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Optimizing CRISPR/Cas9 Transfection Protocol to Generate a Tet-on Inducible Sk-Mel 24 Cell Lines to Better Study ICER

Abdulkader Hallak

Abstract

Inducible cAMP Early Repressor (ICER) is a dominant transcription repressor of the *cAMP Responsive Element Modulator (CREM)* and *cAMP Responsive Element Protein (CREB)* family of transcription factors. ICER isoforms are differently expressed in various cell types and are involved in essential functions including hormonal regulation, circadian rhythm, spermatogenesis, and immune response. In the context of cancer, ICER is proposed as a tumor suppressor in leukemia, prostate, pituitary cancers, and melanoma. In melanoma, ICER is targeted for degradation during tumorigenesis and highly upregulated during regression, further highlighting its potency as a tumor suppressor. Inside the nucleus, ICER targets oncogenes and key cell-cycle regulatory genes such as Cyclin A, Cyclin D, C-fos and Bcl2, yet the full spectrum of its targets has not been fully explored.

The goal of this thesis is to generate a stable inducible cell line which allows better study of ICER at viable conditions, identifying its binding regions and any additional genes it targets. For this study Sk-Mel-24 cells were used as a model of human melanoma. Two plasmids were utilized in each transfection round, all-in-one Cas9 gRNA plasmid and an ICER-containing donor DNA plasmid. The Cas9 gRNA plasmid was used to target the AAVS1 (safe harbor) locus, due to the transcriptional competence and minimal adverse effect of the region. As for the donor plasmid, it carried N-terminus hemagglutinin (HA) tagged ICER ($_{N}$HA-ICER) under the control of Tetracycline-induced expression system. Results from Western blot, immunocytochemistry (ICC), fluorescence microscopy all demonstrate successful doxycycline induction. Furthermore, PCR and sanger data proper integration of $_{N}$HA-ICER into the AAVS1 locus.

Key Words: ICER, Safe HarAAVS1, CRISPR/Cas 9, Hemagglutinin (HA)

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Optimizing CRISPR/Cas9 Transfection Protocol to Generate a Tet-on Inducible SK-Mel 24 Cell Lines to Better Study ICER

by

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OPTIMIZING CRISPR/Cas9 TRANSFECTION PROTOCOL TO GENERATE A Tet-on INDUCIBLE SK-Mel 24 CELL LINES TO BETTER STUDY ICER

A THESIS

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Introduction:

Inducible Cyclic AMP Early Repressor (ICER) is a small DNA binding protein and transcription factor that is well involved in cellular functions such as hormonal regulation, circadian rhythm, spermatogenesis, immune response, and other metabolic functions as well. ICER is a collective name given to four different isoforms, all which originate from the intronic P2 promoter within the CREM gene via alternative splicing (Molina et al., 1993). ICER isoforms consist of conserved N-terminus domain encoded by exon X, followed by a gamma domain encoded by exon gamma (present in ICER I & ICER II and absent in ICER I-gamma & ICER II-gamma), a basic domain encoded by domain H, and a leucine zipper domain encoded by exon I. ICER I & ICER II differ in their exon I sequence giving the two isoforms different DNA binding affinity (Molina et al., 1993) & (Kaprio et al., 2021). The basic domain followed by the leucine zipper forms a DNA binding domain which allows ICER to form dimers while binding DNA.

Under normal conditions, second messengers of the cyclic adenylyl cyclase pathway, such as protein kinase A (PKA), phosphorylate both Cyclic AMP Response Element Binding (CREB) and CREM proteins, both which function as transcription activators of CRE containing genes (Steven et al., 2020). ICER on the other hand, although induced through the same pathway, functions as a dominant transcription repressor by targeting cAMP responsive genes which contain cAMP Responsive Element (CRE) sequence in their promoters, TGACGTCA for example (Laoide et al., 1993). Interestingly, four CRE elements exist in tandem in the P2 promoter, which allows ICER to bind those sequences and autoregulate its own transcription in a negative feedback fashion (Molina et al., 1993).

In terms of physiology, ICER is most known as a potent tumor suppressor (Pigazzi et al., 2008) as it blocks cells in the G1/S (Greco et al., 2017) and G2/M checkpoints (Lamas et al.,

1997). ICER controls those checkpoints by repressing the expression of certain key proteins which are involved in cell cycle regulation such as Cyclin A, Cyclin D and c-fos (Steigedal et al., 2007). Transfection studies using corticotropic cell lines by Lamas et al. (1997) shows that Cyclin A expression was completely absent, and the cells were blocked at the G2/M checkpoint upon expressing sense ICER transcript. It is not surprising, considering that Cyclin A promoter contains a CRE sequence and has been shown to be responsive to adenylyl cyclase pathway signaling (Desdouets et al., 1995).

Another study by Muñiz et al. (2006) demonstrates that Cyclin D2 is regulated, at the transcription level, via a cAMP-PKA-dependent pathway in response to FSH signaling via a CRE element in the promoter. This signaling system, which signals for the progression of the cell cycle towards the DNA synthesis phase, was completely absent in granulosa cells which received ICER treatment, locking the cells at the G1/S checkpoint and preventing the cells from entering the S phase.

In addition to halting cell cycle progression, ICER also induces apoptosis in melanoma (Tomita et al., 2003; Cirinelli et al., 2022) although the mechanism is not fully understood. Melanoma is a rare malignancy that develops in the pigment producing tissues of the skin, also known as melanocytes and is responsible for a vast majority of cancer deaths in the U.S (Garbe et al., 2016). Interestingly, ICER is significantly downregulated in melanoma, not at transcription level but rather at the protein level. In 2013, Healey et al. show that increased H-RasV12G levels, a key protein for the progression and maintenance of melanoma, induce ICER's ubiquitin-mediated degradation while ICER mRNA levels were unaffected. An example of this post-translational modification of ICER has been previously demonstrated as ICER can be mono- or poly-ubiquitinated, which lead to its translocation from the nucleus or degradation by

proteasome, respectively, both which cause loss of function of the transcription factor (Yehia et al., 2001). In summary, we have a basic understanding of ICER's down regulation in melanoma, but very limited knowledge on the full spectrum of ICER's target genes and its relation to tumorigenic phenotype. Taken together, we hypothesize that ICER, as a transcription factor, targets and represses multiple key oncogenes and other key cell-cycle regulating genes.

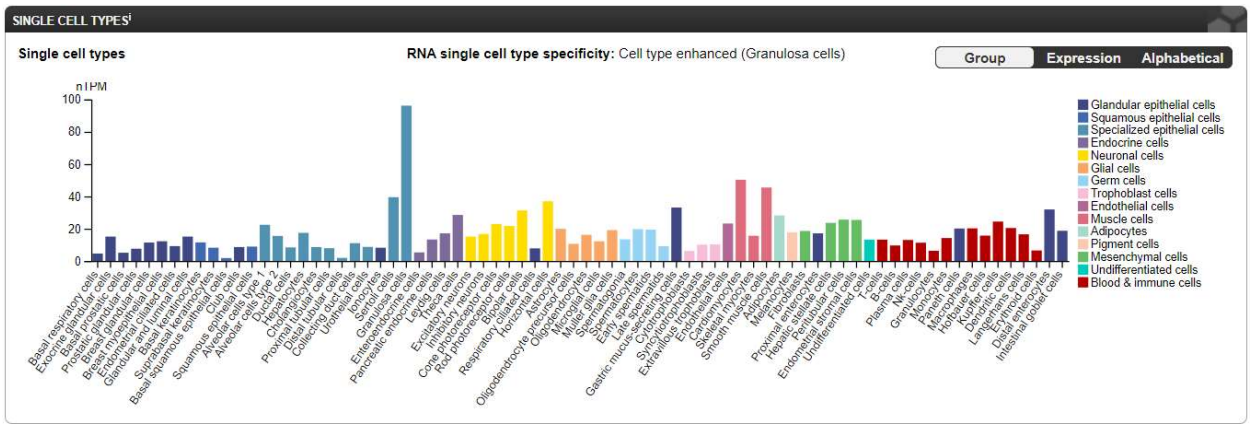
To further investigate this claim, it was necessary to generate model cell lines with controlled expression of ICER at defined levels, high enough to detect the effects on target genes and low enough for the cells to remain viable and minimize the lethal effects of ICER. A plan was devised to generate an inducible ICER-expressing vector using the Tet-on operon. Tet-on system was first introduced by (Goosen & Bujard. 1992). By fusing the tetracycline repressor tetR region of *E. Coli* with the activating region of Virion protein of the HSV virus, Goosen & Bujard (1992) were able to generate a tetracycline-inducible expression system. In the presence of tetracycline, a hybrid transactivator is produced which functions to stimulate the promoter of the tetracycline Operator (tetO) and initiate transcription. Tet-on system not only functions as an "on/off" switch but is also sensitive to tetracycline levels which can modulate the transcription levels, up to five orders of magnitude (Goosen & Bujard. 1992). The Tet-on system with its bacterial origin also provides an advantage in that the system relies minimally, if at all, on eukaryotic endogenous control elements, which is a common problem in eukaryotic inducible systems, thus, providing maximal control over transcription.

To ensure a successful integration and minimal side effects, we picked AAVS1, also known as the "safe harbor" region as our integration site. In human chromosome 19, *PPP1R12C* (protein phosphatase 1 regulatory subunit 12C) gene is a common integration site for the Adeno-Associated Virus (AAV) (Kotin et al., 1992), hence the name AAVS1. Normally, *PPP1R12C*

has an active promoter and is constantly expressed in all cell types (See Figure), and commercial cell lines. Furthermore, no known pathophysiology was associated with PPP1R12C disruption via AAV infection (Smith et al., 2008). Furthermore, integration at this site will help us avoid random gene silencing and/or common epigenetic modifications associated with traditional integration protocols (Tiyaboonchai et al., 2014). Both the transcriptional competence and minimal adverse effect of disrupting the gene make AAVS1 a great candidate for this study.

Figure 1

PPP1R12C RNA expression in eukaryotic cells



The diagram above illustrates PPP1R12C RNA expression across all eukaryotic cell types, diagram produced by protein Atlas: https://www.proteinatlas.org

Finally, the CREM gene, the source of ICER transcripts, was left unmodified in our cell lines to avoid interfering with the physiological functions of the other gene products, such as the CREM protein for example. In order to distinguish endogenous from exogenous ICER expression we decided to use a hemagglutinin tag (HA) tag at the N-terminus sequence of our ICER. Hemagglutinin, also referred to as the “HA” tag is a well characterized epitope tag that is commonly used for molecular labeling purposes and has no known effects on protein function.

As a matter of fact, a recent study by Cirinelli et al. (2022) shows that $_{N}$HA-ICER is more tolerant to ubiquitin-directed degradation compared to the non-tagged ICER.

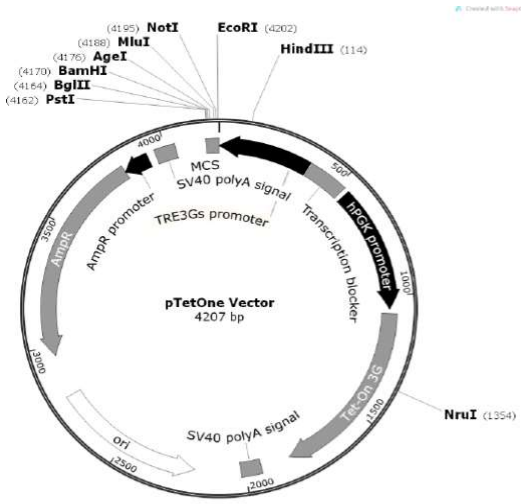
Material and Methods:

Plasmid Construction:

AAVS1 Safe Harbor Site Targeting All-purpose HR Donor Vector 2.0 (AAVS1-SA-puro-MCS) by (System Bioscience, Ca) was used as the donor DNA for our homology recombination approach. The construct came in with a built-in Puromycin resistance cassette, 3' & 5' homology arms, and a multiple cloning site (MCS). A separate Tet-One™ Inducible Expression vector with a hPGK promoter was purchased from Takara Bio, Japan (Figure 2.1). N-terminus HA-tagged ICER ($_{N}$HA-ICER) cDNA plasmid was subcloned into the Tet-One vector which was subcloned into the AAVS1 donor vector comprising the final donor plasmid (see figures 2.2 & 2.3). For CRISPR, all in one pCas9 plasmid was purchased from (System Bioscience, Ca) with the following gRNA (5'-GTCCCCTCCACCCCACAGTG-3').

Figure 2.1

Original Tet-One™ Inducible vector by Takara Bio



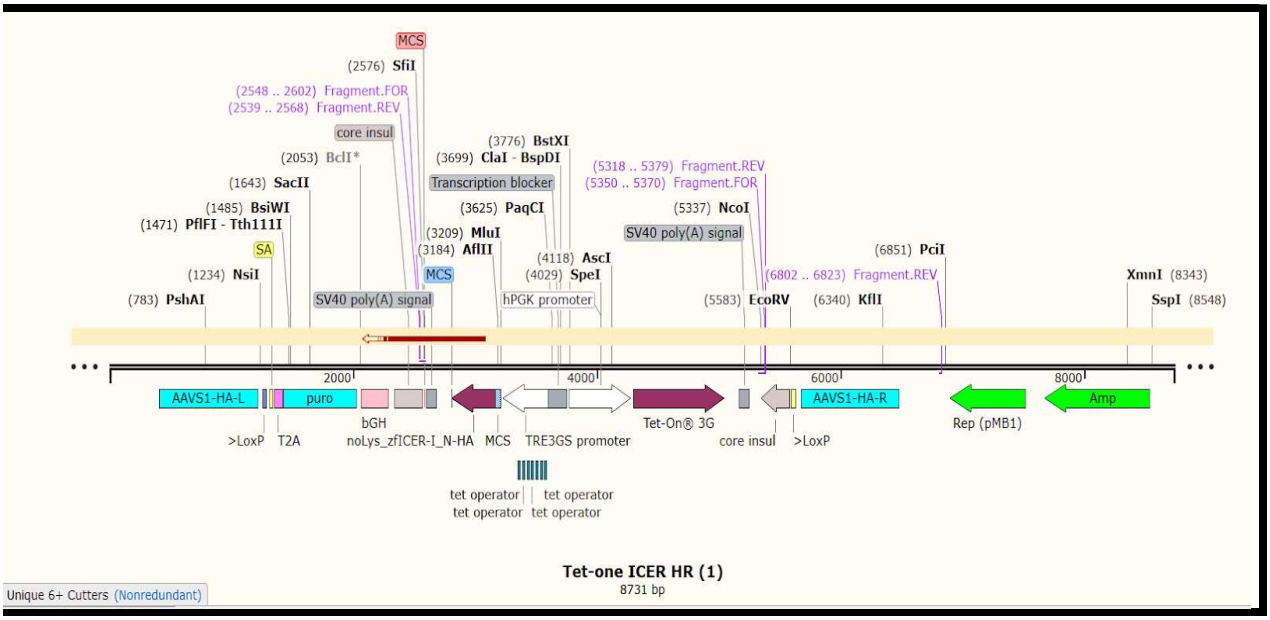
Circular diagram of the original (unmodified) Tet-One™ Inducible Expression vector by Takara Bio, Diagram produced by Snap Gene.

Cell Culture/Transfection:

Commercially available Human melanoma SK-MEL24 by (ATCC, US) were purchased and cultured in 35 mm 6-well plate following the manufacturer's recommendation. Cells were grown in Eagle's Minimum Essential Medium (EMEM) media (ATCC, US) with 10% tetracycline-free Fetal Bovine Serum (FBS) following the suppliers' guidelines. For transfection protocols, pure EMEM media was used in absence of FBS and/or antibiotics. For this experiment, FuGENE® HD Transfection Reagent kit (Promega, US) was used. Each reaction contained transfection Media, pCas9-GFP plasmid, $_{N}$HA-ICER plasmid (eGFP for control), and FuGene reagent. All transfection reagents and plasmids were mixed in 2 mL tubes, then transferred to 35 mm well plates. Cell lines were then incubated for two hours with the transfection reagents, before changing the media once again. At 70 – 80 % confluency post transfection (2-3 days), cells were treated with puromycin for a final concentration of 0.5 ul/mL in media to select for the transfected colonies. It took roughly 30 days before colonies were formed after puromycin treatment. Surviving colonies were then labeled and frozen for later WB and PCR analysis. For the purposes of this study, Sk-Mel 24 cells were also transfected with eGFP and served as control group.

Figure 2.2

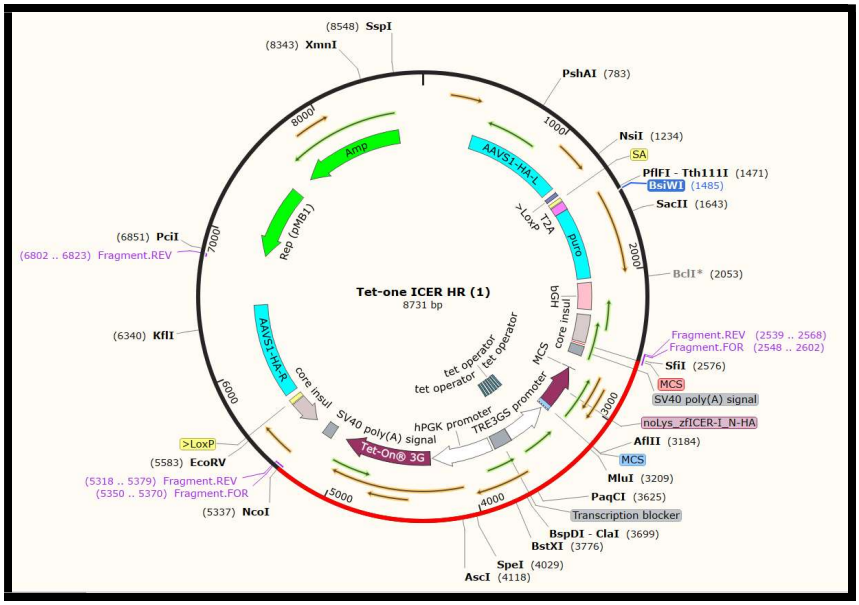
linear Tet-on/HA-ICER construct with the AAVS1 homology arms



Linear diagram of the modified Tet-on/$_N$HA-ICER construct with the AAVS1 homology arms, diagram produced by Snap Gene

Figure 2.3

circular Tet-on/HA-ICER construct with the AAVS1 homology arms



Circular diagram of the modified Tet-on/NHA-ICER construct with the AAVS1 homology arms, diagram produced by Snap Gene

Western Blot & Immunocytochemistry (ICC):

Selected colonies were collected at near 80 – 90 % confluency for WB analysis. First, cells were washed using Phosphate buffer saline (PBS) then incubated in Trypsin for 3 minutes at room temperature. Trypsin was then washed, and 1mL of EMEM+FBS+penicillin media was added to the cell. Tubes were then centrifuged at 300g for 6 minutes to collect cellular fractions. SDS-Page was then followed by Western Blot following Cirinelli et al. (2022) and the antibodies supplier recommendation. The detection and analysis of western blot was performed using IRDye® secondary antibodies and imaged using the Odyssey CLX infrared system. For primary antibodies, rabbit anti-HA tags were used to ONLY detect exogenous ICER expression (Invitrogen, US).

For ICC, selected colonies were washed with PBS before fixating in 4% formaldehyde for 20 minutes. Cells were then washed again with PBS and preheated antigen retrieval buffer (1X NaCitrate pH 8.0) was added and incubated for 10 minutes at 95 C. Cells were then separated from the buffer, washed, and incubated in 0.1% Triton X-100 in PBS for 15 minutes at room temperature. All excess PBS was removed before applying the protein block to block nonspecific binding for 1 hour at room temperature. Mouse anti-HA primary antibodies (Alexa Fluor 594 by Thermofisher) were applied for a final concentration of 1.0 μg/ml, and cells were incubated overnight at 4 C. Cells were then washed again the following morning and allowed to dry at room temperature for 1 hour. One mounting drop was added to each slide and covered with a cover slip before imaging with Nikon® Eclipse Ti inverted fluorescent microscope.

PCR & Sequencing:

PCR was carried out using the Dream Taq Green kit by Thermofischer following the manufacturer's guidelines. The primers (Forward: 5'-CTCAGTCTGAAGAGCAGAGC-3'; Reverse: 5'-CCACTGTTTCCCCTTCCCAG-3') were designed in house and purchased from System Bioscience, Ca. Gel electrophoresis was performed in 1% agarose gel with 10ul of sample in each well. Bands at the region of interest were then excised and sent for sanger sequencing.

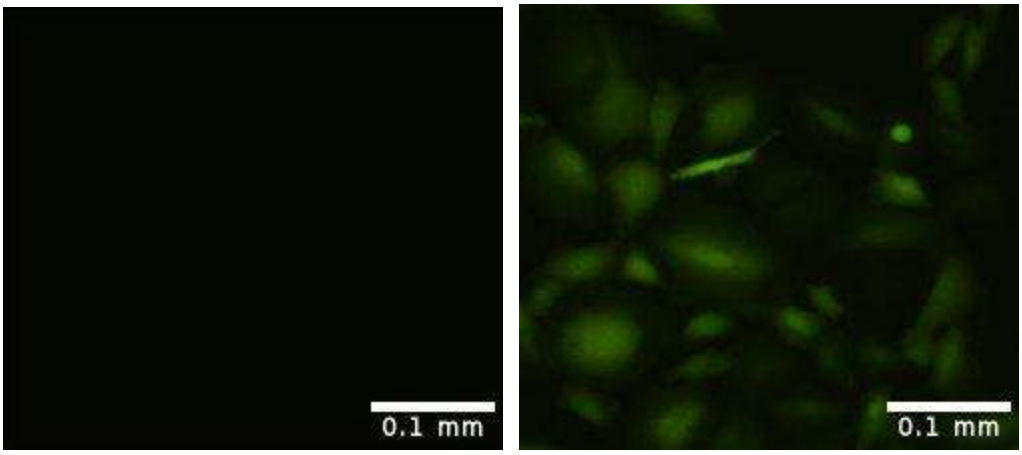
Results:

Successful eGFP expression 48 hours post doxycycline induction:

The first step was to evaluate our protocol by checking for fluorescence signal in the negative control groups. After transfection and puromycin selection, it took roughly 30 days for cells to reach confluency of 70-80 % before inducing with doxycycline. Surviving colonies then received doxycycline treatment, incubated and monitored periodically for maximal fluorescence response. Control Sk-Mel24$_{eGFP}$ (Figure 3) colony for example, showed successful and maximal eGFP induction 48 hours after receiving doxycycline, compared to no fluorescing at all in the absence of doxycycline. Interestingly, increasing doxycycline had no response on fluorescence intensity (data not shown).

Figure 3

Sk-Mel24$_{eGFP}$ control colony before and after doxycycline induction



Sk-Mel24$_{eGFP}$ colony before (left) and after (right) receiving doxycycline treatment under fluorescence microscope at (40x). Right image (after) was taken 48 hours after adding doxycycline

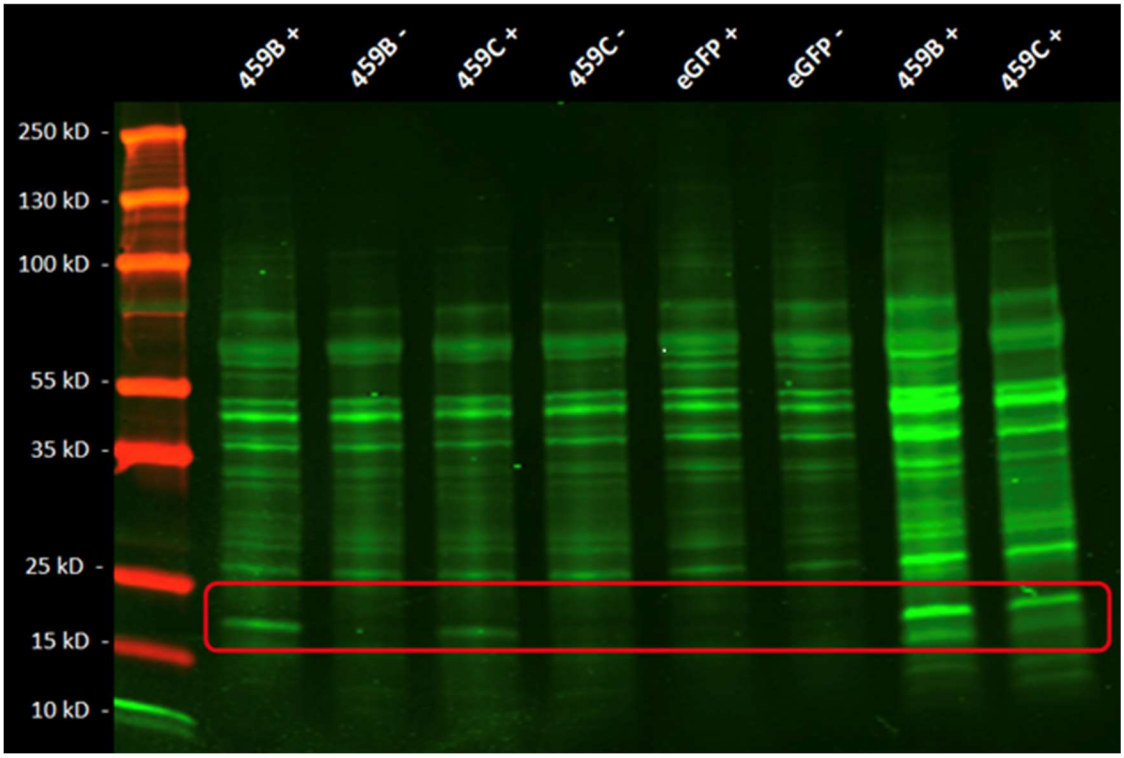
Western Blot indicates successful doxycycline induction:

Like the control colony, selected $_{N}$HA-ICER colonies with decent confluency received doxycycline treatment and were incubated for 48 hours before proceeding to WB. Colonies 459$_{B}$ and 459$_{C}$ were the only surviving $_{N}$HA-ICER containing colonies (459$_{A}$ was a false positive). It is important to note that anti-HA primary antibodies were used in this experiment to avoid incorporating endogenous ICER in the evaluation of this procedure. In figure 4, we see a clear decent band in lanes 2 & 4 which correspond to colonies 459$_{B}$ & 459$_{C}$, respectively, both which received a single doxycycline treatment. Although the band is in the approximately 17 kD region, which is slightly higher than ICER's molecular weight of 13.5 kD, the fusion of HA tag along with ICER being positively charged can explain this deviation. Additionally, that same 17 kD band is completely absent in colonies 459$_{B}$ (lane 3) & 459$_{C}$ (lane 5) which did not receive any doxycycline treatment. For our control, lanes 6 & 7, corresponding to induced eGFP & non-induced eGFP respectively, both which lack the HA-ICER construct, no bands have been

observed in the expected region. Finally, lanes 8 & 9 correspond to induced colonies 459_{B} and 459_{C} (same doxycycline dose) where double the sample volume was loaded to the gel just to compare signal intensity.

Figure 4

Western Blot analysis of colonies $459_{B,}$ $459_{C,\ \&}$ Sk-Mel24$_{eGFP}$



Western blot confirms for the presence of HA-ICER (17 kD) in colonies 459_{B} & 459_{C} after receiving doxycycline treatment while completely absent in the eGFP colony. A (+) sign indicates receiving a single dose of doxycycline and a (-) indicates not receiving a treatment at all. Lanes 2, 3 & 8 correspond to colony 459_{B}. Lanes 4, 5, & 9 correspond to colony 459_{C}. Lanes 6 & 7 correspond to eGFP colonies.

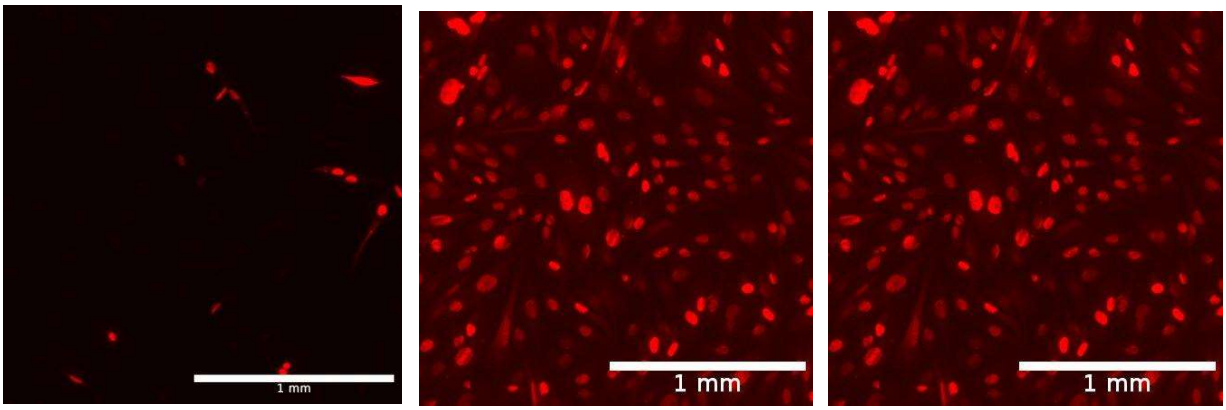
Immunocytochemistry confirms the presence of HA tags in the nucleus of selected colonies:

Fluorescence microscopy post ICC was performed to confirm the presence and localization of HA tags in the selected colonies. For additional control in this experiment, Sk-Mel cells were transiently transfected with $_{N}$HA-ICER and compared against colonies 459_{B} &

459_c (Figure 5). In transient transfection, exogenous DNA is delivered to a limited number of cells which end up expressing HA-ICER, but do not pass on this DNA to their progenitors, in contrast to colonies 459_B & 459_c which are expected to express HA-ICER in all their cells. Figure 5 below shows transiently transfected Sk-Mel (left), 459_B (middle), and 459_c (right) 48 hours after receiving doxycycline treatment. It is apparent that HA tags were present in both 459 colonies, and to a much lesser extent in transiently transfected Sk-Mel cells, and mostly within the nucleus, which is to be expected considering that ICER is a nuclear factor and a DNA-binding protein.

Figure 5

Immunocytochemistry (ICC) analysis of colonies transiently transfected Sk-Mel , 459_B, & 459_C



Immunocytochemistry using anti-HA antibodies confirm the presence of HA tags in transiently transfected Sk-Mel (left), 459_B (middle), and 459_C (right), microscopic images were taken at (10x) 48 hours post doxycycline treatment.

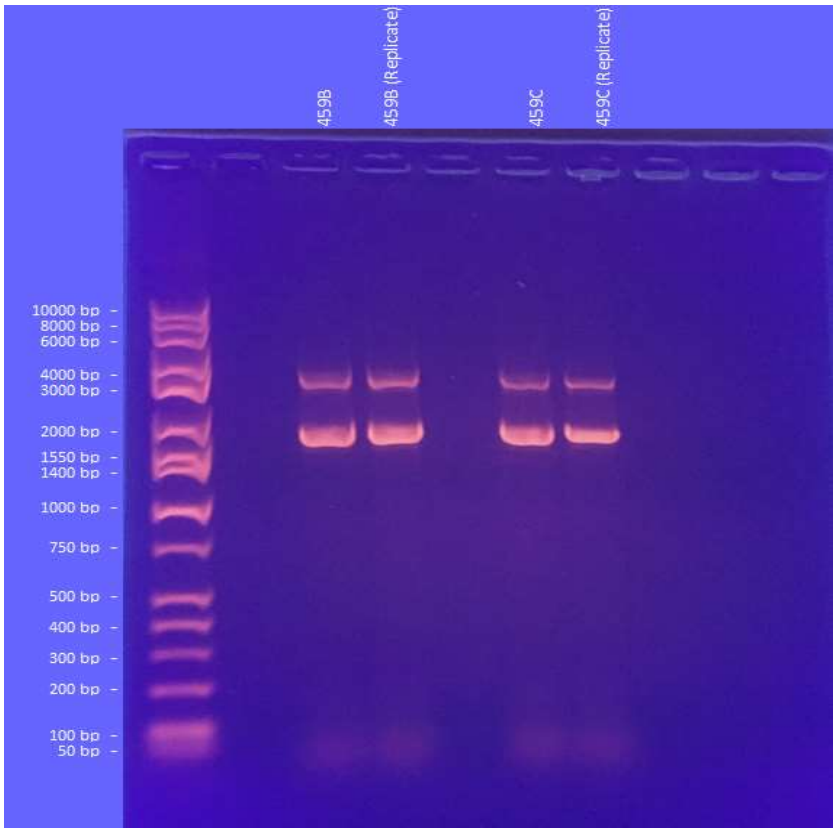
PCR and sanger sequencing confirm successful integration in the safe harbor:

All the previous experiments have indicated a successful induction of HA-ICER upon doxycycline treatment. However, to confirm that this response is indeed due to successful integration of the construct within the safe harbor, PCR was performed as mentioned in the methods section. The primers that were used were specifically targeting the overlap between the

safe harbor region in the genome and the HA-ICER cDNA sequence that we have previously subcloned and transfected into Sk-Mel24 cells. Using SnapGene software, a PCR product of flanking overlap between the two AAVS1 homology arms of our final construct is expected to be around 1750bp. Both colonies 459_B & 459_C underwent PCR in replicates to ensure proper amplification. Interestingly, all four replicates, (459_B in lanes 3/4 and 459_C in lanes 6 /7) have produced fragments around the 3500bp region (explicit data not shown). DNA from each of these band was excised separately and underwent a second round of PCR to produce bands with correct length of 1750bp in figure 6.

Figure 6

Gel electrophoresis of PCR products of colonies 459_B & 459_C



Colonies 459_B (lanes 3&4) and 459_C (lanes 6 & 7) underwent polymerase chain reaction (PCR) then electrophoresed in 1% agarose gel. All four replicates produced the expected band around 1750 bp along with a non-specific band around the 3500 bp region.

Following PCR, Sanger sequencing was the next and final experiment to confirm proper integration of our designed construct. PCR product of 459_{B} (lane 3, figure 6) was excised and sent for sanger sequencing. Sequencing results were then analyzed and aligned against $_{N}$HA-ICER cDNA sequence in SnapGene software and showed perfect alignment (data not shown).

Discussion:

Inducible Cyclic AMP Early Repressor (ICER) is a transcription factor that targets key cell-cycle regulating genes. Although the full spectrum of ICER targets has been fully explored, yet it has been proposed as a potent tumor suppressor in many cancer types including leukemia (Pigazzi et al., 2008), prostate (Mémin et al., 2002) and pituitary cancers (Peri et al., 2001). Additionally, in melanoma, ICER is completely down regulated during tumor genesis and is upregulated during regression (Healey et al., 2013). A major challenge, however, when attempting to study ICER is its low baseline levels in healthy individuals and its lethal effects when in higher concentrations in cancerous cells. Previous attempts to study the protein using transient transfection method have yielded short-lived cell lines. In this thesis, the goal was to generate stable, reliable, and inducible cell lines to control ICER expression within viable levels using Sk-Mel-24 cells as a model to study human melanoma.

Melanoma is a rare, lethal, and highly mutated form of skin malignancy. In melanoma, upregulation of Ras/MAPS signaling pathway has been implicated with ICER down regulation at the protein level as the levels of mRNA remain unaffected. In that same study, Healey et al. (2013) show that ICER is negatively regulated by H-RasV12G, a key inducer of melanoma genesis, via polyubiquitination followed by proteasomal degradation. Moreover, ICER can also be monoubiquitinated at key lysine residues which leads to its translocation from the nucleus to

the cytoplasm and thus loss of function (Mémin et al., 2011). Considering that ICER lacks the P-box domain, a signature phosphorylation site for members of its family, ubiquitin-mediated regulation is the main method of controlling ICER's activity (Folco & Koren, 1997). In addition to generating stable and inducible cell lines, it was equally important to prolong the life of ICER inside the nucleus through the addition of hemagglutinin (HA) tag to the N-terminus. A recent study by Cirinelli et al. (2022) has shown that the addition of hemagglutinin (HA) tag to ICER N-terminus increases its efficiency and increases its resistance to ubiquitination as the latter process follows the N-terminal rule.

The final construct which was used in this study was an expression vector which contained NHA-ICER cDNA under the inducible regulation of the system which was successfully integrated in the safe harbor region. Both experimental colonies, 459B & 459C (and control eGFP colony) were successfully induced to express upon doxycycline treatment. It is worth noting that Anti-HA antibodies were used in this experiment, indicating that present bands correspond to exogenous HA-ICER and do not account for endogenous expression. Although the present bands were slightly higher on the scale, 17.5 kD compared to 13.5 kD MW of ICER, but that could be in part explained considering the fusion HA tag along with positive charge of ICER. Another explanation for this could be ICER's interaction with other proteins. It has been recently reported that ICER has direct interactions with over 28 proteins in Sk-Mel-24 cells to varying extents (Cirinelli et al., 2022). For future WB experiments, it would be more beneficial to use fluorescence-labeled anti-HA primary antibodies as the combination of antibodies used in this experiment produced unspecific background bands. It is worth noting that those unidentified bands were consistently present in non-induced and in control colonies as well further imply their non-specificity,

Although western blot analysis confirmed successful doxycycline induction, it was equally important to confirm that the $_{N}$HA-ICER had successfully integrated in the safe harbor location and not in any other region in the genome. Using primers designed to target the overlap of AAVS1 homology arms and their designated integration site in the genome, PCR results confirm that our construct had integrated successfully. Furthermore, the puromycin resistance gene in the final construct used in this study (figures 2.2 & 2.3) has no built-in promoter and relies on an endogenous PPP1R12C promoter in the safe harbor locus. Having this extra layer of control would have been sufficient to rule out random integration.

The completion of this project lays the foundation for future experiments and allows for up scaling the research process. By the end of this report, all presented data suggest that our $_{N}$HA-ICER has been successfully integrated into the safe harbor location and has been responsive to doxycycline induction as planned. The next step in this project would be to generate a dose-response curve and evaluate Sk-Mel-24 cells' tolerance against $_{N}$HA-ICER. Following that, RNA sequencing experiments can begin under the newly established viable ICER levels.

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