Specific Dinucleotide Repeat siRNAs Decrease Proliferation and Viability of Human Ovarian Carcinomas via a DISE-dependent Mechanism

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Abstract

Trinucleotide repeat disorders manifest as triplet amplifications of specific genetic sequences that exceed a normal range threshold for the affected gene. The most representative example of this class of disorders, Huntington's disease, is characterized by expansion of a CAG repeat in the first exon of the huntingtin gene. Interestingly, Huntington's patients exhibit a dramatic reduction in the incidence rates of nearly all forms of cancer in relation to the general population. Recent evidence suggests that this remarkably low cancer prevalence among the Huntington's population is a consequence of the production of small interfering RNA molecules (siRNA) that selectively target and destroy the products of a subset of genes essential for cell survival, a process referred to as death induced by survival gene elimination (DISE). The current study was conducted to assess the effectiveness of a series of dinucleotide repeat siRNAs to induce DISE in a HeyA8 ovarian cell line. Results indicated that cells transfected with siCU and siUC repeats displayed significantly impaired proliferation and decreased cell survival via live-cell imaging and ATP viability assays, respectively, as compared to scrambled siRNA negative control. Furthermore, survival of cells treated with siCU and siUC were comparable to those transfected with siCAG, a previously validated DISE inducing trinucleotide repeat. Lastly, qPCR data derived from cells transfected with siCU and siUC revealed a 2-8 fold reduction in relative mRNA expression levels for three of four survival genes probed (LPP, CCND1, MYO10) as compared to GAPDH control, suggesting dinucleotide siRNA repeats may be viable cancer therapeutics.

1. Introduction

1.1 Trinucleotide Repeat Diseases

Short tandem repeats, also known as microsatellites or short sequence repeats, are repeated sequences of 1 to 6 nucleotides found in the human genome. These repeats are relatively common. They are often found in introns and the 3' untranslated region of mRNA, but can be found in exons as well. The longer the amount of nucleotides repeated, the less common that repeat is in the genome. By this logic, trinucleotide repeats are less common than dinucleotide repeats; hexanucleotide repeats are the least common while mononucleotide repeats are the most common. Repeats can be caused by mutations during DNA replication because errors are more prone to occur, therefore repeats are often labeled as unstable. Sometimes, diseases are caused by a mutation that creates a short repeated sequence.

Trinucleotide repeat disorders are diseases characterized by unstable triple repeat expansions in the human genome due to mutations. There are two main types of trinucleotide repeat diseases: those in which repeats are found within the exons, and those in which repeats are found within noncoding regions. Thus, these two groups can be termed polyglutamine repeat diseases and non-polyglutamine repeat disease. Examples of polyglutamine diseases include Huntington's Disease, Spinal and Bulbar Muscular Atrophy, and most Spinocerebellar Ataxias. These diseases all show progressive neuron degeneration, which causes a multitude of problems such as a deterioration of one's physical and mental capabilities. Each of the repeats in these disorders are found in different locations in the human genome, and each prompts a different result. For example, Huntington's Disease results in the

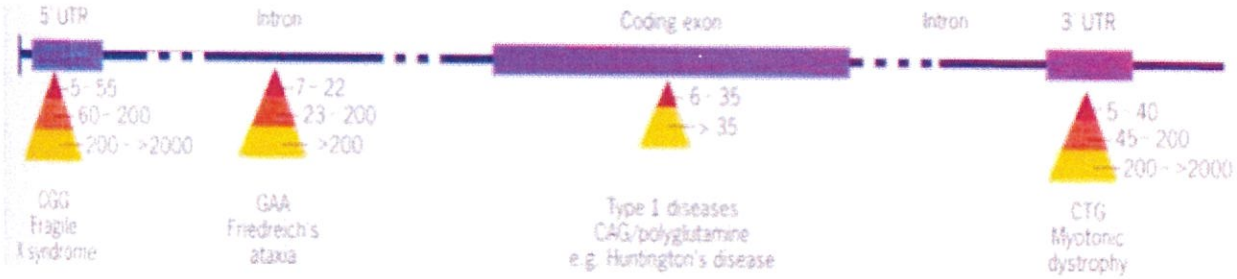

Figure 1. Trinucleotide Repeat Disorders are organized by the general location in a gene (intron, exon, etc.). Triangle portrays the number of repeats in normal individuals (red portion and corresponding number), carriers (orange portion and corresponding number), and patients with the disorder (yellow portion and corresponding number).
Source: Karp, Gerald. *Cell and Molecular Biology: Concepts and Experiments*. John Wiley, 2007. Kramer, Marcel, et al. "Alternative 5' Untranslated Regions Are Involved in Expression Regulation of Human Heme Oxygenase-1." *PLoS ONE*, vol. 8, no. 10, 2013. doi:10.1371/journal.pone.0077224.

transcription of a mutated protein, huntingtin, while other diseases silence transcription of a protein completely. Most of these polyglutamine disorders have the same trinucleotide repeat, specifically a CAG repeat. Examples of non-polyglutamine diseases include Fragile X syndrome, Myotonic Dystrophy, and Friedreich's Ataxia. Repeats can be found in introns or the 3' UTR of the gene. These diseases share less in common, and the specific trinucleotide repeats for each disease vary between the disorders. Similar to the polyglutamine trinucleotide repeat disorders, expression of the protein may be inhibited, or the protein produced may be malfunctioning.

1.2 Trinucleotide repeats and Huntington's Disease

It was previously found that the incidence of cancer in patients with Huntington's disease and other polyglutamine repeat diseases is significantly lower than that of the normal population. In patients with Huntington's disease, the incidence of cancer is around 80% lower than that of the normal population. This fact was originally attributed to the mutated protein, huntingtin, and its effect on the cells. However, the trinucleotide repeat expansions may play a role. In fact, in all polyglutamine trinucleotide repeat disorders, there is a significantly reduced incidence of cancer. Trinucleotide repeats have been found in the mutated genome of many patients with neurological diseases. In huntington's disease, the expressed repeat is a CAG repeat. Further experiments

show that small interfering RNAs based on certain trinucleotide repeats may be toxic to cancer cells, the most toxic being siCAG. These siRNAs dramatically slow cancer cell proliferation. Additionally, further testing with siCAG shows that siCAG preferentially causes DISE in cancer cells, and does not affect normal cells. This may be due to the miRNAs that occur naturally in human cells. Since cancer cells contain less miRNAs uses the RISC, the siRNAs are able to access the RISC in cancer cells, whereas in normal cells, miRNAs are constantly accessing the RISC which prevents the toxic siRNAs from knocking down gene expression.

1.3 Death Induced by Survival gene Elimination (DISE)

Survival genes are defined as genes that are imperative for a cell to live. Elimination of any one of these genes will result in cell death. Death induced by survival gene elimination is a type of cell death that involves the stimulation of more than one apoptotic pathway. This is achieved by the targeting of multiple survival genes simultaneously. One way to induce DISE is by RNA interference. RNA interference (RNAi) is a method of post transcriptional gene regulation in which small RNA molecules are incorporated into the RNA Induced Silencing Complex (RISC). The RISC then seeks the mRNA complementary to the small RNA strand, and either cleaves the mRNA, or causes degradation. This prevents the expression of the gene. In the body, this process includes miRNAs, but synthetic induction of this process involves exogenous siRNAs. The recommended concentration for these siRNAs is between 5nM to 100 nM. siRNAs are often used to knock down a specific gene in order to assess its function in the body. However, if siRNAs are able to knock down a gene imperative to cell functioning, the cell would not be able to survive. The use of non-specific siRNAs can target and degrade multiple mRNAs, resulting in decreased expression of multiple genes. DISE is a promising method to induce death

in cancer cells because of the high mutability of cancer cells. While one target may definitely kill cancer cells at first, the cancer cells can mutate and that precise targeting will not be as effective. On the other hand, if there are multiple death pathways, a cell would have to undergo numerous mutations before it would be immune to DISE. The elimination of one pathway would not stop the process of DISE from occurring, yet only one pathway would be necessary for cell death. Thus, DISE is an avenue for future possible treatments for cancer.

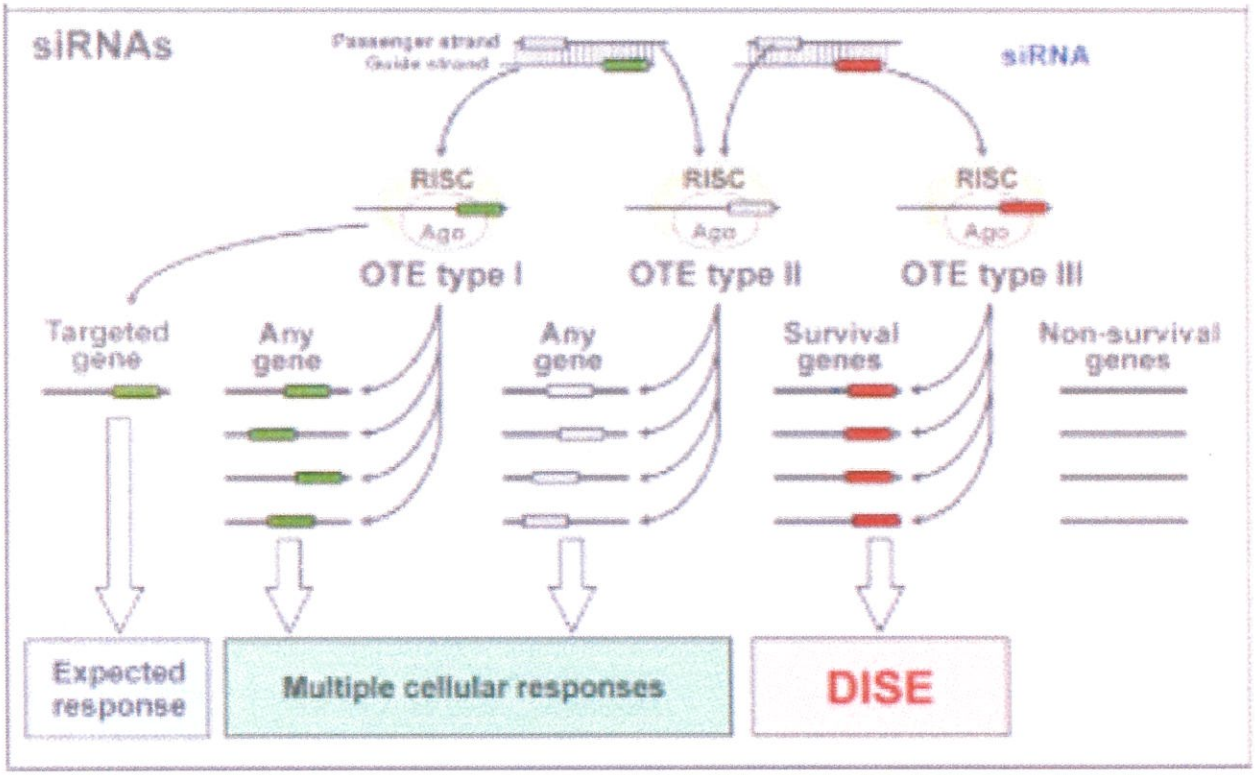

Figure 2. The figure shows the mechanism by which siRNAs impact gene expression. While the targeting of a specific gene leads to a specific response, the targeting of multiple genes leads to a multitude of cellular responses. By targeting various survival genes at once, DISE is observed. **Source:** "Peter Lab." *DISE - a Novel Concept for Cancer Therapy: Peter Lab: Feinberg School of Medicine: Northwestern University*, labs.feinberg.northwestern.edu/peter/research/dise.html.

1.5 Rationale

The role of trinucleotide repeats in the genome, and the ability of trinucleotide repeat siRNAs to hinder cancer cell proliferation, specifically the toxicity of siCAG, is thoroughly being studied. However, there is a possibility that other short repeat sequences can also cause DISE in cancer cells. Since dinucleotide repeat sequences are more common than trinucleotide sequences, I wanted to assess if there were more toxic dinucleotide repeats. Given the frequency of dinucleotide repeats, there are certain dinucleotide repeats in survival genes that can be nonspecifically targeted through RNAi, and thus dinucleotide repeat based siRNAs should be able to effectively knock down survival genes and cause cell death in HeyA8 cancer cells.

2. Materials and Methods

2. 1 Cell Culture and siRNA Reverse Transfection

HeyA8 cells (ATCC TCP-1021™) were defrosted from a supply, and grown in Roswell Memorial Park Institute (RPMI) medium with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine. After cells were cultured to a confluency of around 70% (72 hours), cells were trypsinized, counted, placed in antibiotic free RPMI medium (10% Fetal Bovine Serum (FBS) and 1% L-glutamine), and diluted such that there were 1000 cells per 250 microliters. The siRNAs were mixed with optiMEM and the transfection reagent RNAiMAX (Thermo Fisher Scientific) in different specified concentrations (either 0.01 nM, 0.1 nM, 0.5 nM, 1 nM, or 10 nM). 50 microliters of reagents were plated into each used well of a 96-well plate. The siRNAs included the dinucleotide repeats ACACACACACACACACACA (siAC), CACACACACACACACACAC (siCA), AGAGAGAGAGAGAGAGAGA (siAG), GAGAGAGAGAGAGAGAGAG (siGA) , UCUCUCUCUCUCUCUCUCU (siUC), CUCUCUCUCUCUCUCUCUC (siCU), UGUGUGUGUGUGUGUGUGU (siUG), and

GUGUGUGUGUGUGUGUGUG (siGU), the trinucleotide repeat already found to be toxic CAGCAGCAGCAGCAGCAGC (siCAG), and the non-targeting sequence of known low toxicity UUAGUCGACAUGUAAACCAAA (siNT1). siNT1 acted as a positive control, along with the mock transfection which contained RNAiMAX but no siRNA, cells with optiMEM and the untreated cells. siCAG acted as a negative control because its known toxicity is very high, especially when compared to siNT1. Two OMe groups were added to positions 1 and 2 of the anti-sense strand of each siRNA to allow only the sense strand to load into the RISC.

After 20 minutes, 250 microliters of the cell suspension were plated into each well. For the IncuCyte experiment, the plate was left at room temperature for 30 minutes before it was placed in the IncuCyte machine. For the viability assay, the plate was placed in an incubator at 37 °C with 5% CO_2 until the assay could be performed.

2. 2 IncuCyte Imaging and CellTiter-Glo Viability (ATP) Assay

After transfection cells were scanned and monitored using the IncuCyte ZOOM live-cell imaging system (Essen BioScience), which was contained in an an incubator at 37 °C with 5% CO_2. Two images of each well were taken every four hours and at the starting point. Confluency of each well was calculated by the IncuCyte software. The CellTiter-Glo viability (ATP) assay was performed after the cells were grown for either 72 hours or 120 hours. Old media in each well was replaced with 70 microliters of fresh media, then 70 microliters of CellTiter-Glo reagent (Promega) was added to each well. Plates were covered, shaken for 5 minutes and left at room temperature for 15 minutes. Plates were then inserted into the BioTek Cytation 5, where luminescence was measured. Graphs from the CellTiter-Glo viability (ATP) assay and the Incucyte imaging were student generated.

2. 3 Forward siRNA transfection, RNA isolation and qPCR

HeyA8 cells were seeded into two 6 well plates at a concentration of 50,000 cells per well and cultured overnight before the transfection reagents were added. The siRNAs transfected were siCU, siUC, and siNT1. Three wells were allotted per treatment After a period of 72 hours, cells were lysed with QIAzol. mRNA was isolated using the Qiagen miRNeasy mini kit. Then, cDNA was made using the cDNA reverse Transcription Kit (Applied Biosystems) of highly expressed survival genes in HeyA8 cells that contained the reverse complement repeat sequence (a AG/GA repeat) in the mRNA for that gene. These genes included SLC20A1, MYO10, CCND1, and LPP. The control was GAPDH. After that, qPCR was performed, and data was generated by the qPCR machine.

3. Results and Discussion

3. 1 siRNAs slow HeyA8 cell proliferation

Reverse transfection of various dinucleotide repeat siRNAs was performed and plates were put into the IncuCyte ZOOM live-cell imaging system to track the growth of HeyA8 cells. All of the dinucleotide repeat siRNAs slowed the proliferation of HeyA8 cells. siCAG, which had been previously found to be toxic to HeyA8 cells stagnated growth immediately, while most of the dinucleotide repeats slowed growth, but not to the level of siCAG. siGU treated cells were the first to start to grow out. siUC treated cells grew at first, however cell growth stopped and reached the level of the siCAG treated cells. siUC treated cells did not grow out within the time period monitored, thus indicating the toxicity of siUC is close to the toxicity of the siCAG. Most of the dinucleotide repeat treated cells grew out after a time period. This is likely due to the transient nature of siRNA transfection, which was not apparent in cells treated with siUC or

siCAG. It is possible that siUC kills most cells initially, and thus that is why the effect lasts longer and growth stops. Additionally, siCU should be very similar in its toxicity to siUC due to the fact that it is only a frameshift, so the fact that siUC has a much higher toxicity is intriguing. Thus assays are performed to assess the difference in cell viability between siCAG, siNT, siCU and siUC

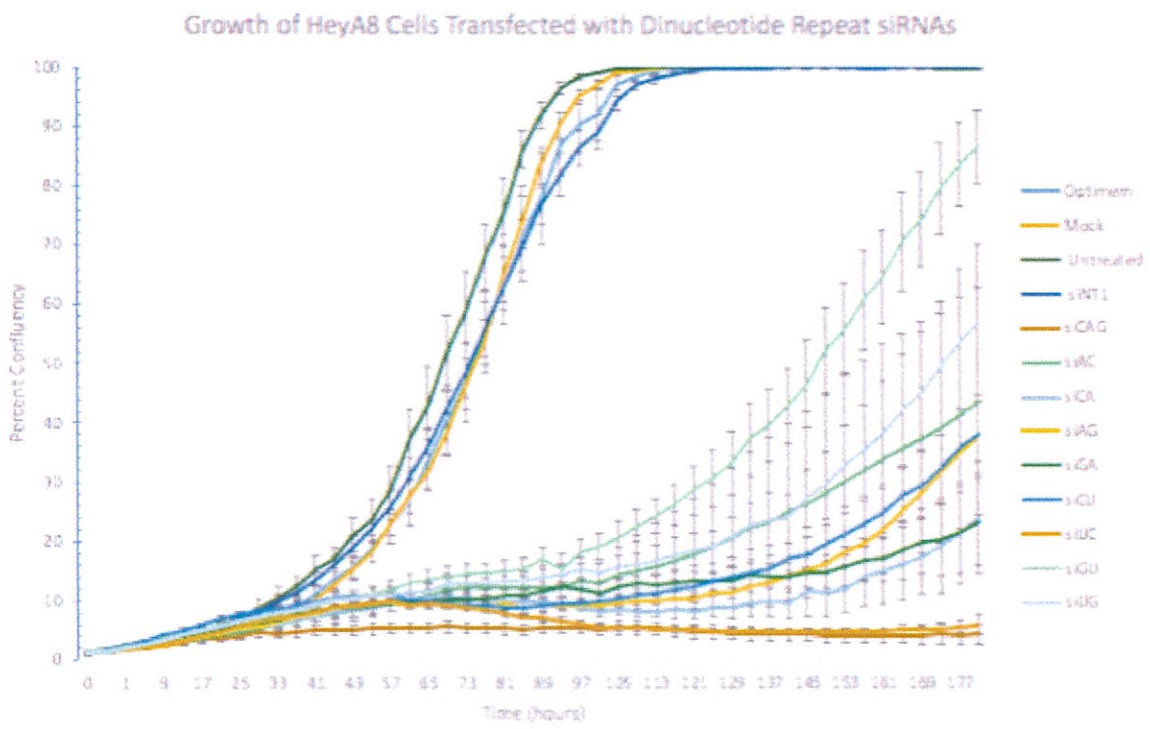

Figure 3. The general trend for the cells treated with the different dinucleotide repeats was an stagnated increase in cell growth. siGU had the least effect on HeyA8 cells, while siUC was the most effective, and comparable to the toxicity of siCAG. siRNAs were at a concentration of 1 nM. Student generated.

Images of siUC and siCU both showed evidence of blebbing, and a decreased amount of cells when compared to the siNT1 treated cells. Blebbing is a common characteristic of DISE, and,

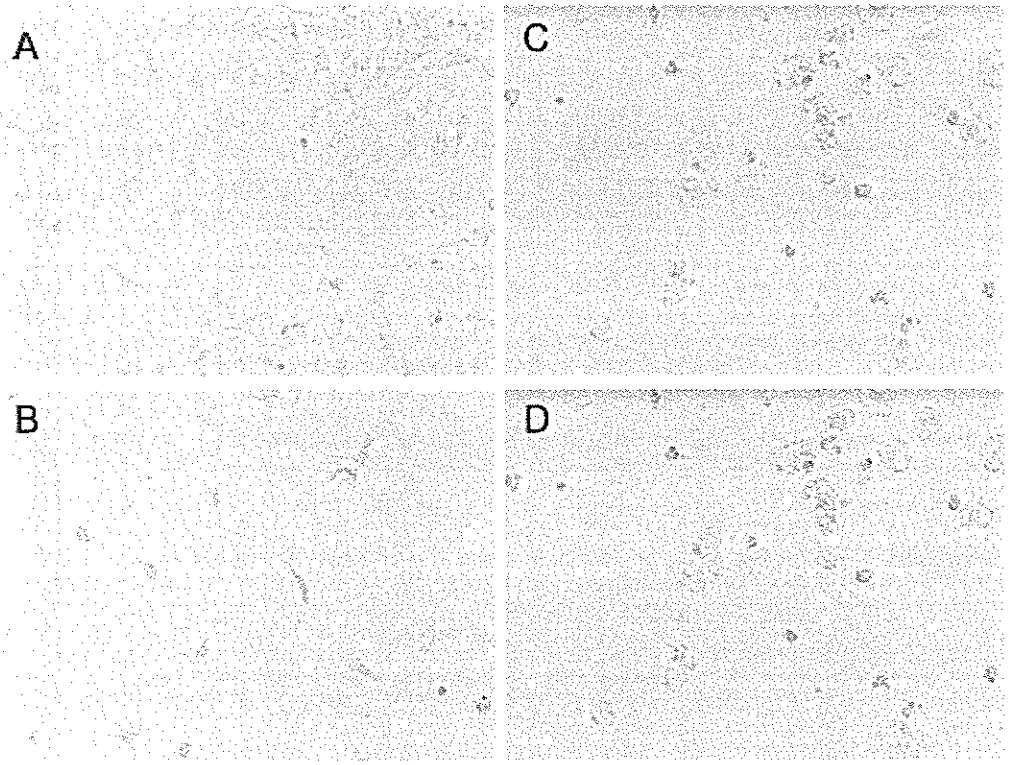

Figure 4. Picture A shows cells treated with siNT1, and the extent to which the HeyA8 cells grew. Picture B shows the sparsity of cells treated with siCAG, and pictures C and D shows the blebbing of cells in cells treated with siCU and siUC respectively. There is an indication of the cytosol of the cell outside of the cell membrane, which is another characteristic in line with previous studies regarding DISE.

therefore, it is likely that both siUC and siCU cause DISE in HeyA8 cells. There is also evidence of cytosol spillage, indicating the burst of the cell, which is another characteristic of DISE. siCAG and the most toxic dinucleotide repeats showed a similar ratio of dead cells to stressed living cells at 72 hours. Most likely, siUC causes DISE, and thus may be a possible avenue for cancer treatment.

3. 2 siCU and siUC reduce cell viability

To test the ability of siCU and siUC to decrease cell viability, the CellTiter-Glo Viability Assay was performed. Additionally, another goal was to assess the effect of different

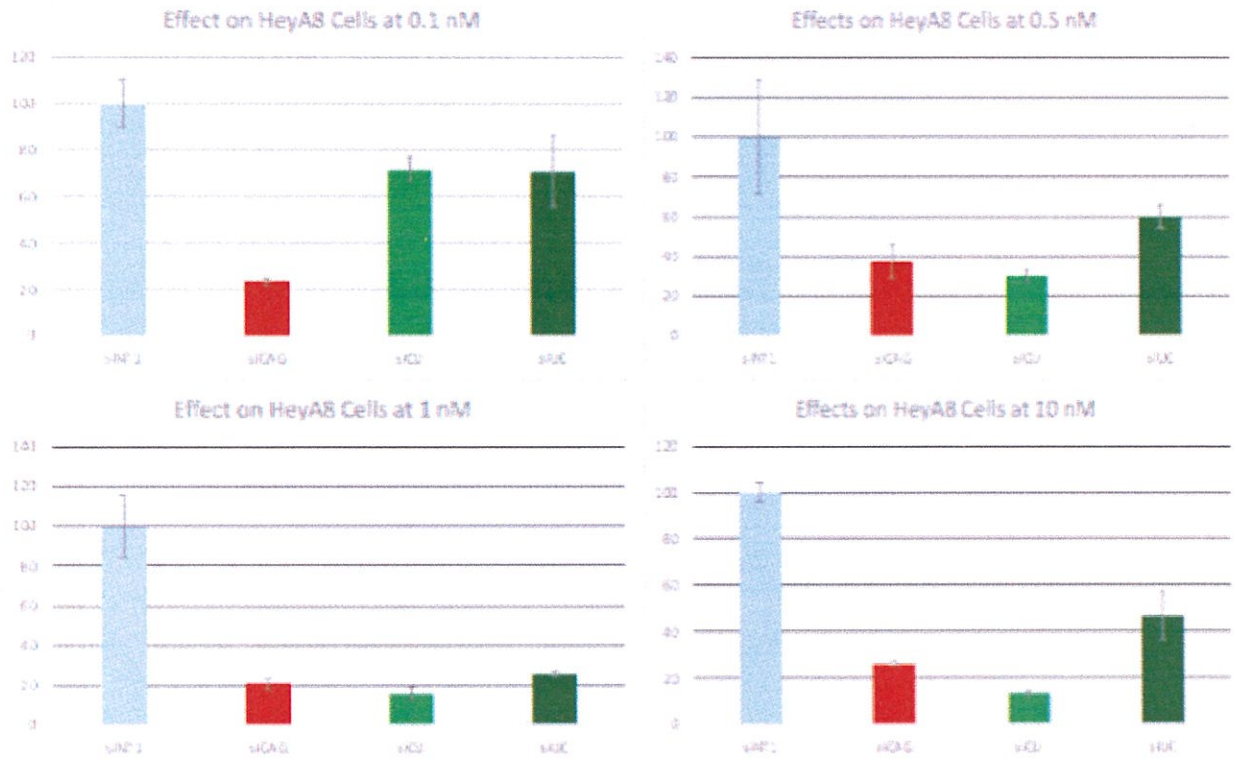

Figure 4. Y-axis is cell viability in comparison to siNT1 treated cells. Different concentrations of siRNAs and the ATP assay results at 72 hours. There is an immediate decrease in viablity for siCAG, while siCU and siUC need a high concentration to have the same effect. siCU seems to decrease cell viability more than siUC, which does not align with the IncuCyte results. Student generated graphs.

concentrations of siCU and siUC on ATP production and therefore cell viability. siCU and siUC did not affect cell viability as much as siCAG in lower concentrations, however both did have an effect that was much more comparable to siCAG when in a larger concentration. When the assay conducted after 72 hours, siCU and siUC were not as effective as siCAG, especially in the lower concentrations. However in the assay conducted after 120 hours, viability of siUC and siCU

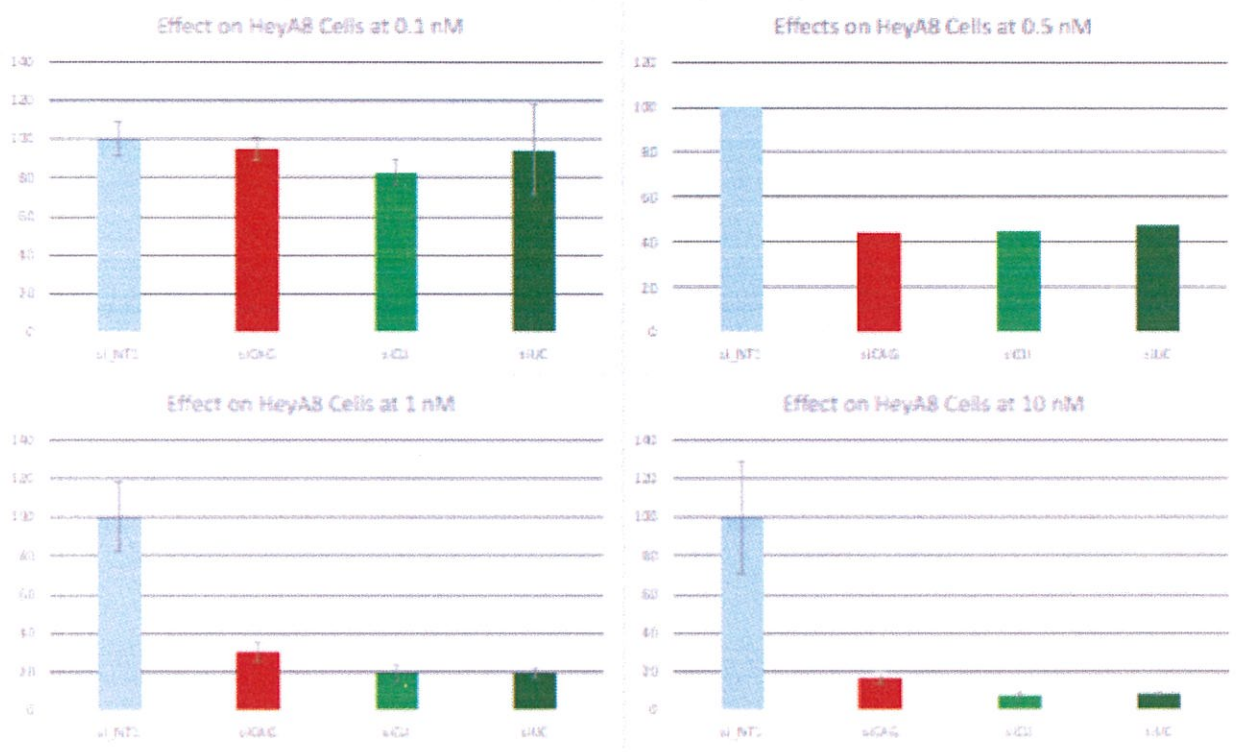

Figure 5. Y-axis is cell viability. Different concentrations of siRNAs and the ATP assay results at 120 hours. The toxicity of siCU and siUC seem to be much more comparable with that of siCAG. siCU seems to decrease cell viability faster than siUC. At higher concentrations, siUC and siCU show less cell viability than siCAG. Student generated graph.

treated cells were comparable to the effects of siCAG on the cells.

3. 3 siCU and siUC knockdown survival genes

SLC20A1, MYO10, LPP and CCND1 are highly expressed survival genes in HeyA8 cells. Further analysis showed that these genes all contain the reverse complement of CU or UC (GA/AG) in either the ORF of the mRNA or the 3'UTR of the mRNA. SLC20A1 and MYO10 expressed the repeat in the ORF of the mRNA, while CCND1 and LPP expressed the repeat in the 3'UTR of the mRNA. To assess whether or not siCU and siUC were able to degrade mRNA and thus decrease the expression of these genes, qPCR was performed. siCU and siUC knocked down three of the four survival genes tested. Out of the four genes tested, SLC20A1 mRNA was

approximately 1.5 times higher relative to GAPDH. There was a 50% decrease of MYO10

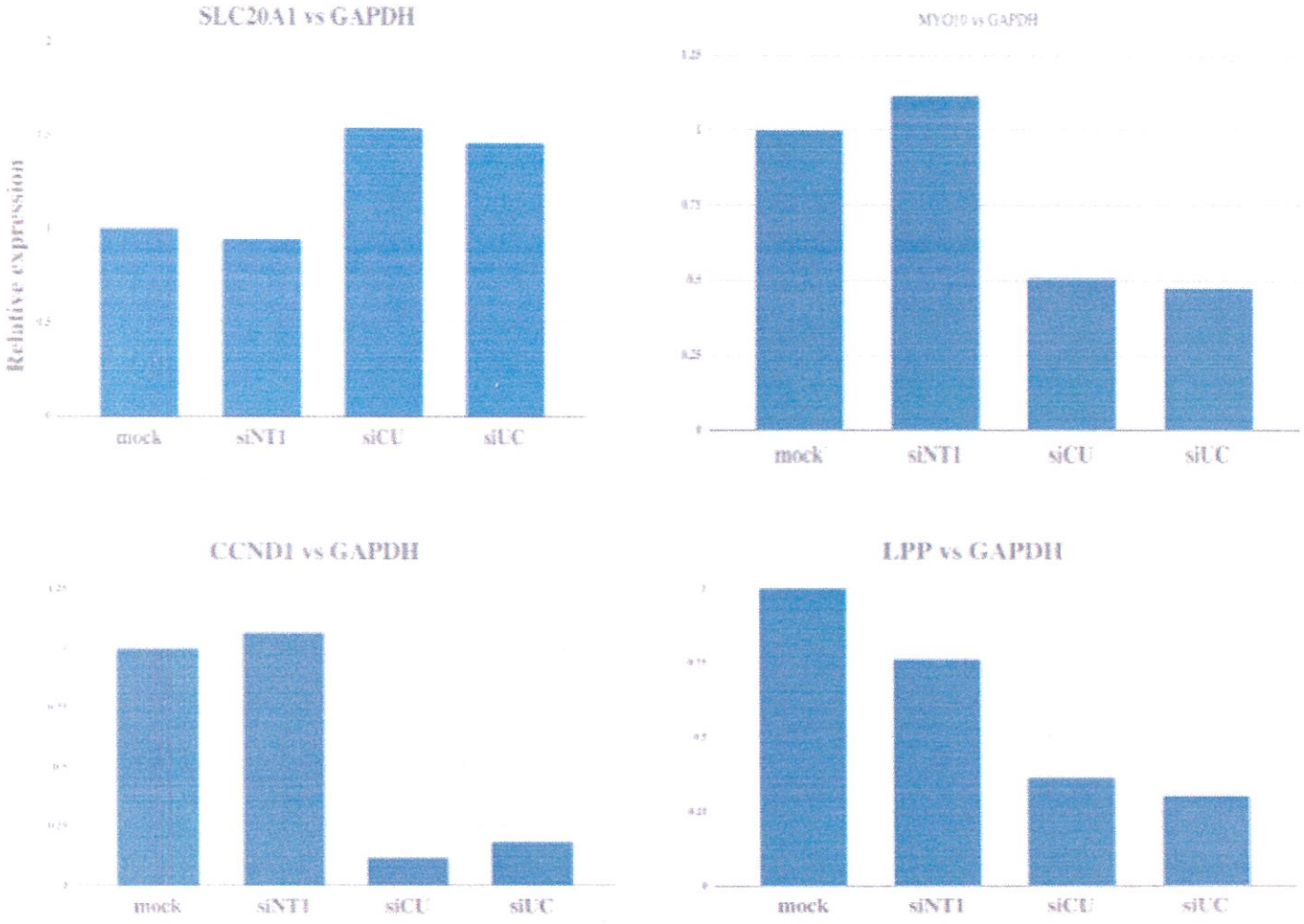

Figure 6. SLC20A1 and MYO10 had repeats in the ORF. SLC20A1 had an increased expression level, whereas the rest of the survival genes had a significantly decreased expression level. LPP and CCND1 both exhibit repeats in the 3` UTR. The expression level of these genes decreased dramatically in comparison to the genes containing repeats in the ORF. Relative expression was compared to GAPDH levels. Student generated image.

mRNA. Both LPP and CCND1 showed decreased expression as well, more so than the decrease of MYO10 expression.

5. Conclusion and Future Research

RNAi can be used as a method to combat cancer cell proliferation, due to the process of DISE. Repeat sequences in the form of small RNAs can be utilized to combat cancer due to their frequency in the genome and therefore their ability to target the expression of multiple survival

genes. Recently, the high toxicity of a trinucleotide repeat associated with Huntington's disease has led to the interest in short tandem repeats as a treatment for cancer. Thus, the toxicity of dinucleotide repeats in siRNA were tested by looking at both the growth curve of cells treated with dinucleotide siRNA repeats, and toxic siRNAs were further assessed to analyze their impact on cell viability. According to the growth curves, siUC was the most toxic dinucleotide repeat showing almost the same toxicity as the siCAG repeat. Its frameshift siCU was more effective in decreasing cell viability as shown by the CellTiter-Glo Assay. Both siCU and siUC were able to knock down three out of the four survival genes tested, where a repeat was found in both the open reading frame and the 3' untranslated region. In the future, it would be beneficial to assess the effect on cell viability of other repeat siRNAs, specifically mononucleotide repeats because they are more common, and thus would possibly provide more apoptotic pathways to target nonspecifically. Furthermore, it would be beneficial to pursue further studies with siUC and siCU to assess if they act like siCAG in that they preferentially kill cancer cells with DISE.

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