The Use of Molecular Beacon siRNA to Induce Degradation of SARS CoV-2 mRNA in Infected Cells

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Background

The use of molecular beacon siRNA technology has exciting therapeutic potential for use in disease. This technology employs the use of siRNA to target specific genes within an organism leading to mRNA degradation. siRNA can be tagged with a fluorophore, resulting in molecular beacon siRNA (Fig. 2).[1] Viewing fluorescence of treated cells is indicative that the siRNA successfully associated with specific genes.

As COVID-19 left a lasting impact throughout the world, it is hoped this technology can be applied to treating this virus. It is hypothesized that the addition of siRNA to cells expressing specific SARS CoV-2 (Fig. 1) mRNA will cause reduction of viral proteins in the cells. To test this hypothesis, Calu-3 cells will be transfected with a plasmid coding for both the envelope (E) protein of the virus and GFP.

The current focus of this research is to successfully transfect and maintain a transfected cell line to then treat with siRNA. To accomplish this, Lipofectamine 3000 was used, and through the process of repeating transfections using the recommended ratio of 1:1, DNA: Lipofectamine, it was found to be ineffective in efficiently transfecting the Calu-3 cell line with the E plasmid. This led to testing different DNA: Lipofectamine ratios to determine the ideal concentrations for successful transfection.

Addition of the molecular beacon siRNA to the Calu-3 cells is expected to induce degradation of mRNA coding for the E protein. Therefore, it is expected that the GFP tagged E protein will be reduced in cells treated with siRNA. The degradation of the mRNA for the E protein will lead to a reduction of proteins; therefore, silencing gene expression. Successful silencing of the gene could then be considered as a therapeutic for COVID-19 due to the suppression of the gene and inhibition of viral particle formation.

Research Question

The overall goal of this research is to investigate if the addition of molecular beacon siRNA to cells transfected with the plasmid coding for the E protein (Fig. 4) of SARS CoV-2 will induce mRNA degradation. In turn, resulting in reduced expression of the E protein. Current work on the project involves achieving a stably transfected cell line for use with siRNA.

If the siRNA prevents expression of the mRNA coding for the E protein, the cell will not produce E protein. Therapeutically, this would render a virus non-functional.

Methods

Preparation for Transfection

Calu-3 cells were grown and maintained in EMEM media (10% FBS, 1% P/S). A kill curve experiment was completed to determine the optimum concentration of G418, which was 0.2 mg/mL (data not shown). The E plasmid was obtained from Addgene (Plasmid #165123) and used to transform One Shot TOP10 Chemically Competent *E. coli* cells (Fisher Scientific, C404010), which were placed onto an LB agar plate containing kanamycin. A single colony was propagated and a standard miniprep purification was performed with 1 µL of plasmid being tested using a NanoDrop 2000 for purity.

Transfection Model Development

Transfections were prepared by plating 47,000 cells per well in a 96-well plate and cells were allowed to grow to 80-90% confluence. Once wells were confluent the process of transfection using Lipofectamine 3000 was performed. The traditional methods for the use of Lipofectamine 3000 uses a 1:1 ratio of DNA to Lipofectamine. This traditional method was found to be insufficient in effectively transfecting the Calu-3 cells. This led to the formation of a multi-ratio experiment; which was completed using ratios of 1:4, 1:8, 4:1 and 8:1. From this the 4:1 ratio was found most efficient.

Stable Transfection

Cells seeded in a 96-well plate were transfected with a 4:1 ratio and treated with G418 for selection (Fig. 6). The transfected cells continued to grow for 3 weeks.

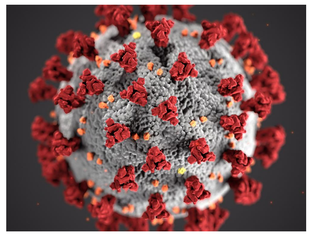


Figure 1. SARS CoV-2.[2] Image of SARS CoV-2, containing all its components, including the spike proteins, represented in red.

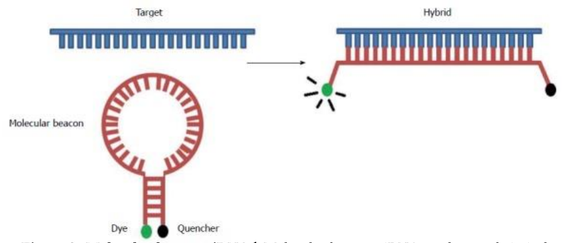


Figure 2. Molecular beacon siRNA.[1] Molecular beacon siRNA produces a hairpin loop that attaches the fluorophore to the quencher when unbound to mRNA. In the presence of mRNA, the siRNA will bind, removing the fluorophore from the quencher to emit fluorescence.

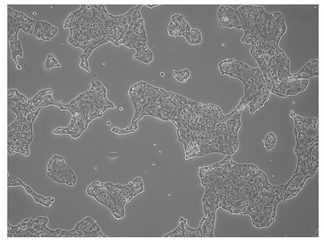


Figure 3. Calu-3 cells.[6] Picture of Calu-3 under 40X magnification. These are what the cells look like under normal conditions when maintained in EMEM in a T-75 flask.

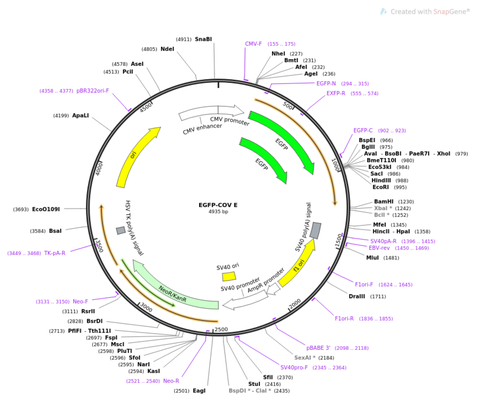


Figure 4. E plasmid.[4] The plasmid used to encode for the envelope protein of SARS CoV-2 consists of 4935 bp. Important features of this plasmid include the superfolder GFP that spans 714 bp to produce fluorescence of the plasmid, as well as NeoR/KanR (795 bp) which confers resistance to neomycin, kanamycin, and G418. This plasmid is also ampicillin resistant, as it contains the 861 bp AmpR gene.

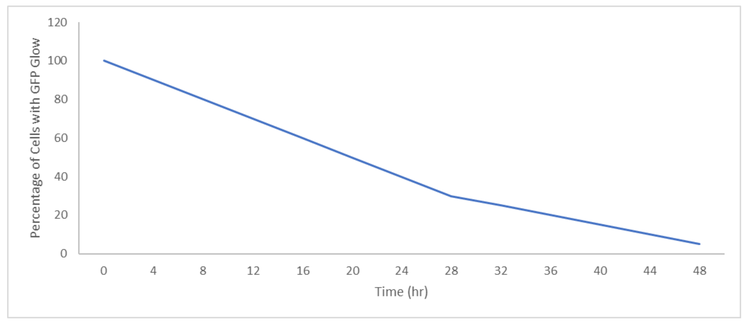


Figure 5. Hypothetical Gene Silencing Curve.[6] Following transfection with siRNA, the change in GFP glow will be measured. The Calu-3 cells containing the E plasmid are expected to have a reduction of GFP production and glow over time after siRNA introduction.

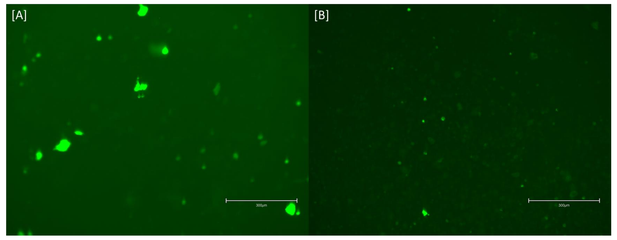


Figure 6. Calu-3 Transfection.[6] A 96-well plate transfected with Lipofectamine 3000. **(A)** The Calu-3 cells transiently transfected with the positive control, GFP. **(B)** The transfection of Calu-3 cells with the E plasmid.

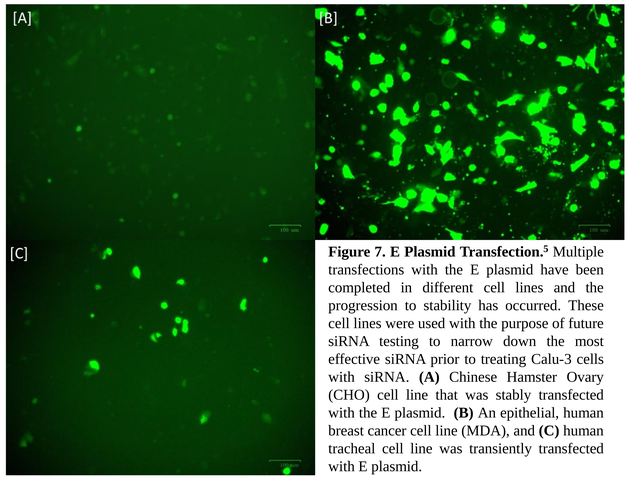
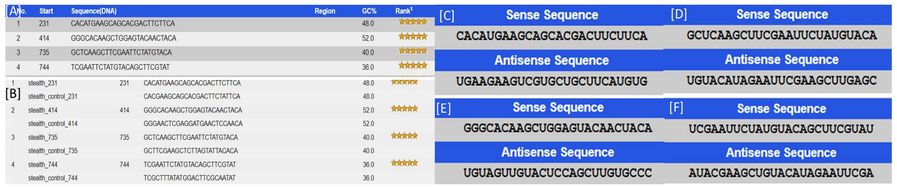


Figure 7. E Plasmid Transfection.[5] Multiple transfections with the E plasmid have been completed in different cell lines and the progression to stability has occurred. These cell lines were used with the purpose of future siRNA testing to narrow down the most effective siRNA prior to treating Calu-3 cells with siRNA. **(A)** Chinese Hamster Ovary (CHO) cell line that was stably transfected with the E plasmid. **(B)** An epithelial, human breast cancer cell line (MDA), and **(C)** human tracheal cell line was transiently transfected with E plasmid.



No.	Start	Sequence(DNA)	Region	GC%	Rank[1]
1	231	CACATGAAGCAGCACGACTTCTTCA		48.0	*****
2	414	GGGCACAAGCTGGAGTACAACTACA		52.0	*****
3	735	GCTCAAGCTTCGAATTCTATGTACA		40.0	*****
4	744	TCGAATTCTATGTACAGCTTCGTAT		36.0	*****

[B]

1	stealth_231	231	CACATGAAGCAGCACGACTTCTTCA	48.0	*****
	stealth_control_231		CACGAAGCAGCACGACTTCTATTCA	48.0	
2	stealth_414	414	GGGCACAAGCTGGAGTACAACTACA	52.0	*****
	stealth_control_414		GGGAACTCGAGGATGAACTCCAACA	52.0	
3	stealth_735	735	GCTCAAGCTTCGAATTCTATGTACA	40.0	*****
	stealth_control_735		GCTTCGAAGCTCTTAGTATTACACA	40.0	
4	stealth_744	744	TCGAATTCTATGTACAGCTTCGTAT	36.0	*****
	stealth_control_744		TCGCTTTATATGGACTTCGCAATAT	36.0	

[C] Sense Sequence: CACAUGAAGCAGCACGACUUCUUCA
Antisense Sequence: UGAAGAAGUCGUGCUGCUUCAUGUG

[D] Sense Sequence: GCUCAAGCUUCGAAUUCUAUGUACA
Antisense Sequence: UGUACAUAGAAUUCGAAGCUUGAGC

[E] Sense Sequence: GGGCACAAGCUGGAGUACAACUACA
Antisense Sequence: UGUAGUUGUACUCCAGCUUGUGCCC

[F] Sense Sequence: UCGAAUUCUAUGUACAGCUUCGUAU
Antisense Sequence: AUACGAAGCUGUACAUAGAAUUCGA

Figure 8. Designed siRNA for the E Plasmid.[3] This is an example of what the siRNA sequences for the E plasmid will be. **(A)** An image of the top four siRNA designs and the rank by predicted effectiveness. **(B)** The title and full sequence of each of the top four along with the matching control sequence. **(C, D, E & F)** The sense sequence and antisense sequence for the top four siRNA designs in sequential order.

Results and Conclusion

Results

Preparation For Transfection

The kill curve demonstrated that 0.2 mg/mL was the ideal concentration of G418 as the cells died after 7 days of treatment.

Plasmid Purification and Digest

The NanoDrop was used to view purity of the E plasmid following purification; purity was found to fall within the 1.8-2.0 range. A restriction digest was done to confirm the purification of the full E plasmid. The digest resulted with three bands of 145 bp, 553 bp and 4,237 bp; confirming the identity of the E plasmid.

Transfection with Lipofectamine 3000

The seeded Calu-3 plate was grown to 80-90% confluence. The cells were transfected using Lipofectamine 3000 in a 4:1 ratio. The cells showed stable growth with G418 treatment and proved that the E plasmid was taken into the cell.

Conclusion

Following plasmid purification and restriction digest confirmation, the 96 well plate was transfected with the E plasmid at the previously determined ideal DNA/Lipofectamine 3000 ratio of 4:1. The cells were treated with G418 media after transfection to select for the cells that successfully expressed neomycin resistance coded for in the E plasmid. During this process fluorescence microscopy was used to determine the uptake of the plasmid into the cells.

Following treatment with G418 media, small areas of minimal glow were observed using fluorescence microscopy (Fig. 6B). These regions were observable for three weeks following treatment with G418, indicating uptake and expression of the plasmid. Results of the sister study occurring at University of Alabama Birmingham which is using alternate cells lines also yielding results indicating cellular uptake of the E-plasmid (Fig. 7).

The next steps will be to isolate transfected colonies and grow a stable transfected cell line. Once achieved, siRNA will be purchased (Fig. 8.) and used to treat the cells. It is expected the number of cells expressing GFP will decrease as the siRNA leads to the degradation of the mRNA coding for the E protein (Fig. 5).

Future Work

1. Stabilize transfection of Calu-3 cells with E plasmid
2. Maintain growth of stabilized cell line
3. Treat transfected cells with siRNA to disrupt viral replication and protein synthesis of the E plasmid
4. Confirm efficacy of siRNA using immunofluorescence
5. Perform Western blot to confirm reduced levels of E protein
6. Investigate the efficiency of targeting N and/or M proteins of SARS CoV-2 and proteins of other similar viruses

References and Acknowledgments

1. Remo, G. et al. (2015). Use of siRNA molecular beacons to detect and attenuate mycobacterial infection in macrophages. *World J Exp Med.*, *5*(3), 164-181.
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4. Addgene, https://www.addgene.org/165123/sequences/.
5. Photos by Dr. Unlap lab.
6. Photos by Brookhart, Dutt, & Winter
6. Bratu, D.P., I. E. Catrina, and S.A. Marras (2011). Tiny molecular beacons for in vivo mRNA detection. *Methods Mol. Biol.*, 714: 141-157.

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