THE ROLE OF CDKN3 IN NEUROBLASTOMA DIFFERENTIATION

by

Veronica Partridge, B.S.

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Committee Members:

Liqin Du, Chair

Karen A. Lewis

L. Kevin Lewis

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LIST OF ABBREVIATIONS

Abbreviation	Description
ALK	Anaplastic lymphoma kinase
APS	Ammonium Persulfate
CD1	Cyclin D1
CDC6	Cell Division Cycle 6
CDK	Cyclin-Dependent Kinase
CDKL5	Cyclin-Dependent Kinase Like Protein 5
CDKN3	Cyclin-Dependent Kinase Inhibitor 3
DKK3	Dickkopf WNT Signaling Pathway Inhibitor 3
DMEM	Dulbecco's Modified Eagle's Medium
E2F	E2 Transcription Factor
G_0	Resting Phase
G_1	Gap 1 Phase
G_2	Gap 2 Phase
GAP43	Growth Associated Protein 43
HCS	High-Content Screen
M	Mitosis Phase
MYCN	N-myc Proto-oncogene Protein
NSE	Neuron Specific Enolase

PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-Kinase
RA	Retinoic Acid
Rb	Retinoblastoma
RNAi	RNA interference
S	Synthesis Phase
siRNA	Small-interfering RNA
SDS	Sodium Dodecyl Sulfate
TEMED	Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane

I. INTRODUCTION

Neuroblastoma is caused by Differentiation Loss

Neuroblastoma is a pediatric cancer that accounts for 7% of childhood cancer diagnoses and 15% of cancer-related deaths in children.[1-2] Neuroblastoma typically occurs in the adrenal gland, and is considered a neuroendocrine cancer due to its origin.[3] This cancer arises from neural crest cells of the sympathetic nervous system that fail to differentiate into mature neurons as shown in **Figure 1**.[4] The defective neural crest cells are able to proliferate indefinitely leading to tumor growth. Since neuroblastoma is a solid tumor cancer, the bulk of the tumors can be removed via surgery, but this can leave behind defective neural crest cells that continue to proliferate, leading to relapse.[5] These residual neural crest cells can be treated using differentiation therapy following tumor removal to reduce the chance of relapse.[4]

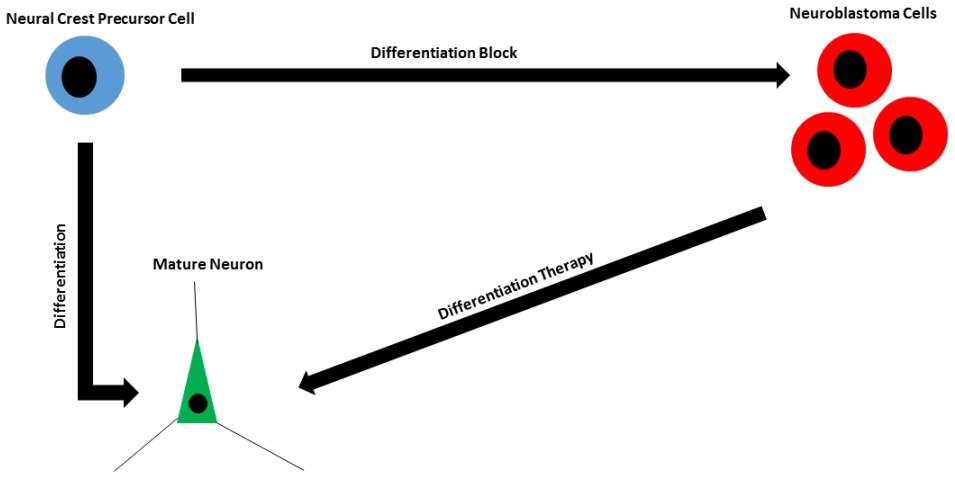

Figure 1. The relationship between neural crest cells, neurons, and neuroblastoma cells.

Neuroblastoma can be Treated with Differentiation Therapy

Differentiation therapy involves using a chemical or biological agent to induce cell differentiation.[6] In the case of neuroblastoma, differentiating agents can force neuroblastoma cells to mature into terminally differentiated neurons, which is also shown in **Figure 1**, thus stopping proliferation and leading to apoptosis.[6] The problem with current differentiation therapy for treating neuroblastoma is that there is only one agent approved for use: 13-*cis*-retinoic acid (retinoic acid). Retinoic acid has improved survival rates but not drastically, which could indicate resistance to retinoic acid treatment in certain strains of neuroblastoma.[7] There is potential to treat neuroblastoma using differentiation-inducing natural extracts such as the aqueous ethanolic extract from *Tinospora cordifolia*, which was shown to cause G_1 arrest by altering the expression of cyclin D1 (CD1) ultimately leading to differentiation.[8] Another extract with the potential to treat neuroblastoma is the aqueous extract from *Withania somnifera*, where the extract was found to cause G_0/G_1 cell cycle arrest along with differentiation thought to be induced via decreased CD1.[9]

In addition to natural products, synthetic compounds have been developed to possibly treat neuroblastoma via differentiation. For example, the synthetic retinoid Am80 which mimics retinoic acid structurally and mechanistically was found to induce more potent differentiation effects. [10] The Am80 treatment resulted in significantly increased neurite length and increased expression of differentiation markers compared to cells treated with retinoic acid.[10] While these compounds have been proven to induce differentiation in the laboratory, they are not currently FDA-approved to treat neuroblastoma patients. In order to develop more effective compounds to treat

neuroblastoma via differentiation-inducing methods, the underlying reason for differentiation loss needs to be understood. In order to elucidate the cause of differentiation block in neuroblastoma cells, cell cycle regulators can be studied to identify novel genes and pathways since cell cycle regulation is coupled to differentiation.

Cell Cycle Regulation is Coupled to Differentiation

The cell cycle is a series of events that govern cell division and growth. There are four major phases of the cell cycle the Gap 1 phase (G_1), the DNA synthesis phase (S), the Gap 2 phase (G_2), and the mitosis phase (M).[11] There is also a G_0 phase which is considered the "resting phase" where the cells will no longer divide and are considered to be quiescent.[12] These quiescence cells are prominently muscle cells, nerve cells and red blood cells which are differentiated and functional.[12] Each phase of the cell cycle is controlled by cell cycle-regulating proteins which are in turn heavily regulated and coordinated to ensure normal cell division and growth.[13-16]

There is evidence suggesting that, in addition to controlling cell division and growth, there is a connection between cell cycle progression and differentiation, however the exact mechanisms are not fully understood.[16-21] For example in terminally differentiated cells, the cell cycle is permanently arrested and will no longer proliferate.[22] One possible explanation for cell cycle arrest leading to differentiation is through cyclin dependent kinase (CDK) inhibition.[23] CDKs are proteins that form complexes with proteins known as cyclins, which activate the CDK, to phosphorylate a target protein leading to cell cycle progression. CDKs and cyclins are able to act as oncogenes in cancer meaning that they can promote cancer by allowing the cell cycle to remain active leading to

uncontrollable cell proliferation.[23] For example, in neuroblastoma cells CD1 and CD1-dependent CDKs such as CDK4 are typically upregulated and have been linked to the undifferentiated phenotype in neuroblastoma meaning that the neuroblastoma cells have little to no neurites.[24-25] Since CDK4/CD1 overexpression is linked to undifferentiated neuroblastoma cells, studies have investigated the effect of downregulating CDK4/CD1 on neuroblastoma cells and found that it leads to reduced cell proliferation, G_1-cell cycle arrest and differentiation.[25-26] The suggested mechanism for the differentiation-regulating function of CDK4/CD1 is that downregulation of CDK4/CD1 leads to increased levels of unphosphorylated retinoblastoma protein (Rb), leading to inactivated E2F, and resulting in G_1 cell cycle arrest along with differentiation.[12, 25] These results show that inhibition of specific cyclins and their associated CDKs can induce differentiation in neuroblastoma.[25]

Another way that CDK inhibition can lead to cell cycle arrest and differentiation is through Cip/Kip proteins. Cip/Kip proteins are capable of acting as tumor suppressor proteins, meaning that they have the potential to stop the progression of cancerous tumors through inhibition of the cell cycle, and some are suggested to have roles in differentiation.[27] For example, p27 is a CDK2 inhibitor that is involved in neural differentiation in mice and *Xenopus*.[28-29] Interestingly, p27 is overexpressed in some undifferentiated neuroblastoma cells; one possible reason behind this is the ability of CD1 to sequester p27.[30] When p27 is sequestered, p27 is unable to inhibit CDKs and downstream differentiation is blocked. In differentiated neuroblastoma cells, CD1 is expressed in lower amounts allowing p27 to inhibit CDKs leading to G_1 cell cycle arrest and the cells being differentiated.[30] Another Cip/Kip protein with potential to be involved in neuroblastoma differentiation is p21, which inhibits CDK1 and CDK2; p21 appears to

be required for the survival of differentiated neuroblastoma cells since it is continuously expressed after differentiation and when p21 is blocked the cells go through apoptosis.[31] Active p21 is required for survival of differentiating neuroblastoma cells.[32] The proposed mechanism for this is that p21 inhibits CDK2 leading to increased expression of unphosphorylated Rb causing E2F inactivation which finally leads to G_1 cell cycle arrest and differentiation.[26, 32]

MYCN is another potential target for differentiation therapy. While it is not directly responsible for regulating the cell cycle, it does affect cell cycle regulators that are involved in neuroblastoma differentiation. In addition, MYCN is a cancer promoting oncogene in neuroblastoma that is a hallmark of highly aggressive and therapy-resistant neuroblastomas when amplified.[33] Forced downregulation of MYCN leads to neuroblastoma differentiation and apoptosis.[33] However, the mechanism by which MYCN regulates differentiation in neuroblastoma is not fully defined. There are multiple pathways involving cell cycle regulators that are potential candidates. For example, one way that MYCN might repress differentiation allowing uncontrollable cell growth is through CDKL5 repression.[34] One study has shown that MYCN inhibition activates CDKL5 leading to G1 phase cell cycle arrest and neuroblastoma differentiation.[34] CDKL5, a CDK-like protein, is upregulated during neuroblastoma differentiation; overexpression of CDKL5 is capable of inducing differentiation and inhibiting proliferation of neuroblastoma cells.[34] Another study performed gene expression microarrays to identify pathways that MYCN may act through cell cycle regulators to effect neuroblastoma differentiation.[35] The study found that MYCN may regulate the expression of two cell cycle regulators, DKK3 and the tumor suppressor p57, to promote

cell proliferation.[35] They found that knocking down MYCN leads to DKK3 inhibition, which allows the WNT signaling pathway to activate ultimately leading to G1 cell cycle arrest and differentiation of neuroblastoma cells.[35] They also found that knocking down MYCN leads to upregulation of p57, which is involved in inhibition of CDK4 and CDK6.[35] While there are some cell cycle regulators with established roles in neuroblastoma differentiation, but have also not been excluded from participating in differentiation pathways.

CDKN3 is a Novel Cell Cycle Regulator

High-content screening (HCS) is a method by which cell cycle regulators involved in neuroblastoma differentiation can be identified.[36] This is performed by plating neuroblastoma cells on 96-well plates, treating the neuroblastoma cells with elements that may induce or suppress differentiation, and analyzing neurite outgrowth. Neurite outgrowth is a quantifying marker for differentiation because it is a measure of the cell body ratio to neurites, which shows how much each cell body has differentiated.[36] Neurite outgrowth is further confirmed with western blot for neuroblastoma differentiation markers such as growth-associated protein 43 (GAP43), neuron specific enolase (NSE) and B-TUBULIN III.[37-39]

This HCS used small-interfering RNA (siRNA) to knockdown known cell cycle regulators. siRNA are used for RNA interference (RNAi) leading to silencing of the target which is summarized in **Figure 2**.[40] The siRNA are transfected into the cell and associate with the RNA-induced silencing complex (RISC) causing the siRNA to unwind.[40] The RISC/siRNA complex will associate with the target mRNA based on the

sequence of the siRNA and RISC will cleave the target mRNA leading to gene silencing.[40]

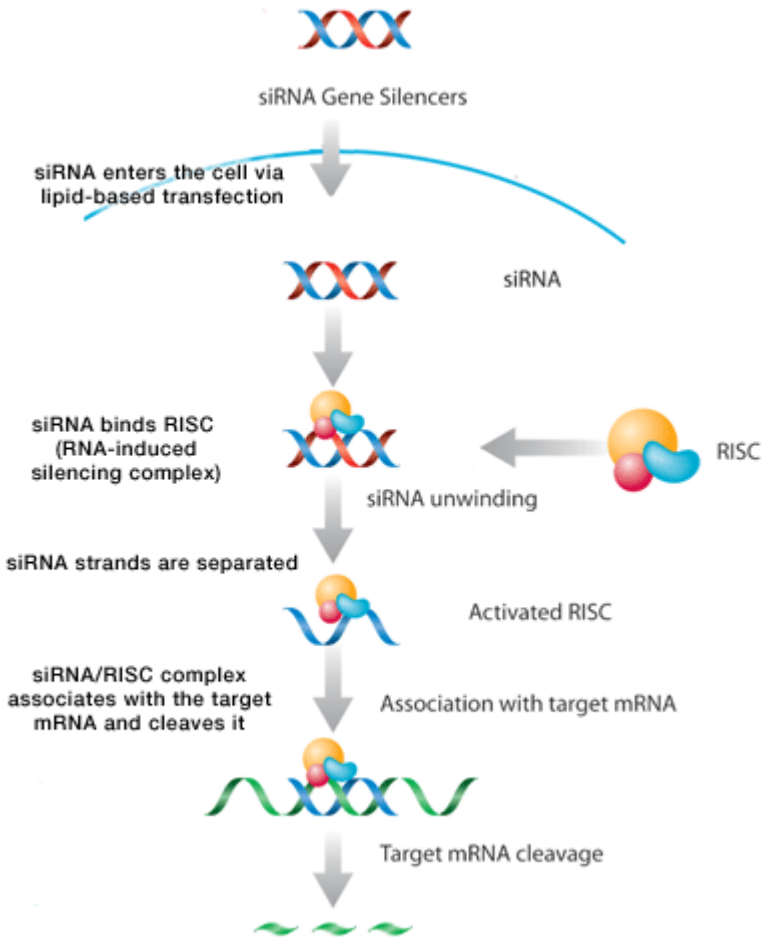

Figure 2. How siRNA work to silence gene expression.

Figure 3 shows the results of the HCS where cell cycle regulators CDK4, CDC6 and CDKN3 showed the most neurite outgrowth.[42] CDK4 and CDC6 have established roles in neuroblastoma and their knockdown is known to cause differentiation or proliferation inhibition in these cells.[26, 43] CDKN3 is a novel cell cycle regulator that has not been previously linked to neuroblastoma differentiation. Interestingly, its knockdown caused the most significant neurite outgrowth of the screen, which makes it a novel target

of interest.

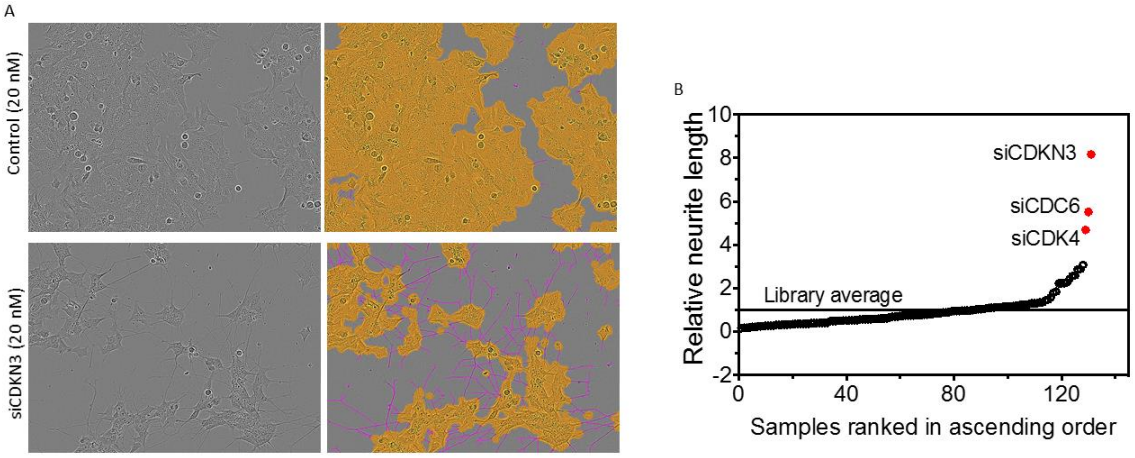

Figure 3. Results of the HCS identifying CDKN3 as involved in neuroblastoma differentiation. (A) Images of the cell bodies (yellow) and neurites (purple) for BE(2)-C cells. (B) The results comparing the siRNA used from the screen.[40]

CDKN3 is a cyclin-dependent kinase inhibitor that is known to inhibit CDK2, and it has been identified as overexpressed in multiple cancers such as non-small cell lung cancer, lung adenocarcinoma, acute myeloid leukemia, breast carcinoma and colorectal adenocarcinoma.[44] In lung adenocarcinoma, high CDKN3 levels are associated with poor prognosis for survival, indicating that CDKN3 potentially acts as an oncogene.[45] Since CDKN3 is overexpressed in many types of cancers, there have been studies pursuing knockdown or silencing of CDKN3 to investigate the potential of CDKN3 to act as an oncogene and therapeutic target. For example, silencing CDKN3 in breast cancer cells leads to cell apoptosis, G_1 cell cycle arrest, and inhibition of cell migration.[46] Another study also found that knocking down CDKN3 reduces proliferation, invasion, and induces apoptosis in ovarian cancer cells.[47] The effect of knocking down CDKN3 in

gastric cancer cells was found to lead to similar results of inhibition of proliferation, migration, and invasion along with G_1 cell cycle arrest.[48] One study involving nasopharyngeal carcinoma found that CDKN3 acts through p27 to promote cell proliferation and tumorigenesis, and that knockdown of CDKN3 leads to increased expression of p27 resulting in cell cycle arrest, apoptosis, reduced proliferation and invasion.[49]

While the majority of the studies on CDKN3 have identified it to act as an oncogene, there are conflicting reports regarding whether CDKN3 is acting as either an oncogene or tumor suppressor in hepatocellular carcinoma. One study, which reported CDKN3 as an oncogene, showed that CDKN3 was overexpressed and promoted tumor cell proliferation.[50] A second study, which reported CDKN3 as a tumor suppressor, showed that inhibiting CDKN3 can promote cell survival in hepatocellular carcinoma and suggested that low CDKN3 levels activated the AKT/PI3K, pathway causing inhibition of p53/p21.[51] Overall these reports indicate that CDKN3 is generally oncogenic in nature in the types of cancer studied to date. However, the role of CDKN3 in neuroblastoma has yet to be determined.

Hypothesis and Specific Aims

Although CDKN3 has been identified as oncogenic in many cancer types, it has no currently known role in neuroblastoma or neuroblastoma differentiation. The results from the HCS suggest that CDKN3 contributes to the proliferation of neuroblastoma cells. To elucidate the mechanisms and roles of CDKN3 in neuroblastoma, this project has three main aims. The first aim was to demonstrate the function of CDKN3 in regulating cell differentiation and cell viability in neuroblastoma cell lines with different

genetic backgrounds. The results from the HCS suggested that knockdown of CDKN3 may lead to differentiation in neuroblastoma cells. RNA interference (RNAi) was used to knockdown expression of CDKN3 in neuroblastoma cells, and the resulting neurite outgrowth measured. Neurite outgrowth will indicate differentiation, which was further analyzed using western blot for established differentiation markers. Additionally, the effect of CDKN3 knockdown on cell viability was studied using MTT assays to measure cellular metabolism. A major hypothesis was that if CDKN3 plays a role in neuroblastoma differentiation and cell survival then siCDKN3-treated cells will be differentiated and display decreased viability.

The second aim of this project was to determine the function of CDKN3 in regulating cell proliferation in neuroblastoma cell lines with different genetic backgrounds (**Table 2**). Cell proliferation was studied using a colony formation assay following treatment with siCDKN3. If CDKN3 is involved in cell proliferation, knockdown of CDKN3 will cause decreased proliferation. The third aim of this project was to determine whether CDKN3 plays a role in mediating the differentiation-inducing effects of retinoic acid. This was done by treating neuroblastoma cells with retinoic acid and then measuring the relative CDKN3 mRNA expression using quantitative PCR. If retinoic acid functions upstream of CDKN3 in the same pathway, CDKN3 expression may go down following treatment. However, if CDKN3 and retinoic acid act through different pathways, there may be no change in CDKN3 expression. To test the hypothesis that they act synergistically, neuroblastoma cells were treated with both siCDKN3 and retinoic acid before evaluating differentiation and cell viability.

II. MATERIALS AND METHODS

General Reagents

Sodium dodecyl sulfate (SDS) and tris(hydroxymethyl)aminomethane (Tris) base were purchased from VWR international. The following reagents were purchased from Fisher Scientific: sodium chloride, potassium chloride, 40% polyacrylamide solution (29:1 acrylamide:bisacrylamide),ammonium persulfate (APS), tetramethylethylenediamine (TEMED), Triton X-100, protease inhibitor cocktail tablets, isopropyl alcohol, trypsin, methanol, 10X phosphate-buffered saline (PBS), acid-phenol: chloroform, dimethyl sulfoxide (DMSO), Lipofectamine RNAiMAX reagent, MTT reagent (catalogue number AC15899-0050), glycerol, bromophenol blue, 2-mercaptoethanol, crystal violet, Super Signal West Pico Chemiluminescent Substrate, Trizol, and the Spectra Multicolor Broad Range Protein Ladder. The Pierce BCA Protein Assay kit was purchased from Thermo Fisher Scientific. Retinoic acid was purchased from Sigma-Aldrich. Powdered milk, separating buffer, and resolving buffer was purchased from Boston Bioproducts. Polyacrylamide gel running buffer and transfer buffer for western blot were purchased from National Diagnostics. Ethanol was provided by the Texas State University Department of Chemistry and Biochemistry.

Oligomers

siCDKN3 oligomers were purchased from Ambion (now Thermo Fisher Scientific), OriGene, which is a pool of siRNA, and Sigma-Aldrich; **Figure 4** shows the sequences, sites, and exons that these oligomers target. The negative control oligomers were purchased from Dharmacon which are a random sequence that has been shown to

target no known human genes by using BLAST.

	siRNA Sequence	Target sites	Exon
Ambion			
siCDKN3-1	GUUUCUCGGUUUAUGUGCU	237-255	3
Origene			
siCDKN3-2A	GGACGAACCAGUGAGCUAAGCUGCG	7-31	1
siCDKN3-2B	AUAAUGGAAGAGCUUACAACCUGCC	358-382	4
siCDKN3-2C	UCACCAGAGCAAGCCAUAGACAGCC	481-505	6
Sigma-Aldrich			
siCDKN3-3	CCAUCAUCAUCCAAUCGCA	423-441	5

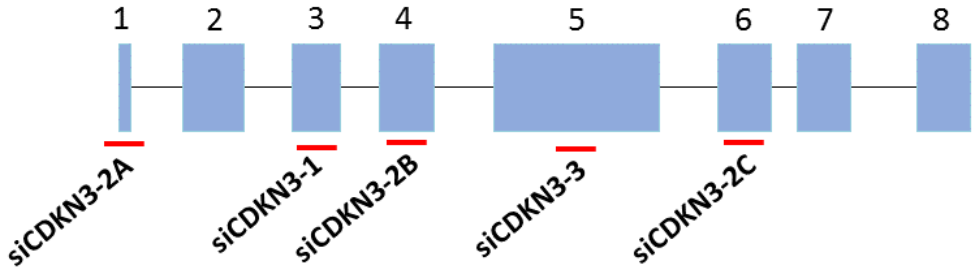

Figure 4. Summary of the target sequence, site, and exon target of each siCDKN3 oligomer tested.

Antibodies

The following antibodies were purchased from Fisher Scientific: growth associated protein 43 (GAP43) catalogue number PA5-34943, neuron specific enolase (NSE) catalogue number PA5-27452, βIII-tubulin catalogue number PA5-25655, calnexin catalogue PA5-34754, and goat anti-rabbit IgG secondary antibody (HRP conjugated) catalogue number 31460. CDKN3 antibodies were purchased from Santa Cruz Technologies (catalogue number sc-475) and Thermo Fisher Scientific (catalogue

number PA5-50929). Streptavidin-HRP (catalogue number 3999S) and goat anti-rabbit IgG biotinylated antibody (catalogue number 14708S) was purchased from Cell Signaling Technology.

Quantitative PCR Reagents

The High-Capacity cDNA Reverse Transcription Kit containing random primers, dNTPs, buffer, and MultiScribe Reverse Transcriptase was purchased from Thermo Fisher Scientific. The RNaseOUT recombinant ribonuclease inhibitor and Maxima SYBR Green/ROX qaPCR Master Mix (2X) were also purchased from Thermo Fisher Scientific. All primers used were purchased from Sigma Aldrich and are listed in **Table 1**.

Table 1. Sequences of primers used for quantitative PCR.

Primer	Sequence
h-GAPDH-F	GAAGGTGAAGGTCGGAGTC
h-GAPDH-R	GAAGATGGTGATGGGATTTC
CDKN3-RT-PCR-F	GCCAGCTGCTGTGAAATAATG
CDKN3-RT-PCR-R	AGATCTCCCAAGTCCTCCATAG

Cell supplies

The sources and genetic variation of each cell line used are listed in **Table 2**. The cells were cultured in a Dulbecco's Modified Eagle's Medium: Nutrient mixture F-12 (DMEM/F-12) purchased from Corning Cell Gro. 10% Equafetal bovine serum (fetal bovine serum equivalent) purchased from Atlas Biologicals was used as additional

nutrients. Penicillin-streptomycin from Fisher Scientific was added to reduce the chance of bacterial contamination in the growth media.

Table 2. Summary of cell lines used.

Cell Line	MYCN	p53	Chromosome mutations	ALK	Source
BE(2)-C	Amplified	Mutated	Transitions (1;2)(p22;p21),(3;17)(p21;q21) Monosomy (17,18) Rearrangements (4,6,9,10,11,and/or 20)	Normal	ATCC*
CHLA-90	Non-Amplified	Mutated	Not available	Mutated	ATCC
SK-N-DZ	Amplified	Normal	Monosomy (10, 11, 13, 14, 19) Missing (2)	Normal	ATCC
Kelly	Amplified	Normal	Deleted (11q)	Mutated	GCCRI**

*American Type Culture Collection
**Greehey Children's Cancer Research Institute

Equipment

For sterile experiments the 1300 Series A2 biological safety cabinet from Thermo Fisher was used to prevent contamination. The Forma series 3 H_2O Jacket CO_2 incubator for growing cells was also purchased from Thermo Fisher. The IncuCyte ZOOM Live Cell Imaging System was purchased from Essen Bioscience.

Software

The IncuCyte Zoom 2016A software was used to image neuroblastoma cells in the incubator and to detect neurite outgrowth. The ImageJ software from the National Institutes of Health was used to quantify westerns and colony formation plates. The

Graphpad Prism 7 software was purchased to analyze data.

Neurite outgrowth of neuroblastoma cell lines

The general procedure was the same for every cell line and treatment except for number of cells plated. The following number of cells were plated for each cell line in a single well on the 96-well plate 2500 BE(2)-C, 3200 SKNDZ, 3000 CHLA-90, and 2500 Kelly. The cells were treated with either 20 nM siCDKN3 oligomers or 20 nM negative control oligomers using the Lipofectamine RNAiMAX transfection reagent. Cells that were to be treated with both siCDKN3 oligomers and retinoic acid were first treated with the oligomer, allowed to grow for a day, then 5 μM of retinoic acid was added to the media. After the cells were treated the 96-well plate was placed into the IncuCyte ZOOM Live Cell Imaging System in the incubator and allowed to grow for 5 days at 37°C with CO_2. During the 5-day period, the cells were imaged every 12 hours under 20X magnification. After 5 days, the images were used to calculate neurite outgrowth (cell body to neurite length ratio) following the programmed definition for each cell line, which can be adjusted by the user if necessary. These numbers were then exported to Graphpad Prism 7 to normalize the data to the control. The images are exported to visualize the neurite outgrowth.

Western blot

The general procedure was the same for every cell line and treatment except for the number of cells plated for control oligos versus siCDKN3 or retinoic acid treatment. Plates to be treated with negative control oligomers were plated with approximately 15,000 cells and plates to be treated with siCDKN3 oligomers or retinoic acid were plated with approximately 30,000 cells. The cells were treated with either 20 nM

siCDKN3 oligomers or 20 nM negative control oligomers using the Lipofectamine RNAiMAX transfection reagent. Cells that were to be treated with both siCDKN3 oligomers and retinoic acid were first treated with the oligomer, allowed to grow for a day, then 5 μM of retinoic acid was added to the media. After being treated the cells were grown for 4 days in the incubator at 37°C with CO_2. Cell lysates were then prepared using 25 mM Tris-HCl buffer at pH 7.4 with 1% Triton X-100.

The protein concentration was determined using a BCA assay. Equal amounts of the cell lysates and ladder were loaded into a 10% SDS-PAGE to separate the different proteins based on size. The proteins were transferred to PVDF membranes and detected using antibodies. The blots were then visualized using SuperSignal West Pico Chemiluminescent substrate and the Molecular Imager ChemiDoc XRS+ imaging system. Once the blots were visualized, ImageJ was used to quantify the blots by measuring the intensity of each band and normalizing the treatment band to the control band. After the ratio of treatment bands compared to control bands is found, the loading differences is considered by dividing by the calnexin results.

Cell Viability

The general procedure was the same for every cell line and treatment except for number of cells plated. The following number of cells were plated for each cell line in a single well on the 96-well plate: 2500 BE(2)-C, 3200 SKNDZ, 3000 CHLA-90, and 2500 Kelly. The cells were treated with either 20 nM siCDKN3 oligomers or 20 nM negative control oligomers using the Lipofectamine RNAiMAX transfection reagent. Cells that were to be treated with both siCDKN3 oligomers and retinoic acid were first treated with the oligomer, allowed to grow for a day, then 5 μM of retinoic acid was added to the

media. The cells were then incubated for 5 days at 37°C with CO_2. Following incubation 15 µl of MTT solution (2.5 mg/ml in 1X PBS) was added to each well with the original culture media present then incubated for 1 hour at 37°C. After confirmation of crystal formation, the plate was spun down and the crystals were dissolved using DMSO. The relative cell viability was determined by taking the difference of the absorbance values at 570 nm and 630 nm. These numbers were then normalized to the control using Graphpad Prism 7.

Colony formation assay

The general procedure was the same for every cell line and treatment except for number of cells plated. The following number of cells were plated for each cell line in a single well on the 96-well plate: 2000 BE(2)-C, 4000 SKNDZ, 2000 CHLA-90, and 2000 Kelly. The cells were treated with either 20 nM siCDKN3 oligomers or 20 nM negative control oligomers using the Lipofectamine RNAiMAX transfection reagent. The cells were then incubated at 37°C with CO_2 for at least 14 days or until sufficiently sized colonies had grown. The colonies were then stained using 0.5% crystal violet, allowing the number and size to be determined by ImageJ after scanning the plates. Graphpad Prism 7 was then used to graph the data.

Quantitative PCR

The general procedure was the same for every cell line and treatment. 2000 cells were plated onto a dish and then the cells were treated with either 20 nM siCDKN3 oligomers or 20 nM negative control oligomers using the Lipofectamine RNAiMAX transfection reagent. Cells that were to be treated with both siCDKN3 oligomers and retinoic acid were first treated with the oligomer, allowed to grow for a day, then 5 µM of

retinoic acid was added to the media. The cells were then incubated for 5 days at 37°C with CO_2. After the 5-day incubation, the RNA was isolated using Trizol. The sample was incubated for 5 minutes at room temperature with Trizol before adding chloroform. The sample was then centrifuged and the aqueous phase was pipetted into a new tube. Next isopropanol was added to the new tube to encourage the RNA pellet to form. Once the RNA pellet was washed with ethanol and dried, the concentration of the RNA was determined using nanodrop. After the RNA had been isolated, the High-Capacity cDNA Reverse Transcription Kit was used to convert the RNA into cDNA in the thermocycler using the following settings: first 25°C for 10 minutes, second 37°C for 120 minutes, and finally 85°C for 5 minutes The Maxima SYBR Green qPCR master mix along with appropriate primers was used to measure the mRNA expression of the cDNA. The settings suggested by the Maxima manual was used in the thermocycler. GAPDH was used as the control for normalizing. The threshold cycle times (Ct) were used with the comparative cycle time method to calculate relative gene expression.[52] Graphpad Prism 7 was then used to graph the data.

Statistical analysis

Data sets were normalized and graphed using the Graphpad Prism 7 program. Normalization involved calculating the ratio of the treated average to the control average. The Graphpad Prism 7 program was also used to conduct unpaired t-tests to define statistical differences. **Table 3** shows the p-values with the symbol meanings from this program.

Table 3. Statistical p-value definitions.

Symbol	Meaning
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

III. RESULTS AND DISCUSSION

The major aim of this project was to determine the role of CDKN3 in neuroblastoma differentiation. This project builds on the high content screen conducted on cell cycle regulators that indicated that knocking down CDKN3 led to significant neurite outgrowth (**Figure 3**). While neurite outgrowth can be an indicator of neuroblastoma differentiation, it is not required nor is it sufficient enough to use alone. Neuroblastoma differentiation must be further confirmed using neuroblastoma differentiation markers (NSE, GAP43, and βIII-Tubulin), cell viability, and cell proliferation. Differentiated neuroblastoma cells will have increased levels of these markers, decreased viability, and decreased proliferation.

CDKN3 knockdown induces cell differentiation and reduces cell survival in BE(2)-C cells

The neurite outgrowth seen in the HCS following CDKN3 knockdown (**Figure 3**) may have been caused by off-targeting effects during the screen. This hypothesis was disproved by using three different oligomers to knock down CDKN3 in BE(2)-C neuroblastoma cells grown in culture. These results are shown in **Figure 5**. **Figure 5A** shows the quantitative PCR results using the oligomers, demonstrating a decrease in relative CDKN3 expression compared to control oligomers. There was also significantly increased neurite outgrowth following treatment with the oligomers (**Figure 5B**) and significantly decreased cell viability (**Figure 5C**) for the BE(2)-C cells treated with siCDKN3. These results show that knockdown of CDKN3 causes neurite outgrowth and decreased viability along with providing strong evidence that the HCS results were not

from off-target effects. Western blot was attempted to measure CDKN3 protein expression levels, but there are no good commercially available antibodies so the results were inconclusive (data not shown). The Ambion siCDKN3 was used for all future experiments due to producing the most profound effect on neurite outgrowth and cell viability along with being the most cost effective.

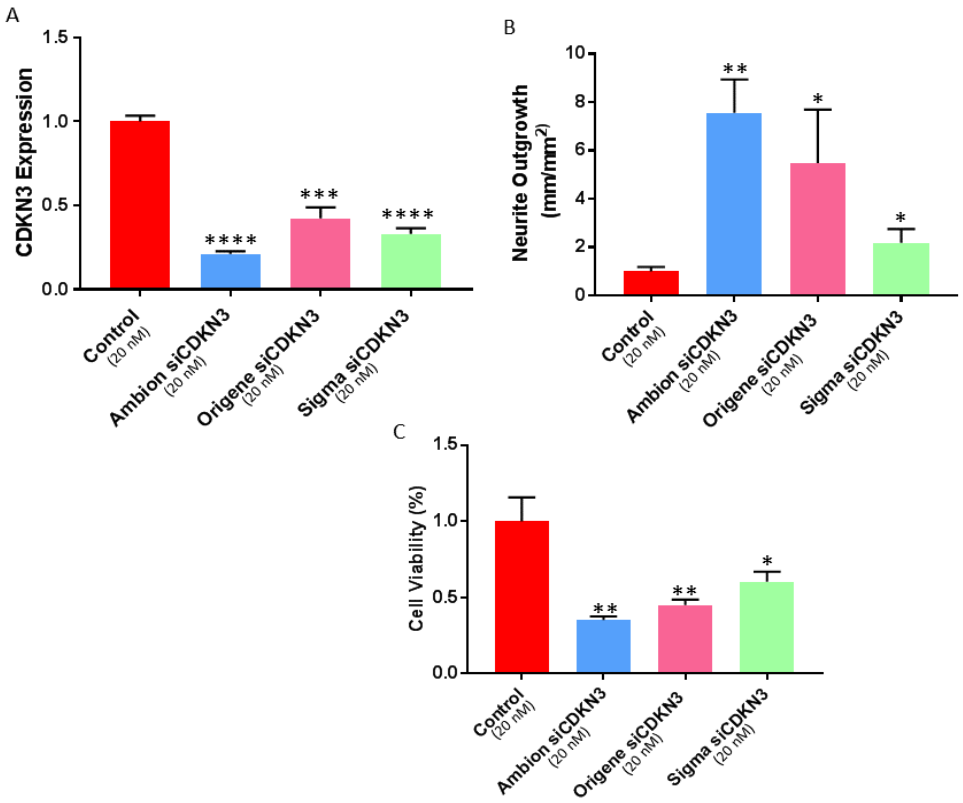

Figure 5. Comparisons of siCDKN3 oligomers effect on BE(2)-C cells. (A) Relative CDKN3 expression with standard deviations and significance. (B) Neurite outgrowth with standard deviations and significance. (C) Cell viability with standard deviations and significance.

Since the HCS used the phenotype of neurite outgrowth to identify CDKN3 as possibly being involved in neuroblastoma differentiation in BE(2)-C cells, the next step was to confirm that siRNA-mediated knockdown of CDKN3 can cause differentiation in BE(2)-C cells. This was done by first confirming the morphological changes associated

with neuroblastoma differentiation following siCDKN3 treatment; these results are shown in **Figure 6A** and **Figure 6B**. The BE(2)-C cells treated with siCDKN3 had significant neurite outgrowth (shown in purple) compared to control cells. Next, western blot was done to confirm differentiation via common molecular markers of differentiation (NSE, βIII-Tubulin, and GAP43) using calnexin as a loading control. The results of the western blot indicate that even after only 4 days of growth there is increased expression of NSE and βIII-Tubulin indicating differentiation of the neuroblastoma cells (**Figure 6C**). Finally, shown in **Figure 6D** are the results showing that BE(2)-C cells treated with siCDKN3 are less viable than control cells which, is another molecular indication of differentiation.

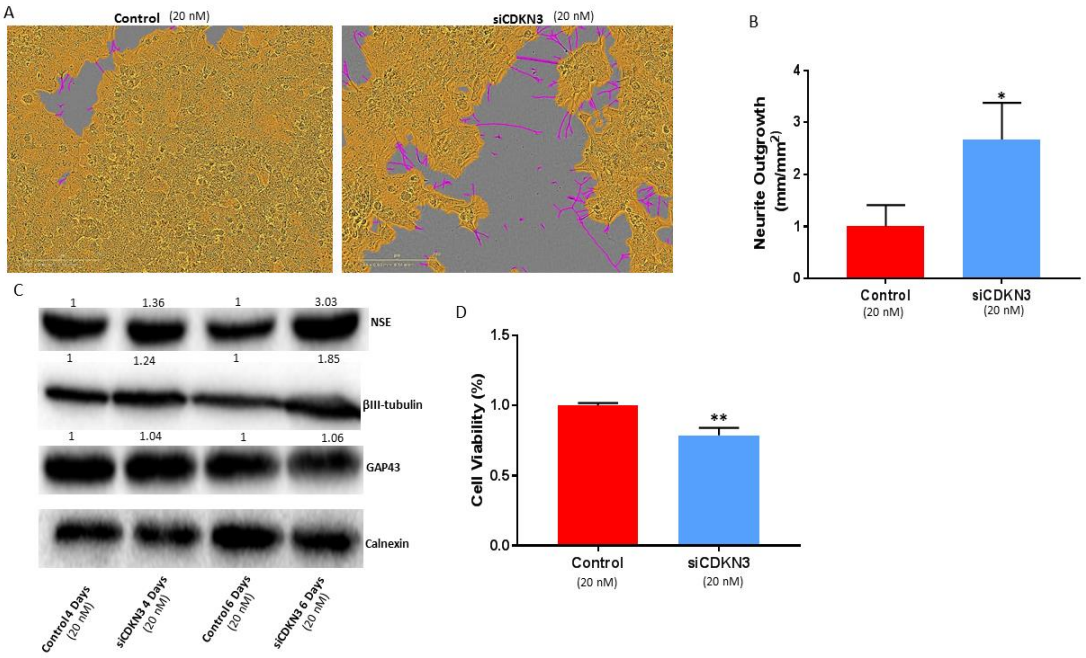

Figure 6. Confirming the effect of siCDKN3 in BE(2)-C cells. (A) Images taken at 20X magnification showing neurites (purple) and cell bodies (yellow). (B) Neurite outgrowth with standard deviations and significance. (C) Quantified western blots for differentiation markers. (D) Cell viability with standard deviations and significance.

CDKN3 knockdown induces cell differentiation and reduces cell survival in additional neuroblastoma cell lines with different genetic backgrounds

In order to test whether the effect of CDKN3 knockdown has a generic effect on cell differentiation, I examined additional neuroblastoma cell lines with different genetic backgrounds. The previous experiment was replicated in three different cell lines: CHLA-90 which has mutated p53 and ALK, SK-N-DZ which has MYCN amplification, and Kelly which is MYCN amplified with mutated ALK.

In the CHLA-90 cells, **Figure 7A** and **Figure 7B** do not show the morphological signs of being differentiated evidenced by the lack of neurite outgrowth. Since it is possible for neuroblastoma cells to be differentiated without neurite outgrowth, western blot was utilized to measure the expression of differentiation markers. The western blot shows an increase of 1.21 fold in NSE, 0.72 in βIII-Tubulin, and 0.34 in GAP43 with the largest increase in NSE. The results of the western blot indicated that despite not showing neurite outgrowth the CHLA-90 cells are differentiated due to the increased expression of the markers (**Figure 7C**).

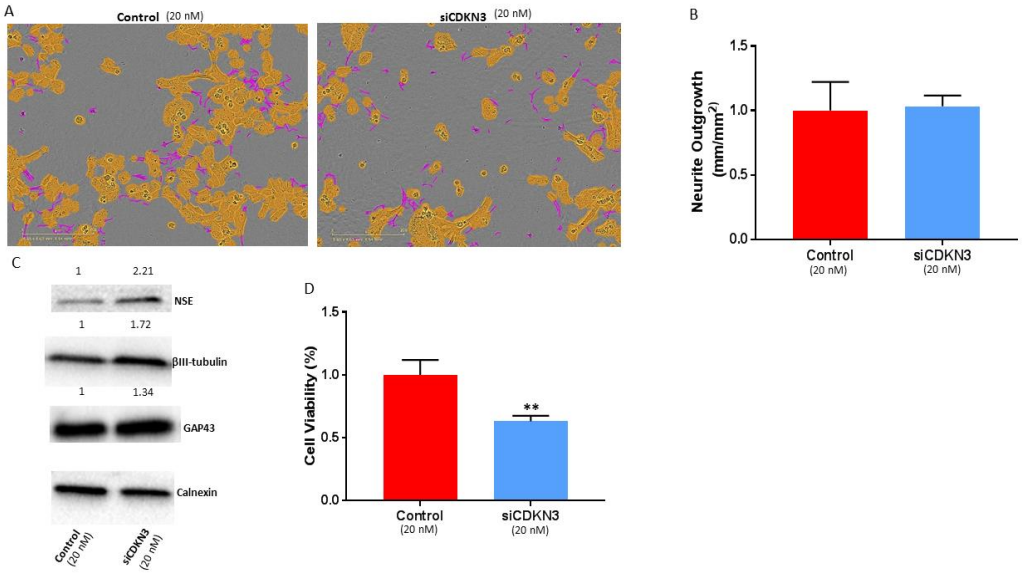

Figure 7. The effect of siCDKN3 in CHLA-90 cells. (A) Images taken at 20X magnification showing neurites (purple) and cell bodies (yellow). (B) Neurite outgrowth with standard deviations and significance. (C) Quantified western blots for differentiation markers at 4 days. (D) Cell viability with standard deviations and significance.

The next cell line tested using previously discussed methods was SK-N-DZ. **Figure 8A** and **Figure 8B** show that this cell line grows spontaneous neurites so neurite outgrowth is a reliable measure of differentiation for this cell line. **Figure 8C** shows the expression levels of the differentiation markers using western blot for siCDKN3 versus control SK-N-DZ cells. The expression levels of NSE and βII-tubulin are increased, which is indicative of differentiated neuroblastoma cells. The siCDKN3-treated SK-N-DZ cells did have decreased cell viability, but it was deemed not significant (**Figure 7D**). It is possible that this cell line grows more slowly, thus needing more time to clearly visualize the effect of siCDKN3 on this line.

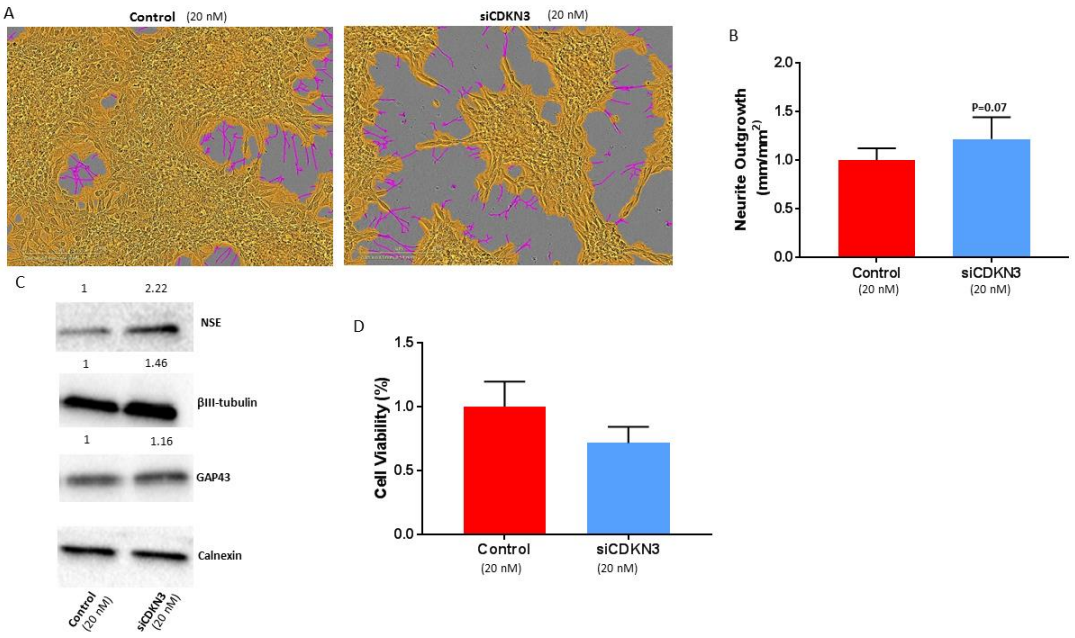

Figure 8. The effect of siCDKN3 in SK-N-DZ cells. (A) Images taken at 20X magnification showing neurites (purple) and cell bodies (yellow). (B) Neurite outgrowth with standard deviations and significance. (C) Quantified western blots for differentiation markers at 4 days. (D) Cell viability with standard deviations and significance.

The final cell line tested for differentiation via CDKN3 knockdown was Kelly. **Figure 9A** and **Figure 9B** show that Kelly cells display significant neurite outgrowth following siCDKN3 treatment. The possible differentiation was confirmed using western blot for the neuroblastoma differentiation markers (**Figure 9C**). The Kelly cells treated with siCDKN3 expressed higher NSE and GAP43, although there was not a marked increase in βII-tubulin levels. **Figure 9D** shows that siCDKN3-treated Kelly cells have significantly decreased cell viability compared to control cells.

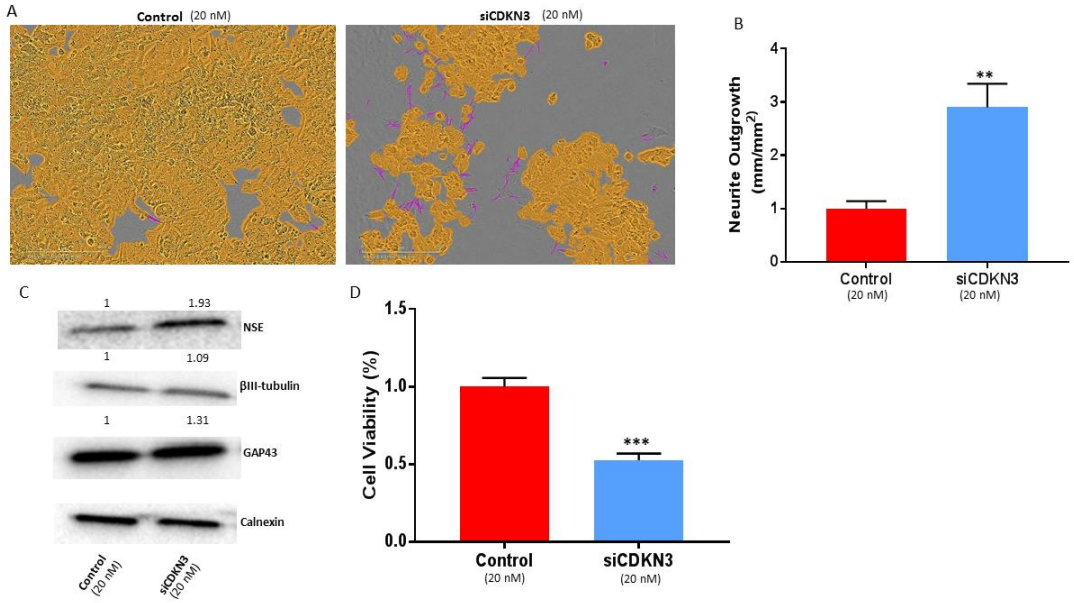

Figure 9. The effect of siCDKN3 in Kelly cells. (A) Images taken at 20X magnification showing neurites (purple) and cell bodies (yellow). (B) Neurite outgrowth with standard deviations and significance. (C) Quantified western blots for differentiation markers at 4 days. (D) Cell viability with standard deviations and significance.

In conclusion, knockdown of CDKN3 is capable of inducing differentiation and decreasing viability in neuroblastoma cell lines that are genetically different. While some cell lines did not have significant neurite outgrowth (CHLA-90 and SK-N-DZ), neurite outgrowth is only a morphological feature of differentiated neuroblastoma cells that is not sufficient nor necessary for the cells to be differentiated. All four cell lines expressed increased amounts of differentiation makers although the specific markers that were increased varied from cell line to cell line. The only cell line that did not have significantly reduced cell viability was SK-N-DZ which was likely because this cell line grows more slowly than the other cell lines tested making it difficult to see short-term effects.

CDKN3 knockdown reduces cell proliferation in neuroblastoma cell lines with different genetic backgrounds

Colony formation assays were next performed to determine the role of CDKN3 in cell proliferation. The first cell line tested was BE(2)-C cells which grew fewer colonies (**Figure 10A** and **Figure 10B**). The smaller number of colonies is indicative of proliferation loss so siCDKN3 can cause decreased proliferation in BE(2)-C cells. Loss of proliferation is an important parameter, because differentiated neuroblastoma cells do not proliferate as much as undifferentiated neuroblastoma cells. The sizes of the colonies, as shown in **Figure 10C**, remained relatively similar. The reason the size remained similar is likely due to the transfection efficiency of the reagent used because some cells will be transfected with sufficient siCDKN3 and some cells will not.

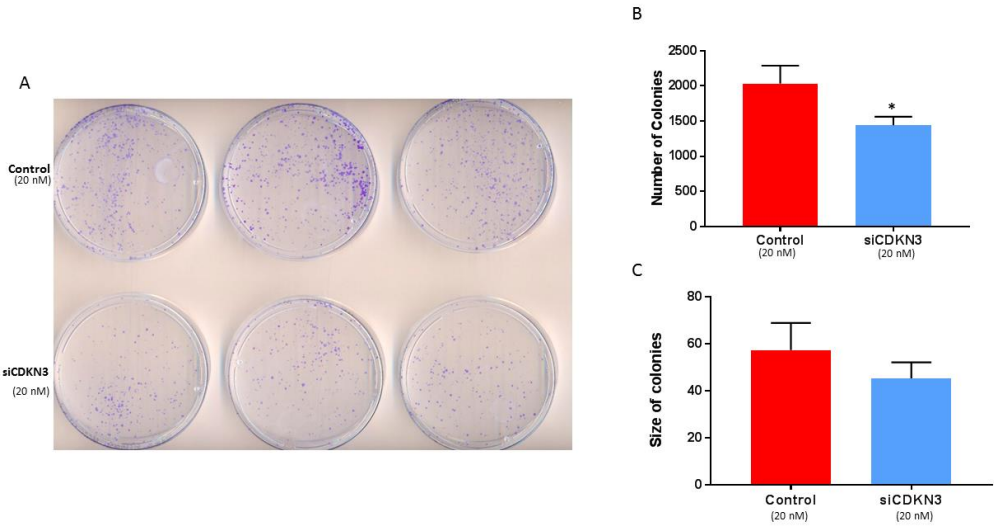

Figure 10. The effect of siCDKN3 on proliferation in BE(2)-C cells. (A) Scanned images of the plated cells. (B) Number of colonies with standard deviations and significance. (C) Sizes of the colonies with standard deviations and significance.

CHLA-90 was the next cell line tested. The siCDKN3-treated CHLA-90 cells had significantly less colonies than the control as shown in **Figure 11A** and **Figure 11B**. These results further confirm that siCDKN3 causes differentiation in neuroblastoma cells since the siCDKN3 would be differentiated leading to this decrease in proliferation. **Figure 11C** shows that the colony sizes are decreased, but not significantly.

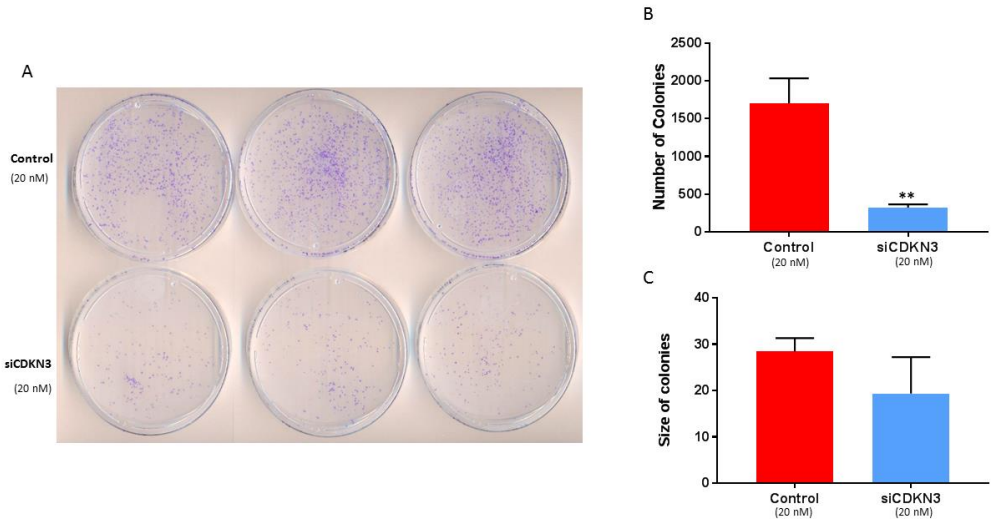

Figure 11. The effect of siCDKN3 on proliferation in CHLA-90 cells. (A) Scanned images of the plated cells. (B) Number of colonies with standard deviations and significance. (C) Sizes of the colonies with standard deviations and significance.

The next cell line, SK-N-DZ, also had significantly decreased colony numbers indicating proliferation loss and differentiation (**Figure 12A** and **Figure 12B**). As previously explained, it is possible that this particular cell line grows at a slower rate than the other cell lines tested, which could have caused the viability results to not be as significant, unlike the other cell lines (**Figure 8D**). **Figure 12C** shows that the sizes of the colonies are not significantly different.

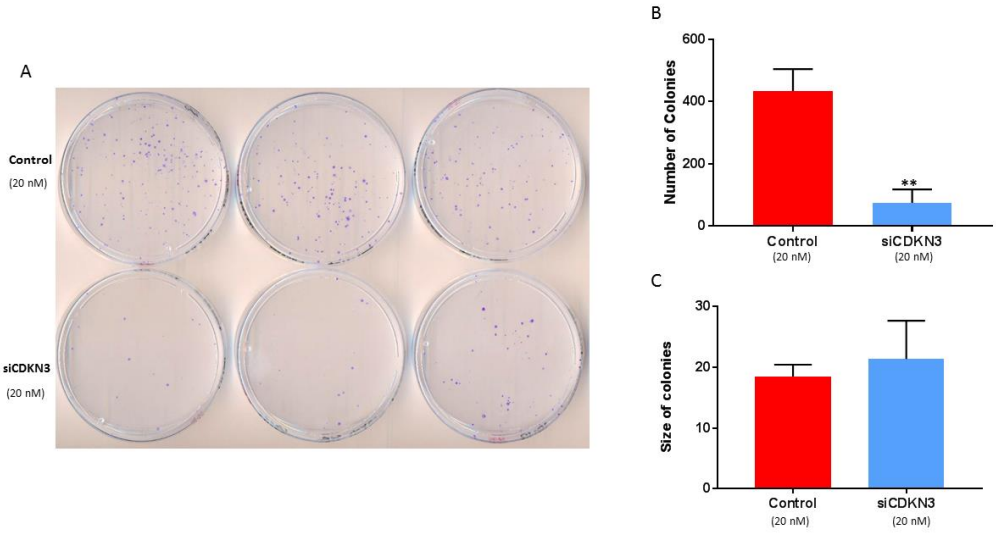

Figure 12. The effect of siCDKN3 on proliferation in SK-N-DZ cells. (A) Scanned images of the plated cells. (B) Number of colonies with standard deviations and significance. (C) Sizes of the colonies with standard deviations and significance.

The final cell line used to determine the effects of siCDKN3 on proliferation was Kelly. **Figure 13A** and **Figure 13B** shows that Kelly cells treated with siDCDKN3 had significantly fewer colonies than the untreated cells. This loss of proliferative ability is likely due to the Kelly cells becoming differentiated. As with the other three cell lines, the colonies produced by the Kelly cells are not significantly different (**Figure 13C**).

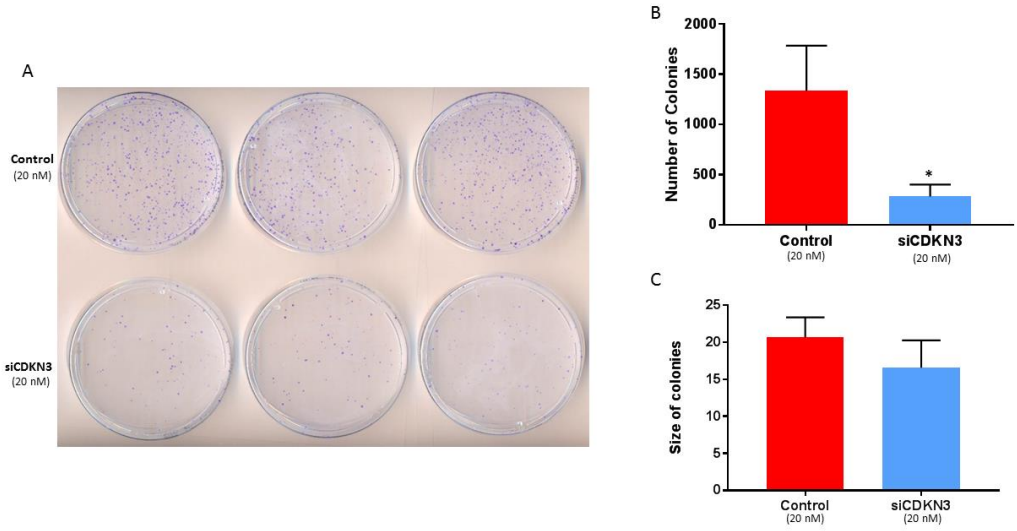

Figure 13. The effect of siCDKN3 on proliferation in Kelly cells. (A) Scanned images of the plated cells. (B) Number of colonies with standard deviations and significance. (C) Sizes of the colonies with standard deviations and significance.

In conclusion, knockdown of CDKN3 reduces proliferation in neuroblastoma cells that are genetically different. Each of the four cell lines had a significantly reduced number of colonies following treatment with siCDKN3 which indicates decreased proliferation. Some cell lines had a slight decrease in the colony size (BE(2)-C, CHLA-90, and Kelly) and one cell line had a slight increase in colony size (SK-N-DZ) which was most likely due to the transfection efficiency of the transfection reagent used. This means that some cells were transfected with the necessary amount of siCDKN3 to see an

effect on proliferation and others were not.

Retinoic acid treatment increases relative CDKN3 mRNA expression in BE(2)-C cells and siCDKN3 slightly enhances the effect of retinoic acid on Kelly cells

The possibility that CDKN3 mediates the differentiation-inducing effects of retinoic acid was tested using dual retinoic acid and siCDKN3 treatments. If CDKN3 is epistatic with retinoic acid-induced differentiation, then the relative expression of CDKN3 may decrease following retinoic acid treatment over the course of five days. However, when BE(2)-C cells were treated with retinoic acid (RA) the amount of CDKN3 mRNA increased (**Figure 14**). This unexpected result is very interesting because it may suggest that CDKN3 overexpression is a possible mechanism by which cells gain retinoic acid resistance. However, this experiment was only performed in one cell line so further studies are necessary.

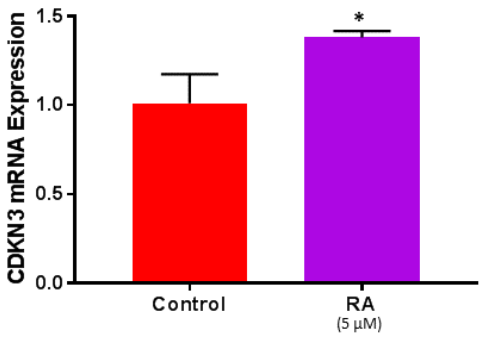

Figure 14. The expression levels of CDKN3 following retinoic acid treatment in BE(2)-C cells. Graph shows standard deviations and significance.

Next the possibility that siCDKN3 combined with retinoic acid would have a synergistic effect on Kelly cells was tested. This was also tested in BE(2)-C cells, however the data was inconclusive and is not shown. The initial neurite outgrowth (**Figure 15A**) indicated that siCDKN3 slightly enhanced the effects of retinoic acid, but not significantly. The western blot for neuroblastoma differentiation markers showed that expression of the markers was slightly increased for the siCDKN3 + retinoic acid treatment, but it is not drastically different compared to the siCDKN3 treatment alone (**Figure 15B**). Finally, the cell viability indicated that the Kelly cells treated with siCDKN3 slightly enhanced the effect of retinoic acid, but it was not significant (**Figure 15C**). All together these results indicate that combining siCDKN3 with retinoic acid treatment is not synergistic.

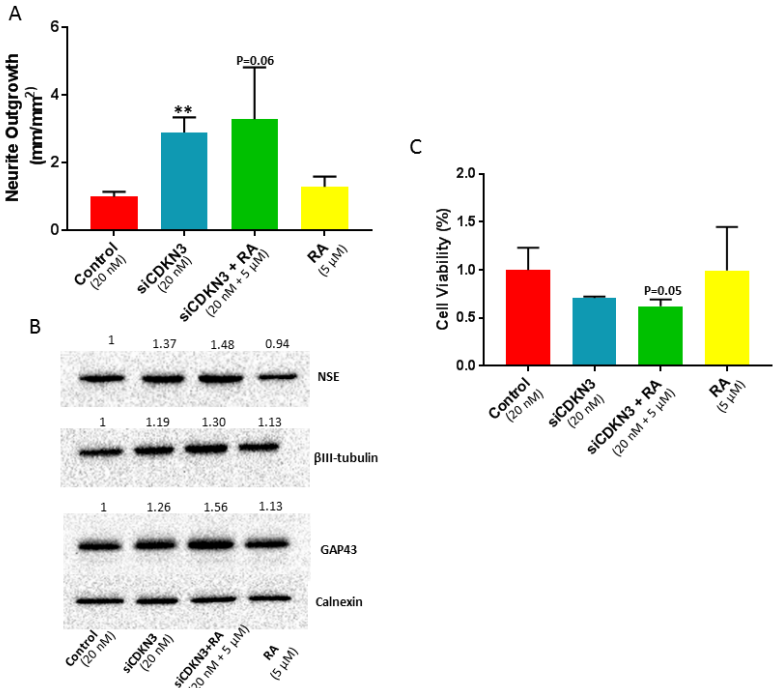

Figure 15. The effect of siCDKN3 combined with RA in Kelly cells. (A) Neurite outgrowth with standard deviations and significance. (B) Quantified western blots for differentiation markers. (C) Cell viability with standard deviations and significance.

In conclusion, the quantitative PCR results disproved the earlier hypothesis that CDKN3 would be downregulated following retinoic acid treatment. The retinoic acid treatment caused increased expression of CDKN3 which could suggest that CDKN3 overexpression is a mechanism by which neuroblastoma cells gain retinoic acid resistance; this experiment was only done in one cell line so further studies are required for confirmation. It was also found that siCDKN3 treatment combined with retinoic acid leads to slightly increased neurite outgrowth, differentiation markers, and decreased viability in Kelly cells. These results were not statistically significant nor did they imply any synergy, but this experiment should be repeated due to the error bars.

IV. SUMMARY AND CONCLUSIONS

The major goal of this project was to determine the role of CDKN3 in neuroblastoma differentiation. Since neuroblastoma arises from neural crest cells that fail to differentiate into mature neurons, one method of treating neuroblastoma involves forcing the neuroblastoma cells to differentiate into neurons which will no longer proliferate causing tumor growth.[3] The problem is that there is only one differentiation agent currently used to treat neuroblastoma: retinoic acid.[6] Retinoic acid has increased survival from this cancer, but there are many neuroblastoma cells that gain resistance to retinoic acid treatment.[6] The reason there is only one differentiation agent used to treat neuroblastoma is because the cause of differentiation loss is not clear.[2] Due to the close relationship between the cell cycle and differentiation it is possible that cell cycle regulators are partially responsible for loss of differentiation making them a possible therapeutic target.[18] One of these cell cycle regulators that has potential to be involved in neuroblastoma differentiation is CDKN3 which was identified in a HCS previously performed by Du *et al.*[42]

The goal of the project was pursued by breaking it down into three aims. The first aim was to demonstrate the function of CDKN3 in regulating cell differentiation and cell viability in neuroblastoma cell lines with different genetic backgrounds. The second aim was to determine the function of CDKN3 in regulating cell proliferation in neuroblastoma cell lines with different genetic backgrounds. The final aim was to determine whether CDKN3 plays a role in mediating the differentiation-inducing effects of retinoic acid and if there is a synergistic effect when combining siCDKN3 and retinoic acid. Overall the

results, which are summarized in **Table 4**, indicate that CDKN3 does have the potential to be involved in neuroblastoma differentiation regulation. The cell lines most affected by siCDKN3 were the cell lines with amplified MYCN: BE(2)-C and Kelly. The CHLA-90 cell line did not have the morphological features of differentiation, but did have the molecular features of a differentiated neuroblastoma cell, so it is possible that this cell line does not grow significant neurites when fully mature. The SK-N-DZ cells did express increased differentiation markers and lowered proliferation, but the viability of the siCDKN3-treated cells was not significantly different from the control cells, which could be due to the rate at which this cell line grows.

One of the most interesting results from this study was that retinoic acid can increase the relative expression of CDKN3. **Figure 16** summarizes this finding showing that neuroblastoma cells treated with retinoic acid leads to increased CDKN3 which may be a mechanism by which neuroblastomas gain retinoic acid resistance, however this is speculative since only one cell line was tested. It was also found that there is no synergy between combining siCDKN3 with retinoic acid treatment.

Table 4. Summary of results for neuroblastoma differentiation.

Cell Line	Neurite Outgrowth	Differentiation Markers	Viability	Proliferation
BE(2)-C	Significant	NSE, βIII-Tubulin	Lower	Decreased
CHLA-90	Not Significant	NSE, βIII-Tubulin, GAP43	Lower	Decreased
SK-N-DZ	Not Significant	NSE, βIII-Tubulin	Not Significant	Decreased
Kelly	Significant	NSE, GAP43	Lower	Decreased

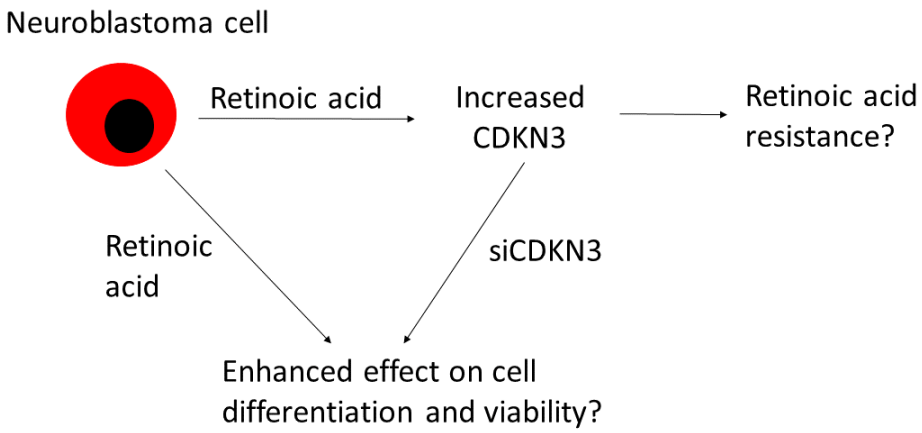

Figure 16. Potential relationships between retinoic acid treatment and CDKN3.

In the future, it would be beneficial to determine whether CDKN3 is responsible for retinoic acid resistance. This can be done by investigating the correlation between CDKN3 expression with retinoic acid sensitivity in a variety of neuroblastoma cell lines with differential retinoic acid sensitivities. This can be tested by measuring the expression levels of CDKN3 in neuroblastoma cell lines that are known to be resistant to retinoic acid treatment and cell lines that are sensitive to retinoic acid. If CDKN3 is responsible for retinoic acid resistance in neuroblastoma then the cell lines resistant to retinoic acid will have higher expression of CDKN3 compared to retinoic acid-sensitive lines. The next part could be to test if overexpression of CDKN3 in retinoic acid-sensitive cell lines can lead to retinoic acid resistance. If CDKN3 overexpression leads to retinoic acid resistance then the previously sensitive neuroblastoma cells will become resistant to retinoid acid induced differentiation.

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