The characterization of miniCoopR cell line clones for the expression of wild type form of ICER

Samantha Moscoso

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Abstract

The transcriptional repressor Inducible cyclic-AMP Early Repressor (ICER) is an isoform of the cAMP-responsive element modulator (CREM) gene. ICER protein has tumor suppressor activity by regulating cAMP-induced transcription. ICER has been found to be effective in providing an anti-tumor effect when in many tumors including melanomas. Melanoma is an aggressive type of skin cancer that begins when there is an overproduction of melanocytes. Using a zebrafish model for melanoma, our laboratory has recently demonstrated that zebrafish that are expressing ICER in their melanocytes succumbed to malignancies at a much faster rate than the control Green Fluorescent protein (GFP)-expressing fish. These fish melanomas were used in our laboratory to generate two fish lines: miniCoopR-EGFP and miniCoopR-HA-wt-ICER. The goal is to use these cell lines to get a better understanding of these unexpected observations. By characterizing the cell line expressing ICER we hope to determine what is causing the malignancies to be increasing. In this thesis, we have characterized these cell lines and demonstrated that they maintain the expected expression of the transgenes, GFP and ICER. We demonstrate that ICER-expressing cell lines grow faster than EGFP control tumor cells. These observations suggested that this ICER-expressing fish melanoma cell line could be used as a model system to further studies the molecular mechanism for unexpected malignancies.
THE CHARACTERIZATION OF MINICOOPR CELL LINE CLONES FOR THE EXPRESSION OF WILD TYPE FORM OF ICER

by

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**Introduction**

According to the American Cancer Society, skin cancer is the most common type of cancer in the United States. About 97,610 new melanomas will be diagnosed in the United States in 2023 (American Cancer Society, 2023). Melanoma is a type of skin cancer that begins in the melanocytes which are the pigment-producing cells. When melanocytes begin to grow out of control, melanoma will develop in the epidermis layer of the skin. Ultraviolet (UV) radiation and exposure from the sun or tanning beds are known to be associated with melanoma formation. While melanomas can develop anywhere on your body, they are most known to develop on your back, arms, legs, and face causing them to be malignant tumors.

BRAF V600E mutations are known to be the activator of melanomas. BRAF is a serine/threonine protein kinase and a form of RAF isoform encoded by a BRAF oncogene (Ascierto et al., 2012). In the BRAF gene at codon V600, the melanoma will get a gain-of-function mutation. This will drive MAPK pathway activation by MEK1/2 and ERK1/2 (Davies et al, 2002; Wan et al, 2004). The MAPK pathway is important for controlling cell growth by regulating gene expression, protein synthesis, nucleotide synthesis, and protein stability by using ubiquitin-mediated and proteasomal degradation (Yehia et al., 2001). BRAF V600E is the most frequent BRAF mutation that is known to drive the proliferation of cancer cells (Ascierto et al., 2012). The mutation will activate the downstream of the MAPK/ERK pathway (Sanchez et al., 2018). This downstream will be caused by the interruption of the interaction between Ras and Raf protein because of the elevated levels of cAMP (Wu et al., 1993). Along with BRAF mutation being an important part of melanomas, cyclin-dependent kinase 2A (CDKN2A) is also found in melanoma. CDKN2A gene is a major melanoma susceptibility gene. CDKN2A provides instructions for the production of several proteins and is
also required for melanogenesis. Two of these proteins are p16 and p14 proteins which function as tumor suppressors. P16 will inhibit retinoblastoma protein phosphorylation through CDK4 and CDK6 which promotes the cell cycle arrest in the G1 phase. P14 will use the p53 pathway to induce the cell cycle arrest (Rossi et al., 2019; Li, Poi, & Tsai, 2011). The CDKs are major contributors to the cells to be able to progress past the checkpoints in the cell cycle. In metastatic melanoma, the combination of BRAF and CDKN2A mutations is common.

Over time, treatment for patients with advanced-stage melanoma has improved. By looking at the BRAF oncogene and the MAPK pathway, BRAF inhibition therapy (vemurafenib and dabrafenib) and MEK kinase inhibition therapy (trametinib) have been developed (Winder et al., 2017). However, with these developments in treatment, patients have developed a resistance to these treatments. This resistance to RAF inhibition can be due to mutation and non-mutational events, as well as microenvironmental resistance. (Winder et al., 2017). While looking at the different inhibition points is necessary, transcription factors and the cAMP pathway carry a huge importance too. The cAMP signaling pathway and MAPK pathway are observed to have a crosstalk that transduces growth-stimulating signals from the cell surface receptor tyrosine kinases (RTKs) (Rodriguez et al., 2014). cAMP pathway activity is dependent on adenylate cyclases (ADCYs) as well as the degradations of PDEs. With the dependency of the two enzymatic components, downstream effectors protein kinase A (PKA) and the cAMP-responsive element binding protein (CREB) are activated. CREB is a ubiquitous expressed transcription factor that binds to cAMP responsive element (CRE) (Rodriguez et al, 2014). It has been revealed that active CREB is suppressed by RAF-MEK inhibition but restored in relapsing tumors (Cirinelli et al., 2022). Both CRE binding and CREM genes can encode for several
isoforms which can act as activators or repressors of cAMP-induced transcription (Yehia et al., 2001).

Inducible cyclic-AMP early repressor (ICER) is an inducible transcriptional factor that comes from the cAMP-responsive element modulator (CREM) gene (Molina et al., 1993). ICER is known for downregulating cAMP-induced transcription by responding to intracellular concentrations of cAMP (Stehle et al., 1993). ICER is small in size ranging from 108 to 120 amino acids making it a good target for small molecule therapy. ICER weighs about 18kDa and has a leucine zipper structure (Poulkes et al, 1991). ICER is observed in vivo and its inducibility is cell-specific. It has been previously demonstrated that in the G1/S and G2/M phases, an arrest will happen due to ICER inhibiting the growth and DNA synthesis because of the high level of cAMP present (Lamas et al., 1997). A study found that depending on nuclear ICER concentration and when apoptotic signals are triggered, ICER can inhibit cAMP-induced apoptosis (Ruchaud et al., 1997). Along with inhibiting the growth and DNA synthesis, ICER inhibits the expression of growth-related genes such as cyclin A, cyclin D1, cyclin D2, and cFos. In a study, the effects that cAMP and ICER have on the growth of LNCaP, PC-3, and DU 145 human cell lines were looked at. It has been observed that cAMP will slow the growth of LNCaP and completely halt the progression of PC-3 and DU 145 cells (Yehia et al., 2001). This shows that the cells that were not expressing ICER grew at a rapid pace compared to the cells that were treated with cAMP or transfected with ICER.

Post-translational modifications (PTMs) could be a cause of the abnormalities that are found in ICER expression in cancer cells. Ubiquitination and phosphorylation are known to be post-translational modifications that can alter the activity of ICER. Research has demonstrated that through inhibition of MAPK Erk 1 protein kinase, cAMP is able to stabilize ICER.
expression in JEG-3 and AtT20 cells. Due to this action, this will cause the phosphorylation of ICER to happen at serine 41 and cause it to be a target for ubiquitination (Yehia et al., 2001). This can cause polyubiquitination and proteasomal degradation to happen since phosphorylation is a prerequisite of both processes. Ubiquitination is a post-translational modification in which the ubiquitin protein gets attached to a substrate protein (Guo et al., 2023). There are two types of ubiquitination a protein can go through: polyubiquitination and monoubiquitination. Polyubiquitination can result in a signal for proteasomal degradation to be created while monoubiquitination can result in a signal for a physiological function to happen (Memin et al., 2011). Some of these physiological functions can be histone functions, transcription regulation, DNA repair, and DNA replication. During the proteasome pathway, ICER is polyubiquitination and degraded (Memin et al., 2011). The change from serine 41 to alanine 41 caused by the site-directed mutagenesis can cause an increase in ICER’s half-life. Cirinelli et al. (2022) found that by adding a hemagglutinin (HA) fusion tag on the N-terminus or C-terminus of ICER, its half-life could be tracked. It was found that due to the N-terminus and C-terminus being blocked, the half-life of N-HAICER was found to be twice as long as the ICER-CHA, as well as the transcriptional repression activity. It was also found that N-HAICER causes apoptosis five times more efficiently than ICER-CHA. The increasing rate of apoptosis is what you would expect to find as a goal in cancer research.

In order to see ICER’s role in melanomas, we have been working with transgenic zebrafish models with Tg(mitfa: BRAFV600E); mitfa(If); p53(If) (Frantz et al., 2020). With BRAFV600E being the most frequent mutation in melanoma patients, the transgenic zebrafish carried this oncogene. A ‘MiniCoopR’ screening strategy was used to develop the Tg(mitfa: BRAFV600E); mitfa(If); p53(If) animals. A strain in which mitfa with a loss of function was
introduced into a tumor-prone Tg(mitfa: BRAFV600E); mitfa(If); p53(If) background. This mutation will allow melanocyte development and formation to be revoked. The zebrafish were introduced to a transgene that carried a wild-type mitfa gene (under mitfa promoter). This will cause the rescuing of melanocytes and melanomas. Tol2 is a transposon that can encode a functional transposase. When Tol2 is injected into the genome of the embryos, it is able to catalyze the integration of the exogenous DNA. This will allow the fish that are injected to be able to transmit the transgene, allowing for a transgenic line to form. Without the Tol2 recombinase, there is no DNA integration. In order to measure the tumor initiation, progression, or abrogation, a companion gene was induced in the rescued animals. Our laboratory was able to generate a different “form” of ICER which is the wild-type(wt) ICER that has been HA-tagged at the N-terminus. This construct is also known as 447A. We also have our miniCoopR-EGFP cells which are known as 446A. After the miniCoopR process, our laboratory euthanized both EGFP and HA-wt-ICER models, and the tumors were removed. The tumors were disassociated enzymatically. The tumors were then added to media and cultured them to prepare them to be characterized (Figure 1).

Figure 1. Generation of primary cultures from the tumor cells. Pictures were taken of both miniCoopR-EGFP and miniCoopR-HA-wt-ICER zebrafish.
Originally the laboratory hypothesized that the ectopic expression of the models will stop melanoma development. We found that the overexpression of ICER caused the animals to succumb to malignancy at a much faster rate than the control (Figure 2). The melanocytes that grew within the EGFP control grew at a stripe pattern, unlike the wild-type zebrafish’s melanocytes which grew in confluent patches. When looking at the pathological analysis of the tumors, the results demonstrated that the miniCoopR-EGFP melanomas are non-invasive and they didn't merge with the muscle. In contrast, miniCoopR-HA-wt-ICER melanomas are invasive and invade the skin and then into muscle (Figure 3). Preliminary characterization has found that both the EGFP and the HA-wt-ICER transgene have a similar mRNA level. Wild-type ICER protein levels compared to EGFP were nine times lower and when incubated with a proteasome inhibitor (PI) in cultured tumors, it precipitated. EGFP does not get altered by the proteasome inhibitor. This supported the hypothesis we had that when HA-wt-ICER is a target for ubiquitin-mediated proteasomal degradation, it will cause the tumors to not respond to the cell growth inhibitory activity that ICER carries.
Figure 2. ICER accelerates melanoma formation in zebrafish. miniCoopR-HA-wt-ICER zebrafish weighted average of 2 independent experiments, n=46. miniCoopR-EGFP zebrafish weighted average of 2 independent experiments, n=33.

Figure 3. Pathological analysis of miniCoopR-EGFP and miniCoopR-HA-wt-ICER tumors. EGFP control tumors are found to be non-invasive while wild-type ICER tumors are invasive. Below the pathological analysis results are photos of both zebrafish and the tumors present. EGFP control tumors are found to be in a stripe pattern and wild-type ICER are found to be in a confluent pattern.

With the preliminary data that our laboratory has found, we are hoping to get an even better understanding of the miniCoopR-HA-wt-ICER cell line. By using miniCoopR to help us, we are able to generate zebrafish clones expressing EGFP and HA-wt-ICER. The tumors grown from these transgenic fish will be removed and the characterization of ICER-expressing cell lines
can begin. My hypothesis is that melanoma tumors are becoming more aggressive because of the expression of ICER and the cells are starting to develop a resistance to ICER.
Materials and Methods

Antibodies

Rabbit polyclonal anti-HA tag antibodies were used as one of the primary antibodies and purchased from Abcam. Mouse monoclonal anti-alpha Tubulin antibodies were used as another primary antibody and were purchased from Abcam.

Western Blot

The tissue culture flask with cells was washed with PBS and PBS was discarded after. Fresh PBS was added back into the flask and cells were scraped to dislodge cells. The mixture was pipetted into microcentrifuge tubes. Cold cell lysis buffer was added to cells with a fresh protease inhibitor mixture. Microcentrifuge tube was incubated on ice. The concentration of protein was measured using a spectrophotometer. 10μL of loading dye was added to lane 1 as the marker. The cell mixture was added in lanes 2 and 3.

DNA Extraction and PCR

DNA was extracted per the Qiagen® DNeasy® Blood and Tissue Kit Quick-Start protocol. MiniCoopR-EGFP (446A) and MiniCoopR-HA-wt-ICER (447A) cells were removed from the wells. The forward HR primer selected for the plasmid amplification was (CATCTTCAGCTGGCCAAGAC) and the reverse HR primer used was (GGGTCTTGTCCTGCTTTTCC). Thin-walled PCR tube was placed on ice and 10X DreamTaq Green Buffer (25μL), forward Primer (0.5μL), reverse primer (0.5μL), template DNA (1μL), and nuclease-free water (23μL) were added. The reaction was placed in a thermal cycler and PCR was performed using the recommended thermal cycling conditions. 10μL of PCR mixture was
added to a 1% agarose gel. Gel was run at 100V for an hour and results were visualized under a UV transilluminator.

Figure 4. Snapgene sequence alignment of miniCoopR-HA-WT-ICER.

**Immunocytochemistry**

ICC was performed using anti-HA fluorescently tagged primary antibodies. Cells were grown on a coverslip in a 12-well tissue culture dish. Cells were treated and media was removed. Cells were washed with phosphate buffer saline (PBS) at room temperature. Cells were fixed with 4% formaldehyde in PBS. Cells were washed three times with PBS and stored in 0.02% sodium azide in PBS at 4°C for several days. Antigen Retrieval Buffer was preheated to 95°C and 1 ml was added and heated again at 95°C for 10 minutes. Antigen Retrieval Buffer was removed and cells were rinsed with PBS three more times. Cells were incubated in 0.1% Triton X-100 in PBS (1ml) for 15 minutes at room temperature. Cells were rinsed in PBS three more times. The remaining PBS was removed and Protein Block (100μL) was added to cover the sample by surface tension. A 1 hour at room temperature incubation period took place and once over cells were washed once with PBS. Primary antibody (100μL) was added and incubated at 4°C overnight. The primary antibody was Anti-HA at a dilution of 1:200. Cells were washed four times with PBS. DAPI (100μL) was added and incubated for thirty minutes at room temperature.
Cells were rinsed four times with PBS. A drop of slow fade mounting medium was added to each slide and covered with a coverslip.

*Growth Curve*

446A and 447A cells were split in a 24-well plate. Media was removed and 250 ml of PBS was added to each well. PBS was removed and 100μL of trypsin was added to the wells. A five-minute incubation period took place. 100μL of media was added to the cells. 3 eppendorf tubes were made for both 446A and 447A. 2 of the 3 eppendorf tubes received 20μL of trypan blue and 10μL of the cells to their corresponding tube. The third eppendorf tube received 10μL of the two eppendorf tubes made before. Overall 6 eppendorf tubes were made. 10μL was added to the hemocytometer and cells were counted. This repeated over the span of five days.
Results

*EGFP expression in miniCoopR-EGFP cells*

MiniCoopR-EGFP and miniCoopR-HA-wt-ICER were analyzed under a brightfield microscope and fluorescence microscope (Figure 5). This was done to see if the expression of EGFP was present in the cells. 446A cells under a fluorescence microscope did fluoresce due to the expression of EGFP being present. 447A cells under a fluorescence microscope should not fluoresce since it was our N terminus HA tag wt-ICER cells.

![Figure 5. Brightfield and fluorescent microscopy images of MiniCoopR-EGFP (446A) and MiniCoopR-HA-wt-ICER (447A) cells. 446A cells fluoresce under the fluorescence microscope.](image)

*PCR analysis demonstrated gene expression*

A PCR analysis was performed to determine the expression of the mitfa promoter HA-ICER transgene with the 447A cells (Figure 6). The mitfa promoter enables DNA-binding
transcription factor activity along with having a positive effect on melanocyte differentiation. Results showed that the transgene was present in the 447A cells as the expected DNA amplification was generated at ~438 base pairs (bp). As expected, 446A cells did not express a band at ~438bp since the PCR was designed to only amplify mitfa promoter-HA-ICER transgene.

Figure 6. PCR image of 446A and 447A cells. 447A is showing that the mitfa promoter HA-ICER transgene is still present in ~438bp.

*Western blot analysis presented HA-ICER expression*

A Western blot was performed using two antibodies, anti-HA antibodies for the determination of HA-ICER and anti-tubulin antibodies for the determination of a housekeeping protein (Figure 7). The determination of the protein expression was the goal for the Western blot. 10.0μL of the cell extract was loaded into the lanes. 447A cells expressed HA-ICER approximately at 18kDa in lane 3 which corresponds to its structural molecular weight of 18kDa. As expected 446A cells did not express the HA-ICER band at ~18kDa. Bands should not be shown for our EGFP cells since anti-EGFP antibodies were not added. Anti-tubulin antibodies were expressed in 446A and 447A cells at approximately 50kDa with similar intensity
demonstrating equal loading of total proteins in each lane. Bands that are asterisked are ubiquitinated ICER slightly present in the 447A cells. The yellow band shown at ~50 kDa is due to the overlay of the anti-tubulin antibodies and a ubiquitinated form of ICER.

Figure 7. Western blot analysis of 446A and 447A. An HA-ICER expression was found in lane 3 which contained 447A cells. Lane 1 contained the marker, Lane 2 contained 446A.

ICC demonstrates the subcellular localization of ICER

ICC was performed on the 447A cells and was fixed with anti-HA fluorescent tagged primary antibodies. This was used to test the expression of transgenic HA-ICER in melanoma cells. The expression of the transgenic HA-ICER was expected to be in the cell’s nucleus and not in the genome like our EGFP cells. Cells that expressed HA-ICER in them were expected to fluoresce under FITC. Figure 8A is demonstrating that all cells are expressing ICER in the nuclei. Figure 8B shows that 447A was treated with DAPI showing the nuclei fluorescing. The DAPI was used to demonstrate that only the cells that were expressing HA-ICER were
fluorescing. Figure 8C is our merged image of both Figure 8A and Figure 8B. Figure 8C gives a better understanding of the localization of the HA-ICER in the cells.

Figure 8A (left), Figure 8B (middle), and Figure 8C (right). DAPI and FITC microscope image with overlay image for 447A cells. Figures 8A and 8B show that HA-ICER is being expressed in the cells and it is being expressed where expected. Figure 8C shows the overlay between the DAPI and FITC to better show localization.

*Growth curve demonstrates that 447A grows at a rapid rate*

A growth curve analysis was conducted over the span of 5 days to determine the growth rate of both 446A and 447A cells (Figure 9). Both cells started at the same amount of cells ($10^5$ cells). The growth curve experiment was done with three independent determinations. The cells were split each day and were moved to a new 24-well plate to prepare for the count. The experiment stayed consistent throughout the 5 days. During the count, it was noticed that the cell count was doubling from the count the day prior. Analysis results showed that 447A was growing at a quicker rate every day compared to 446A. This could be due to the cells growing
resistant to ICER causing them to grow out of control in comparison to our control EGFP. Error bars are showing the standard error within that day’s count.

Figure 9. Line graph demonstrating the average rate of cell growth of 446A and 447A cell lines. The values shown are the average of three independent determinations. Error bars (±/+) represent standard errors.
Discussion

When ICER is present in an active melanoma, its expression is known to decrease due to post-translational modifications. ICER is down-regulated during melanogenesis but during the melanoma regression stage, ICER is up-regulated (Healey et al., 2013). Over time, ICER has been shown to suppress cancer cell growth both in vitro and in vivo (Molina et al., 1993). As we saw in the preliminary data, the tumors that are expressing ICER are increasing the rate of growth compared to tumors that aren't expressing ICER. Due to this, the zebrafish expressing HA-ICER are succumbing to malignancy at a much faster rate. In order to get a better understanding of why these actions are happening, we are looking at the characteristics of the cells expressing ICER. By looking deeper into this, we are able to see if ICER is the main cause of the increased tumor growth as well as if there is a resistance that is being introduced that is inhibiting ICER to function correctly.

In order to get a better understanding of the cell lines, we first took a look at both of our cell clones. By using a brightfield microscope we were able to get a look at our mini-CoopR EGFP cells and miniCoopR-HA-wt-ICER cells. When looking at both cell line clones we were able to see that many cells were present. While both clones had cells present, the shapes of the cells did have minor differences which have yet to be determined. Both cells were then looked at under a FITC microscope. With the miniCoopR-EGFP cells, they fluoresce under the FITC microscope, unlike the miniCoopR-HA-wt-ICER cells which didn't fluoresce. Our miniCoopR-HA-wt-ICER cells should not fluoresce due to them not having EGFP integrated into them. This shows that the EGFP was integrated into the genome of the miniCoopR-EGFP cells which sets up these cells to be our control.
We then had to genotype the cell line clones to determine transgenic ICER cDNA integration. We did that by running a PCR experiment. Our sequence consisted of the mitfa promoter which carried the wild-type mitfa gene. As mentioned before, this allows the recusing of melanocytes and melanomas. Along with the mitfa promoter, we had our forward HR primer as well as our reverse HR primer. We ran a PCR cycle to then determine the expression of the gene. What the PCR results showed was that our 446A cells did not yield PCR product, supporting that the gene was not present. While looking at our 447A cells, we found a clear band at ~438bp. This confirmed that the 447A cells had the gene that we were looking for and when amplified the transgene was still present. This allowed us to move on to our next experiment knowing that the transgene was present.

The expression of the HAn-ICER was looked at by using a Western Blot. The results showed very strong bands within lane 3 which represented our 447A cells. As mentioned before, the Western Blot was run with two antibodies: anti-HA and anti-tubulin. Anti-HA antibodies were used for the determination of HA-ICER and anti-tubulin antibodies were used for the determination of the housekeeping protein. Alpha-tubulin must be used as a loading control. Our Western blot results did show a band for the alpha-tubulin around ~50kDa for both our 446A and 447A showing that the amount of protein was equal. Along with the alpha-tubulin band showing, we had very weak bands of ICER that have gone through post-translational modification. Due to a ubiquitinated form of ICER and alpha-tubulin both appearing around ~50kDa, this caused a yellow band to form due to the overlay. The most important piece of information in this Western blot is the presence of the bands at ~18kDa. ICER is being presented in its structural molecular weight of 18kDa. These bands demonstrate that HAn-ICER is being expressed in the miniCoopR-HA-wt-ICER cell line. The experiment would need to be repeated to ensure that the
band intensity increases or stays the same. Additionally, our ICC results showed us that the 447A clone was successfully expressing HAn-ICER. The ICC results most importantly showed us the subcellular localization of ICER present in the 447A cells. Our goal is to see the ICER expression present in the nuclei of the cells of 447A which was accomplished by looking at the ICC results. The DAPI staining proposed the visibility of the nuclei of the 477A cells present. Our merged image shows a clear view of HAn-ICER being present in the 447A cells. These results and observations demonstrate that HA-wt-ICER cells are expressing ICER and expressing it in the expected location.

The growth curve results confirmed that the HA-wt-ICER cells were growing at a quicker rate compared to the EGFP cells. We started this experiment with the same amount of cells for both 446A and 447A so the count could be as accurate as possible. We saw that over the span of five days, the number of 447A cells doubled compared to the day before. This could be due to the fact that the 446A cells didn't have ICER present so they were growing at a normal rate with nothing to induce the growth. Unlike the 447A cells expressing ICER, they grew at a much quicker rate which could have been due to the ICER present in the cell. This also shows that ICER was not performing the functions it should have and it was most likely because the cells are growing resistant to the functions of ICER. As mentioned earlier, patients undergoing treatment can form a resistance to certain treatments causing them not to work. This experiment could be an example of that since the cells are growing resistant to ICER. This supports the hypothesis that the cells that are expressing ICER are more aggressive and this may be due to the cells growing resistance to ICER.

With these findings, it can give researchers a good starting point for further research. Future experiments could be conducted with the cell lines developed to get a better
understanding on if the same effect will take place for other models. One model example that would give us a good perspective of the effects is using nude mice. By using nude mice models, this could push researchers one step closer to getting a better understanding of how ICER could affect humans. The same experiments mentioned in this paper could be conducted to ensure that the results are as accurate as possible. Nude mice have been used in the past to determine tumorigenicity from a given ICER cell line (Land et al., 1983; Razavi et al., 1998). Along with looking at other models, we can also research how different mutants of ICER would affect the zebrafish or any other models. One way we can do this is by developing cell clones containing ICER during different post-translational modifications like ubiquitination and phosphorylation. Since PTMs can alter the activity of ICER, there is a chance it can have a positive effect on the interaction with melanomas as well as having negative effects. With the development of these cell lines, we can see if it would have any effect on cell growth like HAn-ICER had on the zebrafish. This could also give us a better understanding of ICER and its mechanisms. With the development of the cell clone mutants with ICER during different PTMs, this could be used also with nude mice to see if the same or if any effect would happen compared to the zebrafish.
Conclusion

With the preliminary data received, we found that the zebrafish that are expressing ICER succumbed to malignancies at a much faster rate than the control EGFP zebrafish. By looking at the data gathered by the Western blot analysis, growth curve experiment, and immunocytochemistry we conducted, we were able to support our hypothesis that the expression of ICER is causing melanoma tumors to become more aggressive and to cause more rapid growth. Future experiments could be conducted to get a better look at ICER's interaction in other models like nude mice. As well as determining if the same results will happen for different mutants of ICER under post-translational modifications.
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