Developing a System to Arrest Transformation using a Zebrafish Model for Melanomagenesis

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Abstract

The recent emergence of RNA-guided CRISPR-Cas9 gene editing system adapted from the natural defense mechanism found in bacteria and archaea has made the manipulation of genetic loci more feasible than ever. Using this technique we attempted to eliminate a target sequence of about 20 nucleotides from the promoter of the protein ICER (Inducible cAMP Early Repressor) located just upstream of the start sequence. ICER is a small transcription factor and supposed tumor suppressor protein that comes from the CREM (cAMP Responsive Element) gene. ICER has been found to be absent in tumor cells, marked for degradation by the ubiquitin-proteasomal pathway. By eliminating the target sequence we hope to knockout functional ICER protein in order to observe any possible effects this could have. Essentially it is believed that the elimination of ICER would lead to the generation or acceleration of tumor development. To achieve this we are using the well-characterized zebrafish (*Danio rerio*) melanoma model as a paradigm. Two types of methods are being utilized to insert our Cas9/gRNA construct into zebrafish: transfection via PAC2 cells and direct injection into one-cell stage embryos. In addition, we are using a number of techniques including PCR amplification, sequencing, T7 endonuclease assay, and TOPO cloning in order to provide evidence of target sequence manipulation. The long-termed objective of this study will be to determine whether eradication of ICER will affect the tumorigenesity of wild-type (EK) zebrafish in comparison to the established zebrafish model for melanoma.
MONTCLAIR STATE UNIVERSITY
/ "Developing a System to Arrest Transformation using a Zebrafish Model for Melanomagenesis" /

By
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A THESIS

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

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2015
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Introduction

Melanoma, the most lethal form of skin cancer, arises from mutations within pigment-producing cells called melanocytes located within the epidermis of the skin. In 2014 there were an estimated 76,100 new cases and 9,710 deaths within the United States alone (1). While Melanoma may only account for less than five percent of all skin cancer cases its numbers are on the rise potentially reaching 100,000 by the year 2030 (2). Melanoma genesis is the multi-step process that as a result of genetic mutations results in the formation of Melanoma. The most notorious mutagen related to melanomagenesis is exposure to ultraviolet (UV) radiation via sun exposure, as evident by UV signature mutations within specific genes (3, 8).

The most common mutation found in melanoma is located within the BRAF gene; most notably at codon 600 where there is a substitution of glutamic acid for valine (BRAFV600E) which occurs in over 90% of all BRAF mutations (4, 5). The BRAF mutation alone is not sufficient to initiate melanoma formation, but its prevalence indicates that the BRAF gene plays a crucial role in the formation, progression, and the eventual spread of melanoma (6). This mutation causes the activation of BRAF, a serine/threonine kinase that produces excessive activity along the MAPK pathway (7). Activation of this pathway leads to the phosphorylation of cAMP response element-binding protein (CREB) along with other transcription factors promoting cellular growth (8). This pathway is regulated via the second messenger molecule cAMP. One effect that the MAPK pathway has been linked to in relation to cancer formation is its effect on phosphorylation of the protein, inducible cAMP early repressor (ICER) (8).
ICER, a small nearly complete alpha helical protein, belongs to a large family of transcription factors that mediate the transcriptional response to the cAMP pathway (9). The cAMP pathway is a vital mechanism for the transportation of messages within the cell as a result of external stimuli binding to membrane bound coupled G-proteins. This then stimulates the activation of membrane-associated adenylyl cyclase (AC) converting ATP to cAMP. This conversion results in the dissociation of the regulatory subunit of PKA allowing the catalytic subunit to enter into the nucleus stimulating the transcriptional activators cAMP response element binding (CREB) and cAMP responsive element modulator (CREM) via phosphorylation (10). ICER itself does not have its own individual gene, but in fact is a splice variant of its parent gene CREM (9). CREM contain four promoters, P1, P2, P3, and P4 along with two DNA binding domains, DBD I and DBD II (9). ICER itself is transcribed from the C-Terminus of the CREM protein starting from the P2 promoter and containing one of the two DBDs (9). The binding of phosphorylated CREB to the P2 promoter of CREM results in the transcription of ICER (9). ICER helps to regulate cellular growth by competing with CREM and CREB by binding to cAMP Response Elements (CREs) as either a homodimer or heterodimer thereby acting as a negative feedback mechanism (8, 9).

While not specifically a tumor suppressor protein, ICER exhibits many of the properties of one; which is why the protein is all but absent in human melanoma, in addition to other human cancers including prostate and leukemia (8, 11, 12). Levels of ICER are kept stable in part by intracellular concentrations of cAMP and are eliminated by way of the ubiquitin-proteasome pathway (8, 9, 13). The activation of the MAPK pathway leads to the phosphorylation of ICER at serine residue 41 (Ser41) which results
in the recruitment of ubiquitin thereby tagging the protein for degradation (13). In order for ICER to be fully broken down and degraded via the proteasome, a minimum of at least 5 ubiquitin must bind (14). The loss of ICER allows the cell to continue to grow unchecked leading to tumor formation. An alternative pathway exists that also eliminates functioning ICER but does not degrade it fully. When ICER gets phosphorylated on serine residue 35 (Ser35) instead of Ser41, the proteins becomes monoubiquitinated which causes it to become delocalized into the cytosol instead of degraded via the proteasome (15). This secondary method hints at the possibility that functional ICER could potentially be restored to the nucleus of a tumor cell thereby restoring a normal cell cycle. Research has shown that forced expression of ICER blocks cells at the G1/S and G2/M phases of the cell cycle (8, 11).

The ability to purposely edit a gene in order to elect a desired effect has long been the focus of molecular biology. Through the years there have been progressively improved methods in this regards that have both improved accuracy and reduced the amount of time, money, and effort required to achieve anticipated results making personalized medicine more of a reality. Earlier methods tended to focus on forward genetics and relied largely on mutagenesis to determine a genes function. More recent methods have taken a more reverse genetics approach opting to discover a genes function by observing its phenotypic effect. This was primarily done by utilizing an engineered nuclease composed of sequence specific DNA-binding domains in order to induce a programmed double stranded break (DSB) with the intent to edit the genome (16). Once the DNA has been cut via a site-specific nuclease, there is one of two ways that gene editing could occur. The first way is called nonhomologous end joining (NHEJ),
depending on how the two strands come back together, this could result in the introduction of either small insertions or deletions (indels) at the targeted site. Ultimately this method is very error-prone and could result in the knockout of gene function as a result of a frameshift mutation from the deletion/addition of nucleotides. The second way for a gene to be edited by a site-specific nuclease is called homology-directed repair (HDR). HDR occurs in conjunction with a supplied donor template that allows for the addition or correction of a gene at the targeted site. This method is more direct but requires the engineering of a donor strand in order to elicit the desired results.

Two methods commonly used for gene editing via site-specific nucleases include zinc fingers nucleases (ZFNs) followed later by transcription activator-like effector nucleases (TALENs). ZFNs and TALENs both made use of the nuclease Fkoti in order to induce DBS (16). ZFNs were based off of a common motif found in proteins and each individual zinc finger domain would bind to nucleotide triplets (16). In regards to specificity, ZFNs were able to recognize DNA sequences 9-18bp in length. Although accurate, issues arose due to the fact that not all nucleotide triplets had a corresponding zinc finger limiting sequence options, in addition the production of a zinc finger was a very laborious and expensive task. TALENs while based on the same ideologies differed in their functionality. TALENs were originally found in the plant bacteria genus Xanthomonas and consist of a series of 33-35 amino acids arranged in a repeat domain with each domain recognizing a single nucleotide (16). Different TALENs were distinguished by their unique repeat-variable di-residues (RVDs) corresponding to different bonding specificities (16). Ultimately the same issues befuddled TALENs since
the process of constructing a TALE was a very laborious and expensive task; in addition, two ZFNs or two TALENs were required in order to produce a DSB.

Advances have led to the pioneering of a new method that is quickly revolutionizing genome engineering as a result of its simplicity and cost effectiveness. This new method, termed CRISPR-Cas9, derives itself from the adaptive immune system that is utilized by many bacteria and archaea (17). Short segments of invading virus and plasmid DNA are taken up and inserted as a spacer within areas of CRISPR repeat sequences to be utilized upon a repeat infection. When reintroduced to an achieved pathogen, the CRISPR system will transcribe the stored spacer DNA (protospacer) in addition to a portion of the repeat sequence to form an array referred to as CRISPR RNA (crRNA) (17, 18). The crRNA is able to recognize and bond to its commentary region within the invading pathogens DNA, but in order to elicit the desired silencing effect it will first have to join together and hybridize with another segment of RNA called transactivating CRISPR RNA (tracrRNA). This second segment allows for the formation of a complex along with the nuclease Cas9 (which contains the nuclease domains HNH and RuvC) that induces DSBs at the site directed location (17, 18).

The actual model used for genome editing was borrowed from this naturally occurring process and works relatively the same way with a few modifications. Most notably the engineering of a fused crRNA and tracrRNA into one structure that retained the vital attributes of each individual component; the 20 nucleotide binding sequence within the crRNA and the double stranded section of tracrRNA that binds with Cas9. This new structure, termed guide RNA (gRNA), can now be individually tailored to bind to specific sequences within a genome in order to produce DSBs at desired location (17).
The only limitation for the system is that the target sequence must be 20 nucleotides long and be located directly upstream (5') to a protospacer adjacent motif (PAM) (17, 20). This motif, a three nucleotide segment 5'-NGG-3', is necessary in order to direct Cas9 to cleave the specified target sequence (17, 18). Other versions of PAM exist (i.e. 5'-NAG-3') but are much less proficient in producing the desired results (18). Once attached, Cas9 will then proceed to cut the DNA strand three to four nucleotides upstream of the PAM site, thereby inducing a DSB within the target sequence (19).

The ease of customization and utilization of the CRISPR system is the prominent reason why it was selected to be used on this project; in order to edit the area around the start codon of ICER with the intentions of knocking out the functioning protein while keeping the expression of all other CREM isoforms intact. In this study we will be determining whether the eradication of ICER will affect the tumorigenesity of an established zebrafish model for melanoma. With the deletion of ICER, it is expected to result in increased mortality due to a faster and or higher incidence of tumors; confirming the proposed hypothesis that normal ICER expression is necessary for the maintenance of the non-transformed phenotype. If no melanoma development is observed, then it can be concluded that lack of ICER alone is not sufficient enough to accelerate tumor formation. Although it is possible that other genetic or non-genetic events are necessary to promote tumor formation in an ICER-deficient environment.

Outline of Research

The purpose of the study was to knock out CREM ICER protein from zebrafish using the CRISPR-Cas9 system in order to observe subsequent effects; testing the
hypothesis that normal ICER expression is necessary for the maintenance of non-transformed phenotype.

**ICER Sequence**

CTTCTGAGCTTAAATATAAATATGCAACTGCACATATTTTTTTAAGCAATGAATATAAGGCTTGATGTTTATATAATAATTTAATGAGTCCTGTGTTCTCTCTCTCTCTCACA CACACACACACACACACACACACACACATACATTCTCCAGAGACAGTGTTTT ATTTCCCTGTAGGCTGCTGTGATGTATAGTGATGTCAATGGCCCTTAAATAGT AATCTGAAGAGCCAGAGAGAGAGAGGGGAGGAGGAGGAGGAGAGAGAGAGAT AGGGAAGGAGAGAGAGGTTAAAGGGAAACAGTAAGTGTCACAACTCTAAC AGAGAGTACTAGGAGCGCAGTGAAGAGAGAAACCTCAAGCCAGGAGAGCTGA AGGGAAGAGACAGGCTTTAAATAGGAATCAAGAGGAACACTATCCAACTG GATTACTACAGTATAGAGATGGCAGTGACCGGGAAGAAACTCAACAGGCT GCCAGAGGAGGACATGCGCAGAAGATCAGTCTCGCCGTCGGGTCTCCAGGCTGC CTCCAGGTGTGTGTATGCGATCGTACCCAGGAGGTGACTGACAGGCGCAACC CAAGCAGAGGAGCCACGCCAAGAGAGGATGGTCTGATGAGAGAAGACAG GGAGGCAAGCGGCAGTGTCGCAGAAAAAGAGAAATACGTGAAATGTTGGAGAATCGGGTTGCCGTGCTGGAAAACCAGAACAAGACTCTCATAGAGGA GCTGAAAGCCCTAAAGACATCTACTGCCACAAGCCTGAATAACCCTCACAA ACCTGCTCAAGGACTGTGGATTTCCACAATACCCAGTCTCCTCATTCTACT GCTGACCAGCGCTGGATTATTATCGCT

The sequence highlighted in yellow indicates the target sequence that was used for this study. The sequence in red (ATG) indicates the start codon of ICER. The underlined (TGG) represents the PAM site used for CRISPR editing.

**Materials and Methods**

**Animals**

Wildtype Ek Zebrafish (*Danio rerio*) were graciously donated from the Sabaawy laboratory at Rutgers University’s Cancer Institute of New Jersey in New Brunswick, New Jersey. A total of 12 animals were initially used for this project (6 male and 6 female), between the ages of 3-6 months at initiation of mating. During the course of the project, the progeny of the original 12 animals were also used for mating once they reached the appropriate age.
Animal Care

Animals were cared for according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Montclair State University.

Zebrafish Melanoma Model

Zebrafish embryos of melanoma line Tg(MiftaBRAFV600E);p53 were received from University of Massachusetts and allowed to mature into adulthood.

Transfection of PAC-2 Cells

In a T25cm² culture flask place 270 µL of LS media and 24 µL of FuGENE with 6µL of the experimental vector pCMVCas9GFP(-)atgICER. A separate flask was prepared with the control vector pCMVEGFP. Add PAC-2 Cells. Vortex to amalgamate, then leave it at room temperature for 20 minutes. Incubate for 48 hours at 25°C. Obtain a Las Tek chamber slide system and place in the experimental square 8µl of FuGENE, 2µl DNA, 90µl of media, along with 1 drop of DAPI. The transfected cells should be viewed under a fluorescent microscope. In order to determine the transfection efficiency, the total number of cells that emitted fluorescence were counted and then divided by the total numbers of cells visible. Addition transfections were done the same way with the exception of determination of transfection efficiency. In lieu of DAPI, phase contrast microscopy was used to count total number of cells.

Primers Used

For all PCR amplifications done for this project, a total of four different forward primers were used in addition to a reverse primer. The primers were as follows:
Primer Locations within ICER

CTTCTGAGCTTAAATAAAATATGCAACTGCACATATTTTTTTAAGCAATGAATAAAGCTTGATGTGTTTCTCTCTCTCTCTCTCTCTCTCACAACACACACACACACACACACACACACACACACATACATTCTCCAGAGACAGTGTATTCTCCCTCTGTAGTGCTGACTGTAATCTAGTTGACACAGTCCTGTTTCTCTCTCTCTTTCACAACACACACACACACACACACACACACACACACACACACACATACATTCTCCAGAGACAGTGTGAATTTCCCTGTGAGGCTGCTGTGATGTCATAGTGATGTCAATGCCCTTAATAGTAATCTGACTGAGCGAGAGAGAGAGGGAGGGAGGGAGGGAGAAAGAGAGATAGGGAAGGAGAGAGAGGGTTAAAGGGAAACAGTAAGTGTGCACAACACTCTAACAGAGAGTCGTAGAGCGCGGTAGAGAACACACCTCAGCCAGCGAAGAGCTTTAATAGGAAATCAAGAGGAAACACTATCCCAACTGGATTACTACAGTATAGGATAGCGATGACCGGGAAGAAACCGAGTGACCTGCTGCCAGGAGACATGCCAGCATATCAGCTCCGCGTCGTCGTCAGGGCTGCTCCAGGTTTGTGATCTCACCGACCAGGGCGATGCCAGCCGCCAACACGAGAAAGGTAATTCTCGTCTGATGAAGAACAGGGGAGCGCGCGAGTGTCGCAGAAAAAAGAAAGAATACGTGAAGTGTTTGGAGAATCGGGTTGCCGTGCTGGAAAACCAGAACAAGACTCTCATAGAGGAGCTGAAAGCCCTTAAAGACATCTACTGCCACAAGCCTGAATAACCCTCACAAACACTGCTCAAGGACTGTGTGATTCACACAATACCCGTCTCCTCACTCTACTGCTGCACCGCCTTGATTTTATCGCTCAAAAATAAAATTGTGCAGGGCCGTTTGTGTGTGTTGTT

Primer sequence 2 is highlighted in yellow and represents the most common primer used during this project. Primer sequence 3 is bolded and underlined, Primer sequence 4 located between the red highlighted letter and Primer sequence 5 is located between the green highlighted letters. Reverse primer is highlighted in gray. Targets sequence is highlighted blue with the start sequence for ICER in red font. PAM site is underlined following the target sequence. (Note: primer sequence 1 was not used)

Primer Sequence 2: Forward – AGAAACTCAGCCAGCGAAGA
Reverse – TCTTTTGAGTCGGTCGCTTCT
Primer Sequence 3: Forward – AGCGCGTGAGAGAGAAACTC
Reverse – TCGCGCACTCTCTCTTTGAG
Primer Sequence 4: Forward – GAGCGCGTGAGAGAGAAACTC
Reverse – TCGCGCACTCTCTCTTTGAG
Primer Sequence 5: Forward – AGTAGGAGCGCGGTAGAGAG
Reverse – TCATCCTCGCGCAGCTCTCTC

Extraction and amplification (via Platinum Supermix) of transfected PAC-2 cells DNA

Following transfection, the next step was to amplify the sequence of interest via PCR (using Invitrogen). To extract DNA from transfected cells in the flask, cells were washed with 3ml of cold PBS twice. After the wash, the PBS was removed and 1 ml of it
was added again in order to scrape out the cells. Cells were then transferred into a PCR tube (on ice) and centrifuged at 5,000 RPM for 5 minutes at 4°C. Supernatant was carefully removed and stored on ice. 50µl of cell lysis buffer/Protein degrader mix was added to the tube and PCR was run according to the following parameter in the PCR thermal cycler: 68°C for 15 minutes, 95°C for 10 minutes, and 4°C for hold. The purpose of this was to extract the DNA that was being purified, in this case the transfected PAC-2 cells. In a new PCR tube, add 45µl of supermix, 1µl of 10µM primer mix, and 4µl of template DNA (the purified genomic DNA from the transfected PAC-2 cells). There should be a total of 50µl in the PCR tube. The following steps were done for both control and experimental. The PCR reaction was ran using the following thermal cycle parameters: 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute for a total of 35 cycles. Solution remained in hold at 4°C until removed from apparatus.

**AmpliTaqGold® PCR**

In an empty PCR tube the following components were added: 2µl of cell lystate+1µl of 10µM forward/reverse primer mix, 25µl of AmpliTaqGold® 360 Master Mix, and 22µl of water. In another empty PCR tube the following were added: 1µl of control template, 25µl AmpliTaqGold® 360 Master Mix, 24µl of water. Both tubes should not exceed 50µl. The PCR reaction was run using the following parameters: 95°C for 10 minutes and then a cycle of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds were repeated 40 times. A final extension stage was run at 72°C for 7 minutes following termination of the cycles. Solution was kept on hold at 4°C until removed from apparatus. The reaction was run for about an hour. PCR product was
verified the next day on 1.2% agarose gel following the same protocols as mentioned above.

**Running PCR sample on agarose gel**

Prepared 1.2% agarose gel was loaded on the gel electrophoresis apparatus in the appropriate space with the TAE 1x loading buffer following it. In an empty PCR tube load 5μl of PCR sample and 5μl of ultra-pure water were added along with 1μl of 10x loading dye. This was done for both control and experimental PCR sample. Using a pipet the samples were loaded into the gel and run for 1 hour at 118 volts.

**Analysis of DNA sequence**

With verification of the PCR product, samples were sent for sequencing (both experimental and control) using a forward and reverse primer and were analyzed. Later sequences were analyzed using Chroma software from Technelysium.

**T7 Endonuclease Assay of PCR**

T7 Endonuclease assay was then performed on samples in order to detect more in depth if any gene editing occurred due to Cas9/gRNA assertion. T7 Endonuclease assay works through a process of denaturing/annealing which causes all the DNA strands in the sample to randomly separate and then anneal back together. The hope is that a wild-type strand will anneal to an edited strand thereby causing a "kink" as a result of mismatched bases. The addition of the endonuclease to the sample finds these "kinks" and breaks the strands at the site of the mismatch. Thus when the sample is run on gel, there should be a total of 3 bands visible; the parental band along with 2 addition bands which represent the
2 ends of the strand that was cut by the nuclease at the site of a “kink”. If there is no gene editing occurring then it is expected to see one band, the parental band. Using GeneArt® Genomic Cleavage Detection Kit, 3µl of PCR product along with 1µl 10x Detection Reaction Buffer were added in a PCR tube with 5µl of water for a total volume of 9 µl. This was repeated again for the second tube. The tube was briefly centrifuged in a thermal cycler for reannealing and to ensure no bubbles were presents. The thermal cycler parameters were: 95°C for 5 minutes, 95°C to 85°C decreasing intervals of 2°C per second, and then from 85°C to 25°C decreasing intervals of 0.1°C per second. The solution remained in hold at 4°C until removed from apparatus. After reannealing has occurred 1 µl of detection enzymes were added to one of the tubes and 1 µl of water was added to the other as a control. Both tubes were then incubated at 37°C for 1 hour then vortexed briefly and spun down before placing at 4°C. Following enzymes digestion, 10 µl of water was added to both tubes. 20µl was added to each lane in 2% E-Gel EX Gel using an Egel iBase Power System for 60 minutes at low voltage. 5µl of DNA ladder was also added to lane 1 in order to compare size of cleaved products. The gel was analyzed using a UV trans-illuminator.

**Extraction of DNA from Zebrafish embryos and Transfected Cells**

Zebrafish embryos were injected with the Cas9/gRNA construct via microinjection; while still in the one cell stage (up to 45 minutes post fertilization). Since the embryos are injected compared to transfected, in theory, every cell should then have the target sequence removed since all the cells in the embryos will be decedents from that original cell. We first harvested and amplified our sequence of interest which contained the target sequence via PCR amplification and then verified the results using gel analysis. 50 µl of
buffer/protein degrader mix were added to each of the tubes containing the control and experimental DNA, and placed in PCR machine to start extraction in the following parameters: 95°C for 5 minutes, 95°C to 85°C at decreasing intervals of 2°C per second, then from 85°C to 25°C at decreasing intervals of 0.1°C per second. The solution remained at 4°C on hold until removed from the apparatus. Afterwards 4μl of DNA along with 1μl of primer mix and 45μl of Taq mix were added in a separate tube. This was done for both control and experimental. PCR was run again using the following parameters: 95°C for 10 minutes and then a cycle of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds were repeated 40 times. A final extension stage was run at 72°C for 7 minutes following termination of the cycles. Solution was kept on hold at 4°C until removed from apparatus. Samples were analyzed and run through additional T7 endonuclease assay.

**Animal Mating**

The night before microinjections were to be performed, the animals were separated into designated mating chambers. These are smaller mesh bottom containers that are placed inside the normal animal tank that contain a divider. One female is placed on one side of the divider and two males are placed on the other side and left over night. The divider is lifted first thing in the morning once system light turns on allowing male and female interaction. Eggs that are laid fall through the mesh bottom of the chamber to allow for easier collection and prevention of the eggs from being eaten by the animals.

**Blue water Production**

Used for the storage and incubation of the zebrafish embryos from zygote state until hatching, approximately 48 hours post fertilization. Solution consists of 2 liters of water,
.175 grams of sea salt, and 2 drops of 1% aqueous methylene blue.

**Injection Mold Preparation**

1.5 grams of agar was mixed with 100mL of blue water solution and brought to a boil. The mixture was then allowed to cool significantly before pouring into petri dish. The injection slide mold (Eppendorf), is placed face down in the agar mixture.

**CRISPR Injection Construct Preparation**

Injection solution was prepared as follows: 15 μL of Cas9 mRNA and 1 μL of predesigned gRNA were mixed with 34 μL of sterile water (20). In order to make the injections visible within the nucleus of the embryo, 5 μL of 0.5% phenol red was added.

**Microinjection of Construct**

Eggs were collected immediately following fertilization and were placed in a petri dish full of blue water solution and placed on a warming place to keep at system temperature. The eggs were then lined up individually in the prepared injection mold rolls wiping away excess water to prevent egg rolling within the lanes. Eggs were injected using an Eppendorf microinjecting apparatus with accompanying joystick. Construct was loaded into injection needle via microloader pipette tips following centrifugation. Needle was maneuvered until it just penetrated the surface of the embryo and was visible within the nucleus. Once positioned, the cell was injected at a pressure of 522 hPa for 3.5 seconds with the construct. Cells were verified for successful injection if some red was visible within the nucleus following injection; red coloration slowly dissipated over a course of a few seconds. Around 2 nL of construct was injected into each embryo (19). Once all the embryos on a plate were injected, they were returned back to the blue water petri dish and then placed in an incubator at 28°C for 48 hours or until hatched.
**Genomic Extraction from Inject Zebrafish Adults**

Injected adult zebrafish genomic data was extracted via a small clipping of the caudal fin. This clipping was obtained by taking each fish individually from the tank and placing them in a petri dish containing water then covering the entire fish minus the tail section with dampened gauze. A very small snippet was taken from the top of the caudal fin using scissors placed in a tube that was immediately placed in liquid N₂ to prevent decomposition of the tissue. Following the tail dissection, the zebrafish were returned to a separate smaller individual tank that was numbered accordingly with the samples taken. DNA was extracted and amplified in the same manner that was previously described. Samples were then sent out for sequencing and analyzed.

**TOPO 10 Cloning of PCR Samples**

TOPO cloning of fresh PCR products (from transfected PAC2 cells and injected embryos) were inserted into a TOPO plasmid vector. This vector was then transfected into chemically competent *Escherichia coli* cells via heat shock. The *E. coli* cells were then spread onto one of 4 LB plates and allowed to grow overnight forming colonies. Depending on success of product uptake within the plasmid and intake of plasmid into the bacteria, *E. coli* formed colonies that were either blue or white in color. Colonies that were white in color indicated that plasmid uptake has been successful. Plates 1 and 2 were using bacteria cells featuring vectors containing PCR fragments from the second set of transfections using PAC2 cells. Plates 3 and 4 contained PCR fragments from the injected embryos. Primers for the fresh round of PCR were selected using a screening of all 4 primers used so far for both the transfected cells and injected embryos. Primers 2
and 4 were used for the transfected cell genomic data (plate 1 and plate 2 respectively) and primers 2 and 3 were used for the injected embryo genomic data (plate 3 and plate 4 respectively). Plate 5 was used as a negative control in order to infer background information since no bacteria was spread on it.

**T7 Endonuclease Assay of PCR Samples**

T7 endonuclease assay was utilized for the 4 PCR samples: transfected cell control (EGFP transfected Lac-z), transfected cells experimental (Cas9 GFP (-) AIg ICER), zebrafish embryo control (wild type zebrafish embryo), and zebrafish embryo experimental (injected 24 hours embryo Cas9+gRNA(-)atg ICER), along with a positive control. There will be two tubes for each sample; one without enzymes and one with enzymes (-, +). In total they were 10 samples all together. 1ul of enzyme was added to all positive tubes (tubes 2, 4, 6, 8, 10) and 1ul of H2O were added to all negative tubes (tubes 1, 3, 5, 7, 9). The tubes were then incubated in water bath at 37°C for 1 hour followed by a brief vortex before being spin down and placed at 4 °C. Following enzymes digestion 10ul of water was added to each tube. With a pipette, 20ul was added to each lane accordingly on 2% E-Gel ex gel. Gel was run on the Egel ibase® power system for 60 minutes at low voltage. 5ul of DNA ladder (hilo) was added to lane 1 in order to compare size of cleaved products. The gel was viewed using a UV transilluminator.

**Plasmid DNA Purification Using the QIA prep Spin Mini Prep Kit and a micro centrifuge**
Using a technique called blue/white screening, 25 white colonies from plate 1 and 15 white colonies from plate 3 were obtained. These colonies were separated in tubes containing media and grown over night. Using the QIAprep Spin Miniprep kit DNA was collected and purified from the cells to be sent for sequencing.

Results

First Transfection

To determine transfection efficiency, PAC-2 zebrafish cells were transfected using a vector containing the endonuclease Cas9 in an effort to cut the target sequence with the assistance of gRNA. Only the cells that were deemed brightest were accounted for when assessing efficiency of transfection. Transfection efficiency was determined by taking the number of GFP expressing cells and dividing them by the total number of DAPI stained cells. Therefore the transfection efficiency was determined to be 4.46% (12GFP: 269DAPI) for the experimental vector and 9.87% (31 EGFP: 314 DAPI) for the control vector (Figure 1).

Sequence of interest was amplified by PCR with each of the four groups utilizing one of four primers (2, 3, 4, and 5). Results of PCR amplification can be seen in figure 2. The most notable feature of the gel is the long streaks that appear in both lanes 4 and 5 coinciding with both the experimental and control samples from primer 2. Outside lanes 4 and 5, the rest of the samples looked ideal with a pronounced band around 500bp, as expected. Other bands to note in figure 2 is the faint band located right above the 500bp band (roughly 575bp) in addition to an even lighter band located around 1100bp was found in all the viable samples.
With verification of the PCR product, samples from primers 1, 3, and 4 were sent for sequencing (experimental and control) using both forward and reverse primers. It should be noted that the target sequence when using the reverse primer is the reverse complementary (figure 3 right). A striking feature that was noticed in the sequences was an area of high repeats (not shown). These areas tend to obscure any sequence that is located downstream due to a tendency of the strand to slip; the result is sloppy peaks that prevent any accurate data from being read. This downstream effect can be seen when comparing the target sequences in figure 3. The one to the left is found upstream of the repeat area and therefore has tight concise peaks while the sequence to the right is much less organized. In total, all sequences both control and experimental that were analyzed contained the target sequence.
Figure 2 Gel analysis of PCR product following PCR amplification using each primer for both control and experimental cells. MWM – Molecular weight marker. C – Control. E - Experimental

<table>
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<th>Lane</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<td>C1</td>
<td>E1</td>
<td>C2</td>
<td>E2</td>
<td>C3</td>
<td>E3</td>
<td>C4</td>
<td>E4</td>
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</table>

Figure 3 Target sequence locations in both sequences 3E(F3) with forward primer (left) and 1E(R)(right). The target sequence and reverse complimentary target sequence are highlighted respectively. The reverse primer sequence is more disorganized due to its location within the sequence in addition to being just downstream of an area of high repeats.
**Second Transfection**

Phase contrast microscopy was used in place of DAPI in the determination of transfection efficiency (figure 4 right top and bottom). There was an increase in transfection efficiency from 4.46% to 14% (32 GFP: 224 total cells) for experimental, compared to a decrease from 9.79% to 9% (28 EGFP: 312 total cells) for control.

Following assessment of transfection efficiency, DNA was amplified via PCR. Figure 5 shows the gel analysis. The prominent feature of the gel is the lack of bands in lanes 4 and 5 corresponding to primer 2, a similar issue occurred during the first transfection. The other samples produced a solid band at the expected size of 500bp. After verification of the PCR products the samples were sent for sequencing (excluding primer 2 samples). For simplification, only the experimental samples were sent for
sequencing. Similar to the first transfection, samples 1, 3, and 4 contained the full target sequence in forward samples but only a portion in reverse samples. This was a result of high levels of repeat sequences located upstream of the target site, thereby rendering the data inadequate (Data not shown).

Figure 5 Gel analysis of PCR products following amplification of second transfection. X – Empty lane. MWM – Molecular weight marker. C – Control. E- Experimental

T7 Endonuclease assay was then performed on the samples in order to detect more in depth if any gene editing occurred due to Cas9/gRNA assertion. Despite a loading issue with sample E4, all positive samples (i.e. samples that were treated with the nuclease) clearly expressed the expected 2 bands (350bp and 175bp) along with the parental band (500bp) indicative of strand mismatch (Figure 6). Lane 3 also faintly expressed the same 2 bands. Lastly, two very faint bands of unknown origin were also observed in lanes 6 and 9 just above 100bp.
Embryo Injections

The genomic data from zebrafish embryos injected with the Cas9/gRNA construct via microinjection were analyzed 24 hours post fertilization. DNA was first harvested and sequence of interest amplified via PCR. The results were verified using gel analysis (figure 7). Each sample expressed only 1 band at 500bp as expected. The experimental samples were then sent for sequencing with both the forward and reverse primer. The results were the same as during both transfections, with the target sequence still present in all the samples (Data not shown).
Despite lack of evidence in sequencing data, T7 Endonuclease assay was performed (Figure 8). There were some issues observed with this assay; many of the samples had a streaky appearance, lane 2 (E1+) failed to show any bands despite receiving the nuclease, and lane 3 (E2-) only had a faint smear where the parental band was assumed to be. Regardless of those issues, the rest of the samples appeared fine with all but lane 2 expressing a parental band at 500bp. Lanes 4, 6, and 8 (the positive samples containing the nuclease) expressed the expected additional bands (about 350bp and 175bp) which was very similar to what was seen in figure 7. A second T7 Endonuclease was performed, this time equating the transfected PAC-2 cells (control and experimental), wild-type embryos, and injected embryos on the same gel (figure 9). All positive samples expressed two additional bands that can distinctively be seen with some variability in regards to intensity. The two additional bands in each of the positive samples were around 350bp and 175bp in length which is almost identical to the addition bands observed in figures 6 and 8.
Figure 9 T7 Endonuclease assay comparing control and experimentally transfected PAC-2 cells with injected and wild-type embryos. C – Control transfected PAC2 cell. E – Experimentally transfected PAC2 cell. WT – Uninjected embryo. I – Injected embryo. (+) indicates enzyme was included. (-) indicates water was included. PC – Positive control.

**TOPO Cloning**

Table 1 shows a summary of plate findings from TOPO cloning along with percentage of white colonies (i.e. containing the recombinant DNA). 25 white colonies from plate 1 and 15 white colonies from plate 3 were selected and harvested for sequencing. PCR fragments if incorporated into the TOPO vector can be featured in one of two ways; either forward or reverse orientation. This can be determined by the location of either the primer sequence or its reverse complimentary indicating orientation. In addition, the location of the flanking ends of the TOPO vector within the sequence is vital to locate since it marks the start of where the PCR fragment was inserted. From this we can then analyze the sequences for the presence of the target sequence.
Plates 1 and 3 were selected since both used the same primer (Primer 2) making analysis simpler. A total of 40 sequences were examined with only 26 providing sequencing data. Using the flanking end of the TOPO vector for reference, a common tread emerged placing a majority of the sequences in one of two categories: forward primer or reverse complementary primer. A total of fourteen of the analyzed sequences were determined to contain the forward primer (Figure 10 top). A feature that was common among all the forward primer sequences was the presence of the target sequence. An addition ten was determined to contain the reverse complementary primer (Figure 10 bottom) but in contrast, only four contained the reverse complementary target sequence. While this does seem promising for successful gene editing, one must take into account that the location of the reverse complementary target sequence is located much further downstream leading to the greater possibility that sequencing did not capture enough data to confirm or deny its inclusion. For example, sequence 18 in figure 10 contains the reverse complementary primer and based on the sequence, the reverse complementary target sequence would have been located five nucleotides further downstream from where the sequencing ended.

The last two remaining sequences were unable to be classified into one of the two categories (sequences 20 and 32). While the flanking ends of the TOPO vector were

<table>
<thead>
<tr>
<th>Plate</th>
<th>Estimated Total Colonies</th>
<th>% White Cells</th>
<th>Estimated White Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (P2C)</td>
<td>500-1000</td>
<td>90%</td>
<td>450-900</td>
</tr>
<tr>
<td>2 (P4C)</td>
<td>2-3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3 (P2E)</td>
<td>100-300</td>
<td>80-90%</td>
<td>80-270</td>
</tr>
<tr>
<td>4 (P3E)</td>
<td>50-200</td>
<td>70-90%</td>
<td>35-180</td>
</tr>
<tr>
<td>5 (NC)</td>
<td>30</td>
<td>50%</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 1 Summary of plate findings and amount of white colonies. All counts are estimations based off of counting a section of the plate and then extrapolating for the entire area. NC – negative control. P – primer, 2/3/4 – primer number, E – injected embryo PCR product, C – transfected cell PCR product.
found in both, we were not able to identify a primer nor able to locate the target sequence (either forward or reverse complementary) (Figure 11). Therefore these sequences could not be properly analyzed and most likely resulted from the vector sealing up without incorporating a PCR product.

**Figure 10** Two common motifs found among the TOPO sequencing data. Sample 14 indicative of forward primer insertion and sample 18 representative of reverse primer insertion. Bold red lettering indicates the flanking end of TOPO vector upstream of PCR inclusion. Orange bold lettering indicates the start of PCR fragment and also is the sequence for the primer; in contrast sample 18 contains the reverse primer (in orange). Sequence that is highlighted blue is the target sequence (only in 14).

**Figure 11** Sequencing data of sample 20 from TOPO cloning/cell transformation. Bold red lettering indicates the flanking end of TOPO vector upstream of PCR inclusion. Unlike the other samples, no primer or target sequence could be located.

**Additional Zebrafish Injections**

With the failure to achieve the desired results, further embryo injections were performed. In order to extract genomic information, DNA from zebrafish caudal fin was isolated and then amplified via PCR using primer 2 (Figure 12). A total of fifteen three month old zebrafish were used. Analysis was completed in two parts: samples 1-7 and
samples 8-15 (sample 3 was redone due to streaky results). The expected band of 500bp was only visible in samples 1-9 and 13. Additional unknown bands of 200bp are noticeable in samples 8 and 10.

Prior to sending out for sequence analysis, samples 11, 12, 14, and 15 were rerun through PCR since they failed to show during gel analysis (figure 12). Once completed and verified via gel analysis (data not shown), all fifteen samples were sent out for sequence analysis. Examination of the sequences veiled that all the samples contained the entire target sequence along with an intact start codon for ICER indicating that no genome editing occurred at the desired site.

A third set of 32 injected zebrafish were analyzed once they reached approximately three months of age. Tail biopsies were performed in order to extract genomic information from the adult animals and then amplified via PCR. PCR products were verified by gel analysis (data not shown). Every sample with the exception of sample 21 expressed the expected 500bp band indication successful PCR. Sample 21 was too low to be included in the gel analysis (therefore water was loaded into its corresponding lane) but was sent for sequencing along with the 31 other samples.

Sequencing results were much similar to the two previous attempts with 31 of the 32 samples containing the target sequencing along with a fully intact start codon therefore making them wild type. The only exception was sample 12 which did not contain any fragment of the target sequence indicating either a positive result or procedural error (figure 13). Further analysis using the reverse primer reveled that sample 12 also is wildtype contrary to initial beliefs.
Figure 12 Gel analysis of PCR products from samples 1-15 of genomic extraction of caudal fin DNA from three month old zebrafish. Top row in table indicates lane number, while the bottom row indicates sample. MWM – Molecular weight marker. PC – Positive control.

| M | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| MWM | 1 | 2 | 3 | 4 | 5 | 6 | 7 | PC | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 3 |

Figure 13 Sample 12 from the third analysis of injected zebrafish embryos. DNA was extracted via the caudal fin of three month old adult zebrafish and amplified via PCR using primer 2. No traces of the target sequence were able to be located.

Discussion

During the transfection of the PAC2 cells, the transfection efficiency of 4.46% was much lower than what was expected potentially attributing to the lack of results; although when transfection efficiency was increased to 14% following subsequent transfection the results did not fare much better. Issues were also run into whilst analyzing the sequences. Areas of high repeats were found to be located downstream of the target sequence. While this does not have an effect on the forward primers, the reverse primer information is rendered relatively useless as a result of strand slippage. Sequencing technology tends to lose accuracy the more repeats that consecutively follow...
one another, resulting in sloppy downstream data that runs the risk of false positives during analysis. An alternative reverse primer that ultimately avoids this repeat sequence could help alleviate this issue which ultimately has relegated half of our sequencing data to obscurity.

Following a second failed transfection it was hypothesized that the construct failed to work due to the wildtype strands overwhelming the strands that due contain Cas9 induced indels; in essence hiding them from detection during sequencing analysis. A T7 endonuclease assay was performed specifically looking for any "kinks" or mismatches following reannealing that would of arose from Cas9 editing regardless of a miniscule amount. Results from figure 6 were promising indicating that some gene editing had occurred due to the presence of addition bands being visible at around 325bp and 170bp outside of the 500bp parental band. While these values were not identical to the theoretical values of 367bp and 113bp, they were close enough to warrant further investigation.

The next stage of the project was the direct injection into one cell stage zebrafish embryos (up to 45 minutes post fertilization) with the gRNA/Cas9 construct. Theoretically the percentage of edited cells should be higher since all cells will be descendants from the original injected cell but alas this was not the case. Once again sequencing data failed to provide evidence that neither the target sequence nor the start sequence of ICER had been edited in any way. Possible explanation for the outcome could be centered around injection issues that resulted in not all of the embryos within a sample being injected, thus allowing the wildtype sequences to regain superiority and overwhelm the edited strands. Additionally, errors within the injection method and
preparation stage could have also played a part. T7 Endonuclease assay was performed to further analyze the samples and once again revealed that some gene editing potentially occurred with the presence of additional bands at approximately 350bp and 175bp in addition to the parental band at 500bp.

A third T7 endonuclease assay was run in order to factor out if the assay itself could potentially be faulty. In order to verify this, transfected PAC2 cells (control and experiment), wildtype embryos, and injected embryos were all run on the same gel with specific interest in the control PAC2 cell and wildtype embryo samples. These samples in particular even when presented with the nuclease should theoretically express only the 500bp parental band since they were not exposed to Cas9. Unfortunately this was not what was observed (figure 9). These results question the validity of the previous findings since it is apparent that the nuclease is working but in a nonspecific way, cutting strands regardless of indel presence.

TOPO cloning followed by blue/white screening was performed as an alternative method in order to provide sequencing data of a successful gene editing. A major concern following the analysis of the plates was that plate 2 had less colonies than the negative control (plate 5) despite never being in contact with *E. coli*. As a result, colonies from plates 1 and 3 were selected for sequencing. From the 40 samples that were analyzed only sample 20 was of interest since it lacked a primer sequence and target sequence. The only recognizable feature was one of the flanking ends of the TOPO vector. Possibilities for this result include that the sequence joined back together without being included into the vector (unlikely since the other side of the flanking repeat was not visible), there was an
error with sequencing, or somehow the PCR fragment got included in a such a way that
the beginning of the sequence where the primer would have been located got eliminated.

Further zebrafish embryo injections were performed to further refine the method
and to achieve the desired result but ultimately we were unsuccessful in our attempt to
create a zebrafish knockout for ICER. As was discussed here there were many factors
that potential had an effect on the results. Firstly, off-target binding of the gRNA/Cas9
construct would have resulted in gene editing occurring but not in the desired location.
Issues with microinjection method itself such as high viscosity within the construct
resulted in numerous clogged needles preventing proper injection. Additional, with
multiple RNA components being used (Cas9 mRNA and gRNA); the sensitivity of RNA
to degradation could also potentially pose an issue. Even through the construct was
prepared using aseptic techniques and handled with care, contamination cannot be
entirely ruled out.

During the process of microinjecting the zebrafish embryos, it was noticed that
the embryos that were either injected or awaiting injection in the transfer petri dish were
becoming much too cold in relation to their ideal 28° C. To address this, a warming plate
was used and the embryos not currently on the injection mold were placed on the
warming plate. Additionally, the blue water solution was also heated in a hot water bath
before being used on the embryos. Another problem that occurred dealt with tank system
regulations; most notably a sharp drop in temperature that resulted in the loss of the first
two sets of injected juvenile zebrafish. The issue was later corrected by placing additional
water heaters in the system to better provide thermoregulation. Water pH and
conductivity additionally were found to be in deviation from the expected range but daily system checks were put in place to prevent this from happening again.

**Future Studies**

While ultimately unsuccessful in generating a legitimate zebrafish knockout of ICER, the results that were generated along with possible issues suggests strongly that the process is plausible. Refinement of the accuracy of the gRNA could help to eliminate any potential off target binding that might be occurring. If a similar site is located elsewhere within the genome, alternations could be made to make the process more site specific.

Alternatively, different ratios among the components of the construct (specifically gRNA and Cas9 RNA) could be utilized to see whether different proportions are needed in order to elicit the proposed desired effect. Limitations do exist since it has been shown that increased gRNA and Cas9 concentrations tend to result in an increase of off-target binding (21, 22).

Upon proper identification of a successfully edited zebrafish, an ICER knockout specific model can then be generated. From here, observations can be made as to whether the eradication of ICER promotes tumorigenesis when compared to wildtype zebrafish. Additionally, ICER will be knocked out from a transgenic zebrafish lineage that contains the mutated gene most associated with human melanoma (Tg(mitfa:BRAF(V600E)); p53 -/-) to create an additional zebrafish model. This particular model will be used to determine whether ICER deletion results in accelerated development of melanoma since the mutated gene is already presence.
If sufficient correlation exists then the next step would be to access whether the reconstitution of ICER expression hinders the tumorigenesis process in both the transgenic and wildtype ICER knockout models. A number of studies have been performed that forced the expression of ICER within cells that had previously lost ICER expression and therefore were transformed (11, 13). When ICER expression is reintroduced within the cell there is an inhibition of DNA synthesis, cell growth, growth-related genes (i.e. cyclin A, cyclin D2, cyclin D1, and cFos), anchorage-independent growth, and tumor formation (8, 11, 13). Thus supporting the notion that reintroduction of ICER within a human tumor cell might one day offer a potential therapy for treating cancer by restoring the non-transformed phenotype.
References


