Oklahoma State University Honors Thesis:

Determination of the Tumor Suppressor Properties of INI-1 in a Human Cancer Cell Line

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Abstract:

Rhabdoid tumors have historically been characterized by their histological phenotype, but genetic investigations have revealed a common characteristic: the loss or mutation of the INI-1 gene. INI-1 (SMARCB1) is a tumor suppressor gene located at 22q11.2 which encodes a subunit of the SWI/SNF chromatin remodeling complex. Originally identified in the Atypical Teratoid Rhabdoid Tumor (AT/RT) found in pediatric patients, they have been implicated in the pathogenesis of numerous tumor types. However, few, if any, studies have investigated the actual tumor suppressing properties of this gene. *The goal of this study is to investigate and determine if the deletion of the INI-1 gene within the human genome leads to a tumorigenic phenotype which can be identified in future studies using assays measuring cell proliferation and growth.* This report details the creation of INI-1 deficient cell lines that can be used in future studies. These experiments aim to establish a causal relationship between INI-1 loss and tumor progression and eventually assist with the development of therapies to treat INI-1 deficient tumors.

Acknowledgements:

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Introduction

Overview

Atypical Teratoid Rhabdoid Tumor (AT/RT) is a very rare and aggressive pediatric cancer of the central nervous system that develops in the brain or spinal cord. Scientists determined that the defining characteristic for this cancer is a lack of the SMARCB1 gene which encodes for the human INI-1 protein. While the exact function of INI-1 in the human body is not known, it plays a role as one of the twelve subunits of the SWI/SNF chromatin remodeling complex. For cells to store their genetic information efficiently, they utilize proteins known as histones that act as a spool for DNA to wrap around and condense. This condensed form of DNA is known as chromatin, but for the cells to access the information stored in these complexes, enzymes like SWI/SNF are utilized to open the condensed DNA for expression. The highly aggressive and fast-growing nature of AT/RT cancer has raised questions about the exact function of INI-1 within cells and if it plays a role in regulating cell proliferation. While AT/RT tumors have been studied in the past to research such functions, without a controlled cell line, it would be impossible to determine if any results were caused by INI-1 or another cellular mutation that has yet to be determined. To test this hypothesis, an INI-1 deficient cell line must be developed. The current best-practice technique for DNA editing is CRISPR-Cas9, a gene editing tool that can be used in living cells. With this technology, it is possible to knockout the INI-1 gene in living cervical and breast cancer cell lines, HeLa and MCF-7 cells respectively, that can then be cultivated and studied. Once these cells have a confirmed knockout of the INI-1 gene, to be confirmed by western blots, proliferation assays can be conducted to monitor and quantitate the rate of growth in the edited cells versus the controls.

INI-1 Structure

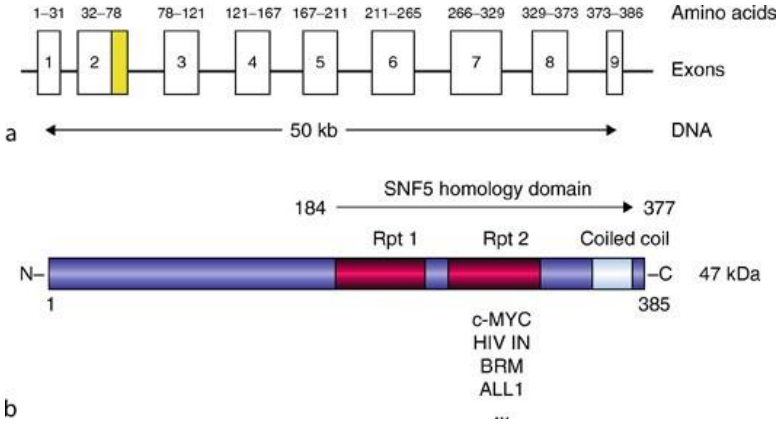

Figure 1- Structure of INI-1 protein with labled repeats, coiled coil, and binding regions. The boxes indicate the various coding exons of the gene with the corresponding number of residues. (Bourdeaut et al).

Integrase interactor 1 (INI-1), also known as SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1 (SMARCB1), is an important component of a chromatin remodeling complex (Parker et al., 2020). Weighing approximately 47kDa and consisting of 385 amino acid residues, INI-1 is located on chromosome 22.q11.23 in humans. As with most members of the INI-1/SNF5 family, this protein is characterized by two imperfect 60-amino-acid repeats that are followed by a coiled coil. INI-1 contains multiple binding domains responsible for interactions with DNA, HIV-1 integrase, and MYC. Tandem repeats within the sequence facilitate multimerization which impacts its arrangement and function, especially in circumstances where it forms dimers, tetramers, or octamers.

Residues 184 to 377 have been labeled as SNF5 homology domain due to crosslinking studies of yeast complexes (Allen et al., 2015). The region spanning between residue 187 and 243 has been shown to enhance DNA transcription via interactions with HIV-1 integrase (Parker et al.). Structurally, INI-1 is comprised of 16 alpha helices, 14 beta strands, and 2 beta turns which facilitate interactions with DNA and other proteins. Post-translational modifications, such as phosphorylation and cross-link modifications involving SUMO2, further regulate its activity (Dixit et al., 2021). Understanding these structural elements is crucial for determining INI-1's role in chromatin remodeling.

INI-1 Function

INI-1 is one of the main subunit proteins of the SWItch/Sucrose Non-Fermentable ATP-dependent chromatin remodeling complex (SWI/SNF). Within the complex, this protein plays a large role in epigenetic regulation as well as cell cycle progression and crosstalk between signaling cascades (Kalimuthu and Chetty). INI-1 is heavily involved in chromatin remodeling and is thought to have roles in transcription activation and repression. Studies have shown that lack of gene expression of this protein leads to decreased presence of SWI/SNF proteins whereas reintroducing the protein increases the levels of the complex, revealing its potential role as a complex stabilizer. While the exact function of INI-1 within the SWI/SNF complex is unknown, identification of SMARCB1 mutations within different cancer types has led scientists to recognize it as a tumor suppressor gene (Kohashi & Oda, 2017).

Even though scientists do not have a comprehensive understanding of the tumor suppressor properties of INI-1, they have connected its presence to numerous pathways that can lead to cancer. Studies revolving around INI-1 in metastatic rhabdoid tumors (MRTs) have shown that the presence of the protein repressed Cyclin D1 transcription which inhibits the action of cyclin dependent kinase in the G1 stage of the cell cycle through histone deacetylation (Kalimuthu & Chetty, 2016). In the sonic Hh pathway, SMARCB1 prevents the transcription of oncogenes that can lead to gliomas which reduces downstream target genes. Other research shows that INI-1 activates regular enhancers involved in differentiation with no effect on super enhancers. When it is absent, genes regulated by the regular enhancers are barely detectible but restored with the reintroduction of INI-1 (Wang et al., 2017).

INI-1 also can bind to nucleosome acidic patches which is essential for unwinding DNA. The mutations in the C-terminus alpha-helical domain impair the protein’s ability to generate

accessible DNA segments and activate critical genes, despite still targeting chromatin normally (Valencia et al., 2019). This shows that mutations don't always eliminate the presence of INI-1 in the SWI/SNF complex but can still affect the remodeling function.

SWI/SNF Chromatin Remodeling Complex

The SWI/SNF complex plays a major role in controlling chromatin structure which translates into regulation of gene transcription in eukaryotes (Chen et al., n.d.) Nucleosomes make up the basic structural unit of DNA where approximately 147 base-pair DNA wraps around the histone octamers. Various chromatin remodelers edit the histone components which alter the position of nucleosomes. SWI/SNF is one of four families of ATP-dependent chromatin remodeling complexes. There are two central catalytic subunits within SWI/SNF, SMARCA4 or SMARCA2, along with 10-13 different associated subunits (Dreier & de la Serna, 2022). SWI/SNF can alter the accessibility of chromatin and regulate gene expression by enhancing the binding of transcription regulations through making the chromatin more accessible. The various subunits all work to cooperate with other epigenetic regulators to activate enhancers. Most of the subunits have unknown purposes due to the lack of information regarding this complex, but scientists have determined that ARID1A and ARID1B contains nuclear receptor binding motifs, SMARCA2, SMARCAB1, and SMARCA4 all

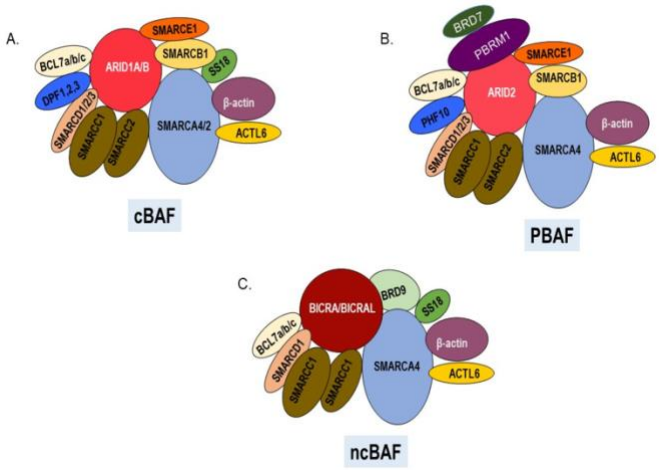

Figure 2-SWI/SNF chromatin remodeling complex. All three complexes contain central ATPase, and various subunits. (Dreier et al.)

deal with ATP dependent chromatin remodeling, and ACTL6A and ACTL6B are actin-like proteins (Tang et al., 2010).

INI-1 Mutations

Complete loss of INI-1 had historically been linked to numerous types of pediatric and adult sarcomas. Most of these cancers are MRTs or epithelioid sarcomas (ES) which result from biallelic deletions. MRTs typically appear before the age of three and are highly aggressive tumors with elevated mortality rates. ESs are typically categorized into two groups, distal and proximal, and can develop almost anywhere in the body (Parker et al., 2020)

Germline mutations are specific genetic alterations that occur within the DNA of germ cells which can be inherited. 25-30% of MRTs have germline alterations which match the anatomical location of their tumors. The most common of these abnormalities includes point or frameshift mutations within the gene followed by heterozygous loss and duplications or deletions of the exons within the gene (Eaton et al., 2011). Studies have shown that essentially all patients who exhibit two primary tumors have a germline mutation. These mutations have also been connected to familial schwannomatosis cases even though INI-1 has not been shown to be the primary cause of this disease (Kalimuthu & Chetty, 2016).

While most diseases associated with INI-1 deficiency are tumor related, mutations in this gene have also been connected to Coffin-Siris syndrome, a genetic disorder characterized by developmental delays, speech impediments, hypertrichosis, and agenesis of corpus callosum. The mutations leading to this disease are typically either missense or in-frame deletions which is similar to the germline mutation in schwannomatosis, yet these patients rarely develop tumors.

CRISPR Cas-9

Clustered regularly interspaced short palindromic repeats (CRISPR) and the associated protein, Cas-9, is the most accurate and efficient method of editing the genome of a living cell (Asmamaw & Zawdie, 2021). The CRISPR system refers to a system of short, repeated DNA sequences found in prokaryotes which is a type of adaptive immunity as a defense against viruses or bacteriophages.

CRISPR/Cas-9 genome editing is divided in three distinct steps: recognition, cleavage, and repair. Using single guide RNA (sgRNA), Cas-9 is directed to the designated target sequence in the gene of interest through base pair complements. The nuclease makes a double strand break upstream of the chosen target which triggers local DNA melting, a mechanism that is not yet understood. This break triggers the cells own repair pathways to fix the DNA fragments, typically by nonhomologous end joining. This base mechanism can be used for three purposes: deletion, disruption, and correction (A et al., 2020). For deletion, two different sgRNAs can be used to remove a length of DNA from the genome. Disruption is the basic level of editing where the entire gene is not removed but inactivated. The final use is for correction where an added DNA template is used alongside the CRISPR/Cas-9 machinery which allows the cell to correct a specific gene or even replace it with a new one.

For this experiment, a plasmid containing sgRNAs for INI-1, puromycin resistance, and the Cas-9 enzyme was used to knockout INI-1 in human cells. Using the disruption technique along with the correction technique for confirmation, the sgRNAs will guide the CRISPR system to the INI-1 gene in the cells, cut open the DNA, leading to nonhomologous end joining which causes a premature stop codon within the gene. While this process does not remove the entire gene from chromosomes, the stop codon prevents the cells from producing INI-1. The specific plasmid contains

a puromycin resistance factor, so we can confirm CRISPR knockout by introducing puromycin in the cell media. Any cell that absorbed the plasmid would resist the antibacterial.

Goals

The goal of this project is to develop INI-1 deficient cell line which can be used for further investigations into cell proliferation and protein expression. Future studies will be able to confirm the CRISPR Cas-9 knockout of this gene.

Materials and Methods

sgRNA Determination

The guide sequences needed to control the CRISPR/Cas-9 machinery was designed using CRISPOR, an online software that determines the best primers for specific genes. sgRNAs were chosen based on their MIT and Doensch scores. All selected RNAs scored greater than 80 for MIT and 50 for Doench. Sequences were also chosen based on minimizing the off-target sites for INI-1. CRISPOR results were based on the INI-1 sequence from the National Center for Biotechnology Information database (NCBI, SMARCB1: **NM_001007468.3**). The following table contains the forward and reverse sequence of the chosen sgRNAs.

Sequences		
sgRNA #9	120 Forward	5’ -AACTACCTCCGTATGTTCCGAGG- 3’
sgRNA #9	120 Reverse	5’ -AAACCGGAACATACGGAGGTAGTTCCA- 3’
sgRNA #11	327 Forward	5’-CACCATGGAGCTACCTCCGTATGTTCCG- 3’
sgRNA #11	327 Reverse	5’ -AAACCGGAACATACGGAGGTAGTTCCAT- 3’

sgRNA #2	1196 Forward	5’ -AAACTCCGGAACACGGGCGATGCGCCAT- 3’
sgRNA #2	1196 Reverse	5’ -CACCATGGCGCATCGCCCGTGTTCCGGA- 3’

sgRNA Cloning

Plasmids were obtained containing the forward and reverse oligos of each of the selected sgRNA sequences which were annealed into a pX459 plasmid also containing a puromycin selection marker. A solution of 11.5 µL Nanopure water, 1 µL pX459 plasmid (100 ng), 1.5 µL 10X Restriction Buffer, and 1 µL BbsI Enzyme was made to digest the plasmid. The solution was placed in a 37℃-water bath for one hour and then moved to a 65℃ bath for 20 minutes. The final solution was left at room temperature.

The next step in this process was annealing the primers. A solution of 7.0 µL Nanopure water, 1.0 µL 10X T4 Ligation Buffer, 1.0 µL Forward sgRNA primer, and 1.0 µL Reverse sgRNA Primer was mixed for each of the individual sequences. These mixtures where then placed in a 95℃-water bath for five minutes. After that time, the bath was turned off, and the mixtures were left to cool in the room temperature bath overnight. The primers were then diluted to a ratio of 1:200 in nanopure water.

The annealed primers needed to be ligated into the digested plasmid using 8.0 µL of nanopure water, 7.0 µL of the digested plasmid, 2.0 µL of the 10X T4 Ligation Buffer, 2.0 µL Annealed Primers, and 1.0 µL of T4 DNA Ligase (New England Biolabs). This mixture was heated in a 37℃ bath for 5 minutes and then stored in the fridge.

The sgRNA plasmids were transformed into DH5α competent *Escherichia coli* cells and grown in ampicillin petri dishes. 5 µL of the ligation and 20 µL were placed in microfuge tubes and mixed. The cells were then incubated on ice for 30 minutes. The bacteria were then shocked by

placing them directly into a 42℃-water bath for 40 seconds. They were then returned to ice for 2 minutes. 175 µL of Super Optimal Broth with glucose was added to each tube and incubated on a rocker at 37℃ for one hour. This process was completed a second time with a positive control uncut pX459 plasmid. The bacterial cultures were spread onto ampicillin petri dishes with aseptic technique and incubated over night at 37℃. Isolated colonies were collected and placed in tubes with 5 mL of Luria broth. The samples were left in the shaker at 37℃ for 16 hours.

Finally, the sgRNA plasmids were ready be isolated from the cell and quantified. 1.5 mL of each of the cultures was centrifuged for 4 minutes at 4000 rpm to pellet the bacteria. The supernatant was discarded. 100 µL of pH 8.0 Tris, EDTA with RNase A was added to the pellet and resuspended. 200 µL of SDS in NaOH was added, and the sample was mixed until uniform. 150 µL of 3M Potassium Acetate, pH 5.0 was added and once again inverted. The microcentrifuge tube was then centrifuged for 10 minutes at 13,000 rom. The supernatant was saved, and the pellet discarded. 600 µL of isopropanol was mixed with the supernatant and placed in an ice bath for 10 minutes. The sample was then centrifuged for 10 minutes at 13,000 rpm. The supernatant was discarded. 500 µL of 70% ethanol was added to the pellet without disturbing it. The sample was then centrifuged for 5 minutes at 13,000 rpm. The supernatant was removed, and the sample was briefly spun again to remove the rest of the ethanol. 50 µL of nanopure water was added to resuspend the pellet.

sgRNA Confirmation

Now that the plasmids were assembled, it was necessary to check the sequence to make sure that it was correct. 1 µL Cutsmart Buffer (New England Biolabs), 0.5 µL NcoI (New England Biolabs), 4.5 µL nanopure water, and 4 µL of the isolated DNA was combined and incubated at 37℃ overnight. This digest was run on a 1.5% agarose gel with 1X TBE buffer and 3 µL EtBr. The

wells were loaded with 4 µL of orange G dye (Sigma), 2 µL diluted DNAA, and 4 µL nanopure water. The gel was run at 100 V for 40 minutes. A photo was taken using the BioRad ChemiDoc XRS+ with Image Lab Software.

In order to confirm the sequence of each plasmid, a 0.75% gel was run with the plasmids against a high mass ladder to determine approximate concentration. The isolated plasmid was measured using a nanodrop on the double stranded DNA option and the measured concentration was diluted to 125 ng/µL. The wells of the gel were loaded with 4 µL of orange G dye (Sigma), 2 µL diluted DNA, and 4 µL of nanopure water. The gel was run at 100 V for 35 minutes then imaged with the BioRad. The plasmid band intensity was compared to the intensity of the mass ladder to determine accurate concentration. After properly diluting samples down to 50 ng/µL, the samples were sent for sequencing.

Cell Cultivation Media

The HeLa cells used in this project were grown in Dulbecco's Modified Eagle's Medium media (Sigma). Media was made by combining one vial of DME/F12 containing Hepes, 1.2g of Nabicarb, and 900 mL of nanopure water. This solution was mixed in cell-specific glassware and mixed until homogenous. Using a pH meter, the media was titrated to a pH of 7.2 using 1 M sodium hydroxide. Under a sterile hood, 100 mL of 10% Fetal Bovine Serum and 10 mL of Pen/Strep were added before filtering through a bottle-top filter.

A second media was made following the same protocol and adding 2.5 mL of puromycin to the media before filtration.

Cell Cultivation Maintenance

HeLa cells were maintained in an incubator at 37°C and 5.0% carbon dioxide. Each plate underwent media change every three to seven days based on cell confluency. For media changes, old media was vacuumed off the plates and replaced with 10 mL of fresh media with a sterile pipet. New media was heated to approximately 37°C before plating. Plates were placed back inside the incubator. All cells were grown on 10 cm CellStar plates.

Once plates reached 100% confluency, they underwent splitting. Plates were removed from the incubator and placed in the sterile fume hoods. Old media was removed with a sterile tip attached to a vacuum. Plates were rinsed with 10 mL of warm 1x phosphate buffered saline (PBS; 8g NaCl, 0.2g KCl, 0.92g $Na_2HPO_4 \cdot 7H_2O$, 1 L Nanopure H_2O). PBS was removed and 1 mL of warm trypsin (Sigma) was added to the plate. Plates were returned to the incubator for approximately 10 minutes or until the cells were visibly detaching from the plate base. Once the cells were suspended, they were either introduced to a new plate or removed for destruction. 10 mL of warm fresh media was added, and plates were maneuvered to evenly distribute cells.

HeLa cell stocks were created by freezing down cells from five 100% confluent plates. Old media was removed from the plates, and they were split according to previous protocol. Once cells were incubated with the trypsin, 2 mL of warm fresh media was added. The suspended cells were placed in conical tubes. Cells were pelleted using low G force centrifuge. Supernatant was discarded. Cells were resuspended using 5 mL of media and set on ice for 10 minutes. 5 mL of ice-cold freezing media (3 mL Cell Culture Media, 5 mL Calf Serum (filtered), and 2 mL DMSO (unfiltered)) was added to the cells. Cell solution was mixed and aliquoted into freezing vials with 1 mL per vial. Vials were then placed in Styrofoam freezing container in the -80°C freezer.

Cell Counting

Confluent plates needed to undergo cell counting to assure a proper cell concentration for CRISPR/Cas-9 editing. Plates were split using standard protocol. Suspended cells were diluted to 10 mL total solution using warm fresh media. 10 µL of the cell solution were placed on a hemacytometer and the average cell count was taken. The average cell count was multiplied by 10,000 to determine the number of cells per mL of solution. 700,000 cells per well was required for the CRISPR system Lipofectamine 3000.

Transfection

HeLa cells were placed in 6-well plates with at least 700,000 cells in each well. In accordance with Lipofectamine 3000 protocol, both high and low concentration protocols were run. High concentration wells were incubated with 125 µL of opti-MEM medium (Gibco) and 7.5 µL of lipofectamine 3000. Low concentration wells were incubated with 125 µL of opti-MEM medium and 3.75 µL of lipofectamine 3000. Both protocols received 125 µL of opti-MEM medium, 2.5 µg of each sgRNA, 5 µL of P3000 (Invitrogen) reagent in a 1:1 ratio which were mixed and incubated for 15 minutes prior to inoculation with 250 µL of the total mixture of the HeLa cell wells.

After a 24–48-hour incubation with the Lipofectamine 3000 solution, the media was changed on the cells. Media containing puromycin was used to confirm knockout. The media was changed back to the normal media without puromycin after an unedited control plate displayed complete cell death. Regular maintenance was continued.

Colony Isolation

To develop cell lines that were genetically identical, it was necessary to isolate cell colonies. HeLa cell plates were lysed according to standard splitting protocol. Suspended cells were diluted

to a total volume of 10 mL with fresh media. 10 μL of the solution were placed on a hemacytometer. The average cell count of the quadrants was multiplied by 10,000 to approximate the number of cells per mL. This estimation was used to perform a series of serial dilutions to have approximately 1 cell per 10 μL of solution. 10 μL of the cell solution was placed in a well of a 96 well plate along with 990 μL of warm fresh media. Cell wells were monitored for growth. Wells with more than one distinct cell colony were discarded. Wells with one distinct colony were allowed to proliferate and were moved to a 6-well plate once confluency was reached.

Cell Extracts

Cells extracts were prepared for gels using a RIPA cell lysis protocol. Cells were lysed from plates using normal splitting protocol. Suspended cells were placed in microcentrifuge tubes. Cells were spun at 2,000xg for 5-7 minutes at 4°C. The supernatant was discarded. Ice-cold 1x PBS was used to wash the cells, and 2,000x g for 5-7 minutes in the centrifuge was repeated. Ice-cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM pH 8.0 Tris-HCl, to 10 mL Nanopure water) was added in a 1:1 ratio. Tubes were agitated for 30 minutes at 4°C. Next, tubes were centrifuged at 13,300 rpm at 4°C for 30 minutes. The supernatant was saved, and the pellet was disposed.

SDS-PAGE Gel

The SDS-PAGE gel mold was assembled after thorough cleaning. A solution of 6.00 mL 30% acrylamide, 3.75 mL 1.5 M Tris buffer pH 8.8, and 5.00-mL nanopure water was mixed in a flask. 150 μL of 10% SDS stock solution was added to the flask and swirled gently. 7.5 μL TEMED

was added to the flask and swirled. A stock solution of ammonium persulfate was made by combining 0.1 g of ammonium persulfate with 1.0 mL of nanopure water. 150 μL of the 10% ammonium persulfate solution was added to the flask and swirled gently. A plastic transfer pipet was used to transfer the gel solution up to a mark 1.8 cm from the top of the glass plate. Water was added to the top of the glass plate to level the gel surface. The gel was left for 30-45 minutes to solidify. Once it has solidified, remove the layering water.

Next is the preparation of the stacking gel. A solution of 1.3 mL 30% acrylamide, 0.8 ml 1.5 M Tris buffer pH 8.8-, and 7.9-mL nanopure water. Before stacking the gel, add 100 μL of 10% SDS and 10 μL TEMED and swirl. Add 10 μL of ammonium persulfate and swirl. Gently add the gel to the top of the solidified page using a plastic transfer pipet without introducing bubbles. Place comb in the space between the two plates without trapping air bubbles. Leave the gel to solidify.

Cell extracts were prepared for the gel using 4 μL and 10 μL samples for each cell type being sequenced. 5 μL 20% SDS and 13 μL 4X sample buffer were mixed with each the 4 μL and 10 μL amounts and boiled for 90 seconds. Cell extracts were loaded into the gel with a protein ladder. The gel holding core was placed into the buffer chamber and filled with running buffer until the shorter glass was submerged. Pour running buffer into the outer buffer chamber until it is level with the other side. Put the chamber lid in place and run it at 140 V for approximately 40 minutes or until the blue band reaches the bottom of the gel.

Once the band reaches the bottom of the gel, disconnect the power and separate the glass slides. Use a razor blade to slide the gel from the glass. Was the gel in a large weigh boat three times for 12 minutes in 50 mls of water on a rocker. After the last wash, add 25 ml of gel code

blue and leave it covered on the rocker overnight. Using a BioRad imager, take a photo of the gel to view the bands better using visible light.

Western Blot

Submerge a PVDF membrane in 100% methanol for 30 seconds and then move to transfer buffer for 2 minutes. Pour the buffer into the weigh boat with the gel. Place the PVDF paper on top of the gel without bubbles. Pour methanol into original container and cover. Wet one piece of filter paper with transfer buffer and place it on top of the membrane and remove any air bubbles. Place the second filter paper in the transfer buffer. From the anode to the cathode, the stack should be filter paper, gel, membrane, filter paper. Make sure that no bubbles are present in the sandwich and close the transfer unit, running it at 14 V for 30 minutes. Soak PVDF membrane in 100% methanol for 30 seconds. Wash the membrane in TBS pH 7.4 for 2 minutes. Pour off the TBS buffer and add 25 ml of TBS containing 5% skim milk and incubate at room temperature for 45-60 minutes. Cut strips out of the membrane, marking which side was against the gel during transfer, and place in primary antibody on a rocker at 4°C overnight. Remove membrane from vial and wash three times with 50 ml TBS for five minutes each. Wash once in 25 ml of TBS with 5% skim milk for 5 minutes at room temperature on a rocker. Pour off milk solution and place strip in secondary antibody and agitate for 75 minutes at room temperature. Remove from secondary antibody and wash three times with 50 ml TBS for 5

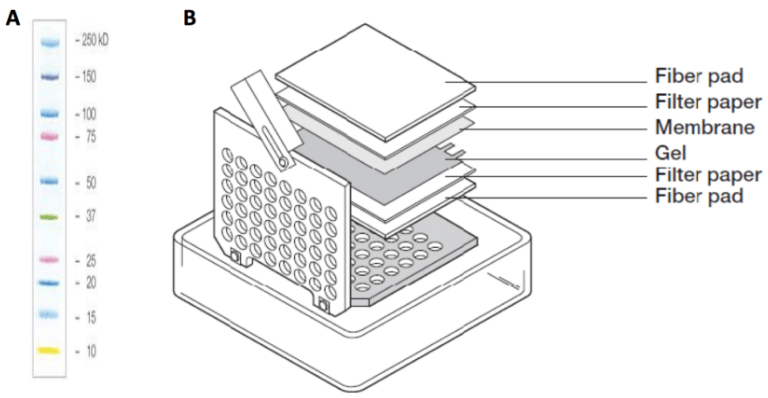

Figure 3- Western Blot sandwich order. Without this specific orientation, the proteins can move in the wrong direction and not end up on the membrane. (Theriault)

minutes each. Rinse briefly in RO water. Place strip in 10 ml of visualization buffer. Incubate at 37°C under reduced light with gentle agitation until lines are visible, up to 6 days of processing at room temperature.

Results

sgRNA Selection and Cloning

Four different sgRNA sequences were identified for the purpose of this experiment, 92, 120, 327, and 1196. 327 and 1196 were processed in the Introduction to Biochemistry Lab course. 92 and 120 were identified later on with the intention of having additional sgRNAs for the transfection of cells. These sequences were selected based on the MIT and Doench scores with consideration for the lowest amount of off target effects. 327 and 1196 were obtained as isolated plasmids, but concentration calculations for sequencing were still required. Samples with concentrations above 50 ng/µL were preferred because they only required a simple dilution to send to sequencing. The same technique was used on sequenced 92 and 120, but no clones from the 92 primer were able to be identified.

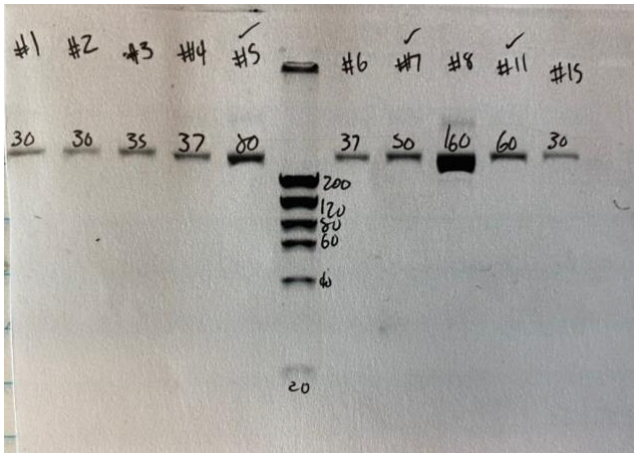

Figure 4- Gel electrophoresis of 327 and 1196 plasmids against a mass ladder to determine accurate concentrations for DNA sequencing.

CRISPR Editing of HeLa Cells

In order to study the effects of INI-1 deficiency in human cell lines and to confirm that INI-1 deficiency was the primary cause of rhabdoid tumors, it was necessary to knockout the INI-1 gene using CRISPR/Cas-9. Six-well plates were seeded with approximately 700,000 HeLa cells based on the cell concentration determined by the hemocytometer. The three different sgRNAs were transfected into HeLa cells using Lipofectamine 3000. The cells were treated with puromycin media to kill of any cells that were not properly transfected. OG HeLa cells were also treated with puromycin media as a control. Once no cells were remaining on the control plate, puromycin media was removed from the delta HeLa cells. When comparing the cells using high versus low lipofectamine, there was no noticeable difference in the efficiency of the transfection.

Once cells were growing properly, the colony isolation protocol was followed for each of the sgRNAs to develop distinct cell lines. 96-well plates were maintained until colonies formed. Each well was assessed for multiple colonies, and only single colony wells were maintained. After some weeks of growth, single cell colonies were moved into 6-well plates to facilitate growth. After months of cultivating the cells, only cells from sgRNAs 120 and 327 stayed alive and free from contamination. Cell lines are 11C, 11D, 11E, 9A, and 9B. All cell lines were frozen down to facilitate future cultivation and testing, and cellular extracts were prepared for Western Blot analysis.

Western Blot

Western blots were conducted to confirm the knockout of INI-1 in the developed HeLa cell lines. Different concentrations of the cellular extract were utilized in order to get the clearest bands for the SDS-PAGE. When comparing 4 μL, 6 μL, and 8 μL to the standard protein ladder, they

were all a bit darker than the ladder, but 4 µL yielded clear bands that had the best contrast between the different bands, indicating its potential as the optimal concentration for protein detection.

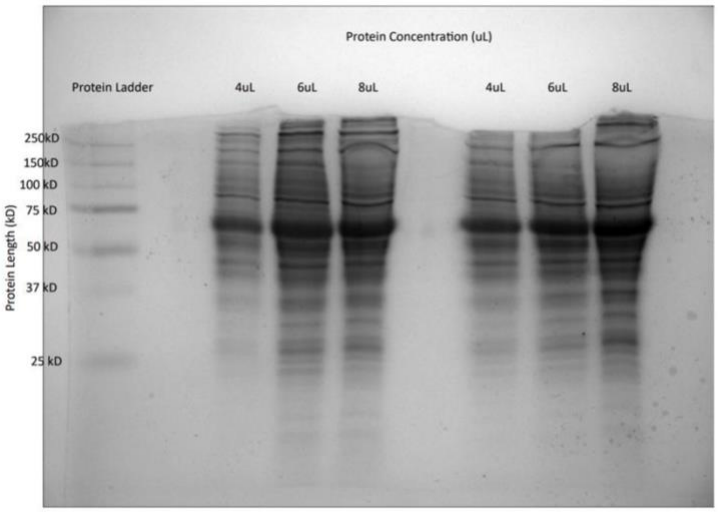

Figure 5- SDS-Page comparing different concentrations of cellular extract against protein standard ladder.

The western blot presented many problems with this research. After a few failed attempts, it was determined that we were using the wrong visualization buffer combination with the primary and secondary antibodies. Once this was fixed, we started having trouble with running straight SDS-PAGEs, and the standard ladder was not showing up properly, so there was no way to compare the loaded samples to a standard. Due to complications with the western blot, we were unable to definitively determine the knockout of INI-1 in the HeLa cell lines.

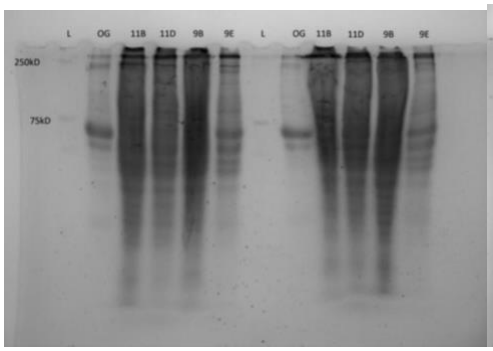

Figure 6- SDS-PAGE with very faint ladder

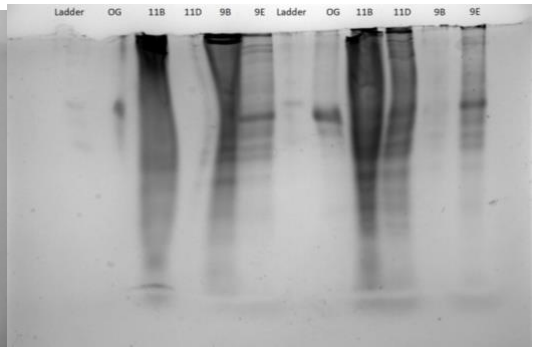

Figure 7- SDS-PAGE with wavy lines.

Discussion

Western Blot Optimization

The optimization of Western blotting procedures is a critical aspect of confirming the genetic makeup of the CRISPR edited HeLa cells. In order to progress with this line of investigation, it is imperative that we determine the problems with the past Western blots in order to run proper ones. Previous SDS-PAGE gels ran well, but the last collection of gels was all problematic for different reasons. One of the challenges encountered in Western blotting experiments is the variability in results, which we attributed to inconsistencies in reagent quality and concentration. Despite successfully running SDS-PAGE gels in previous experiments, recent attempts have yielded varied outcomes, indicating a need to reassess the quality of reagents. By remaking all reagents, including buffers and blocking solutions, we aim to eliminate any potential sources of variability stemming from deteriorated or improperly prepared reagents. The next part of optimizing Western blots focuses on the sample qualities. While we worked with different amounts of the samples, the actual concentration of protein in each sample was never determined. By utilizing a Bradford Assay, we can precisely quantify the protein content within the cell extracts. This allows us to load equal amounts of protein of each sample onto the gel, ensuring uniformity and consistency across the gels.

HeLa Cell Line Future

Once we have confirmed the INI-1 knockout in the cell lines, several future research avenues will open including the exploration of tumor suppressor properties through cell proliferation assays. These assays will offer valuable information into whether the absence of INI-1 triggers and increase in proliferation rates, a characteristic feature of many cancer cells. By

conducting these assays, we can gain insights into how the cellular landscape responds to the loss of INI-1, a crucial protein in cell cycle regulation. One way to pursue these findings is using Orangu assays, a sensitive and non-cytotoxic method for quantifying viable cell numbers. These assays use a water-soluble salt which is reduced by dehydrogenase activity to produce an orange dye. The intensity of the dye directly corresponds to the number of living cells in a sample. By using the assay alongside other assays, we can obtain more data concerning the effects of INI-1 loss on cell viability and proliferation. In addition to cell proliferation assays, we can also work on gene expression analysis which would allow us to identify downstream targets and pathways affected by INI-1 loss which could help identify future therapeutic targets.

CRISPR Clinical Implications

CRISPR Cas-9 has become a staple in genetic modification of living cells, but the future of the technology is not solely found within a laboratory. At the end of 2023, the FDA approved the first ever therapy utilizing CRISPR technology to treat sickle cell disease in humans. There are currently very few treatments for sickle cell anemia even though it is one of the most common monogenic diseases worldwide. While there are four FDA approved drugs that help reduce acute complications, the only curative therapy is hematopoietic stem cell transplantation from a donor which can be very difficult to match (Park & Bao, 2021). This therapy was created by editing stem cells from healthy donors with CRISPR Cas-9, targeting the *BCL11AA* erythroid-specific enhancer (Frangoul Haydar et al., 2021). Patients were then able to receive autologous CD34+ cells that targeted this enhancer and showed high levels of allelic editing in their bone marrow a year later. CRISPR technologies for human therapies is rapidly expanding. One research group looking into cervical cancer is attempting to use CRISPR edited cells to target proteins that would lead to cell

death in human papillomavirus (HPV) positive cells, protecting infected patients from cancer (Hu et al., 2014). These advancements in CRISPR therapies marks a significant milestone in the advancement of genetic medicine, offering hope for more effective and targeted treatments across a range of genetic disorders.

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