confirm that the RNA is in frame with the GFP, eliminating out of frame cloning as a source of error. We hope to determine the lowest GFP dose that relays a good fluorescent signal. If the dose is above the GFP saturation limit, then it is harder for an introduced siRNA to decrease the signal from the overly abundant GFP mRNA. Hence, our goal was to determine the GFP doses which would eventually be used to screen siRNAs. The fact that only one independent experiment with two replicates was performed in each cell line presents a limitation and this experiment will be replicated to achieve a greater accuracy of results. One potential disadvantage of this technique is that no amplification of fluorescent signal using GFP occurs. Alternative reporter assays can also be used: the SARS-CoV-2 gene fragments can be fused with a luciferase reporter gene to produce better detection via the luciferase enzymatic reaction, which has been used recently in siRNAs silencing SARS-CoV-2 reporter constructs. (26)

Lastly, we performed early reverse transfection experiments. The first set of experiments provided data on HEK293T cells twenty-four hours after setting four conditions for negative control screening (Fig. 5). Two non-targeting siRNAs (scrambled siRNAs that do not target the SARS-CoV-2 genome) were used to reverse transfect HEK293T cells to evaluate whether the siRNAs exhibited any toxicity. Given that there are few cells to observe in Figure 5, it is difficult to determine toxicity visually or qualitatively. Thus, toxicity will be measured quantitatively in the future with a WST-1 assay. Hydrogen peroxide or Poly-I:C (a long, double-stranded RNA) will be used as a positive control to show cells death. Still, the absence of conclusive visual differences among cells in each condition steers us towards the cautious conclusion that there were no dramatic effects from the transfection reagent, nor from the transfection reagent with the siRNA negative controls. Given that transfection reagents and foreign RNAs can have toxic effects on cells via hybridization-dependent off-target effects, it is important to ensure that added concentrations are optimal before further screening. (16) We will also screen all efficacious test siRNAs for potential toxicity with the WST-1 metabolism assay. This will confirm that any decrease in GFP expression originates from siRNA targeting of the construct, as opposed to cell toxicity, which would also lead to a loss of GFP expression. The preliminary readouts for the test siRNAs targeting the M, N, and E constructs showed that some siRNAs appear to be active, although more replications are needed to determine accuracy of the observed effects. We will also need to confirm that there is no sequence-dependent toxicity caused by these siRNAs.

For future research, alignments of the second half of the SARS-CoV-2 genome will be made to calculate sequence conservation for the entire genome. For the constructs that did not produce adequate GFP signals in either VeroE6 cells or HEK293T cells, we will collect fluorometer readings in the near future after cloning the S and F1–F3 inserts into smaller fragments. If GFP fluorescence levels are significantly higher than the background, test siRNAs will be evaluated against the aforementioned constructs at varying concentrations. Inhibition of GFP signal will be monitored, with the expectation of observing a GFP signal decrease by some of the siRNAs. Following the optimization of GFP readout, the efficacy and safety can be tested. Cell metabolism assays can be used to eliminate siRNAs with cytotoxic effects in mammalian lung epithelial cell culture; this assay has been used in RNA therapies against HIV-1. (27) The efficacy and toxicity of the generated siRNAs can also be measured by their ability to target SARS-CoV-2 replication, inhibit SARS-CoV-2-induced cell death in a WST-1 cell metabolism assay, and independently affect cell metabolism. Specific constructs expressing viral mRNAs under a CMV promoter can also be created to verify the inhibition of the CoV-2 mRNA by qRT-PCR, which was previously performed for Zika Virus RNAs. (28) Inhibition of coronavirus protein production can also be confirmed using polyclonal antibodies that are anti-Capsid and anti-Spike. Thus, the next arm of this project is to screen the siRNAs for activities against SARS-CoV-2 production. There has been a great need for the development of therapies against the SARS-CoV-2 virus, and siRNAs are attractive candidates due to the simplicity in design and administration. Here, we used sequence conservation to confirm that our siRNAs target viral sequences have remained highly conserved throughout the course of the pandemic and began testing the binding of these siRNAs targets within SARS-CoV-2. This study aims to give health services an upper hand on the current pandemic as well as for future outbreaks of related coronaviruses.

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