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CRISPR/CAS9 as a Gene Editing Tool to Delete the Transcriptional Repressor Inducible cAMP Early Repressor from the Zebrafish Genome

Madhumalini Gnanasekar
Montclair State University

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Abstract

Genome editing has become an important tool in identifying the specific function and role of a gene in an organism. With the advent of genome editing using clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) systems, editing a gene has become much easier and less expensive. This CRISPR/Cas9 system uses a small 20 nucleotide long guided RNA (gRNA), which along with Cas9 will bind to the target site and cleave it. In this research, CRISPR/Cas9 system was used to knock out the inducible cAMP early repressor (ICER) promoter sequence in zebra fish. ICER has anti-proliferative activity and acts as a tumor suppressor. In Ras-induced melanoma, ICER protein is being targeted to degradation. Knocking out the ICER gene will help us to establish the tumorigenicity of ICER in melanomas.

A gRNA, specific towards the ICER promoter sequence was designed in an attempt to cleave it with Cas9. Plasmids pDR274(-)atgICER and pMLM3613 were used to generate gRNA and Cas9mRNA via *in vitro* transcriptions. To check the efficiency of designed gRNA *in vitro*, PAC-2 cell lines were transfected with a plasmid that expressed both gRNA and Cas9. The results demonstrate the ability of Cas9 to cleave the target sequence. In the future, this plasmid could be used to perform microinjection in zebra fish embryos with the generated gRNA and Cas9mRNA.

MONTCLAIR STATE UNIVERSITY

**“CRISPR/CAS9 as a gene editing tool to delete the transcriptional repressor
Inducible cAMP Early Repressor from the zebrafish genome”**

By

Madhumalini Gnanasekar

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

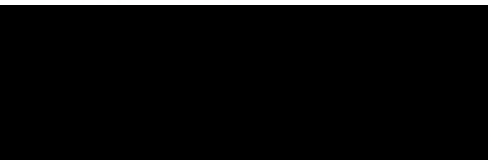
Master of Science

August 2014

College of Science and Mathematics

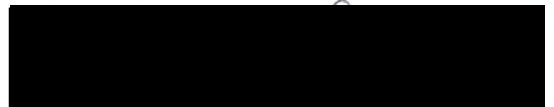
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Thesis Committee:



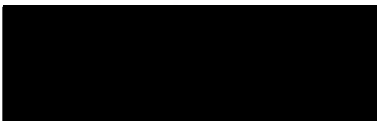
Carlos A. Molina, Ph.D.

Thesis Sponsor



Sandra D. Adams, Ph.D.

Committee Member



Kirsten J Monsen, Ph.D

Committee Member

**“CRISPR/CAS9 as a gene editing tool to delete the transcriptional repressor
Inducible cAMP Early Repressor from the zebrafish genome”**

A THESIS

Submitted in partial fulfillment of the requirements
For the degree of Master of Molecular Biology

by

MADHUMALINI GNANASEKAR

Montclair State University

Montclair, NJ

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TABLE OF CONTENTS

ABSTRACT.....	1
SIGNATURE PAGE.....	2
TITLE PAGE.....	3
ACKNOWLEDGEMENTS.....	4
TABLE OF CONTENTS.....	5
LIST OF FIGURES.....	6
INTRODUCTION.....	7
OUTLINE OF RESEARCH.....	12
MATERIALS AND METHODS.....	15
RESULTS.....	23
DISCUSSION.....	32
FUTURE STUDIES.....	33
REFERENCES.....	35

LIST OF FIGURES

Figure 1: BsaI digestion of pDR274.....	25
Figure 2: Confirmation of gRNA sequence ligation into the vector pDR274.....	25
Figure 3: DraI digestion of vector pDR274(-)atgICER and PmeI digestion of vector pMLM3613.....	26
Figure 4a: Short run - RNA gel of <i>in vitro</i> transcribed gRNA Cas9mRNA.....	26
Figure 4b: Long run - RNA gel of <i>in vitro</i> transcribed gRNA and Cas9mRNA.....	27
Figure 5a: PAC-2 cells transfected with pCMVEGFP.....	30
Figure 5b: PAC-2 cells transfected with pCMVCas9GFP(-)atgICER.....	30
Figure 6: PCR amplification of ICER.....	30
Figure 7: Cleavage assay on PCR products.....	31

Introduction

A critical role in biology is to determine the function of each gene¹. Gene editing is one fascinating tool that enables us to study and determine the function of an individual gene in an organism. Earlier, gene functions were determined using forward genetics screening methods like chemical mutagenesis and transposon mediated mutagenesis^{2,3}. Even though these methods were initially successful, each had its own procedure to determine the mutated site and the whole process is laborious and time consuming⁴. With the advent of new and affordable sequencing methods, the complete genome sequence of an organism is more readily available. This led to the study of specific gene function using reverse genetics techniques. Some of the reverse genetics techniques include 1) partial gene knock down by RNAi^{5,6}, 2) virus induced gene silencing⁷, 3) insertional mutagenesis and 4) chemical mutagenesis/ targeted induced local lesions in genomes (TILLING). All of these methods have some limitations. For example, gene silencing using RNAi has unpredictable off targets and the result varies between experiments^{8,6}. Methods like TILLING need large mutant populations and are expensive⁷. The demand for more precise study on genes and their functions, led to the development of new gene editing techniques, which are more effective and less expensive.

In the past decade, a new genome editing approach emerged that enabled a gene to be edited using engineered nucleases^{2,6,9}. This approach relies on DNA repair that occurs when a double stranded DNA is cleaved. The two main types of DNA repair mechanism to repair double stranded breaks are non-homologous end joining method (NHEJ) and homologous recombination repair (HR)². Non-homologous end joining

repair could result in nucleotide insertion or deletion (indels), which in turn may affect the gene function ¹⁰. The genome editing tools like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) cut the DNA at specific sites and stimulate DNA repair mechanisms such as, NHEJ repair and HR, allowing study of gene function ⁶.

Zinc finger nucleases are a combination of a zinc finger domain and *FkoI* nucleases. Zinc finger domains are a small yet common motif observed in proteins, especially in DNA binding proteins ⁶. A different Zinc finger domain binds to a different nucleotide triplet in the major groove of the DNA. This nucleotide specificity of this zinc finger domain along with the nucleases *FkoI* allows precise cleavage within the target gene. Even though this method was successful, it had a few limitations, a) the corresponding zinc finger domains have not been discovered for all nucleotide triplets, b) production of zinc finger nuclease is highly labor intensive, time-consuming and very expensive ⁶.

Like ZFNs, another effective tool for genome editing with relatively rare off target effect is TALENs. TALEN is a combination of TALE and *FkoI* nuclease. TALE (transcription activator-like effector) was first discovered in plant pathogens *Xanthomonas sp* ² and it has a DNA binding domain with 30 -35 tandem amino acid repeats that recognize a single, specific nucleotide in DNA ^{2,8}. The amino acid residues within this repeats are highly conserved and changes within these amino acid residues contributes to its different binding specificity. The successful outcome in gene editing using TALE depends on the tandem repeats. At the same time, constructing a TALE with many repeats is laborious and time consuming.

In spite of the site specific cleavage achieved using the above ZFNs and TALENs, the discovery of type II CRISPR/Cas system emerged as a more efficient gene editing tools. This CRISPR/Cas system, also called the RNA guided nucleases genome editing tool, has been proven to be very simple, cost effective, less time-consuming and can be applied on all organisms ^{11, 12, 13, 14}. In this system, a small piece of processed RNA guides Cas nuclease to specifically cleave the DNA to which it binds.

Type II CRISPR/Cas system is an adaptive immune mechanism used by many bacteria against invading pathogens, especially viruses ^{15, 16, 17}. In this process, bacteria incorporates a short sequence of a genetic element from the invading pathogen into its genome, at a unique site called the clustered regularly interspaced short palindromic repeats or CRISPR loci. This CRISPR is composed of short tandem repeats of approximately 30-40 base pairs (bp). The sequence from the invading pathogen called the spacer/protospacer is incorporated between the CRISPR repeats ^{18, 19, 20}. Following an attack, the CRISPR responds to the invading pathogen, by transcribing and processing the protospacer sequence at the CRISPR locus to produce CRISPR RNA (crRNA). The crRNA consists of the sequence from the invading pathogen and it is flanked by part of the CRISPR repeat. This crRNA hybridizes with the trans-activating RNA (tracrRNA) and forms a complex with the Cas9 protein (Cas9 protein contains RuvC nucleases and HNH nuclease domain). The crRNA then binds to its complementary target DNA and directs Cas9 nuclease to cleave the DNA ^{21, 22}. Overall, genome editing using CRISPR/Cas9 requires only two components: a small crRNA specific towards the target site and a Cas9 enzyme to cleave the target site. This eliminates the complication of

designing multiple zinc fingers as required by ZFNs and repetitive amino acid sequence as required by TALENs at target site^{23, 24}.

Researchers have used this CRISPR/Cas system from *Streptococcus pyogenes* (*S.pyogenes*) to edit the genome in recent years. This was done by expressing the Cas9 protein and a small guided RNA called gRNA (a fusion between crRNA and tracrRNA) complementary to the target DNA that is to be cleaved. Recent *in vitro* and *in vivo* studies have successfully employed this mechanism in a few model organisms like zebra fish, fruit flies, and mice^{10, 11, 25}. The only constraints with this system are: the size of the gRNA should be 20 nucleotides long and the target site should be immediately followed by a protospacer adjacent motif called PAM. The PAM sequence is necessary for gRNA to direct Cas9 to cleave the target DNA^{21, 26, 27}. The most recognized PAM sequence is 5'-NGG-3'. Sometimes a PAM site with 5'-NGA-3' is also recognized, but with less efficiency. Thus, Cas9 protein could be directed to cleave any DNA site in the form of N20-NGG, by simply creating a complementary gRNA^{15, 23, 28, 29}. This form of gene editing tool has made it very easy for scientists to study and determine the function of specific genes. This type II CRISPR/Cas9 gene editing tool was used in this study to edit the promoter of the induced cyclicAMP early repressor (ICER) in zebra fish cells.

Melanoma is a skin cancer that is rapidly growing in western countries. It develops in the melanocytes located in the epidermis of the skin^{32, 33}. It is caused by mutations in *B-Raf*, *N-Ras* and *H-ras* by UV exposure. Mutations, in these proto-oncogenes, leads to loss of cell cycle control and results in uncontrolled cell proliferation. In melanocytes, increased levels of cyclic AMP (cAMP) are observed, which positively regulates cell growth and differentiation. Increased levels of cAMP regulate the

transcription of many cellular genes. This signaling pathway requires the activation of transcription factors by protein kinase A (PKA). PKA phosphorylates many transcription factors like CREB (cyclicAMP- response element binding protein), CREM (cyclicAMP – response element modulators) and the ATF gene family^{32, 30, 33}. Upon phosphorylation by PKA, these transcription factors activate CRE-containing genes. CREM is a unique gene that generates gene activators and repressors by alternate splicing³⁰. One of the products of the CREM gene that is not activated by PKA is the ICER protein. The CREM gene consists of two promoters P1 and P2. Both P1 and P2 are induced by cAMP^{20, 31}. ICER is a product of P2, which is not regulated by PKA. ICER acts as a potent inhibitor of cAMP inducible responses. When the cAMP level increases within a cell, ICER binds to CREs on the CREB/CREM gene as a homodimer or heterodimer and negatively down regulates cAMP gene expression³¹. This function of ICER is important to manage cAMP genes, and in keeping the delicate balance that is necessary for proper gene regulation. This negative down regulation of ICER prevents the cancer cells from growing in an anchorage – independent manner^{30, 37}. Recent studies have demonstrated that increased cAMP levels contribute to melanocyte growth and differentiation, thus resulting in melanoma^{32, 33, 34}. In the case of Ras induced melanoma, ICER has been targeted for degradation by Ras, thereby preventing the down regulation of cAMP genes. This indicates the significant role of ICER as a tumor suppressor in preventing melanoma. By knocking out the ICER promoter sequence using CRISPR/Cas9 system in zebra fish, it may be possible to establish the role of ICER in melanoma.

Outline of Research and Designing gRNA

The aim of this study was to knock out the ICER gene promoter. The first step was to identify the potential target site for gRNA. ZiFiT Targeter¹⁰ was used to identify the potential target site for gRNA. This was done by submitting the ICER sequence to the ZiFiT website. Out of four suggested target sites, the site that contained part of the ICER promoter, with adjacent PAM sequence was selected.

ICER Sequence

```
CTTCTGAGCTTAAATAAATAAATATGCAACTGCACTATTTTTTTAAGCAATGA
ATATAAGCTTGTATGTTAATATAAAATGAGTCCTGTTTCTCTCTCTCTTTTCA
CACACACACACACACACACACACACACATACTTCTCCAGAGACAGTGTGTT
ATTTCCCTGTGAGGCTGCTGTGATGTCATAGTGATGTCAATGCCCTTAATAGT
AATCTGACTGAGCGAGAGAGAGAGGGGAGGGAGGGAGGGAGAAAGAGAGAT
AGGGAAGGAGAGAGAGGGTTAAAGGGAAACAGTAAGTGTCACAACCTAAC
AGAGAGTCAGTAGGAGCGCGTGAGAGAGAACTCAGCCAGCGAAGAGCTGA
AGGGAAGACAGAGCTTAAATAGGAAATCAAGAGGAAACACTATCCCAACTG
GATTACTACAGTATAGAGATGGCAGTGACCGGGGAAGAAACCGAGTCAGCT
GCCACAGGAGACATGCCAGCATATCAGATCCGCTCGCCGTCGTCAGGGCTGC
CTCCAGGTGTTGTCATGGCATCGTCACCAGGGGCGATGCACAGCCCGCAACC
CAACGCAGAGGAGGCCACGCGCAAGAGAGAAGTCCGTCTGATGAAGAACAG
GGAGGCAGCGCGGAGTGTCGCAGAAAAAAGAAAGAATACGTGAAGTGTTT
GGAGAATCGGGTTGCCGTGCTGGAAAACCAGAACAAGACTCTCATAGAGGA
GCTGAAAGCCCTTAAAGACATCTACTGCCACAAGCCTGAATAACCCTCACAA
AACTGCTCAAGGACTGTGTGATTCACACAATACCCGTCTCCTCACTTCTACT
GCTGCACCGCCTGGATTTTATCGCT
```

The sequence in Yellow indicates the gRNA target sequence .The sequence in red color (ATG) indicates the start codon.

The research was done in two stages:

Stage 1- Production of mRNA for in vivo gene alterations study

Two separate vectors, pDR274 and pMLM3613, were used to express gRNA and Cas9. Both gRNA and Cas9 sequence were under the control of the T7 promoter. Previous studies^{15,23} suggested that the T7 promoter requires a pair of Guanine or Adenine residues at the 5' end of the transcript. So the gRNA sequence should be in the form of 5'-GG/AA-N18-NGG-3'.

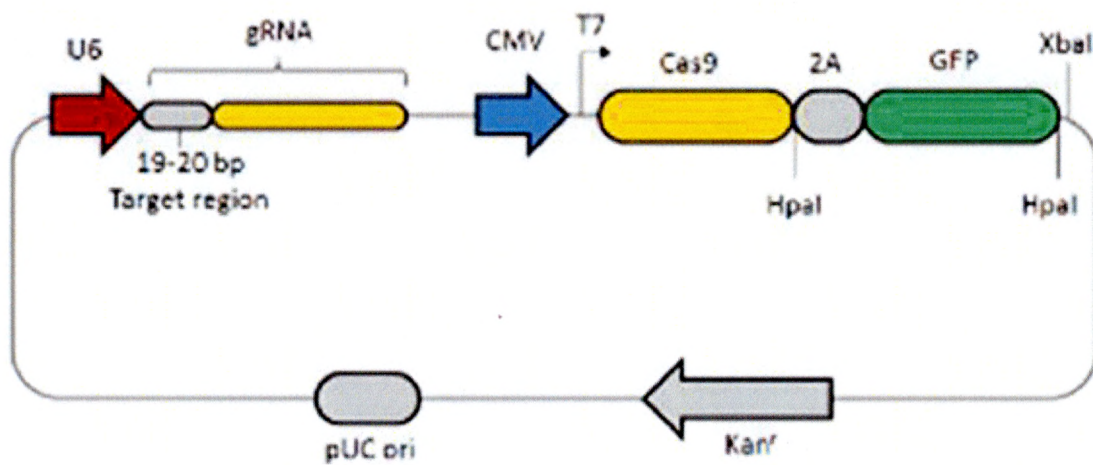
The following sequence 5' – GGATTACTACAGTATAGA - 3' was cloned in to the vector pDR274. pDR274 harboring the 5' GGATTACTACAGTATAGA -3' gRNA sequence was transcribed *in vitro* following the MAXIscript® kit manufacturer's protocol (Life Technologies, Grand Island, NY, USA). Cas9 sequence, under the control of the T7 promoter in the vector pMLM3613, was transcribed *in vitro* following the mMessage mMachine T7 ultra kit manufacturer's protocol (Life technologies, Grand Island, NY, USA). Since Cas9 is an enzyme a 3' poly (A) tail and a 5' cap were added to the mRNA sequence. The mRNA of both gRNA and Cas9 was purified by lithium chloride precipitation and stored.

Stage 2 - In vitro study of CRISPR/Cas9 system in zebra fish cells

A single vector system that carried both gRNA and Cas9 sequences along with the sequence for GFP expression was used. This single vector system enhanced the chance of equal delivery of all CRISPR/Cas components into the cells. The presence of the GFP sequence in the vector made it easy to determine the percentage of transfected cells using a fluorescent microscope. When the construct was transfected into the zebra fish cells, and if the gRNA bound specifically to the target site, it would direct the Cas9

nuclease to cleave the target DNA. Cleavage by Cas9 nucleases on the target sequence could be determined by amplifying the ICER sequence using PCR and performing a T7 endonuclease assay on the amplified DNA. A positive result would indicate the efficiency of CRISPR/Cas9 system on the ICER sequence in zebra fish. In the future, Cas9mRNA and gRNA (generated in stage 1) could be used to perform microinjection in zebra fish embryos.

pCMVCas9GFP(-)atgICER



pCMVCas9GFP(-)atgICER contains the sequence for gRNA, Cas9 and GFP. U6 promoter drives gRNA expression and CMV promoter drives Cas9 expression. pCMVCas9GFP(-)atgICER was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

Materials and methods

Identifying gRNA target site

The ZiFiT Targeter (<http://zifit.partners.org/ZiFiT/>) was used to identify the potential target site for gRNA. The ZiFiT site also gives the list of oligonucleotide sequences necessary for cloning the target sequence into the pDR274 vector.

Target site - 5' – GGATTACTACAGTATAGA - 3'

Oligonucleotide sequence 1 - 5'- TAGGATTACTACAGTATAGAGA – 3'

Oligonucleotide sequence 2 - 5' – AAAGTCTCTATACTGTAGTAAT – 3'

Stage-1

pDR274atg(-)ICER construction.

E.coli cells containing pDR274 plasmid were purchased from Addgene (Cambridge, MA, USA). These bacterial cells were grown on an agar plate containing kanamycin, and then plasmid pDR274 was isolated following QIAprep® spin Miniprep protocol (Qiagen, Valencia, CA, USA). pDR274 contains the T7 promoter. To this the 20 nucleotide target sequence (gRNA) was incorporated as following the protocol detailed below.

pDR274 digestion and DNA extraction from agarose gel

Ten microliters of pDR274 were mixed with 2 µL of 10x BufferG, 2 µL of Bsal (Thermo fisher scientific, Waltham, MA, USA), and 6 µL of double distilled water and incubated at 37°C for 1 hour. The reaction mixture was then run on 1% agarose gel at 100V for 1 hour. The DNA was extracted according to the QIAquick® gel extraction kit

(Qiagen, Valencia, CA, USA) protocol. The band of interest (approx. 2147bp) was excised from the gel, placed in 1.5mL micro centrifuge tube and weighed. To this QC buffer was added in the ratio of 1:3 (Gel: QC buffer). The solution was then placed on a heating block at 50°C for 10 minutes, mixing every 3 minutes. To this isopropanol was added in the ratio of 1:1 (Gel: Isopropanol). The solution was then placed on a DNA specific column and purified according to the manufacturer's protocol. DNA concentration was measured with the Nanodrop spectrophotometer.

Ligation of oligonucleotide (gRNA) into pDR274

The forward and reverse oligonucleotides were reconstituted (1.5µg/µL) and were mixed together in the ratio of 1:1. The oligonucleotides were annealed by placing them in a beaker containing water at 94°C and were then allowed to cool to less than 30°C on the bench. Then oligonucleotides were allowed to cool to room temperature overnight. Following annealing, different dilutions of inserts (annealed oligos) were prepared (1:5, 1:10, 1:50, 1:100). DNA ligation was done following manufacturer's protocol (DNA ligation Kit ver. 1 Manual, Takara, Clone Tech Laboratories, Mountain view, CA, USA). Five microliters of linearized vector pDR247 (extracted from the gel) was mixed with 5µL of insert at various concentrations (1:5, 1:10, 1:50 and 1:100). To this 50 µL of solution A and 10 µL of solution B were added. The solutions were incubated at 16°C for 30 minutes.

DNA transfection and sequencing

DNA transfection was done following One Shot® Top10 competent cell transformation protocol (Invitrogen, Carlsbad, CA, USA). Five microliters of ligate was

added to 50 μ L of One Shot® Top10 competent cells (1 vial of cells for each dilution). The vials were incubated on ice for 20 minutes. Following incubation on ice, the vials were incubated in a water bath at 42°C for 30 seconds. Then the vials were immediately placed on ice. To this 250 μ L of pre-warmed S.O.C medium was added and the vials were kept in a shaking incubator for 1 hour at 225RPM. Following transformation, 200 μ L of the mixture from each vial were spread on labeled LB agar plates containing kanamycin, and were incubated at 37°C overnight. Following incubation, plasmids were isolated from the transfected cells according to the QIAprep® spin Miniprep protocol (Qiagen, Valencia, CA, USA). Once the plasmids were isolated, the DNA samples were sent to GeneWiz (South Plainfield, NJ, USA) for sequencing to check for the proper ligation of target sequence (gRNA) in to pDR274 vector.

In vitro transcription of Cas9mRNA and gRNA

Restriction Digestion of pDR274(-)atgICER and pMLM3613

The expression vector, pMLM3613, containing the gene for Cas9 was purchased from Addgene (Cambridge, USA). These bacterial cells were plated on agar dishes containing kanamycin. Following incubation, pMLM3613 were isolated from the bacterial cells. Both pDR274(-)atgICER and pMLM3613 were isolated following QIAprep spin Miniprep protocol (Qiagen, Valencia, CA, USA). Additionally, pDR274(-)atgICER was linearized with DraI (Invitrogen, Carlsbad, CA, USA). In a 1.5mL microcentrifuge tube, 12 μ L of pDR274(-)atgICER, 2 μ L 10x buffer B, 1 μ L of DraI, and 5 μ L of ultra-pure water were combined and incubated at 37°C for 5 hours. Similarly, pMLM3613 was linearized with PmeI (Invitrogen, Carlsbad, CA, USA). In a 1.5mL

microcentrifuge tube, 11 μ L of pMLM3613, 6 μ L ultra-pure water, 2 μ L 10Xbuffer B and 1 μ L of PmeI were combined and incubated at 37°C for 5 hours. The digested samples were then mixed with 2 μ L of loading buffer and run on 1% agarose gel (containing ethidium bromide) at 100 volts for 60 minutes in 1xTAE buffer. The bands were observed under gel dock.

Production of Cas9mRNA and gRNA

The linearized plasmid pDR274(-)atgICER and pMLM3613 were extracted from the gel according to the QIAquick® gel extraction kit protocol (Qiagen, Valencia, CA, USA), and the concentration of the plasmids were measured with Nanodrop spectrophotometer. The linearized plasmids pDR274(-)atgICER and pMLM3613 were used as templates for gRNA and Cas9mRNA *in vitro* transcription. pDR274 (-)atgICER was transcribed following MAXIscript® kit manufactures protocol (Life Technologies, Grand Island, NY, USA) . In a 1.5 mL microcentrifuge tube, 80 μ L of nuclease free water, 10 μ L of pDR274(-)atgICER, 2 μ L of 10X transcription buffer, 1 μ L of 10 mM ATP, 10 mM CTP, 10 mM GTP, 10 mM UTP each, 4 μ L of T7 (15U/ μ L) enzyme were added and mixed thoroughly. The solution was incubated at 37°C for 1 hour. Following incubation, 1 μ L of TURBO DNase (2U/ μ L) (Life Technologies, Grand Island, NY, USA) was added and incubated at 37°C for 15 minutes.

The expression vector, pMLM3613, was transcribed following the mMessage mMachine T7 ultra kit manufacturer's protocol (Life Technologies, Grand Island, NY, USA). In a 1.5 mL microcentrifuge tube, 20 μ L of nuclease free water, 10 μ L of T7 2X NTP/ARCA, 2 μ L of 10X T7 reaction buffer, 6 μ L of pMLM3613 and 2 μ L of T7 (15U/

μL) enzyme were added. The solution was incubated at 37°C for 1 hour. To this, $1\ \mu\text{L}$ of TURBO DNase ($2\text{U}/\ \mu\text{L}$) was added and incubated for 15 minutes at 37°C . Following transcription a 3' poly (A) tail was added according to the of mMessage mMachine® T7 ultra kit protocol (Life Technologies, Grand Island, NY, USA). To the incubated solution, $20\ \mu\text{L}$ of mMessage mMachine® T7 ultra reaction, $36\ \mu\text{L}$ of nuclease free water, $20\ \mu\text{L}$ of 5X E – PAP buffer, $10\ \mu\text{L}$ of MnCl_2 and $10\ \mu\text{L}$ of ATP solution were mixed together. The solution was incubated at 37°C for 40 minutes. After the addition of the 3' poly (A) tail and the 5' cap, the Cas9mRNA was recovered by lithium chloride (LiCl) precipitation and was re-dissolved in RNase free water. The concentration of both gRNA and Cas9 mRNA were measured with Nanodrop spectrophotometer.

Stage-2

pCMVCas9GFP(-)atgICER

The vector, pCMVCas9GFP(-)atgICER (the gRNA sequence was cloned into the vector pCMVCas9GFP by Sigma, to generate pCMVCas9GFP(-)atgICER) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The vector contains the gRNA, Cas9 and GFP sequence. The gRNA was expressed under the U6 polymerase III promoter and Cas9 was expressed under the CMV promoter.

Transfection of pCMVCas9(-)atgICER in PAC-2 cells.

Two PAC-2 cell culture flasks were used. One PAC-2 cell culture flask was transfected with pCMVCas9GFP(-)atgICER using FuGENE HD transfection reagent (Promega, Madison, WI, USA) and the other flask was transfected with pCMVEGFP. Briefly, $6\ \mu\text{L}$ of pCMVCas9GFP(-)atgICER, $270\ \mu\text{L}$ of LS media and $24\ \mu\text{L}$ of FuGENE

were combined, and added to the PAC-2 cells. Similarly, 20 μ L of pCMVEGFP, 256 μ L of LS media and 24 μ L of FuGENE were combined and added to the second PAC-2 cell culture flask. This served as a control. Both the flasks were incubated at 25°C for 48 hours.

Genomic DNA Extraction

Following 48 hours of incubation and prior to genomic DNA extraction, the cells were observed under fluorescent microscope and the transfection efficiency was determined. Genomic DNA was extracted using GeneArt [®]Genomic cleavage Detection Kit (Life technologies, Grand Island, NY, USA). Briefly, the transfected PAC-2 cells were washed three times with 1mL of 0.1% PBS. After a final wash 500 μ L of 0.1% PBS was added and then the cells were scraped. The cells were then transferred into 1.5mL centrifuge tube and were centrifuged at 2000 g for 5 minutes at 4°C. The supernatant was removed, and to the pellet, 50 μ L of cell lysis buffer and 2 μ L of protein degrader were added and the pellets were re-suspended. The entire solution was then transferred into PCR tubes, and cells were lysed in the thermal cycler. The thermal cycler parameters for lysis were: 68°C for 15 minutes, 95°C for 10 minutes and 4°C on hold.

Primers Used

ICER SEQUENCE

CTTCTGAGCTTAAATAAATAAATATGCAACTGCACTATTTTTTTAAGCAATGA
ATATAAGCTTGTATGTTAATATAAAAATGAGTCCTGTTTCTCTCTCTTTTCCACA
CACACACACACACACACACACACACACATACATTCTCCAGAGACAGTGTGTT
ATTTCCCTGTGAGGCTGCTGTGATGTCATAGTGATGTCAATGCCCTTAATAGT
AATCTGACTGAGCGAGAGAGAGAGAGGGAGGGAGGGAGGGAGAAAGAGAGAT
AGGGAAGGAGAGAGAGGGTTAAAGGGAAACAGTAAGTGTCACAACCTAAC
AGAGAGTCAGTAGAGCGCGTGAGAGA[■]AAAC[■]CAGCCAGCGAAGA[■]CTGA
AGGGAAGACAGAGCTTTAATAGGAAATCAAGAGGAAACACTATCCCAACTG

GATTACTACAGTATAGAGATGGCAGTGACCGGGGAAGAAACCGAGTCAGCT
GCCACAGGAGACATGCCAGCATATCAGATCCGCTCGCCGTCGTCAGGGCTGC
CTCCAGGTGTTGTCATGGCATCGTCACCAGGGGCGATGCACAGCCCGCAACC
CAACGCAGAGGAGGCCACGCGCAAGAGAGAAGTCCGTCTGATGAAGAACAG
GGAGGCAGCGCGAGTGTCGCAGAAAAAAGAAAGAATACGTGAAGTGTTT
GGAGAATCGGGTTGCCGTGCTGGAAAACCAGAACAAGACTCTCATAGAGGA
GCTGAAAGCCCTTAAAGACATCTACTGCCACAAGCCTGAATAACCCTCACAA
ACACTGCTCAAGGACTGTGTGATTCACACAATACCCGTCTCCTCACTTCTACT
GCTGCACCGCCTGGATTTTATCGCTCAAATAATAATTGTGCAGGGCCGTTTGT
TTGTTTGT

Primers Used

Primer set 1: Forward Primer - AGTAGGAGAGAGTGAGAGAG
Reverse primer - TAATAATGTGTGCAGGGCCGT

Primer set 2: Forward Primer - GAGCGCGTGAGAGAGAACT
Reverse primer - TAATAATGTGTGCAGGGCCGT

Primer set 3: Forward Primer - GAAACTCAGCCAGCGAAGAG
Reverse Primer – TAATAATGTGTGCAGGGCCGT

These three sets of primers were used to amplify the ICER sequence.

Yellow sequence - Indicates the target site in ICER sequence where forward primer 1 anneals in ICER sequence. Red letters – Indicates the start and end of the target site in ICER sequence where primer 2 anneals. Green letters – The start and end of the target site in ICER sequence where forward primer 3 anneals. Purple – Indicates the target site in ICER sequence where reverse primer anneals.

PCR Amplification

Three different sets of primers were designed to amplify the ICER sequence.

OligoAnalyzer 3.1 (<http://www.idtdna.com/analyzer/>) was used to design primers for PCR. A total of six reactions were prepared. Briefly, 2 μ L of genomic DNA, 1 μ L of 10 mM forward primer, 1 μ L of 10 mM reverse primer, 25 μ L of AmpliTaqGold360 Master Mix (GeneArt ®Genomic cleavage Detection Kit, Life technologies, Grand Island, NY,

USA) and 22 μL of water were combined together in a PCR tube and PCR reaction was run. The thermal cycler parameters were: 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, 40 cycles of 55°C for 30seconds, 40 cycles of 72°C for 30 seconds, 72°C for 7 minutes and 4°C on hold. Three microliters of the PCR product, 1.3 μL of 10x loading buffer and 10 μL of water were combined in a PCR tube and were run on a 2% agarose gel. Five microliters of DNA ladder (HiLo, Minnesota Molecular Inc, MN, USA) was run in parallel to compare and estimate the fragment size and DNA concentration of the products.

Cleavage Assay

The amplified PCR samples were subjected to a cleavage assay. This was done following the GeneArt® Genomic Cleavage Detection Kit (Life technologies, Grand Island, NY, USA). Briefly, 3 μL of PCR product, 1 μL of detection reaction buffer and 5 μL of water were combined in a PCR tube. These solutions were then kept in the thermal cycler for re-annealing. The thermal cycler parameters are: 95°C for 5min, 95°C to 85°C at the rate of (-2°C)/seconds, 85°C to 25°C at the rate of (-0.1°C)/seconds, 4°C on hold. Following re-annealing, 1 μL of detection enzyme was added to the entire mixture and the samples were incubated in a water bath at 37°C for 1 hour.

Agarose gel electrophoresis

Following enzyme digestion, to each enzyme digested sample, 10 μL of water and 2 μL of 10x loading buffer were added. The samples were then run on a 2% agarose gel at 100v for 60 minutes. Five microliters of DNA ladder (HiLo, Minnesota Molecular Inc, MN, USA) was run in parallel to compare the size of the cleaved products.

Results

Stage1

Inserting gRNA sequence into pDR274/ construction of pDR274(-)atgICER

The goal of this project was to knockout the ICER promoter by using a gRNA specifically targeting the ICER promoter sequence followed by the cleavage of Cas9. In order to produce the vector, pDR274(-)atgICER, plasmid pDR274 was linearized and analyzed by agarose gel electrophoresis. The band of size 2147 (Figure 1) was excised and pDR274 was extracted. The concentration of the plasmid was measured with the NanoDrop spectrophotometer. The total yield of pDR274 was 200 ng/ μ L. This pDR274 was ligated with gRNA oligonucleotides and the constructs were transfected. The plasmids extracted from the transfected cells were sequenced. The sequenced data suggest the successful insertion of gRNA into pDR274. The inserted gRNA oligonucleotide is highlighted in yellow (Figure 2).

In vitro transcription of Cas9mRNA and gRNA

The vector, pDR274(-)atgICER, was digested and analyzed by agarose gel electrophoresis. Two bands of approximate size 1885 bp and 282 bp were observed (Figure 3). The band of size 282 bp that contained the gRNA sequence was excised for DNA extraction. The concentration of the DNA was measured with a Nanodrop spectrophotometer and the yield was 1 μ g/ μ L. The extracted DNA was transcribed into RNA and concentration was measured. The total yield of gRNA was 634.2 ng/ μ L.

The vector, pMLM3613 was digested and the band of size 8000 bp was extracted (Figure 2) and the concentration of the DNA was measured. The total yield was 2.2

$\mu\text{g}/\mu\text{L}$. The extracted DNA was transcribed into RNA and the RNA concentration was measured. The yield of Cas9mRNA was $1 \mu\text{g}/\mu\text{L}$. The RNA was analyzed by a RNA gel electrophoresis (Figures 4a and 4b).

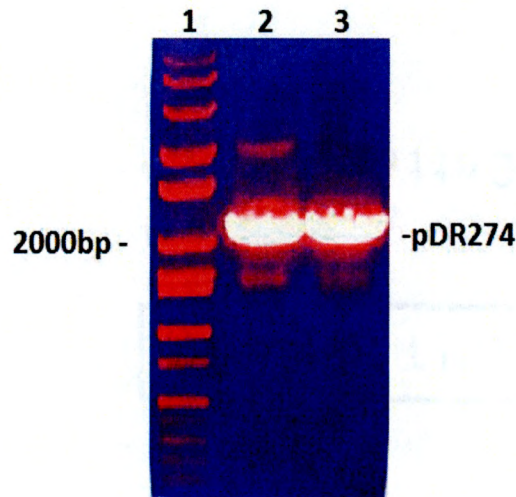


Figure 1 - Lane 1-Molecular weight marker (HiLo). Lanes 2 and 3 - pDR274 (~2147) digested with BsaI.

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CCCGTGTA AACGACGGCCAGTTTATCTAGTCAGCTTGATTCTAGCTGATCGTGGACCGGAAGGTGAGCC
AGTGAGTTGATTGCAGTCCAGTTACGCTGGAGTCTGAGGCTCGTCCTGAATGATATGCGACCGCCGGAGG
GTTGCGTTTGAGACGGGCGACAGATCCAGTCGCGTCTCGTCTCGATCCAAGCTTTTTAAAAGCACCGA
CTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATTCTAGCTCTA
TAGGATTACTACAGTATAGAGACCTATAGTGAGTCGTATTAGCTAGCGGTGCGAGCGGATCGAGCAGTGTCCG
ATCACTACTGGACCGCGAGCTGTGCTGCGACCCGTGATCTTACGGCATTATACGTATGATCGGTCCACGA
TCAGCTAGATTATCTAGTCAGCTTGATGTCATAGCTGTTTCTGAGGCTCAATACTGACCATTTAAATCA
TACCTGACCTCCATAGCAGAAAGTCAAAGCCTCCGACCGGAGGCTTTTGACTTGATCGGCACGTAAGAG
GTTCCAAC TTCCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGATTTTCAGG
AGCTAAGGAAGCTAAAATGAGCCATATTCAACGGGAAACGTCTTGCTTGAAGCCGCGATTAAATCCAAC
ATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTGGGCAATCAGGTGCGACAATCTATC
GATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGT
TACAGATGAGATGGTCAGGCTAAACTGGCTGACGGAATTTATGCCTCTCCGACCATCAAGCATTTTATC
CGTACTCCTGATGATGCATGGTACTCACCCTGCGATCCCAGGGAAAACAGCATTCCAGGTATTAGAAG
AATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTCTCGCCGGTTGCATTTCGATCC
TGTTTGTAATTGTCCTTTTAAACGGCGATCGCGTATTTTCGTCTCGCTCAGGCGCAATCACGAATGAATAAC
GGTTTGGTGGTGCGAGTGATTTTGTATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAA

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Figure 2 - Confirmation of gRNA sequence in pDR274 vector. Yellow highlights the gRNA sequence.

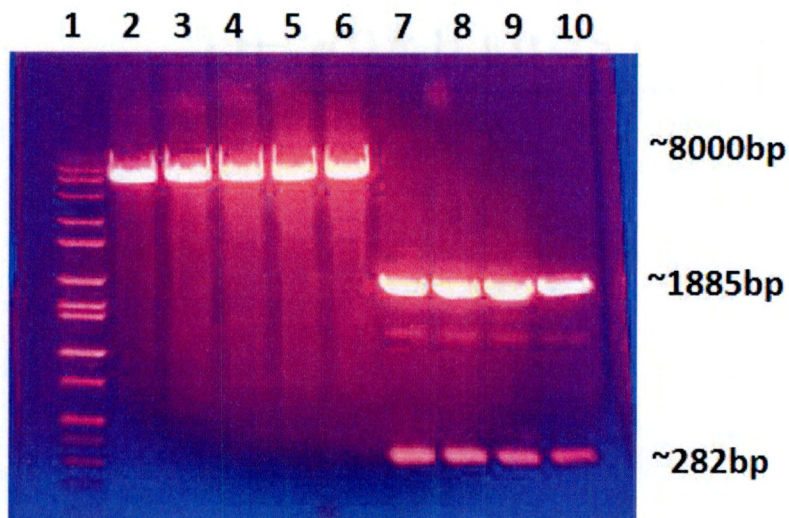


Figure 3 - Lane 1- molecular weight (HiLo), Lanes 2-6 – pMLM3613 digested with PmeI and the band displays the successfully linearized plasmid. Lanes 7-10 – pDR274(-)atgICER digested with DraI sample and displays 2 distinct bands. The band ~ 282 bp codes for gRNA sequence.

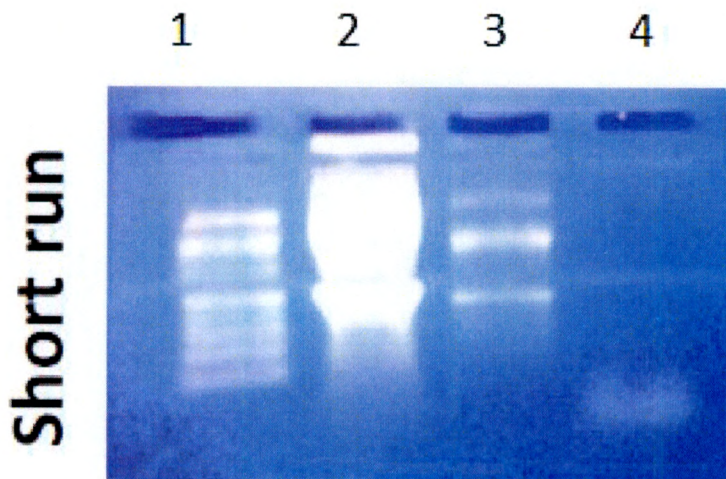


Figure 4a - Cas9mRNA and gRNA were transcribed *in vitro* and was run on RNA gel. Lane 1- Molecular weight 0.5,1,1.5,2,2.5,3,4,5,6 and 9kb, Lane-2—Cas9RNA after poly(A) tail, Lane-3—Cas9 RNA before poly(A) addition, Lane-4 – gRNA (the band is faint).

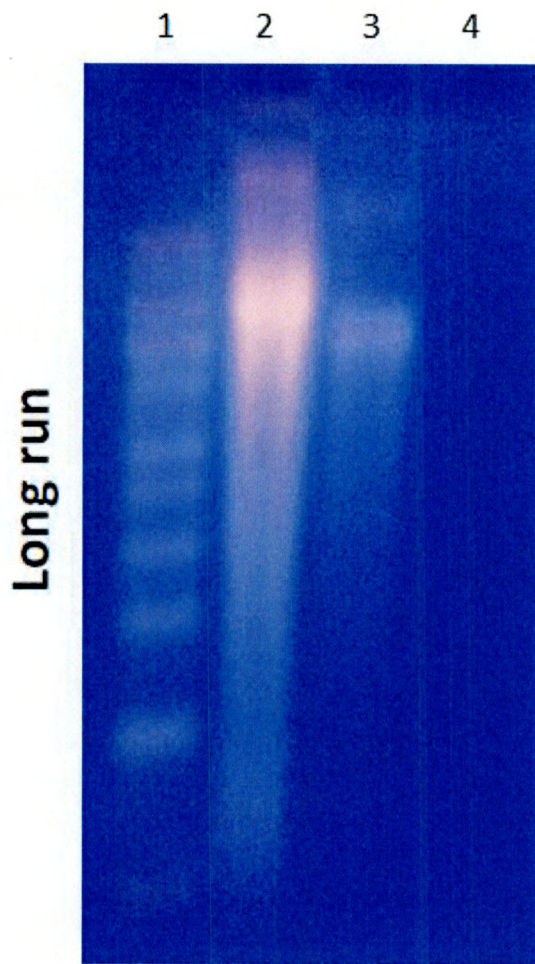


Figure 4b - Cas9mRNA and gRNA were transcribed *in vitro* and was run on RNA gel. Lane 1- Molecular weight 0.5,1,1.5,2,2.5,3,4,5,6 and 9kb, lane-2—Cas9RNA after addition of poly(A) tail and smeared band was observed, Lane-3—Cas9 RNA before poly(A) addition, Lane-4 – gRNA band is not visible.

Stage -2

Transfection of PAC-2 cell line with pCMVCas9GFP(-)atgICER and pCMVEGFP

The transfected cells were observed under a fluorescent microscope. The percentage of transfection was determined by counting the total number of cells and the number of cells that emitted fluorescence. The percentage of transfection in the control flask (PAC-2 cells transfected with pCMVEGFP) and the test flask (PAC-2 cells transfected with pCMVCas9GFP (-)atgICER) was 14.2% and 8.7%, respectively (Figures 5a and 5b). Overall, the transfection efficiency was very low in both test and control flasks.

Table 1 - Percentage of Transfection in PAC-2 cells

PAC-2 Cells Transfected plasmid	Total Number of cells	Fluorescent cells	% Transfection
pCMVCas9GFP(-)atgICER	114	10	8.7
pCMVEGFP	140	20	14.2

Genomic DNA Extraction and Amplification of ICER

Total genomic DNA was extracted from test (PAC-2 cells transfected with pCMVEGFP) and control (PAC-2 cells transfected with pCMVCas9GFP(-)atgICER) cells. The ICER sequence was amplified with PCR. Gel electrophoresis of PCR products gave a strong band of size 350 bp with all 3 sets of primers (Figure 6).

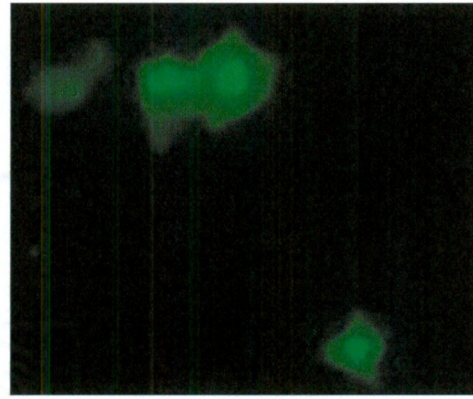
Cleavage Assay

The PCR products were cleaved with detection enzymes and were analyzed by agarose gel electrophoresis. The bands obtained in each lane were compared with a

positive control. Samples amplified with primer set 1 and primer set 2 did not have three bands as was visible in the positive control. The samples amplified with primer set 3, had three bands similar to that of the positive control (Figure 7). This indicates that the cleavage assay was successful and the ICER promoter sequence was knocked out. The cleavage band that appeared in lane 3(+) was very faint. This suggests that the transfection efficiency had a major effect on the band strength since only 8% of cells were transfected.



5a



5b

Figure 5a - Control- PAC-2 cells transfected with pCMVEGFP (viewed under fluorescent microscope – 400x).

Figure 5b - Test- PAC- 2 cells transfected with pCMVCas9GFP (-)atgICER (viewed under fluorescent microscope – 400x).

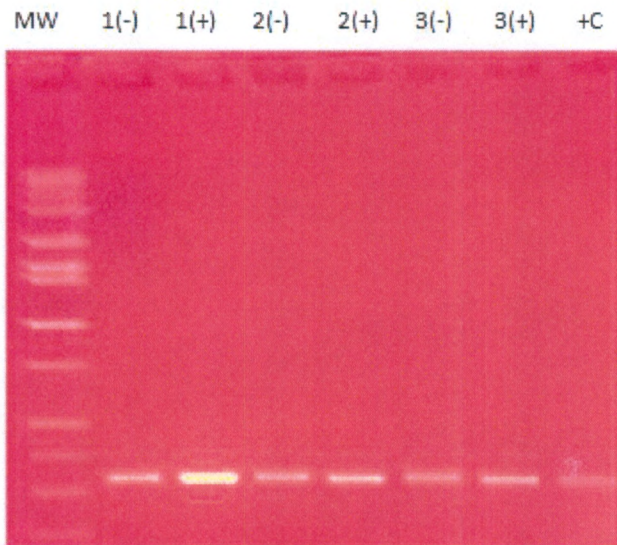


Figure 6 - Gel display the PCR amplification. The band of ~350bp indicates that the ICER sequence has been amplified. 1(-) and 1(+), 2(-) and 2(+), 3(-) and 3(+) indicates PCR amplification with primer set 1, 2, and 3. +c indicates positive control (sample from the gene art kit)

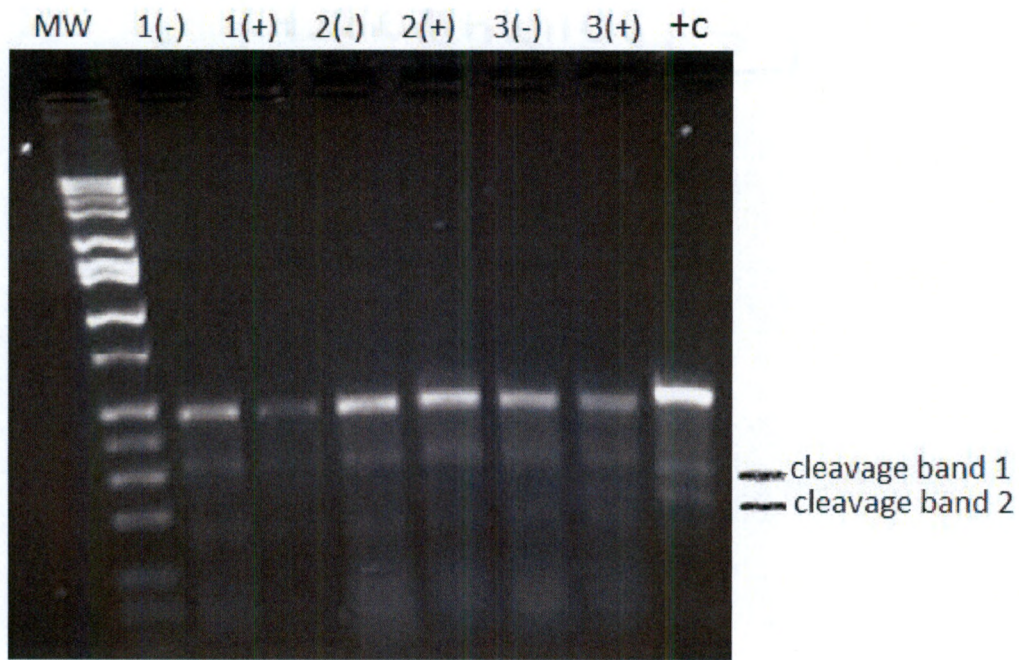


Figure 7- Gel displays the cleavage assay. Lane 1(-) and 1(+), Lane 2(-) and 2(+) displays one band. This indicates that the cleave assay is not positive and ICER promotes sequence is not cleaved. Lane 3(+) displays cleavage bands similar to that of the +C, and the 2nd cleavage band is visible, but it is very faint.

Discussion

The vector pCMVCas9GFP(-)atgICER successfully knocked out the ICER promoter sequence *in vitro*, in PAC-2 cell lines. At the same time, the cleavage bands obtained, after the cleavage assay, were not very strong. One of the major reasons behind this was the transfection efficiency in PAC-2 cell lines. The percentage of transfection in the PAC-2 cell line with a ratio of 8:2 was only 3% and the cleavage assay result was not positive. In an attempt to increase the transfection efficiency, a ratio of 10:2 was used, and the percentage of transfection was increased by 5.2%. The cleavage assay was successful, but the cleavage bands were very faint. The other factor that would have had an effect on the cleavage assay was the percentage of indels formed. It was demonstrated that indel mutation less than 2% at the target site will not be cleaved by cleavage enzymes¹⁵. Since the overall transfection in PAC-2 cells was only 8.2%, it could have had a major effect in the percentage of indels formed. The other factor that might have had an effect on the cleavage assay results was the specificity of Cas9 nucleases. Cas9 cleaves the target at the PAM site, and acts efficiently if the PAM sequence is NGG. The PAM site at the 3' end of the gRNA used in this research is NGA and this might have reduced the specificity of Cas9.

The other major challenge in this research was to produce a sufficient amount of gRNA by *in vitro* transcription. The initial concentration of gRNA after *in vitro* transcription was around 43.2 ng/ μ L. It was believed that lithium chloride worked better on RNA samples greater than 300 bp and the gRNA used in this research was only 282 bp. In an attempt to increase the gRNA yield, the gRNA was transcribed *in vitro* again and the RNA was precipitated with ammonium acetate. The gRNA yield increased and

the concentration of gRNA was around 634.2 ng/ μ L. This gRNA concentration should be sufficient to perform *in vivo* experiment in zebra fish embryos.

Future Studies

In the future, the gRNA and Cas9mRNA generated *in vitro* could be used to perform an *in vivo* transfection in zebra fish embryos. While performing the microinjection, it would be good to try various concentrations of gRNA and Cas9mRNA, since the difference in concentrations may have an impact in the percentage of indel formations²³. Future studies should also concentrate on the factors that affect the transfection efficiency and Cas9 nuclease's specificity. It is necessary to optimize the transfection in PAC-2 cells by trying different transfection ratios. A few recent studies have demonstrated nucleofection as the better transfection method for PAC-2 cell lines³⁵.³⁶ If possible, transfection efficiency in PAC-2 cells could be studied using different transfecting reagents, and the reagent that gives better efficiency could be used in future research. Since in this research the bands obtained after the cleavage assay were very faint, the DNA sequencing should be done on the PCR products to confirm the presence of an indel mutation in the target sequence. Since there is a possibility that NAG located next to the 3' end of the gRNA at the target site might have had an effect on Cas9 cleavage, in the future, designing a gRNA that satisfies the NGG requirement next to the 3' end may give better results. This could be achieved by extending the current gRNA by 3 bases at the 3' end.

These suggested changes, especially optimizing the transfection efficiency and increasing Cas9 specificity, may increase the effect of CRISPR/Cas9 system in knocking

out the ICER promoter sequence in zebra fish cells (*in vitro*) and in zebra fish embryos (*in vivo*). Knocking out the ICER promoter sequence in zebra fish models may establish the tumorigenicity of ICER in melanomas.

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