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Understanding the Role of DNA Methylation in Carbon Black-Induced Endoplasmic Reticulum Stress Response Elements (ERSE)

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Summer Undergraduate Research in Biology
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Objectives

1. Grow and subject different cell lines to Carbon Black nanoparticle stress.
2. Use bisulfite conversion to detect methylation of cytosines in purified genomic DNA
3. PCR amplify promoter regions of ERSEs and confirm amplification through polymerase chain reaction (PCR) for use in sequencing applications.
4. Analyze methylation changes of ERSE promoters by Sanger Sequencing, looking at the differences of methylation on CpG sites.

Further Directions

I. Use the same process to amplify the remaining 25 primer pairs, using higher concentrations of isolated DNA.
II. Produce more replicates for each treatment with each primer pair in order to discover methylation changes rather than methylation variation at different C-G sites.
III. Introduce Illumina Sequencing to enhance understanding of CB-induced stress related methylation patterns at the singular strand level.
IV. Treat LUHMES neurons and other cell types to see if gene expression changes in various other cell types and illuminate whether each response is cell specific or general across most cell types.
V. Use fluorescence microscopy to study organelle morphology changes when exposed to CB stressors.

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Introduction

Carbon Black (CB) is an ultrafine nanoparticulate, 20-50 nm in size, that is a pollutant produced by the combustion of fossil fuels. CB is small enough to be suspended in the air of polluted environments.[1] In 2017, over 1600 nano-particulate containing consumer projects were registered in the global market.[3] Nanoparticulates plague our lives in many different ways. The production of coal, quartz, diesel production, and the utilization of asbestos provides constant exposure to these ultrafine nanoparticulates.[3] When inhaled, CB's minute size allows it to enter the body and enter into the cellular cytosolic space via transmembrane transport.[2] Cellular accumulation of CB nanoparticulates have been shown to induce decreased cell division, stress signaling, pro-inflammatory signaling and apoptosis.

In some cases, nanoparticles have been shown to induce the unfolded protein response (UPR), an adaptive and potentially apoptotic signal emerging from the endoplasmic reticulum (ER). In this process, cellular stress can result in the accumulation of misfolded proteins in the endoplasmic reticulum (ER). Consequently, the cell initiates a response through various pathways (IRE1, ATF6, PERK) to aid in proper protein folding. Germane to this process is the activation of various pro-survival genes found within the nucleus of the cell. However, if the cell is subjected to chronic UPR stress, pro-apoptotic signaling cascades will begin transcription of pro-apoptotic genes leading to cell death. Clearly gene transcription in the nucleus is a central mediator of the URP signaling response pathway, yet the role of epigenetic regulation on this process has not been studied in detail.

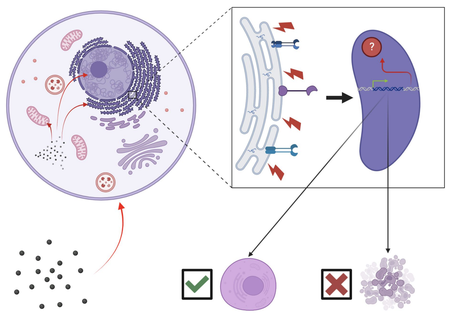

Figure 1. Nanoparticles can initiate the UPR and trigger the expression and repression of pro-survival and pro-apoptotic genes

Transcription can be regulated in many ways, one of those being DNA methylation on cytosine, which can alter promoter activity.[4] Promoter regions are found within all genes and are usually located 100-1000 bp upstream of the first exon.[8] Cytosine methylation is associated with decreased expression of a gene. Studies have previously focused on the interplay between the UPR and chromatin changes as it related to cancerous cells and numerous other diseases as well as how nanoparticles induce ER stress.[5,6] However, studies have not aimed at understanding the role of DNA methylation as it relates to CB-induced stress. **Our hypothesis is that CB induces epigenetic modification of promoter elements for stress response genes through the process of DNA Methylation.**

Methodology

1. **Growth and Treatment of Cells:** Two cell types were selected for the study of this topic: LUHMES neurons and THP1 macrophages. Cell types were selected due to previous literature suggesting the effect of CB nanoparticles on cellular stress of mouse offspring.[7] As a result, human cell lines were used for the subsequent experiments to further illuminate the effect of CB on these cells in humans. Treatments were prepared using different dosages of CB100 as well as a DNA Methyl-Transferase Inhibitor ($DNMT_i$). Figure 2

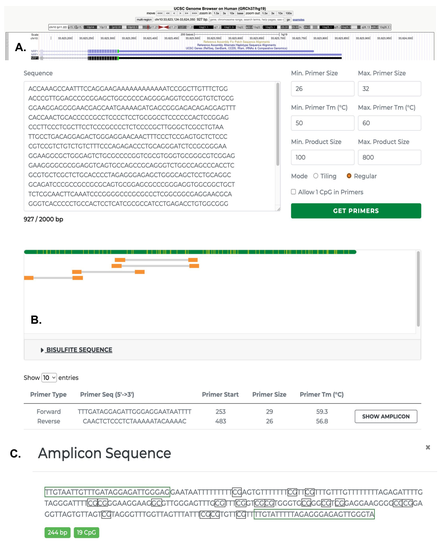

Figure 3. (A) Selection of 800 bps containing the NRP1 promoter. (B) Input and readouts of primer pairs of the bisulfite converted DNA. (C) Amplicon sequence showing both the primers and the region to be amplified.

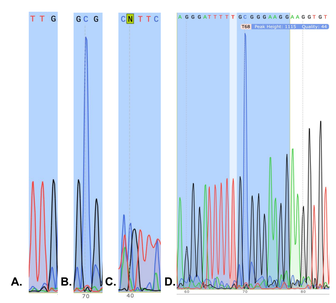

Figure 6. (A) Bisulfite converted sample 2 at site 4 showing demethylation (T-G). (B) Methylation shown at site 5 (C-G) of sample 2. (C) Too much noise to decipher a base in sample 5. (D) Overall view of a chromatograph with low noise.

2. **Design of Primers to Interrogate CpG Sites:** To understand gene expression, a set of 10 genes were selected based on the presence of ERSE motifs (High G-C content) within their promoter regions. Further, these genes were sorted into five categories: ER stress, general signaling, oxidative stress, proteasome-related, and nanoparticle induced. Promoter regions selected for amplification were synthesized by selected 800 bp ahead of the first exon DNA was processed by a bisulfite-specific primer design tool by Zymo Research. The primers were selected based on the amount of C-G's found within the amplicon. Primers needed to be at least 26 bp's in size in order to account for loss of a base after bisulfite conversion. Primers were ordered as oligonucleotides. (Figure 3)

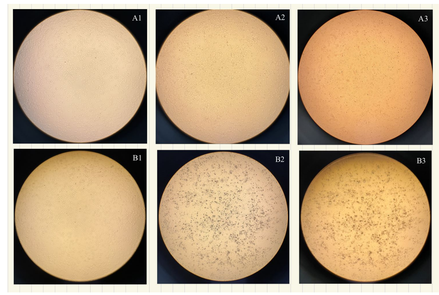

Figure 2. (A) Different treatments of THP1 cells right after treatment (1-Mock, 2-CB 50 ug/mL, 3-CB 100 ug/mL). (B) THP1 cells 24 hours after treatment (1-Mock, 2-CB 50 ug/mL, 3-CB 100 ug/mL).

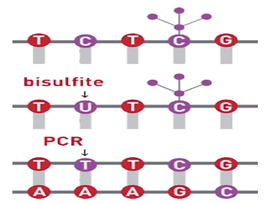

Figure 4. Process of Bisulfite conversion.

3. **Bisulfite Conversion Steps:** Bisulfite Conversion is the process by which methylated cytosines can be distinguished from unmethylated cytosines.[9] A combination of chemicals converts unmethylated cytosines into uracils, while leaving the methylated cytosines unchanged.[9] In this way, after PCR amplification, unmethylated cytosines are converted into thymine and methylated cytosine bases remain cytosines (Figure 4). By taking the original DNA and comparing, one can determine methylation changes.

4. **Analyzing Broad Methylation Variation:** Sanger Sequencing was used on purified bisulfite converted DNA to understand broad methylation changes within a certain treatment of DNA. Treatments included Mock, CB50, CB100, and 5-Aza (a $DNMT_i$). By comparing the original strands from the samples of different treatments, one can decipher broad methylation changes at different cytosine-guanine sites. (Figure 5) (Figure 6)

```
11_f1      T-G-TGGGAAGGAAGGTGTTGGGAGTTTGTGTTTTGGTTGTGTGGGTGTG
1_Bisulf   C-G-CGGGAAGGAAGGCGTTGGGAGTTTGCGTTTCGGTCGCGTGGGTGCG
1_f1       T-G-TGGGAAGGAAGGTGTTGGGAGTTTGTGTTTTGGTTGTGTGGGTGTG
2_f1       T-G-CGGGAAGGAAGGTGTTGGGAGTTTGTGTTTTGGTTGTGTGGGTGTG
5_f1       TCGTCGGGAAGGAAGGTGTTGGGAGTTTGTGTTTTGGTTGCGTGGGTGTG
6_f1       C-G-TGGGAAGGAAGGTGTTGGGAGTTTGTGTTTTGGTTGTGTGGGTGTG
             *  ********** ********** **** *** * ******* *
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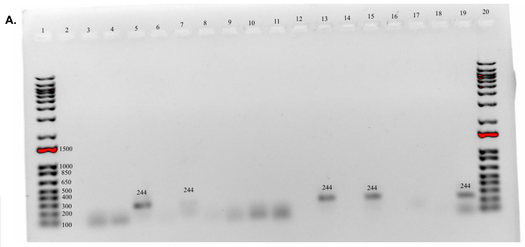
Figure 5. T-Coffee Alignment readout showing each amplicon matched with the expected amplicon sequence (* = matching sequences; highlighted region = C-G site in which there is a difference from the original amplicon).

Results

After isolating the DNA and performing bisulfite conversion, DNA was treated with various primer pairs in order to amplify different promoter regions. Of the ten genes analyzed, NRP1 and NHIP showed amplification of promoter DNA regions after bisulfite treatment. Below is a representative gel of PCR amplification of the NHIP and NRP1 promoter. There were three replicates (lanes 2-11; 12-19 and 22-23; and 24-33) consisting of amplified DNA from 5 different treatments. These five treatments were *Mock + 5-Aza* (2-3; 12-13; 24-25), *CB50* (4-5; 14-15; 26-27), *CB50 + 5-Aza* (6-7; 16-17; 28-29), *CB100* (8-9; 18-19; 30-31), and *CB100 + 5-Aza* (10-11; 22-23; 32-33). After running the gels at 74 volts and 65 mAmps for 97 minutes, results indicated that the primer pair of NRP1 [1] was successful and amplified correctly as indicated by a 244 bp band as predicted by the amplicon (Figure 7).



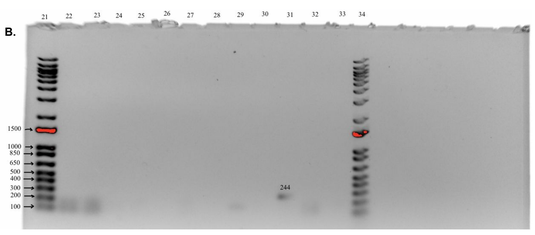


Figure 7. (A) Part one of the gel, replicate 1 (2-11) show successfully amplified NRP1 gene (244 bp product) in wells 5 and 7. Replicate 2 (12-19; 22-23) shows amplified bands in well 13, 15, and 19. (B) Part two of the gel. Replicate 3 (24-33) shows a successfully amplified band at well 31.

Sanger Sequencing is a method used to ascertain the nucleotide sequence of a sample of DNA. It cannot tell you strand by strand but can give you the average of all samples. Sanger sequencing utilizes chain-terminating ddNTPs that illuminate differently when treated with laser excitation.[10] Our DNA treated samples were subjected to a column clean-up and then sent off for sequencing analysis. The chromatograph readout is shown below (Figure 6). By studying each C-G site inferences on methylation changes and variation can be assessed. Data was compiled into a table and show that C-G sites 2-6, 8-10, and possibly 17-19 are sensitive to CB dosage and DNMT inhibition. Contrastingly, C-G sites 7 and 11-16 show no variation in response to CB amount and DNMT inhibition. The table is shown below (Table 1).

	TTTTTC GAGTG	TTTCGTTCGTTT		AGGGATTTTCGCGGGGA AGG		GGAAGGC GTTG	GAGTTT GCGTT	TTCGGTCGCGTGGG		
CpG Island	1	2	3	4	5	6	7	8	9	10
Sample 5	N/A			T (mix/mainly methyl)	T (mix/mainly demethyl)	T/C	T (demethyl)	T/C	T/C	C
Sample 1	N/A	T/C	T/C	T/C	T/C	T/C	T	T	T	T
Sample 6	N/A		methyl (hard to tell)	C (mix)	T (mix)	T (demethyl)	T (demethyl)	T	T	T
Sample 2	N/A	T	T	T	C	T	T	T	T	T
Sample 11	N/A	T	T	T	T	T	T	T/C	T/C	T/C
	GTGCGGGCGTCGGAGG			AAGGGGCGCGGAGG		GTGTTAGT CGTAGGGT	TAGTTTATTTCGGCG TGT		GTTCGTTTT GTATT	
CpG Island	11	12	13	14	15	16	17	18	19	
Sample 5	T	T	T	T	T	T	T	T (busy)	T (could be C)	
Sample 1	T	T	T	T	T	T	A (not reliable)	C (not reliable)	C (not reliable)	
Sample 6	T	T	T	T	T	T	N (weird)	C (weird)	C	
Sample 2	T	T	T	T	T	T	T	T	T (really busy)	
Sample 11	T	T	T	T	T	T	T (very busy)	T (less busy, some C)	C	

Table 1. Methylated and Unmethylated CpG Sites (19 sites). Sites with variation are highlighted in yellow and orange. Sites 17-19 were hard to determine so conclusions are drawn with less confidence. Sample 5 was Mock + 5-Aza; Sample 1 and 6 was CB50 treated; Sample 2 was CB50 + 5-Aza treated; and Sample 11 was CB100 treated.