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Generation of a CCL2 knockout using CRISPR/CAS9 and lipid mediated transfection in CT-26 murine colon carcinoma cells

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Abstract

CCL2 is an inflammatory mediator that is released by tumor cells to activate and direct immune cell species, especially macrophages, to inflammatory sites within the body. The goal of this project was to successfully generate knockout the CCL2 ligand gene using a CRISPR/Cas9 complex delivered via lipid mediated transfection. The sgRNA and Cas9 mRNA were introduced into the cells via lipid-mediated transfection. The cells were incubated for 4 days, before being analyzed using PCR and gel electrophoresis. We expected to see one band on the first gel and two bands on the second gel. Two bands appeared on the first gel and 2 bands appeared on the second gel. This indicates that the target sequence was amplified, but the transfection efficiency was too low for the enzyme to detect cleavage. We conclude that lipid mediated transfection is an efficient method for introducing foreign genetic material into CT-26 cells.

Introduction

Colorectal cancer is the third most commonly diagnosed cancer and the third leading cause of cancer related deaths in both men and women in the United States[1]. Colorectal tumors develop from growths of epithelial colon cells, called polyps, that spontaneously undergo genetic modifications to become carcinomas. These genetic modifications promote proliferation and inhibit apoptosis within the polyp. For tumor progression, it is necessary that the microenvironment promote inflammation and have infiltrative properties[2]. Treatment for colorectal cancer depends on the stage. In Stage 0 and Stage I, cancerous cells have not grown outside the colon wall. The cells are surgically removed, and the patient typically does not need to undergo chemotherapy. For more advanced stages, II-IV, the cells have grown outside of the treatment wall and metastasized to other regions of the body. Treatment includes chemotherapy to shrink tumors around the body. Shrunken tumors are then removed surgically, and more chemotherapy is performed[3].

The downfall of conventional methods of treatment is that they are nonspecific, which then results in system toxicity, insufficient drug concentration in tumor cells and the development of tumor cells are that drug resistant[4]. Chemotherapy drugs inhibit various stages of the cell cycle to stop cell division and induce apoptosis. In the process, both cancerous and normal cells are killed. To prevent healthy cells from being affected, new cancer therapies are being developed that are specific to cancer cells. This method of targeted therapy employs chemical compounds that inhibit proliferation and metastasis by suppressing specific proteins involved in tumorigenesis[5]. Side effects due to off-target delivery are also minimized, which increases the overall effectiveness[4]. The class of targeted therapy we are interested in is monoclonal antibody therapy. Monoclonal antibodies (mABs) can disrupt cancer cells in three ways: inhibiting protein function and

downstream signaling, antibody-dependent cytotoxicity, and complement-dependent cytotoxicity[5].

Tumors often contain a mixture of fibroblasts, epithelial, and immune cells. Macrophages are the most abundant immune cell present in the microenvironment and are called tumor-associated macrophages (TAMs) when they infiltrate the tumor mass. There are two types of macrophages: M1 and M2. The M1 phenotype is anti-tumor and pro-inflammatory; it mediates the destruction of cancerous tissue. In contrast, the M2 phenotype is pro-tumor and anti-inflammatory. They work on both the tumor microenvironment and tumor cells to promote cell proliferation and genetic instability, invasion of tumor cells into the surrounding tissue, forming fibrous tissue deposits, and angiogenesis[6]. They also produce growth factors and chemical signals, called chemokines, to strengthen the inflammatory response[2].

Chemokines (CKs) are inflammatory mediators that are released by a variety of cells, such as endothelial and epithelial, to activate and direct more immune cell species to inflammatory sites within the body. When CKs are secreted by cells, they travel through the body to other tissues, such as the brain, blood, and colon, that are expressing the receptor protein on the surface of those tissues. Ligand binding induces G-protein coupled signal transduction pathways that induce chemotaxis of monocytes[7]. Chemokine C-C motif ligand 2 (CCL2) is one of the five members of the monocyte chemoattractant protein (MCP) family. Though other members of the MCP family are able to signal through the chemokine C-C motif receptor 2 (CCR2) protein, CCL2 has the highest potency when activating the signaling pathways to induce cells of the monocyte lineage to the site of inflammation, especially TAMs[8]. CCL2 is shown to be involved in both early and late steps in metastasis. Initially, CCL2 guides cancer cell migration when it interacts with the CCR2 receptor. Binding initiates a signaling cascade to express metalloproteinases and induce

intravasation of cancer cells into the circulation. Extravasation from the circulation occurs when the cancer cells interact with TAMs, which are highly influenced by CCL2. Later in metastasis, CCL2 preferentially attracts cancer cells, stimulates their proliferation and enhances their survival[7].

The effects of CCL2 can be prevented by setting up a monoclonal antibody blockade, in which the anti-CCL2 antibody binds to the CCL2 ligand and prevents it from binding to the CCR2 receptor. However, antibodies are not as specific as was once believed and are able to interact with other antigens[9]. In order to study the effects of adding an antibody when its preferred ligand is not present is to generate a knockout of the ligand gene, add in the antibody, and see if any promiscuous binding occurs. The ligand gene we eliminated was the CCL2 gene in a CT-26 cell line, which is a mouse colon carcinoma fibroblast.

To generate a knockout of the CCL2 gene in CT-26 cells, a prokaryotic genomic editing system was used and delivered via lipid mediated transfection. Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) is a section of DNA that includes repeating nucleotide sequences and spacers between the repeating sequences. The CRISPR system originated in bacteria and archaea as an RNA-based bacterial adaptive immune system used to modify DNA. It combines a CRISPR-associated protein 9 (Cas9) with a CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA). The two RNA structures combine to form a single-guide RNA (sgRNA) that attracts the Cas9 protein to the protospacer adjacent motif (PAM) sequence at the beginning of the cleavage site. The PAM sequence is where the sgRNA and target DNA pairing occurs and helps mediate the double-stranded DNA cleavage[10]. When the sgRNA-DNA pairing has occurred, the Cas9 protein can also bind to the complex and cause a double-stranded break (DSB) to occur within the target DNA. The DNA is repaired via non-homologous end-joining

(NHEJ) by recognition of the DSB, bridging the ends of the DNA to promote stability and process them, and direct ligation of the ends to repair the DSB[11]. Repairing DNA via NHEJ is more likely to cause a frameshift mutation, either insertion or deletion, at the cleavage site. The creation of a mutation is the goal of the Cas9 complex and the Cas9 protein will continue to make DSBs in the DNA until a mutation occurs during repair[10] (Fig 1).

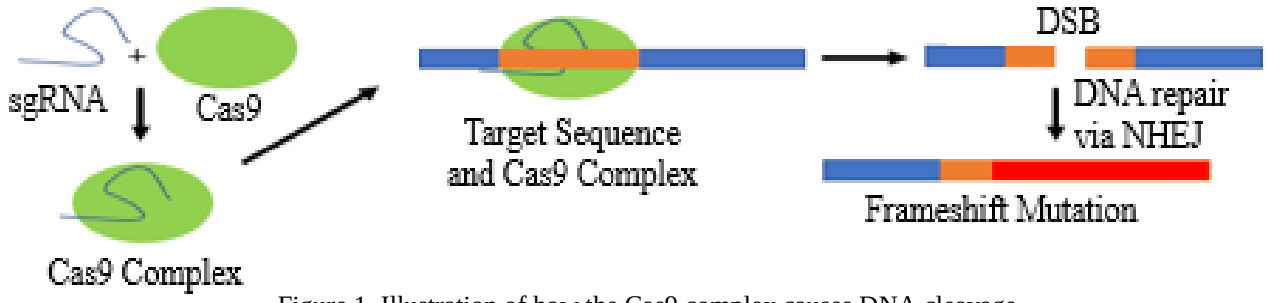

Figure 1. Illustration of how the Cas9 complex causes DNA cleavage.

Since the Cas9 protein and sgRNAs are not natural to mammalian cells they must be delivered into the cell. Cas9 can either be introduced as mRNA or as an already transcribed, functional protein. Although both mRNA and protein require assistance getting through the cell membrane, the protein is bulkier than the mRNA and often has low potential to penetrate the cell membrane[12]. The protein is also in an unprotected state and would quickly be cleared by endosomal proteases or neutralized by antibodies[13]. Transfecting mRNA is slightly easier and allows for the Cas9 protein to be transiently expressed, which increases the time that the protein is transcribed within the cell and minimizes the possibility of off-target genomic modifications since it is only expressed for a short period of time[14].

There are three methods in which foreign material can be delivered into a cell: electroporation, viral delivery, and transfection[15]. Electroporation is the use of pulsatile high-voltage electrical currents to cause pores to form within the cell membrane. Cells are suspended in a buffer solution containing the material that needs to enter the cell, while the electric current is

applied the material can flow through the pores in the cell membrane. When the electric current ceases, the pores close and the cell membrane repairs itself[16]. Although this method is useful for moving large amounts of foreign material, it is not suitable for all cell types as it can cause stress to the cells and cytotoxicity[16,17]. Viral delivery of foreign material can be via retrovirus, lentivirus, adenovirus, or adeno-associated virus. They also are able to deliver large amounts of foreign material into the cell at once and have high efficiency rates[17]. However, the virus can integrate into the host genome and cause insertional mutagenesis[18]. There is also potential for the virus to cause an immunogenic reaction within the cell[17].

Genetic material can be transfected into the cell in multiple fashions, but the most common form is lipid mediated transfection. Nucleic acids, such as Cas9 mRNA and sgRNA, are highly anionic have difficulty passing through the hydrophobic region of the lipid bilayer[16]. Lipofectamine reagents are cationic lipids that encapsulate the genetic material and guide it through the cell membrane, while protecting them from degradation and escape[13,16] (Fig 2). Although the quantity that can be delivered via lipid mediated transfection is more limited than other methods, there is less potential for immunogenic reactions, cytotoxicity, and cellular disruption[16].

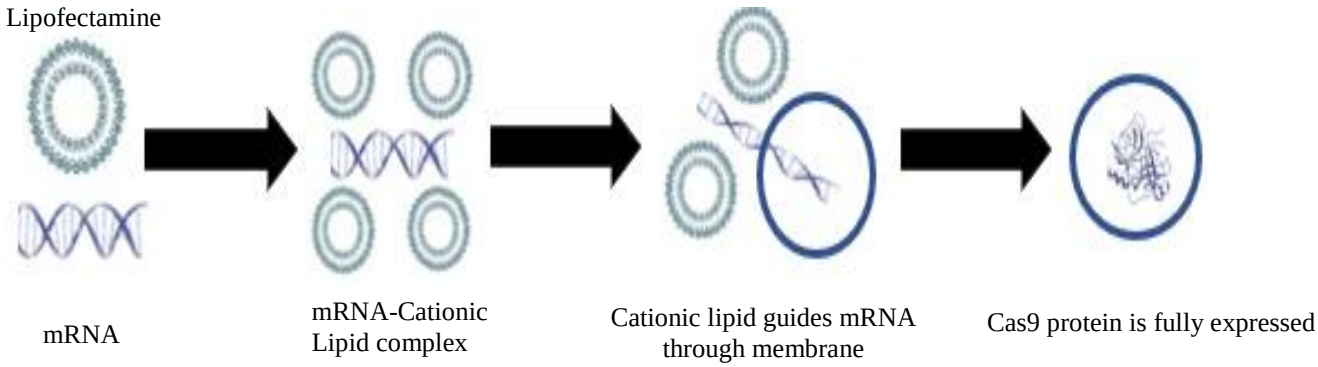

Figure 2. Schematic of lipid mediated transfection.

Transfection efficiency is dependent on several factors, including the chemical properties and mechanism of uptake of the lipid complex, the intracellular delivery route, and the type of cell being transfected. The cell line we are working with, CT-26, is known to be difficult to transfect. The uptake of foreign material into the cell happens more slowly in CT-26 cells due to an overproduction of heavily glycosylated proteins, called mucins[19]. The mucins act as a physical barrier and have a tendency to bind to cationic complexes to induce aggregate formation. The aggregates cause the complexes to have difficulty moving through the cytoplasm and entering the nucleus. As well as, being easily shunted into endo-lysosomal pathways for degradation[19].

To minimize the limitations of CT-26 cells, the medium used to culture the cells did not contain any serum or antibiotics. Certain serum proteins can inhibit the formation of the RNA-lipid complexes and reduce the amount of RNA that enters the cell[20]. Serum starvation also synchronizes cells to the G0 phase and ensures that the progress through the cell cycle at the same rate[21]. Antibiotics were eliminated from the medium as well as they are known to lower the transfection efficiency. Removing antibiotics also prevent the cells from becoming cytotoxic due to the increased permeability of the cell membrane to foreign material[20,22].

Materials and Methods

Cell Culture

The CT-26 cell line should be cultured in serum free medium and cultured without antibiotics to increase the transfection efficiency. Cells should be fed every 3 days and passaged when they are about 70% confluent to maintain a healthy population. When the 6-well plate is ready to be prepared, the cells should be counted and seeded to a density of 100,000 cells per well

with 2 mL of media per well. Incubate the cells at 37 °C and 5% CO_2. The well plate will be ready to use when the cells are approximately 60% confluent.

Preparation of Stock sgRNA Solution

Remove the tube of concentrated sgRNA from the freezer and bring to room temperature. Centrifuge the tube at 4,000 RCF for 5 minutes. Add 30 µL of 1 TE buffer to create a 100 µM stock solution, this is equal to 1.3 mg/mL. Vortex the tube to resuspend the gRNA and centrifuge the tube for 1 minute at 4,000 RCF. Incubate the tube at room temperature for 30 minutes to allow the sgRNA to dissolve. Use the vortex to thoroughly mix the solution and centrifuge again for 1 minute at 4,000 RCF.

Transfection of CT-26 Cells

In a sterile cell culture fume hood, take out two small centrifuge tubes. Tube 1 will consist of the Cas9 mRNA/sgRNA solution. The reagents must be added exactly in the order described: 125 µL of Opti-MEM I medium, 2.5 Cas9 µL mRNA nuclease, 1.25 µL sgRNA, and 12.5 µL Cas9 Plus reagent. Remove the tube from the hood and vortex to thoroughly mix. In Tube 2, add 125 µL Opti-MEM I medium and 7.5 µL Lipofectamine CRISPRMAX reagent. Pipette the contents of Tube 1 into Tube 2, vortex the solution to mix. Incubate the transfection reagent for 15 minutes at room temperature and add 250 µL of transfection reagent to each well. If the bottom of the well is not completely covered, add more Opti-MEM I medium until it is. Incubate the cells at 37 °C and 5% CO_2 for 4 days.

Harvesting and Lysing Transfected Cells

Remove 6-well plate from the incubator. Remove media and wash with 500 µL of PBS. Add 1 mL of trypsin and incubate at 37 °C and 5% CO_2 for five minutes. Check the cells under the microscope to ensure that they are detached from the bottom of the well. Add 1 mL of Opti-MEM I medium and transfer the contents to a centrifuge tube. Centrifuge the cells at 200g for 5 minutes at 4 °C. Remove the supernatant. In a separate tube, mix 288 µL cell lysis buffer with 12 µL of the protein degrader. Add 50 µL of the cell lysis/protein degrader solution to the pellet and resuspend. The remaining cell lysis/protein degrader solution can be stored at -20 °C. Transfer all the resuspended pellet into a PCR tube and run the program (Table 1) in a thermal cycler.

Table 1. Cell lysis thermal cycler program.

Temperature	Time
68 C	15 minutes
95 C	10 minutes
4 C	Hold

PCR Primer Design

The PCR primers were designed based on the following guidelines from the genomic cleavage detection kit. Primers must be between 18 and 22 bp long, contain 45-60% guanine-cytosine (GC) content, yield amplicons between 400-500 bp long, and have melting temperature ™ greater than 55 ºC. The target sequence cannot be in the middle of the amplicon, to ensure that two distinct bands would form. Online software from Primer3Plus was used to design the PCR primers. We tested two primers. For the first primer (Fig 3A) the forward primer sequence is cccactcacctgctgctact (20 bp, Tm 60.5, GC 60%) and the reverse primer sequence is aaaatggatccacaccttgc (20 bp, Tm 59.8, GC 45%). With this primer, the total amplicon length is 470 base pairs (bp) and after cleavage detection we expect two bands of lengths of approximately

40 and 430. The forward primer sequence for the second primer (Fig 3b) is cccactcacctgctgctact (20 bp, Tm 60.5, GC 60%) and the reverse primer sequence is atggatccacaccttgcatt (20 bp, Tm 60.2, GC 45%). With the second primer we expect a total amplicon size of 473 bp and after cleavage detection the two bands should be approximately 41 bp and 434 bp. The location of the primers in relation to the target sequence is shown below (Fig 3).

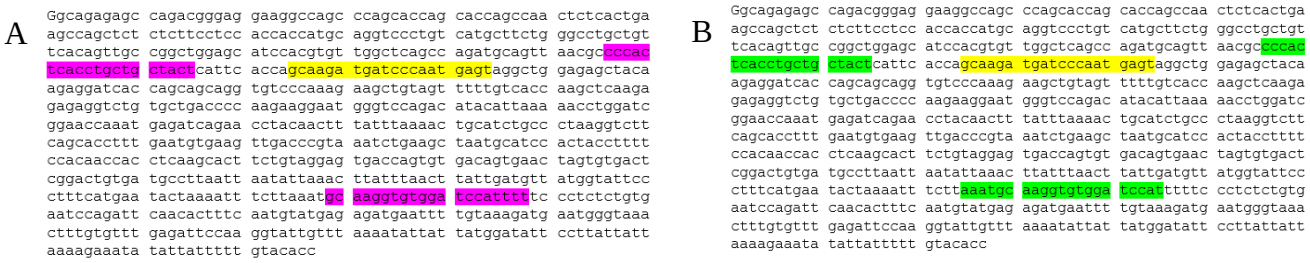

Figure 3. Gene sequence for CCL2 and locations of the primers in relation to the target sequence (A) primer 1 and (B) primer 2.

DNA Amplification via PCR

After the program is completed, vortex the cell lysate. In a new PCR tube add the reagents in the order as listed: 2 µL cell lysate, 10 µL SYBR Green dye, 1 µL 10 µM Forward/Reverse primer mix, 25 µL AmpliTaq Gold360 Master Mix, and 12 µL water as provided by the kit. The total volume in the PCR tube will be 50 µL. The PCR reaction will be performed as specified in Table 2.

Table 2. PCR description for amplification of the target DNA sequence.

Stage	Temperature	Time	Cycles
Enzyme Activation	95 C	10 minutes	1 X
Denature	95 C	30 seconds	40 X
Anneal	57 C	30 seconds	
Extend	72 C	30 seconds	
Final Extension	72 C	7 minutes	1 X
Hold	4 C	Hold	1 X

Verification of the PCR Product

Remove an agarose gel tray from the package and place in the E-Gel iBase Power System. Load one well with 20 µL with a 1 kB DNA ladder. In a PCR tube combine 3 µL of the PCR product with 7 µL loading buffer and 10 µL water. Vortex to mix and load the entire sample onto the gel. Run the gel for 30 minutes. View the product with a UV transilluminator. It is expected that one band that is approximately 470 bp long is seen.

Denaturing and Re-annealing of DNA

The PCR product will be denatured, and fragments are re-annealed with and without indels to form heterogeneous DNA duplexes. In a new PCR tube, combine 2 µL of the PCR product with 1 µL Detection Reaction Buffer. Add 6 µL of water and briefly centrifuge to eliminate bubbles. The reannealing reaction will take place as described in Table 3.

Table 3. PCR description for denaturing and re-annealing of amplicons to form heterogeneous DNA duplexes.

Stage	Temperature	Time	Temp/Time
1	95 C	5 minutes	-
2	95-85 C	-	-2 C/sec
3	85-25 C	-	-0.1 C/sec
4	4 C	-	Hold

Enzyme Digestion

The detection enzyme identifies the heterogenous DNA duplexes that are formed during the re-annealing process and cleaves that ones that contain indels. If there are no indels, then the detection enzyme will not cleave the DNA. Add 1 µL of the detection enzyme to the tube and incubate at 37 ºC and 5% CO_2 for one hour. After incubating, vortex the solution and briefly centrifuge at 4,000 RCF for one minute.

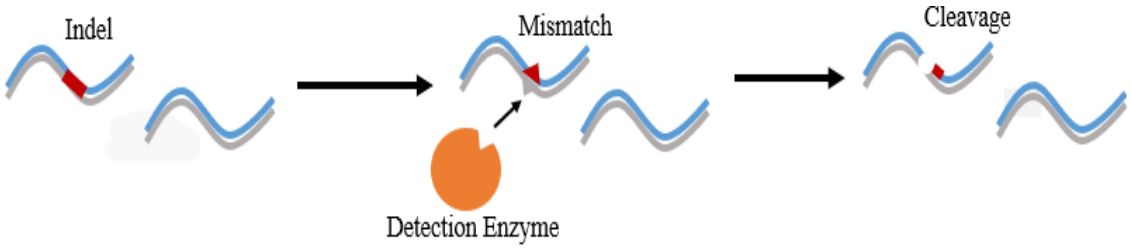

Figure 4. Schematic describing how the cleavage detection enzyme cleaves DNA. After the DNA has been denatured and re-annealed, the DNA forms heterogeneous duplexes (mismatches). The enzyme detects the mismatches and cleaves the DNA.

Cleavage Detection Analysis

Dilute the sample with 10 µL water. Obtain a new agarose gel tray and place in the E-Gel iBase Power tray. In one well, add 10 µL of 1 kB DNA ladder. Add all 20 µL of the sample to a separate well. Run the gel for 30 minutes and view under a UV transilluminator. It is expected that two distinct bands will be seen on the gel as described in the PCR primer design section.

Results

Verification of PCR Amplification

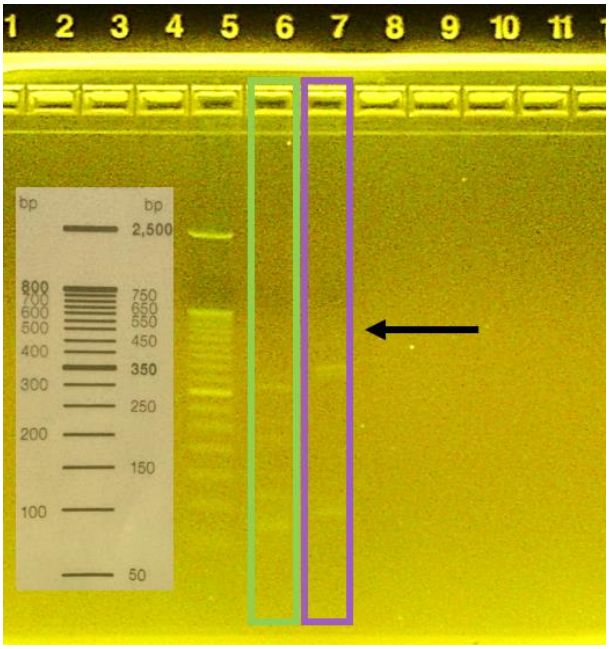

Figure 5. Gel electrophoresis image verifying the size of the PCR amplicons.

Figure 5 shows the results of the PCR amplification of the gene sequence. From this reaction it was expected that a single distinct band of approximately 470 bp would be seen, the expected value is shown by the black arrow in the figure. Lane 5 contained the 50 bp DNA marker. Lane 6 is color coded green and contained primer one, which showed two faint bands of approximately 375 and 80 base pairs long. Lane 7 is color coded purple and contained primer two, which showed two bands of approximately 450 and 100 base pairs long. It is unclear exactly what caused additional bands to appear, but there are several possibilities.

First possibility is that the program used to design the PCR primers did not search any genetic databases to check that the primer sequence was unique to the intended target sequence. There could have been other sequences on a different gene that could have been similar enough to the intended target and caused binding to occur[23]. The second possibility is that the GC content of the forward sequences was too high. If the GC content is too high, the primer may bind non-specifically to other sections of DNA. It is suggested that the optimal GC content percentage should be as close to 45% as possible. Both forward primers had GC contents of 60%, which was the maximum value as stated by the guidelines in the genomic cleavage detection kit. The third possibility is that the ramping speed of the thermal cycler was too slow and caused non-specific band formation. When the ramping speed is too slow, it allows more time for spontaneous annealing and binding to occur[24].

Cleavage Detection Enzyme Analysis

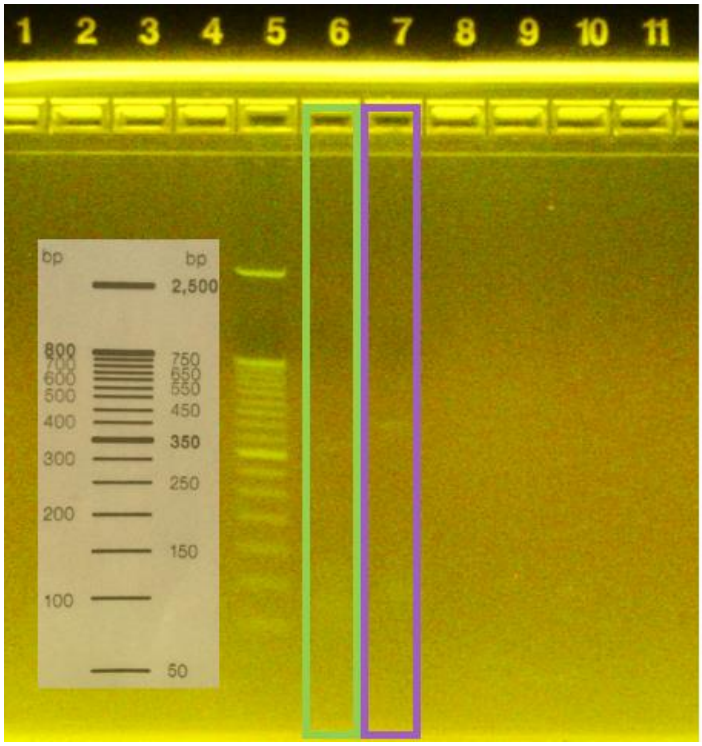

Figure 6. Gel electrophoresis image of PCR amplicons after the cleavage detection enzyme has been applied to the samples.

Figure 6 shows the number of bands after the detection enzyme was applied to the amplified DNA. It was expected that two distinct bands of approximately 30 and 440 bp would be seen. Lane 5 contains the 50 bp DNA ladder. Lane 6 is color coded green and contained primer one. Lane 7 is color coded purple and contained primer 2. For both lane 6 and 7 the bands are in the same positions as they were before the cleavage detection enzyme was added and appear to be fainter. This indicates that no cleavage occurred.

If a frameshift mutation had occurred from the CRISPR/Cas9 complex, the enzyme would have cleaved the DNA. Not obtaining the two expected bands is shows that the CRISPR/Cas9 complex was unable to reach the nucleus and make incisions in the DNA to induce a CCL2 gene knockout[25]. This is most likely due to mucin overproduction within CT-26 cells that caused lipid

aggregation and prevented the CRISPR complex from reaching the nucleus. Mucins tend to gravitate towards the surface of the cell, so when the lipid complexes attempt to enter the cell they immediately come into contact with the mucins. The mucins cause aggregation and the aggregates are then shunted into endosomal pathways to be removed from the cells as waste products. The mRNA and sgRNA associated with the lipofectamine is also shunted into the pathway[19]. Even if there was a small amount of the CRISPR complex that made it to the nucleus to induce a knockout, it would not be sufficient enough for two distinct bands to appear.

Discussion

Conclusions

We have shown that lipid mediated transfection is an inefficient method in which to transfect a CRISPR/Cas9 complex to induce a gene knockout. A more efficient method for transfecting CT-26 cells needs to be found before the effects of unknown antibodies can be determined. Since CT-26 cells are known to have an overproduction of mucins that cause lipid aggregate formation, it would be better to use an electroporator to move the genetic material inside of the cell. An electroporator machine uses pulsatile high-voltage electrical currents to cause pores to form within the cell membrane while the cells are suspended in a buffer solution that contains the genetic material that needs to be transfected into the cell[16]. This cell line tends to be hardier than other cells and would most likely be able to withstand the harsh conditions of the electroporator[19].

Future Directions

However, electroporation machines are expensive to buy and maintain. A more cost-effective method would be to abandon the CT-26 cells in favor of an easier to transfect cell line. A cell line that is known to be easy to transfect is the SK-BR3, which is a human breast adenocarcinoma cell line. The SK-BR3 cells do not over produce mucins, so the rate of internalization of the transfected material is much higher than that of the CT-26 cells[19].

Transfection is never one hundred percent efficient. There will always be some cells that remain non-transfected within the well-plate. When a method of transfection has been established and upon verification shows that at least 60% of the cells do not have the CCL2 gene, it can then be considered successful. The knockout cells will need to be separated from the wild type cells. This should be done using flow cytometry without fluorescence. The addition of fluorescent dyes is also not completely efficient, and it is likely that the dye could tag a wild type cell. The exact methods of how to sort for the transfected cells will need to be determined after a successful knockout is generated.

Acknowledgements

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