Genetic Editing Out the Tumor Growth Suppressor Gene TRM9L in Colorectal Cancer Models Using CRISPR-Cas9

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Genetic editing out the tumor growth suppressor gene TRM9L in colorectal cancer models using CRISPR-Cas9

An honors thesis presented to the Department of Nanoscale Science, University at Albany, State University of New York
In partial fulfillment of the requirements for graduation with Honors in Nanoscale Science and graduation from The Honors College.

Philip Blatner
Research Mentor and Advisor: Thomas Begley, Ph.D.

May, 2017
Clustered regularly interspaced short palindromic repeats (CRISPR) is a precise genetic engineering tool for genome editing. CRISPR utilizes guide RNA (gRNA) to find specific DNA sequences followed by a Cas9 nuclease to cut the DNA at a specific site. TRM9L is a tumor growth-suppressor gene that restricts the growth of some colorectal cancer cells by upregulating LIN9 expression. TRM9L expression is lost in some late stage colorectal cancers and cancer models (SW620). SW480 colorectal cancer cells express TRM9L and these cells are considered to be at the beginning of colorectal cancer development. The goal of my project was to use genetic engineering approaches to remove TRM9L from early stage colorectal cancer cells (SW480) and to then determine the effect on tumor growth. In this project, CRISPR constructs were synthesized, hybridized, and ligated to an expression vector. The hybridized vector underwent bacterial-facilitated replication and was then harvested via plasmid purification. The plasmids were gene sequenced and then transfected into a growing culture of SW480 cells. After a month of plasmid-inclusion growth, the cell cultures will be analyzed for growth effects. My work is important because it will develop new tools for use in colorectal cancer research, a disease that claims over 50,000 lives each year.
Acknowledgements

I would like to thank my advisor, Dr. Thomas Begley, for never giving up hope on me regardless of the situation.

I would also like to thank Uli Begley and Khadijah Onanuga for their mentorship and guidance throughout the entirety of the project. This study would not have any substantiability if it were not for them.
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**Background**

Yeast Trm9 is a tRNA methyltransferase enzyme that modifies the wobble uridine and helps prevent damage-induced cell death\(^1\). Trm9 catalyzes the modification of wobble bases in specific tRNA anticodons, and promotes increased levels of important damage-response proteins\(^2\). The *TRM9* gene was first identified in 2003 when researchers showed the deletion of the F6 gene (later called TRM9) lead to hypomethyl esterification of specific tRNA bases\(^2\). The human tRNA methyltransferase 9-like (hTRM9L) protein is a homolog of yeast Trm9. TRM9L is a tumor growth suppressor that acts as control for LIN9 expression by influencing the translation of specific proteins involved in the regulation of LIN9. It was discovered when researchers studying hTRM9L expression in SW620 (colorectal cancer) cells achieved a 93% knockdown of LIN9 in the cells and then consequently observed a reversal of the hTMR9L tumor growth suppression phenotype in the SW620 expressing cells\(^3\).

![Figure 1](image-url)

**Figure 1.** hTRM9L’s role in a cellular stress response by increasing LIN9 expression.

Image retrieved from reference (3)
The TRM9L protein affects a cell’s ability to restrict cancer growth, as TRM9L deficient cells have been shown to proliferate while TRM9L proficient cells are in a cell-replication arrest state\(^4\). The importance behind studying the TRM9L gene is that the evaluation of its expression and removal from the cell will help researchers define the genetic programs associated with aggressive colorectal cancer cells, which may promote better avenues for treatment.

Colorectal cancer originates in the epithelial cells of the colon or rectum when mutations occur in the APC, TP53, and TGFB genes. APC (adenomatous polyposis coli) gene is a tumor suppressor gene that prevents the buildup of β-catenin protein. Mutation in APC leads to an accumulation of β-catenin proteins which translocate to the nucleus and bind to DNA activating the transcription of proto-oncogenes\(^5\). Mutation in the TP53 gene produces defective p53 proteins responsible for controlling cell division and killing cells that begin to divide rapidly\(^6\). TGFB is responsible for producing a superfamily of proteins that regulate growth inhibition which fails after a mutation in the gene. TGFB also controls a portion of helper T cell differentiation in that the failure of proper production of TGF-β1 proteins inhibits the differentiation of helper T cells, making the cell less recognizable by the body as hazardous\(^7\).

Starting in the mucosa (inner) layer of the organ, colorectal cancer progresses through multiple stages begins as a polyp and develops into a metastasized tumor. The advancement of the tumor growth is depicted below in Figure 2.
Figure 2. Progression of colorectal cancer from Stage 0 to Stage IV.


The characteristics of each stage are as follows: Stage 0 – The cancer cells begin to divide in the mucosa. Stage I – The cells invade the muscular layer of the colon/rectum but have not spread into nearby tissues/lymph nodes. Stage II – Cancer has grown through the wall of the colon/rectum and begun to spread into nearby tissues, but not to the lymph nodes. Stage III – Cells are now spreading into nearby lymph nodes, but remain localized and have not disseminated to distant regions of the body. Stage IV – Cancer is now metastasizing into multiple different portions of the body making treatment exponentially more difficult.⁸

Clustered regularly interspaced short palindromic repeats (CRISPR) acts as an immune system for prokaryotic cells⁹. CRISPR acts by cutting the genetic material of a foreign invader and selectively splicing the foreign DNA into its own genome for future reference by the cell,
working as an immune history/memory. The origin of CRISPR began with Francisco Mojica in 1993 when his group identified interrupted repeats of DNA in the genome of two archaeal species he was studying: *Haloferax volcanii* and *Haloarcula quadrata*. In collaboration with Ruud Jansen, Mojica coined the term CRISPR in one of Jansen’s reports in 2002 to clear the confusion of multiple acronyms circulating at the time. Mojica began to identify many of these interrupting repeats as snippets from the genomes of bacteriophages leading him to hypothesize that CRISPR is an adaptive immune system for prokaryotes. Alexander Bolotin in 2005 identified Cas9 and the PAM, protospacer adjacent motif, sequence when studying the bacteria *Streptococcus thermophiles*. Dr. Bolotin noted Cas9, a large protein, showed nuclease activity, and that many of the spacers shared a common sequence at one end called a protospacer adjacent motif (PAM), which is required for target recognition. In 2012, Jennifer Doudna and Emmanuelle Charpentier reported that two of the targeting RNA segments, crRNA and tracrRNA, could be fused together to create one single, synthetic guide (known as gRNA) simplifying the CRISPR system drastically. A year later, Feng Zhang demonstrated that the CRISPR-Cas9 system could be adopted for eukaryotic organisms as they successfully performed genome cleavage in human and mouse models. They also showed that the system could drive homology-directed repair (HDR) in the genome which is important for gene conversion, and that the system could target multiple genomic loci. Recently, Cpf1 was discovered in *Francisella novicida* producing a new system, CRISPR-Cpf1 contrasted to CRISPR-Cas9, which caused a staggered cut in a genome as compared to a blunt cut produced by Cas9. Also, Cpf1 only requires crRNA inside of the synthesized gRNA strand. The staggered cut is important as it
makes gene conversion much simpler and smoother for a cell by being less disruptive to the surrounding genes\textsuperscript{11}.

Inside the CRISPR complex, gRNA must be properly formulated to target the correct region of genomic DNA. gRNA consists of two parts: crRNA, which finds the desired sequence, and tracrRNA, which is complementary to crRNA so it combines with it to form an RNA duplex and turn it into gRNA. The gRNA goes into the nucleus of a cell bound to the Cas9 protein, and will bind to the corresponding DNA sequence so that Cas9 may cleave the genome at the PAM site, which is a 2-6 base pair DNA sequence immediately following the gRNA’s target sequence. PAM’s characteristic sequence is 5’-NGG-3’, where N is any nucleotide. Different Cas9 proteins have different PAM sequences, but the most common is the NGG sequence derived from \textit{Streptococcus pyogenes} bacteria\textsuperscript{12}. Once cut, the open-ended DNA may now be manipulated to delete a certain gene or insert a new gene. The deletion of a gene occurs when the cut ends are incorrectly repaired causing an indel mutation, thereby inactivating the corresponding protein once translated. To achieve a deletion, the DNA repair pathway needed is called non-homologous end joining (NHEJ). NHEJ which does not use a template for the repair process, in contrast to homologous directed repair (HDR) which does use a parental strand as a template. NHEJ will remove nucleotide overhangs near the cut site until two sufficient blunt ends are achieved with the ends then rejoined through DNA ligation which leads to a frameshift mutation. Figure 3 is a schematic detailing how the CRISPR-Cas9 complex interacts with the target DNA sequence long with the repair process the cell can use if there is a homologous
A CRISPR-Cas9 complex seeks target strands of DNA and cleaves at the desired site directed by the gRNA. Once used to eliminate bacteriophages, it now has been repurposed to selectively splice in and out genes from human genomes. Some examples of this include the generation of transgenic mice by the disruption of five genes: Tet1, 2, 3, Sry, and Uty. The simplicity of CRISPR also allows for a new form of gene function analysis on a genome-wide scale, ultimately a more effective version of genetic screening. Duchenne Muscular Dystrophy, what was once
a severe muscle-degenerative disease that had little to no treatment now has the potential for a cure by means of an exon knock-in, a specialized technique that is only capable with CRISPR\textsuperscript{16}.

The goal of my study was to achieve a TRM9L knockout in human colorectal cancer cells (SW480) using CRISPR. So far, we have successfully generated and sequenced the required CRISPR constructs including the plasmid that contains the gRNA, Cas9 gene, and GFP gene. The next aim is to culture SW480 cells and add these constructs to observe the effects of a TRM9L knockout. Future work may include reorganizing the procedure to target similar but different genes in the genome such as LIN9.

**Materials and Methods**

The first goal of my work was to identify four characteristic DNA sequences in the TRM9L gene for targeting by CRISPR gRNA. This initial step was important because we wanted to select sections of DNA that were found only in the TRM9L gene, to avoid unwanted off-target activity. TRM9L gene exons were retrieved from NCBI using a FASTA sequence download of the TRM9L (KIAA1456) gene and can be observed below in Figure 4.
Random 20 base pair long nucleotide segments beginning with ATG (start codon) were identified in the gene and analyzed using the crispr.mit.edu database. We chose to start our nucleotide analysis with ATG because an ATG sequence indicated the start of a new open reading frame, therefore, cutting with or after an ATG would successfully cut out a section of a protein from an exon. The crispr.mit.edu database compares the nucleotide sequences to the rest of the human genome to determine how frequently 20 base-pair (bp) sections of those sequences appeared in the rest of the genome. The sequences were then given a score from 0-100 with the higher the score meaning the less often it was found. Sections that had a score of over 85 (meaning that the sequence would lead to very few off-target interactions) were saved for use in the experiment. An example of this is shown below in Figure 5.

**Figure 4.** The FASTA format of the TRM9L gene which contains five exons and four introns.
For each 20 bp strand selected, the 5’-3’ complement was written, and then rewrote in reverse as each strand would be synthesized in the 5’-3’ fashion. Eight oligonucleotides were developed, four selected 20 basepair sequences and the respective four reverse compliment sequences, with the eight sequences sent to Integrated DNA Technologies (Coralville, IA) for chemical synthesis (Table 1).

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<tr>
<td></td>
<td>AAA CGG AAC GAA ACG AAT TTC CTA CC</td>
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**Table 1.** Eight oligonucleotide sequences sent to IDT to be synthesized 5’-3’.

The oligonucleotides needed to be hybridized and cloned into a destination vector. A plasmid is a circular piece of DNA that acts as a vector. A plasmid can operate independently of the genome (i.e., autonomous replication and segregation into dividing cells), but is also able to insert and combine with the genome. Plasmid pX458 (Addgene, Cambridge, MA) was chosen for its successful use in previous experiments by Feng Zhang’s group, and because it contains
ampicillin as a selectable marker in *E. coli* and offers green fluorescent protein (GFP) for tracking in mamalian cells. A map of pX458 is shown in Figure 6.

![Figure 6](image_url)

**Figure 6.** Detailed plasmid pX458 gene map describing different primer/gene regions. Image retrieved from reference (17)

To start, pX458 was first acquired (through lab mentor Khadijah Onanuga) and digested. To digest the plasmid, 1 µg pX458 was mixed with 1 µL FastDigest *BbsI* (Thermo, Fredrick, MD), 1 µL FastAP (Thermo), 2 µL 10XFastDigest Buffer, and enough µL of ddH2O to produce a 20 µL solution. The solution was then placed in 37°C for 30 minutes. A 0.8% agarose gel was prepared using 50 mL of TAE buffer (Tris base, Acetic acid, and Ethylenediaminetetraacetic acid) and 0.4g of agarose. For loading the lanes in the gel, Lane 1 contained 20µL of 10kb molecular ladder, Lane 2 had 6µL of loading dye mixed with 14µL of digested plasmid solution, and Lane 3 had 6µL of loading dye with the remaining plasmid solution. The 0.8% gel was then run using TAE buffer and 1 µL EtBr for 1 hour at 120V. The gel was placed under UV light and small cuts were made in the gel to excise the plasmid DNA in Lanes 2 & 3 around the 10kb level marked by the ladder in Lane 1.
Gel extraction was performed with a QIAquick Gel Extraction kit from Qiagen\textsuperscript{18} (Louisville, KY), according to the supplier protocol. First, the gel slices were weighed in a micro-centrifuge tube and then 3x the volume of the gel was added in Buffer QG (100mg ~ 100µL). The solution was incubated at 50°C for 10 minutes with periodized vortexing every 2-3 minutes. After checking to reassure that the color of the mixture is yellow, 1 gel volume of isopropanol was added to the mix. The mixture was applied to a QIAquick spin column in a 2 mL collection tube and then centrifuged for 1 minute at 13,000 rpm. The flow through was discarded and 0.5 mL of Buffer QG was added. The sample was then centrifuged for another minute and 0.75mL of Buffer PE was added as a wash and then the tube was centrifuged for 1 minute. Lastly, after the flow-through was discarded, the spin column was placed in a new micro-centrifuge tube with 50 µL of Buffer EB added to the center of the column and centrifuged for one last minute. A summary of DNA gel extraction is shown in Figure 7.

![Gel Extraction Diagram](Image retrieved from reference (18))

**Figure 7.** DNA in agarose bound to a spin column, washed with multiple buffers, and eluted.

The oligonucleotides were re-suspended in a solution of ddH\textsubscript{2}O by following IDT’s Specification Sheet to produce a final concentration of 100 µM. The oligonucleotides were
phosphorylated using T4 polynucleotide kinase (T4 PNK) in the following recipe: 1 µL of Oligo 1 (100 µM), 1 µL of Oligo 2 (100 µM), 1 µL of 10X T4 Ligation Buffer (NEB, Ipswich, MA), 6.5 µL ddH₂O, and 0.5 µL of T4 PNK (NEB). The reaction mixture was placed in a thermocycler for annealing: 37°C for 30 minutes, then 95°C for 5 minutes followed by ramping down to 25°C at 5°C/minute.

To insert the oligonucleotides into the plasmid, the now double-strand oligo sequences needed to be ligated into the plasmid vector using DNA Ligase. The ligation reaction was prepared using a solution of 50 ng of digested pX458, 1 µL oligonucleotide duplex in a 1:250 dilution, 5 µL 2X QuickLigation Buffer (NEB, Ipswich, MA), 1 µL of Quick Ligase (NEB), and XµL of ddH₂O which produced an 11µL solution that then sat on the lab bench for 10 minutes.

To ensure only our undigested, ligated plasmid vectors were available for transformation, the 11 µL from the ligation reaction was mixed with 1.5 µL of 10X PlasmidSafe buffer, 1.5 µL 10 mM ATP, 1µL PlasmidSafe exonuclease (Epicentre, Madison, WI). The reaction was incubated at 37°C for 30 minutes, then 70°C for 30 minutes. This process would effectively remove any un-ligated/digested plasmids by cleaving individual nucleotides from the linear DNA at either the 5’ and 3’ end. Next, the remaining circular DNA was transformed into *E. coli*. Epicentre’s One Shot Stbl3 bacteria were cultured in One Shot vials with our plasmids according to supplier recommendations. First, the One Shot vials were thawed on ice. 1-5 µL of plasmid DNA was added to the vials and then incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42°C and then placed on ice for 2 minutes. 250 µL of S.O.C. medium (Super Optimal Broth) was added to each vial and then shook horizontally for 1 hour at 225 rpm.
and 37°C. 25-100 μL of the culture from each vial were spread out on 100 μg/mL LB + ampicillin plates and grown overnight at 37°C.

To ensure the characteristic sequences were cloned into the vectors, plasmid DNA was sent to Genewiz (South Plainfield, NJ) for DNA sequencing. To prepare the plasmid, three colonies were taken from each plate (grown in previous section) and placed into 5 mL of LB + ampicillin broth producing a total of 12 new growth solutions. The vials were left to grow for approximately 16 hours in a shaking incubator. The solutions were centrifuged at 13,500rpm for 10 minutes at 4°C and then decanted leaving the cell pellet at the bottom. We then used a Qiagen Plasmid Extraction kit to harvest our plasmid from the bacteria cells. 250 μL of p1 buffer was pipetted into each vial to resuspend the cells. 250 μL of p2 buffer was added to lyse the cells. P3 buffer is acidic and 300 μL of p3 was added to neutralize the basic p2 buffer. It is important to note that p2 buffer causes the solution to turn blue and that proper incorporation of p3 buffer should allow the solution to return to a translucent color. After the inclusion of p3, bacterial chromosomal DNA precipitates out leaving this fluffy white material floating in solution as seen below in Figure 8.
The vials were centrifuged for another 10 minutes at 4°C at 13,500 rpm. The liquid was pipetted out from each vial and individually applied to Qiagen filter columns where a bind, wash, and elution process took place. The vials were centrifuged for 1 minute at 4°C and 13500 rpm and flow-through was discarded. 750 µL of PE buffer was used to wash the column, spun for 1 minute, and flow-through discarded. The tube was then centrifuged again for 1 minute and the flow-through was discarded. The plasmid DNA was eluted with 45 µL of HyPure warm water into a new Eppendorf tube.

A sample from each DNA elution, 20µL, was taken and sent to GeneWiz (South Plainfield, NJ) for sequencing. After the company completed the sequencing and determined the nucleotide base order of the genetic material sent to them, they compiled it into separate text documents and sent to us. We used the text files to evaluate whether our plasmid was inserted. With the genetic information in hand, we BLASTed or Aligned the genetic sequence

**Figure 8.** Chromosomal DNA precipitating out of solution appearing in fluffy, white form.
with our original pX458 plasmid. In this comparative process, the program assesses the input DNA and compares them to a control sequence (the original plasmid) to find regions of local similarity. In this way, the program can align small portions of matching genetic information to deliver a report of the overall alignment and matchup of the two genetic sequences.

**Results**

To first ensure the initial plasmid sample was pure, gel electrophoresis was performed on the digested pX458 solution to verify plasmid size (Figure 9).

![Figure 9. Agarose gel analysis of digested pX458 plasmid.](image)

A single band resulted at the expected size of about 10 kb as the band is approximated around 9.2 kb. Using a gel extraction kit allowed us to purify the plasmid band from the gel.
After inclusion of the plasmid into the bacterial One Shot Stbl3 E. Coli cultures – the cells were grown and spread on LB + ampicillin plates. Four plates grown overnight in a 37°C incubator (one plate for each respective vector), each experimental LB + Ampicillin plate showed colony growth as seen in Figure 10. We included positive and negative controls during this trial to ensure it was our plasmid that induced the results by having one plate with no streaked bacteria, and one plate streaked with bacteria that had not been exposed to our recombinant vector. No colonies grew on either control plate (data not shown).

![Figure 10. Four LB + Amp plates that contained Stbl3 cultures transfected with respective recombinant pX458 plasmids.](image)

After the Plasmid Purification step, the concentration of DNA in each vial was tested to ensure the DNA had been successfully harvested from cells and was now in solution. A NanoDrop Spectrophotometer was used to measure the DNA concentration in ng/µL by placing
a single microliter of sample on the spectrophotometer and having the program run the test under ‘Nucleic Acids’. Nanodrop concentration data is shown in Table 2.

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**Table 2.** Concentration of DNA in ng ng/µL corresponding to its sample number.

I was able to take the results I received from GeneWiz and use an Alignment tool to compare the original pX458 vector with genetic sequence of the recombinant vector. The Alignment tool lays out in order each respective genetic sequence 5’-3’ and then determines the number of mismatch base pairs (where the nucleotides in different pieces of DNA are not the same), and calculates the percentage of mismatches after accounting for frameshifts that may occur due to the mismatches. An alignment score under 100% indicates there were modifications made to the genetic sequence of the plasmid, and after checking with the mismatches with our oligonucleotide sequences – it can be determined that those base pair differences can be attributed to the inclusion of the desired oligonucleotides (Figure 11).
Discussion

Overall, the effectiveness of the CRISPR technique is unable to be determined as the concluding portions of the experiment have yet to be undertaken. CRISPR is regarded as a precise technique that can specifically alter the genome of a desired organism with few undesirable interactions, and so this hypothesis is sought to be evaluated in the coming term.

Should CRISPR not operate as expected in the experiment, there are other techniques that produce a similar outcome with RNAi (RNA interference) which are essentially RNA strands that target and neutralize mRNA strands preventing gene translation after transcription. RNAi would be capable of negatively manipulating (aka deletion/prevention) TRM9L expression comparable to the outcome of CRISPR, making it a plausible alternative technique.
Pitfalls and Limitations

Completing these experiments was a great learning experience not just because of the novel techniques and new procedures, but also because it exposed many things I had not been conscience about. For example, it was not clear in the first steps of digestion which buffer to use because the recipe called for a different medium than what was in stock. Knowing one of the available buffers was an appropriate substitution, I took the one that seemed most likely correct instead of seeking scientific clarification. A buffer lacking BSA was chosen when a buffer with BSA was required holding the experiment back an interval of time due to the incorrect usage of a buffer.

A simple misunderstanding that spiraled into a semester long conundrum was the difference between agar and agarose. Knowing one is used to make plates and one is used to make gels is common knowledge, however, I failed to realize there was a significant difference between the two and that the selection of the ingredient would effectively alter the entire efficacy of the experiment. I had chosen wrong for multiple months.

The last issue to touch on is the expectation of time in the laboratory. Simple logic could help generate a time table for many of the experiments and procedures that will be undertaken, however, doing so lends a serious underestimation of experiment success rates and reagent reliability – especially for the first time running through a new procedure. I could often guess how long I should be in lab for any given portion of the experiment, but I constantly failed to take into consideration what it might mean to my plan should a portion of my experiment fail or I did not have the proper reagents available to me. Doing so held back my
progress for quite a bit of time which is why I learned it is important to give amble time to both the procedures and the overall progress of the experiment.

**Current Work in the Field**

CRISPR is a popular topic in biomedical research currently due to its recent discovery and the DNA editing potential that it offers. Many groups are pioneering work in this field, such as the Rotello group at the University of Massachusetts. The Rotello group wanted to focus on how to improve actual CRISPR-Cas9 entry into the nucleus. Currently there are few effective methods that will successfully transfer the complex into the cytosol and then into the nucleus as well without being degraded. The Rotello group achieved a highly efficient translocation of CRISPR-Cas9 by coupling a complexed Cas9 (had a gRNA bound to it) with carrier gold nanoparticles to generate nanoassemblies. This method allowed for a 90% delivery rate of Cas9 proteins into the nucleus and is important because it offers a new, effective method to efficiently transport Cas9 complexes into the nucleus without experiencing degradation. The new approach opens possibilities into studying genome dynamics and more effective CRISPR treatments in eukaryotic organisms\(^1\).

The Ohta group used the CRISPR-Cas9 system to better explore colorectal cancer by selectively causing mutations and deletions in known hallmark genes. The group was attempting to explore the extent to which each genes pathology contributed to the development of colorectal cancer when mutated by individually targeting each gene. This work is important because they are offering new information on which genes are critical in colorectal carcinogenesis, which yields more descriptive information for researchers attempting to
discover cures or treatments for this type of cancer, by informing them which genes are worth focusing on. The Ohta group showed that mutations in the tumor suppressor genes APC, SMAD4, and TP53 formed tumors after implantation, but these cells require additional molecular lesions to exhibit more invasive behavior\textsuperscript{21}.

When devising a CRISPR-mediated experiment, it is important to determine the expected efficiency of the CRISPR-Cas9 model with the targeted genetic site to evaluate whether the model can be predicted to work as intended. The Liu group in Harvard successfully derived a new sequence model which predicts gRNA efficiency in CRISPR-Cas9 knockout experiments. They assessed the DNA sequence features which contribute to gRNA efficacy over multiple designs to discover new features indicative of efficiency such as a preference for cytosine at the cleavage site. Their work helps improve others CRISPR research because it allows researchers to now better determine their chance of a successful completion of their experiment based on their designed CRISPR model\textsuperscript{22}.

CRISPR-Cas9 systems have been more popular in cancer research as of late, as scientists attempt to narrow down and interact with specific genes and encoding exons, to evaluate the protein’s relationship with cancer development. The Vakoc group focused CRISPR-Cas9 mutagenesis on exons encoding functional protein domains which lead to a higher proportion of null mutations, and had substantially increased the potency of negative selection during cellular assays. Their work provides a replicable process to carry out the same type of analysis on the identification of protein domains that sustain cancer cells and which are suitable for drug targeting\textsuperscript{23}. 

While CRISPR research is becoming highly prevalent, the practice is still currently constrained by the need of a specific PAM sequence for the CRISPR-Cas9 nuclease function to operate correctly. The Zheng group sought to address this very problem, and successfully showed that the commonly used Cas9 protein (SpCas9 from *Streptococcus pyogenes*) can be modified to recognize alternative PAM sequences by altering the evolution of the bacterium. Their work increases the ease of use and functionality of many other experiments by making CRISPR targeting easier and more specific. They proved the efficacy of the new altered PAM sequences by editing hard to target endogenous gene sites in both zebrafish and human cells, sites which were not targetable by SpCas9\textsuperscript{24}.

Genetic screens are considered one of the best tools for identifying genes that are responsible for diverse phenotypes in expressing organisms. Researchers conducted a genome-wide CRISPR/Cas9-mediated loss-of-function screen in tumor growth and metastasis in mice. After mutagenizing a non-metastatic mouse cancer cell line with a genome-scale library of gRNAs, they showed that the mutant cell pool rapidly generated metastasized tumors when transplanted into mice. The developed screening method revealed genes regulating lung metastasis in mice, and highlighted the use of Cas9-based screening as an effective method to systematically assay gene phenotypes in the evolution of cancer\textsuperscript{25}.

Editing other organisms’ DNA could be beneficial to humans by reducing the negative impact these organisms can have on our environment. Editing approaches could range from antimicrobial-resistant bacteria, to the reduction of virus-spreading insects. The Nolan group of researchers experimented with a CRISPR-Cas9 treatment and *Anopheles gambiae* (mosquito species) to target gene drive systems to reduce these insects’ ability to spread malaria. After
identifying three separate genes, they showed the CRISPR-Cas9 disruption to the gene loci had a transmission rate to progeny at about 91.4-99.6%. Their findings conclude that targeting just one loci meets the minimum requirement for a gene drive targeting female reproduction in the insect population to propagate, showing that further research into the topic could severely reduce and suppress mosquito populations ceasing the transmission of malaria.²⁶

While many researchers are making CRISPR more efficient and easily practicable, there are also many researchers focusing on reducing the negative side effects of a CRISPR experiment. The Joung group is focusing on reducing off-target effects that results when a Cas9 protein reacts to more DNA sequences than intended. By modifying the SpCas9 protein to generate SpCas9-HF1, a high-fidelity variant, the function of the new protein retained all on-target activities comparable to wild-type SpCas9 with over 85% of sgRNAs, however, all typical off-target events or interactions were nonexistent or undetectable. Research like this increases the potential efficacy and plausibility of future CRISPR-Cas9 therapy treatments by increasing the specificity and accuracy of the treatment as they decrease the chances of an adverse reaction with the DNA.²⁷

Further increasing the efficacy of potential CRISPR therapeutic treatments, the Anderson group experimented with delivery methods of CRISPR-Cas9 systems to target cells to try improving the bioavailability of these treatments. They generated a new delivery system by combining lipid nanoparticle-mediated delivery of the Cas9 mRNA and gRNA along with a repair template with adeno-associated viruses as they evaluated the ability for these injections to treat and repair diseased genes in adult mice. They showed a correction efficiency of over 6% when targeting a mouse model with human hereditary tyrosinemia. A single application to the
mice generated fumarylacetoacetate hydrolase (Fah)-positive hepatocytes by correcting the causative Fah-splicing mutation. Their findings increase the potential utility of therapeutic Cas9-based genome editing for a variety of DNA-based diseases.

The end goal of most CRISPR research is to improve or understand more about the CRISPR process so that it can be used as a therapeutic treatment in humans. The Liu group recently published a paper where they tried first-hand repairing genetic abnormalities in a single-cell embryo to attempt for one of the first time ‘designer babies’. The disease they were attempting to treat was a mutation called G1376T in the G6PD enzyme which commonly causes favism, a disorder where eating certain foods such as fava beans can cause the destruction of red blood cells. Their method was to use CRISPR to cut out the mutated genes while also splicing in the correct genetic sequence. While their success rate was low in repairing these mutations, a rate less than 1 in 10, it is still encouraging to receive results showing that the treatment may be capable with more research and development.

Conclusions

In conclusion of the work, it can be deemed that the pX458 plasmid was successfully engineered to include the targeting double stranded DNA specific to the hTRM9L gene in SW480 cells. The experiment has been halted at the characterization step after the SW480 cells were transfected with the recombinant pX458 plasmid, verified through GFP bioluminescence.

Future Plans

The next steps after the transfection of the SW480 cells using an EZ480 transfection reagent via a normal transfection protocol, we will grow a separate culture of SW480 cells to
use as a control for the experiment, and then use GFP luminescence to evaluate the degree of plasmid inclusion by the cells as GFP was included in the pX458 plasmid, and we can sort the GFP expressing cells by fluorescence activated cell sorting (FACS). To determine the effect of TRM9L deletion, we will carry out live-dead assays with trypan blue staining and then contrast the growth of the edited SW480 cells with the control SW480 cells. After observing the effects of eliminating TRM9L from colorectal cancer cells, it might prove interesting to try modifying the procedure to insert more copies of TRM9L into the genome. By increasing the concentration of TRM9L in the genome, the cancer cells may be less able to downregulate these genes allowing for some transcription, and since TRM9L is a tumor suppression gene, possibly halt the growth of these cancer cells.

TRM9L is one of many genes of interest in the genome that relate to cancer growth. By utilizing the same principles implemented in this experiment, the target could be replaced to observe the effects of the elimination of another gene. A few examples include targeting LIN9, the tumor suppression gene that TRM9L directly upregulates when expressed, and observe the effects of when this gene is knocked out from the cell. Another is the APC gene which is also classified as a tumor suppressor gene due to its function in controlling how often a cell divides, how it attaches to other cells, and how a cell moves within a tissue. The MLH1 gene codes for a DNA mismatch repair protein, a gene where should a mutation occur, often does lead to colon cancer. Lastly, the TGFBR2 gene codes for a cell membrane receptor responsible for signal transduction that may also lead to colorectal cancer should a mutation occur as the gene plays a role in regulating cell division.
References


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