Cadmium-Induced Transcription by NFATc2 in Breast Cancer Cells

Natalie Miller

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Thesis Advisor:

Dr. Jennifer Kugel, Department of Chemistry and Biochemistry

Honors Council Representative:

Dr. Joseph Falke, Department of Chemistry and Biochemistry

Committee Members:

Dr. James Goodrich, Department of Chemistry and Biochemistry

Dr. Jennifer Martin, Department of Molecular, Cellular and Developmental Biology

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Abstract

Although breast cancer is the second leading cause of cancer death among women in the United States, many of the mechanisms behind breast cancer development are unknown. It is known, however, that mis-regulated gene expression contributes to cancer development. Gene expression is tightly regulated at transcriptional initiation both by the formation of the pre-initiation complex and assembly of a host of other transcription factors, which act to promote expression of specific genes. NFATc2 is one such transcription factor implicated in breast cancer progression. This protein is regulated by intracellular calcium levels that may be induced by exposure to the environmental carcinogen, cadmium. This project aims to understand how exposure to cadmium may promote breast cancer cell invasiveness and proliferation via the NFATc2 transcription factor. MDA-MB-231 breast cancer cells exposed to 9 μM cadmium chloride ($CdCl_2$) showed increased expression of some NFATc2-regulated genes, specifically COX-2 and IL-8. This response was shown to be cell type specific. Preliminary knockdown of NFATc2 by siRNAs show NFATc2 dependence for the cadmium-induced increase in COX-2 and IL-8 transcription. Cadmium may induce transcription of many genes regulated by NFATc2 that are important to breast cancer progression and invasiveness, which further experiments need to address.

Introduction

Breast Cancer

Cancer has become the second leading cause of death among adults in the United States. In 2016 alone, the American Cancer Society predicts more than 1.6 million people will develop cancer, and nearly 600,000 will die because of their illness.[1] Given current rates of incidence, increasing survival rates, and increasing treatment costs, cancer costs are estimated to exceed $172 billion dollars by the year 2020.[2] Cancer, then, has become a significant burden on the United States, both economically and in terms of lives lost.

Aside from skin cancers, breast cancer is the most common form of cancer in women. About 1 in 8 women are anticipated to develop breast cancer in their lifetime and, in 2016, breast cancer will account for approximately 29% of all new cancer cases in women.[1,3] Following lung cancers, breast cancer is the second leading cause of cancer death.[4] Although incidence and mortality rates of nearly all cancers (including breast) have decreased in the last decade, cancer remains a significant problem and mortality from the disease is still too high.[1,3]

It is well known that cancers can arise from mutations in DNA, although the cause of these mutations can vary widely and, in many cases, is not known at all. Non-genetic risk factors also contribute to cancer initiation and progression. Breast cancer has been linked to a variety of factors such as age, heritable mutations, obesity, birth control, and environmental factors.[5] Heritable genetic mutations include those in the BRCA1 and BRCA2 genes, which have been identified to cause 5-10% of breast cancers. However, not all cancers arise from purely genetic reasons. A variety of naturally occurring substances have long been identified as carcinogens (compounds capable of causing cancers). Environmental substances, such as cadmium, may induce mutations or trigger changes to the cell that result in cancer. The mechanisms by which

environmental substances contribute to cancer are varied and often not clearly understood. My lab is interested in understanding how specific environmental carcinogens impact transcriptional regulation in breast cancer cells.

Transcription by RNA polymerase II

For proper growth, development, and viability of a cell, expression of genes is tightly regulated. One of the most tightly regulated steps in gene expression is transcription. Transcription of protein-coding genes is the process by which DNA is copied into mRNA. In eukaryotes, the mRNA undergoes processing to make the copied mRNA functional for translation into a protein. Initiation of transcription is the primary regulatory point for differential gene expression.[6] This important step is predominantly regulated by the large number of proteins that must come together to transcribe a specific sequence.

Eukaryotes have three distinct RNA polymerases that are responsible for transcription: Pol I, Pol II, and Pol III. Pol II is the polymerase involved in the transcription of mRNAs. Transcription is initiated at core promoters, which contain elements such as the TATA sequence 25 to 30 nucleotides upstream of the transcriptional start site (called the TATA box) and the Inr sequence that surrounds the start site of transcription. To initiate transcription, a complex of general transcription factors and Pol II (called the preinitiation complex, PIC) assembles at the promoter (Figure 1). TFIID and TFIIA first bind the TATA box and Inr. TFIIB binds next, forming a complex that later recruits TFIIF and Pol II. After Pol II is recruited to this promoter complex, TFIIE and TFIIH also associate and are required for initiation. TFIIH is responsible for unwinding the helix to melt the DNA surrounding the start site of transcription. Mediator is also

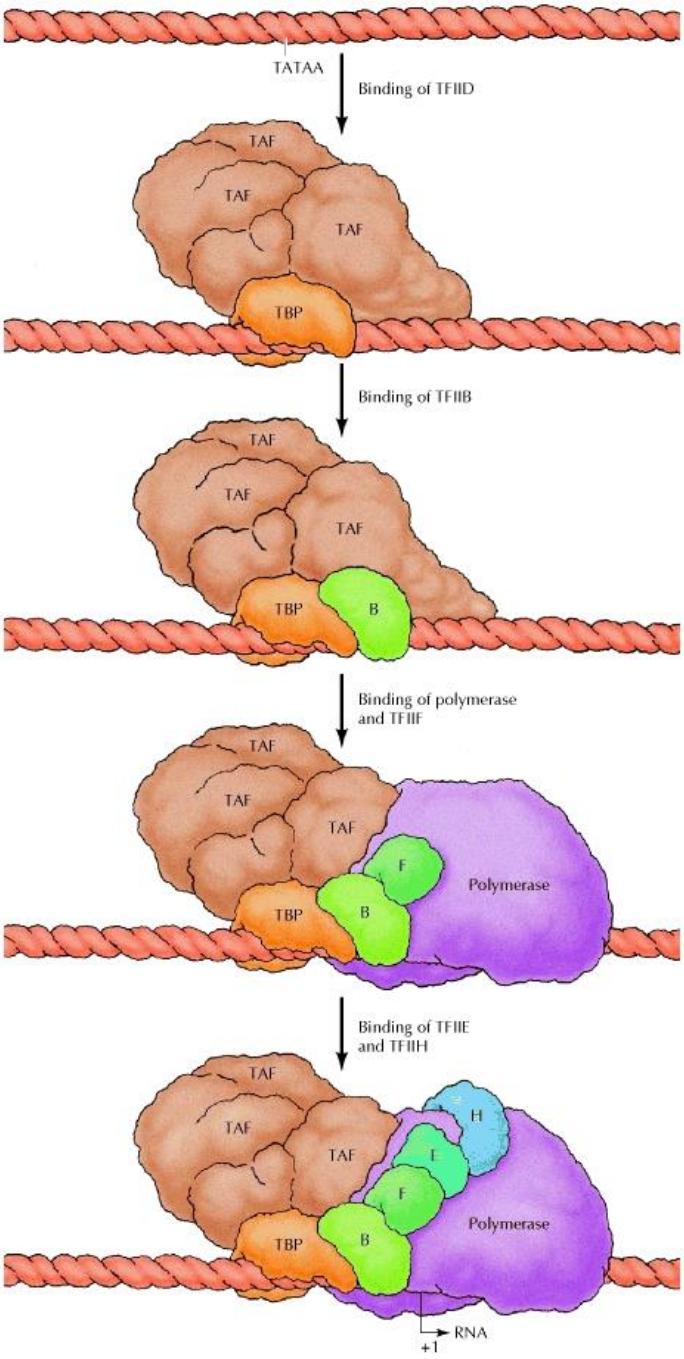

Figure 1. Assembly of preinitiation complex in eukaryotes. TFIID and TFIIA (not pictured) associate with the TATA box on the promoter DNA, followed by binding of TFIIB and TFIIF. Polymerase is recruited to the promoter, then TFIIE and TFIIH are recruited to the complex as well.[8]
Cooper GM. 2000. Eukaryotic RNA Polymerases and General Transcription Factors.

an important protein in the assembling of this complex, stabilizing PIC formation due to its large size, and aiding recruitment of Pol II via the C-terminal domain of its largest subunit.[7]

Those six general transcription factors are the proteins that associate with Pol II to drive transcription of all genes. Transcriptional activators and repressors provide gene-specific control by binding to specific *cis*-regulatory sequences that are present in promoter regions (near where the PIC forms) and distal enhancers (which can be thousands of kilobases away). It is the transcriptional activators that allow for gene-specific expression by promoting recruitment of the PIC to the promoter at the correct gene at the correct time via interaction with specific transcription factors.[9] DNA looping allows transcriptional activators bound to enhancers to act from long distances (Figure 2). NFATc2 is one such transcriptional activator, which I studied during my project.

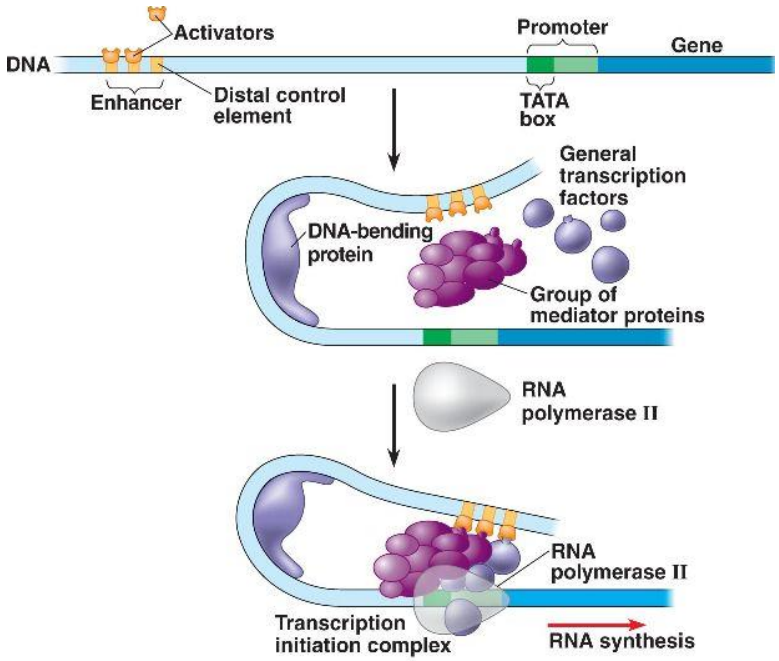

Figure 2. Activators associating with the PIC. Transcriptional activators that bind enhancer regions work by looping the DNA to interact with the PIC at the promoter of a specific gene.

Urry LA., Cain ML., W Steven A., M Peter V., Jackson RB., Reece JB. 2013. Campbell Biology in Focus. 1st Edition. New York (NY): Pearson Education, Inc.

NFATc2 is a transcription factor regulated by calcium

NFATc2, also known as NFAT1, belongs to the Nuclear Family of Activated T Cells (NFAT). These transcription factors were originally identified as regulating interleukin transcription in activated T-cells, hence the name, but have since been identified in almost all cell types.[10] Five proteins belong to the NFAT family: NFATc2, NFAT2, NFAT3, NFAT4, and NFAT5. As transcription factors, NFAT proteins have a highly conserved DNA binding domain that strongly resembles that of the REL-Family Transcription Factors.[10] It is this domain, called the REL-Homology region (RHR) that allows all NFAT proteins to bind specifically to DNA.[10] Except for NFAT5 (which is responds primarily to osmotic stress in T Cells), NFAT proteins are regulated primarily via calcium signaling.[10]

To activate NFATc2, receptors associated with calcium signaling (such as the T cell receptor (TCR) in T cells or receptor tyrosine kinases) must be bound. This binding induces activation of phospholipase C-γ (PLC-γ).[11] PLC-γ then hydrolyses phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol.[10] IP3 then activates release of stored calcium from the ER, the release of which triggers opening of calcium release-activated calcium channels (CRAC channels) to maintain the increased levels of cytosolic calcium.[12] At this stage, calcium binds calmodulin, which then activates calcineurin. This serine-threonine phosphatase dephosphorylates multiple serine residues on the NFATc2 regulatory domain.[11] NFATc2 has 14 phosphorylated serine residues within three conserved serine-rich motifs, and calcineurin removes 13 of them. Dephosphorylation of NFATc2 exposes a nuclear localization signal and potentially increases affinity of the RHR for DNA.[10,13] This allows NFATc2 to enter the nucleus where it interacts with a variety of other proteins to initiate transcription (Figure 3).

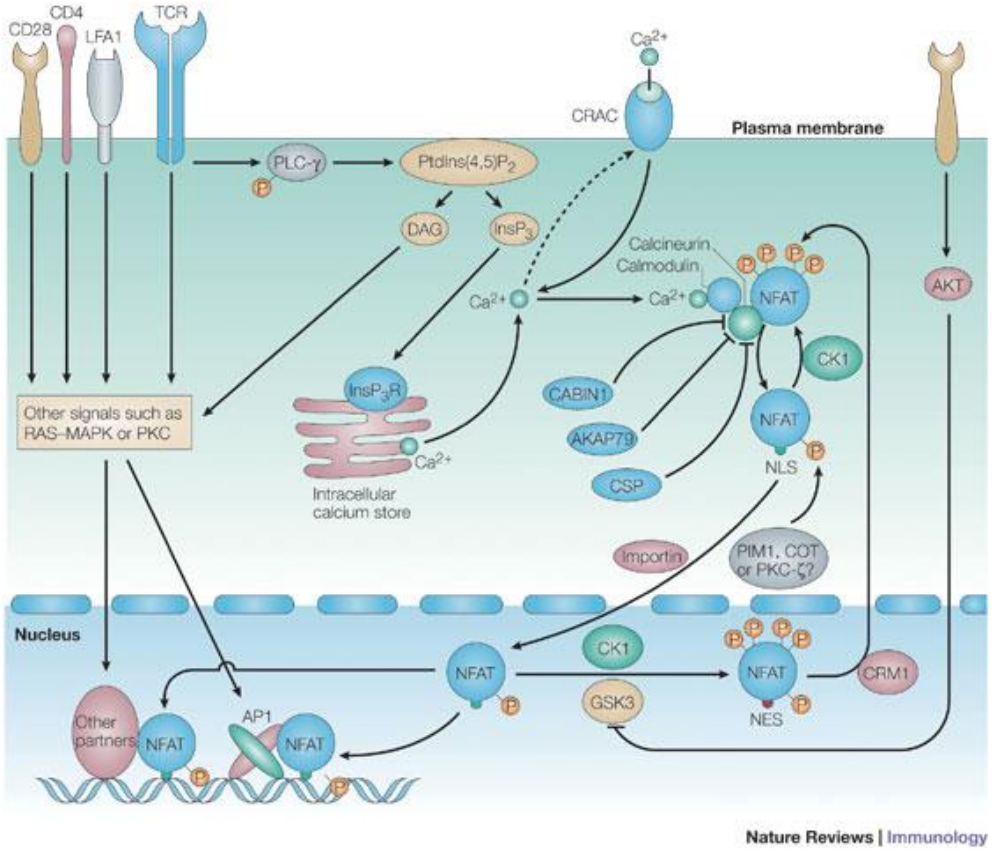

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Figure 3. NFAT activation by calcium signaling. After a surface receptor has been stimulated, PLC-γ gets activated. This ultimately results in an influx of cytosolic Ca^{2+}, which binds calmodulin. Calmodulin then activates calcineurin, which dephosphorylates sites on NFAT to expose the nuclear localization signal and hide the nuclear export signal. NFAT then enters the nucleus and activates transcription.

Macian F. 2005. NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5:472–484.

NFAT proteins have frequently been identified as interacting with the AP-1 family of proteins in order to activate transcription.[10,14] These AP-1 proteins bind as homo- or heterodimers cooperatively with NFAT at composite sites.[14] This interaction is best characterized in immune cells. In the case of IL-2 transcription, NFATc2 has even been shown to recruit cJun homodimers despite the lack of an AP-1 site.[15] In addition to several interleukin genes, COX-2 has been identified as having adjacent NFAT and AP1 sites it its promoter region.[16]

NFATc2 and Cancer

NFAT transcription factors regulate many essential genes, making it reasonable to think these may be involved in cancer development. Although the exact role of NFAT in cancer is unknown, a growing body of evidence suggests that NFAT is crucial to many carcinomas and may promote cell transformation.[17] NFATc2 has been linked to more invasive breast cancer phenotypes and may promote metastasis of breast cancer cells.[18,19] Several proteins such as COX-2, GPC6, ENPP2 and IL-8 have each been linked to promoting breast cancer cell migration and invasiveness, and all are thought to be regulated by NFATc2.[16,20,21,22] Additionally, NFATc2 has been shown to increase expression of MDM2 and promote inactivation of p53 in breast cancer cells, further increasing proliferation and reducing the cell's ability to undergo apoptosis.[23] NFATc2 has been shown to induce many genes that regulate cell migration and proliferation, both of which are cellular processes that are typically enhanced in cancerous cells. This strongly suggests that NFATc2 contributes to breast cancer progression and invasiveness.

Although NFATc2 has been linked to cancer progression, some studies indicate that NFATc2 also has tumor suppressing functionality. NFATc2 and NFAT4 knockdown mice have T cells that are resistant to apoptosis, defective in FasL induction, and whose proliferation increases, suggesting a tumor suppressing function.[24] These contradicting roles suggest that NFAT may act as a tumor suppressor and oncogene in untransformed and cancer cells, respectively.[25] As studies of NFAT function move beyond immune cells and into different cell types, complex regulation is likely to be revealed.

Exposure to known environmental carcinogens (specifically arsenite, benzo[a]pyrene, nickel, and vanadium) has been shown to activate NFAT.[23] In bronchial cells, this exposure

increases expression of COX-2, which contributes to cell-survival in an NFAT dependent manner.[23] This induced expression of COX-2 in bronchial cells could suggest that other environmental carcinogens are capable of activating NFAT and changing patterns of gene expression. If other NFATc2 regulated genes involved in migration and proliferation (such as GPC6, ENPP2, and IL-8) are upregulated in response to carcinogens, it is possible that environmental carcinogens are promoting cancer migration and invasiveness via activation of the NFATc2 transcription factor.

Cadmium is an environmental carcinogen that can mis-regulate calcium signaling

Cadmium is a heavy metal frequently isolated from zinc ores.[26] It's often found as a mineral and frequently combines with other elements to form cadmium oxide, cadmium chloride, and cadmium sulfide.[27] Although it is naturally found in soil and rocks, it is most often used to produce batteries, pigments, coatings, and plastics.[26,27] Cadmium has long been identified as a carcinogen, targeting the cardiovascular, developmental, gastrointestinal, neurological, renal, respiratory, and reproductive systems. Evidence suggests that cadmium's mutagenicity stems from its ability to inhibit mismatch repair.[28]

Cadmium has also been specifically linked to breast cancer progression. Several epidemiologic studies have indicated that increased urinary cadmium is associated with increased breast cancer rates, although they were unable to comment on the possible mechanisms behind such incidence. [29,30] Other studies have shown that cadmium interacts with ERα, inducing growth of breast cells in an estradiol-independent manner.[31,32,33] Cadmium has also been implicated in other cancers such as kidney, pancreas, and bladder cancers.[34] The mechanisms by which cadmium might influence progression of cancer are still being unraveled.

Cadmium has also been shown to significantly disrupt calcium signaling. It is capable of entering the cell via a variety of channels or transporters often utilized by other essential metals.[35] Many of these pathways also involve calcium channels--for example, in electrogenic cells, cadmium may enter through voltage-dependent calcium channels or in non-electrogenic cells, it may enter through store-operated calcium channels.[35] Once inside the cell, cadmium can disrupt calcium homeostasis in a variety of ways. By blocking Ca^{2+}-ATPase, cadmium prevents calcium from both leaving the cell and entering the ER, thus increasing intracellular calcium levels.[35] Additionally, interaction of cadmium with a GPCR promotes activation of PLC, generating IP3 capable of signaling Ca^{2+} release from the ER.[35]

This ability of cadmium to increase cytosolic calcium levels strongly correlates with the beginning steps of NFAT activation. This suggests that the environmental carcinogen, cadmium, could induce NFATc2 nuclear localization. In fact, a previous undergraduate student in the lab showed that cadmium can cause NFATc2 to go to the nucleus. Thus, this thesis seeks to identify if cadmium induces expression of genes involved in breast cancer cell progression and proliferation via the NFATc2 transcriptional activator.

Methods

Cell Culture

MDA-MB-231 breast cancer cells were utilized for the majority of the experiments. These cells were maintained in L-15 media supplemented with 10% FBS, 2 mM GlutaMAX, and 1X penicillin/streptomycin and incubated at 37°C with 0% CO_2. Jurkat T cells were maintained in RPMI media supplemented with 10% FBS and 1X penicillin/streptomycin and incubated at 37°C in 5% CO_2.

Cadmium Treatment

Prior to treatment, MDA-MB-231 cells were trypsinized with 0.25% trypsin and plated in a 6-well plate at approximately 500,000 cells/well. Cells were allowed to incubate at 37°C for 24 hours. The cells were then given either fresh media (mock treatment), media supplemented with 50 ng/µL PMA and 1 mM Ionomycin, or media supplemented with 9 µM $CdCl_2$. The cells were then incubated for two hours before being scraped and frozen at -80°C or used directly for RNA extraction. For experiments with Jurkat T cells, cells were counted and plated in 12-well untreated plates at a concentration of 500,000 cells/mL. After a 24 hour incubation, 50 ng/µL PMA and 1 mM Ionomycin or 9 µM $CdCl_2$ were then added to the appropriate wells. Cells were incubated for two hours, spun down, and then frozen at -80°C or directly placed in RiboZol for RNA extraction.

RNA Isolation and cDNA Synthesis

RNA extraction using RiboZol reagent (Amresco, Inc., Solon, OH) was performed per the manufacturer's instructions, with only minor alterations. RNA was isolated from treated cells with 200 µL of RiboZol reagent per well. After five minutes of incubation, 40 µL of chloroform was added and the solution was vortexed vigorously for 15 seconds followed by a 2-3 minute incubation at room temperature. Samples were then spun at 13,200 rpm for 15 minutes at 4^{o}C. The upper aqueous layer was then extracted to a new tube. To this layer, 100 µL of 100% isopropanol and 1 µL of GlycoBlue was added and incubated at room temperature for 10 minutes. This was followed by a 10 minute spin at 13,200 rpm at 4^{o}C. The supernatant was removed, leaving the pelleted RNA in the tube. 100 µL of 70% ethanol was then added to the

tube, vortexed, and spun at 13,200 rpm at 4ºC for an additional five minutes. Again, the supernatant was carefully extracted and the remaining pellet was vacuum dried for five minutes. The pellet was resuspended in 12 µL of Mill-Q water and incubated at 55ºC until fully dissolved.

After isolation, 5 µL of whole cell RNA was DNase-treated using 1 uL of DNase and 1X DNase buffer. RNA was converted to cDNA in a 20 µL reaction containing 1X RT buffer, 0.5 mM dNTPs, 3 µM random hexamers, 0.01 M DTT, and 0.5 µL of in-house RT enzyme.

RT-qPCR

RT-qPCR was used to analyze gene expression in breast cancer and Jurkat cells. 3 µL of cDNA was used with 11 µL SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 0.2 µM of forward and reverse primers, and ultra-pure water per reaction. Primers for RT-qPCR were as follows (5' to 3'):

GAPDH: Forward: GAACATCATCCCTGCCTCTACT, Reverse: ATTTGGCAGGTTTTTCTAGACG

IL-8: Forward: GCAGTTTTGCCAAGGAGT, Reverse: CACTCTCAATCACTCTCAGTTC

COX-2: Forward: TGAGCATCTACGGTTTGCTG, Reverse: TGCTTGTCTGGAACAACTGGC

β-Actin: Forward: TGGGCATGGGTCAGAAGGATTCC, Reverse: GAAGGTGTGGTGCCAGATTTTCTCC

MT1A: Forward: GCAAATGCACCTCCTGC, Reverse: CACAGCAGCTGCACTTC

ENPP2: Forward: GAGCAGAAGGATGGGAGG, Reverse: GTCACAGCGACAATCAGG

NFATc2: Forward: CCAAGACGAGCTTGACCTC, Reverse: CATAGTCCAGGACATCATCG

Knockdown Cell Lines - Lentivirus

NFATc2 knockdown cell lines were made in MDA-MB-231 breast cancer cells using lentivirus expression of shRNA against NFATc2. To generate the viral particles, 300,000 HEK293T cells were plated in each well of a 6-well plate. The following morning the media was changed to transfection media (lacking penicillin/streptomycin) and the HEK293T cells were transfected with shRNA plasmid (concentration 100 ng/µL), Packaging Vector Mix (containing packaging plasmids), and PEI in Opti-MEM media. The media on the cells was replaced with complete media 12-14 hours post-transfection. Two days later, viral supernatant was collected from the HEK293T cells, filtered, and stored at -80°C or added directly to MDA-MB-231 cells. Knockdown cells were then selected for by puromycin addition for the duration of cell growth.

Knockdown using siRNA Transfection

NFATc2 was knocked down using siRNAs that contained a pool of NFATc2 specific sequences (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). INTERFERin® reagent (Polyplus-transfection, New York, NY) was used to transfect MDA-MB-231 cells with the NFATc2 siRNA. Protocols were as given by Polyplus-transfection. To summarize, cells were plated at a density of 300,000 cells/well. 24 hours after plating, cells were treated with 15 nM siRNA. 24 or 48 hours following transfection, cells were treated for two hours with 9 µM $CdCl_2$ or mock treatment. Cells were then scraped, RNA isolated, and gene transcripts analyzed with RT-qPCR.

Results

COX-2 and IL-8 Transcription are Induced by Cadmium

To ultimately understand if cadmium was capable of inducing transcription via NFATc2 in breast cancer cells, I first asked if cadmium could increase transcription of genes known to be regulated by NFAT. I treated wild-type MDA-MB-231 breast cancer cells with 9 μM $CdCl_2$ for two hours. I assessed mRNA levels of specific genes to monitor transcription. RT-qPCR analysis of COX-2 and IL-8 (genes known to be regulated by NFATc2) revealed that expression increased upon cadmium exposure (Figures 4 and 5). PMA and Ionomycin were utilized as positive controls as they are commonly used to promote cytokine signaling. PMA is a known tumor promoter and activator of protein kinase C (PKC) while ionomycin increases intracellular Ca^{2+} levels, meaning they should be able to promote strong IL-8 transcription.[37,38] Although COX-2 expression did not increase quite as significantly with cadmium as it did with PMA/Ionomycin treatment, expression did increase significantly over expression in untreated cells (Figure 4). IL-8 demonstrated a far more robust cadmium response, increasing over 50-fold compared to wild-type IL-8 expression (Figure 5).

Two other genes, ENPP2 and MT1A did not show increased expression after cadmium exposure (Figure 6 and 7). ENPP2 is also regulated by NFATc2, so it was anticipated that it's expression would follow that of COX-2 and IL-8.[22] MT1A is a metallothionein protein that functions to bind to heavy metals such as cadmium. MT1A and another related metallothionein, MT2A have been shown to be highly inducible by zinc, cadmium, mercury, and copper.[36] The fact that an increase in MT1A expression with cadmium exposure was not observed was unexpected (Figure 7). However, induction of these genes is highly metal and time specific, so it is possible that two hours at 9 μM was simply not enough time or concentration to see a

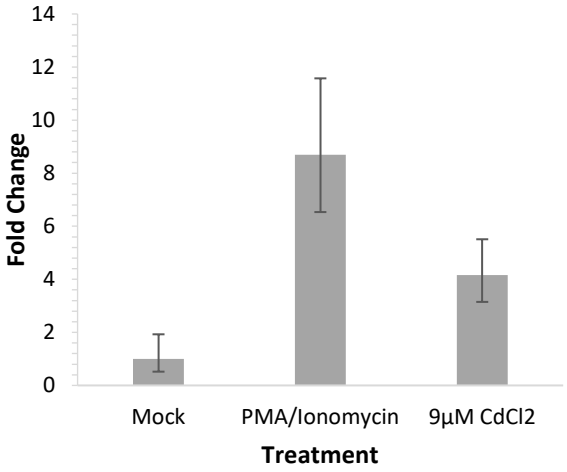

Figure 4. Fold Change in Cox-2 Expression. Changes in Cox-2 gene expression were observed in MDA-MB-231 cells after two hours of mock treatment, treatment with 50ng/µL PMA and 1mM Ionomycin, or treatment with 9µM $CdCl_2$ normalized to GAPDH expression. Bars represent the average of two biological replicates and error bars are the range.

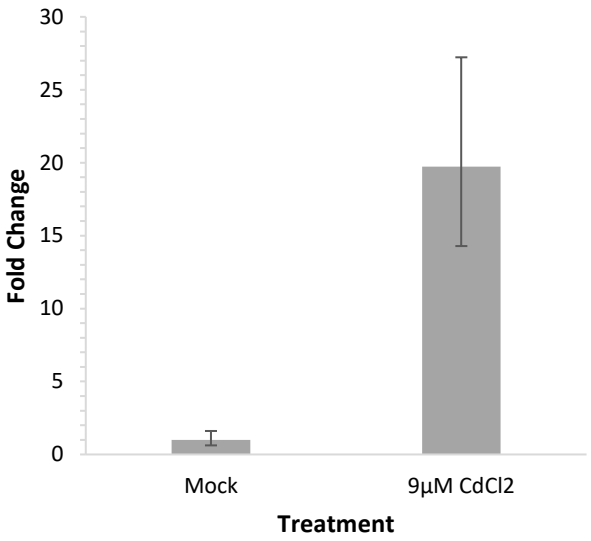

Figure 5. Fold Change in IL-8 Expression. Changes in IL-8 gene expression were observed in MDA-MB-231 cells after two hours of mock treatment and or treatment with 9µM $CdCl_2$ normalized to GAPDH expression. Bars represent the average of two biological replicates and error bars are the range.

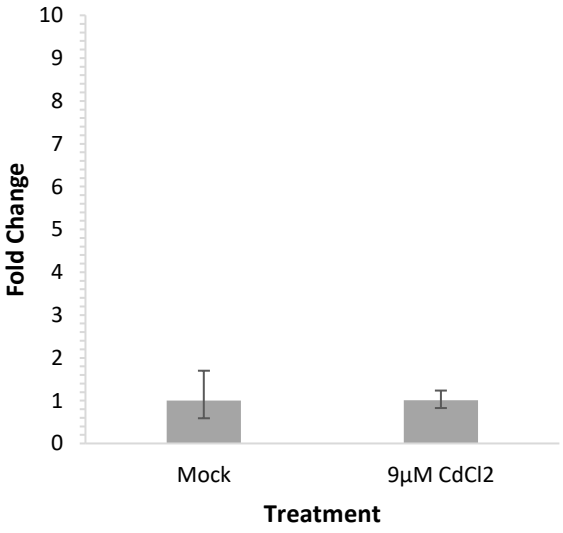

Figure 6. Fold Change in ENPP2 Expression. Changes in ENPP2 gene expression were observed in MDA-MB-231 cells after two hours of mock treatment and or treatment with 9µM $CdCl_2$ normalized to GAPDH expression. Bars represent the average of two biological replicates and error bars are the range.

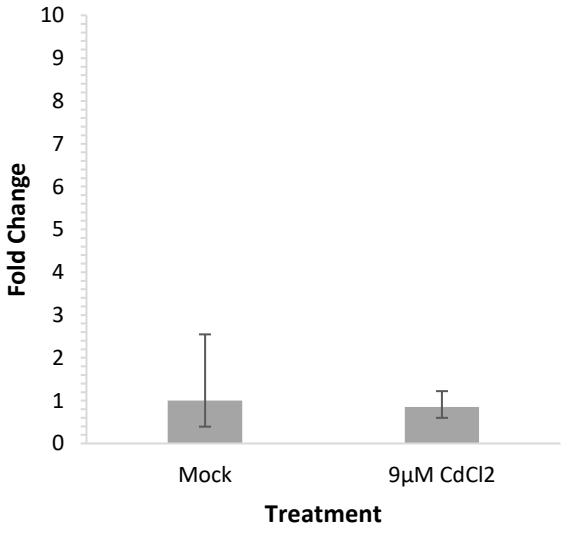

Figure 7. Fold Change in MT1A Expression. Changes in MT1A gene expression were observed in MDA-MB-231 cells after two hours of mock treatment and or treatment with 9µM $CdCl_2$ normalized to GAPDH expression. Bars represent the average of three biological replicates and error bars are the standard deviation.

measurable effect.[39] These data suggest that cadmium promotes expression of some, but not all, NFATc2 regulated genes.

Knockdown of NFATc2 in MDA-MB-231 Cells

To test whether the cadmium-induced expression is indeed regulated by NFATc2, an NFATc2 knockdown cell line was created. To do this, NFATc2 shRNA was inserted into a lentiviral plasmid, which was then transfected into HEK-293 cells with packaging plasmids. The genes necessary for viral replication are split into several plasmids, one containing the gene of interest (i.e. the shRNA), one or two containing viral structural proteins, and another containing a VSV envelope protein. HEK-293 cells are then used to package the plasmids into a recombinant virus. Two days after transfection, the media from the HEK-293 cells (containing the recombinant virus) is collected, filtered, and put on the MDA-MB-231 cells. After this viral transduction, selection with puromycin would eliminate the cells that did not receive the lentivirus, leaving only the cells expressing the gene of interest. A Turbo-GFP plasmid was utilized as a control and indicates that transfection efficiency into the HEK-293 packaging cells was high (Figure 8). After several days of selecting the MDA-MB-231 cells in puromycin, the NFATc2 knockdown cells stopped growing. Both GFP and scrambled shRNA cells acted as controls, and those MDA-MB-231 cells grew well in the puromycin-selective media, suggesting that the problem was likely not with the lentiviral transduction protocol.

Since the creation of stable knockdowns proved ineffective, a method to transiently knockdown NFATc2 expression was sought out. siRNAs specific to NFATc2 (Santa Cruz Biotechnology, Inc) were transfected into MDA-MB-231 cells using INTERFERin® reagent (Polyplus-transfection). The efficiency of this method was first tested with FITC labeled control

siRNAs (Santa Cruz Biotechnology, Inc). The transfection efficiency needs to be high enough for the impact of NFAT knockdown to be observed in a population of cells. MDA-MB-231 cells were plated such that on the day of transfection they were 30-50% confluent, as per protocol, and then transfected with FITC siRNA. FITC was observed in the cells at 24 hour time points following transfection, with 24 hours appearing to achieve the greatest efficiency with the least cell death (Figure 9).

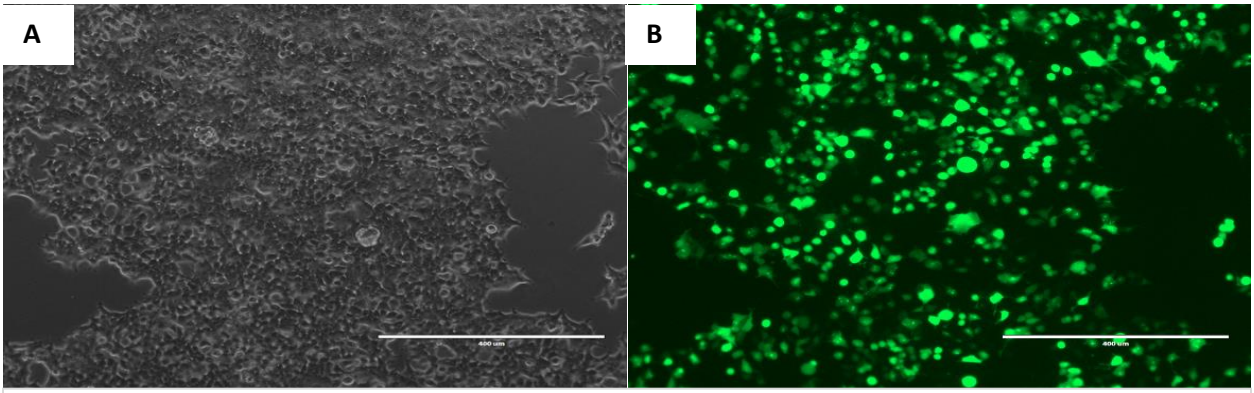

Figure 8. Lentiviral Packaging in HEK-293T cells. GFP visualization in HEK-293T cells transfected with TurboGFP at 10X magnification. **A**. Cells under transmitted light **B**. Cells under GFP filtered light. HEK-293T Cells were utilized to package lentivirus containing NFATc2 shRNA, control shRNA, or TurboGFP for the knockdown of NFATc2 in MDA-MB-231 breast cancer cells.

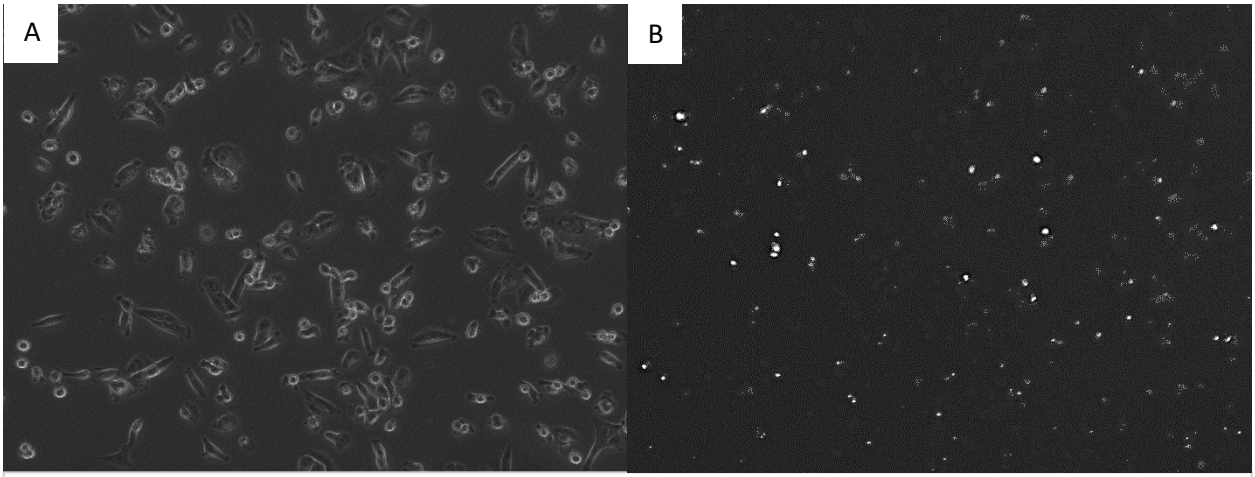

Figure 9. FITC siRNA transfection. MDA-MB-231 cells were transfected with 25nM FITC siRNA using INTERFERin reagent. Cells were visualized 24 hours after transfection under **A**. transmitted light and **B**. a GFP filter to monitor efficiency at 10X magnification.

Following this optimization, MDA-MB-231 cells were transfected with NFATc2 or control siRNA and incubated for 24 hours. Cells were then treated for two hours with 9 μM $CdCl_2$ or mock treatment, scraped, and RNA was isolated. RT-qPCR of IL-8, COX-2, and NFATc2 was performed. When normalized to β-actin, it appears that NFATc2 expression was knocked down in cells treated with NFATc2 siRNA compared to a control scrambled siRNA (Figure 10). I showed in Figures 4 and 5 that IL-8 and COX-2 expression increased with cadmium. If cadmium is inducing IL-8 and COX-2 expression via NFATc2, these genes should be less responsive to cadmium exposure in the NFATc2 knockdown cells. It appears that both COX-2 and IL-8 expression decreased in the NFATc2 siRNA treated cells compared to cells treated with the control siRNA (Figures 11 and 12). After exhaustive attempts to knockdown levels of NFATc2, this was the first success, therefore, the experiment needs to be repeated. If this is indeed the result, it would appear that cadmium is upregulating COX-2 and IL-8 in an NFATc2 dependent manner.

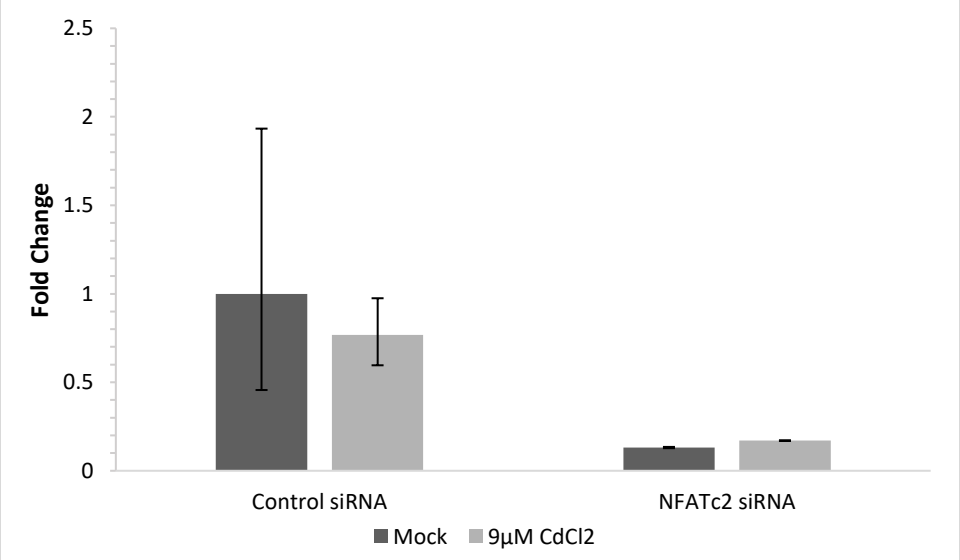

Figure 10. Fold Change of NFATc2 in Knockdown Cell Lines. MDA-MB-231 cells were transfected with 15nM control siRNA and NFATc2 siRNA using INTERFERin reagent. 48 hours after transfection with siRNAs, cells were mock treated or treated with 9μM $CdCl_2$ for two hours. Fold changes in NFATc2 when normalized to β-actin expression. Bars represent the average of two biological replicates and error bars are the range.

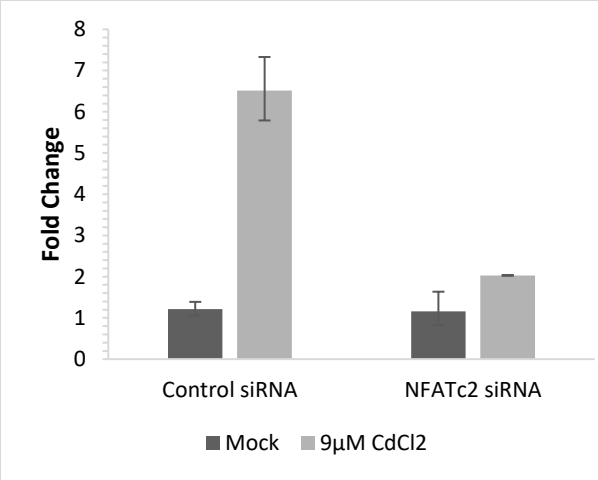

Figure 11. Fold Change of COX-2 in Knockdown Cell Lines. MDA-MB-231 cells were transfected with 15nM control siRNA and NFATc2 siRNA using INTERFERin reagent. 48 hours after transfection with siRNAs, cells were mock treated or treated with 9µM $CdCl_2$ for two hours. Fold changes are normalized to β-actin expression. Bars represent the average of two biological replicates and error bars are the range.

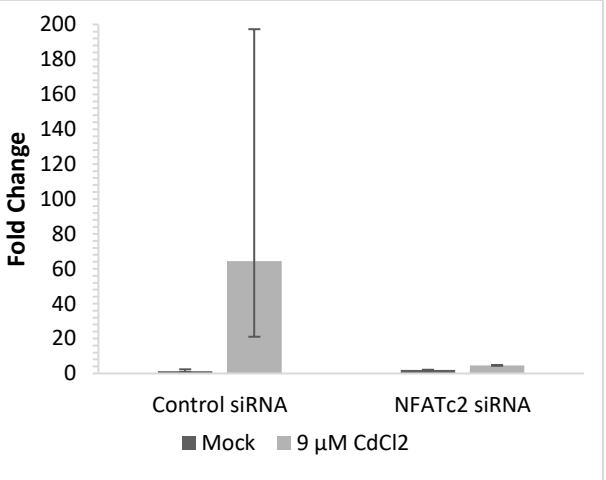

Figure 12. Fold Change of IL-8 in Knockdown Cell Lines. MDA-MB-231 cells were transfected with 15nM control siRNA and NFATc2 siRNA using INTERFERin reagent. 48 hours after transfection with siRNAs, cells were mock treated or treated with 9µM $CdCl_2$ for two hours. Fold changes in IL-8 when normalized to β-actin expression. Bars represent the average of two biological replicates and error bars are the range.

Cadmium Induced Expression is Cell Type Specific

I next asked if the cadmium induced increase in transcription is specific to breast cancer cells, or if it also occurs in Jurkat T cells. I chose Jurkat cells since NFAT was first discovered in T-cells, and its expression and the genes it regulates are well defined in this cell type.

Jurkat T cells were treated in the same manner as MDA-MB-231 cells. Cells were treated for two hours with mock treatment, 50 ng/µL PMA and 1 mM Ionomycin, or 9 µM $CdCl_2$. RT-qPCR analysis of IL-8 showed that, as in MDA-MB-231 cells, treatment with PMA and Ionomycin dramatically increased expression. PMA/Ionomycin increased IL-8 expression 8755-fold relative to wild-type, while cadmium did not increase expression above normal (Figure 13). In biological replicates, PMA and Ionomycin induced IL-8 transcription 6500 to 25300-fold, but again cadmium did not induce IL-8. This result is entirely different from what was observed in MDA-MB-231 cells. PMA/Ionomycin still acted as a positive control, increasing expression of IL-8 in

both Jurkat and MDA-MB-231 cells, but cadmium exposure only increased expression of IL-8 in the breast cancer cells and had no impact in T-cells. This suggests that cadmium responsiveness is cell type specific.

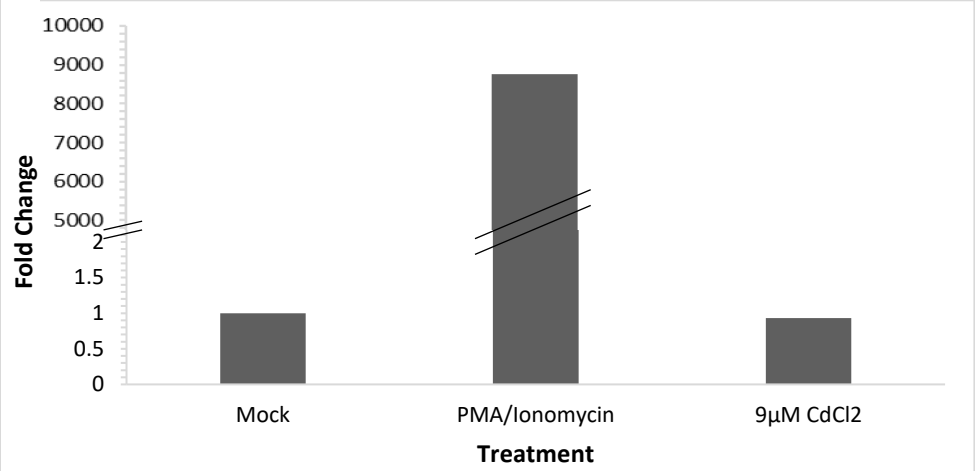

Figure 13. Fold Change in IL-8 Expression. Changes in IL-8 gene expression were observed in Jurkat T-cells after two hours of mock treatment, treatment with 50ng/µL PMA and 1mM Ionomycin, or treatment with 9µM $CdCl_2$ normalized to GAPDH expression. Bars represent the average of four biological replicates and error bars are the standard deviation.

Discussion

This project sought to determine if the environmental carcinogen, cadmium, promoted breast cancer proliferation via the NFATc2 transcription factor. To test this, cells were first treated with 9 µM $CdCl_2$. Treatment of MDA-MB-231 cells induced transcription of some genes regulated by NFATc2. Then, to test whether this increase in expression was dependent on NFATc2, knockdown cell lines were attempted. Results were promising, and suggested that NFATc2 knockdown eliminated upregulation of COX-2 and IL-8 in response to cadmium. However, experiments with Jurkat T cells (in which NFATc2 transcriptional activation is well characterized) did not show the same increase in the NFATc2 regulated IL-8 gene that breast

cancer cells did. These results suggest that cadmium-induced expression of NFATc2 regulated genes is likely to be cell type specific.

Experiments show that cadmium is capable of inducing expression of some genes known to be regulated by NFATc2. IL-8 and COX-2 were shown to be repeatedly inducible by cadmium exposure (Figure 4, 5). However, not all NFATc2 regulated genes were cadmium responsive. MT1A and ENPP2, both of which have been linked to increased growth and proliferation in breast cancer cells,[40] were not responsive to cadmium exposure (Figure 6, 7). This could suggest that cadmium responsiveness is gene specific, likely because other transcription factors are involved.

My primary challenge was showing that the increased expression of these genes depends upon activation of NFATc2. Stable NFATc2 knockdown was unsuccessful. NFATc2 knockdowns have been done before, however these were oftentimes done in a background of first stably overexpressing NFATc2. [16,19,21] Since lentiviral transfection worked for GFP and the control shRNA, my technique was effective. If NFATc2 is critical to MDA-MB-231 growth and viability, knockdown of NFATc2 could greatly reduce the ability of these cells to proliferate under normal cell culture conditions. Perhaps this is why the lentiviral knockdowns did not grow.

The NFATc2 knockdowns via siRNA were promising. I found that levels of the NFATc2 transcript were knocked down, and the cadmium-induced increase in IL-8 and COX-2 was repressed in these cells. It was very difficult to find conditions where NFATc2 was knocked down, so this result has not been repeated, which will be a goal of future experiments. During these experiments, I also observed changes in levels of control RNAs such as GAPDH, β-actin, and 18S rRNA. This could indicate variable cell numbers during plating, or possibly relate to the cadmium treatment. Cadmium has been observed to induce apoptosis in a variety of cell types by

Ca^{2+} overabundance.[35,40,41] Although I optimized the concentration and treatment time to avoid this scenario, it is possible that cadmium treatment also altered cell number in my experiments. These will be points of optimization in future experiments.

The lack of induced IL-8 expression by cadmium in Jurkat T cells, taken with the results showing induction of COX-2 and IL-8 expression in MDA-MB-231 cells, indicates that cadmium may have a cell-type specific effect. It is unclear why cadmium may promote NFATc2-driven transcription in breast cancer cells but not T-cells, but this is not necessarily inconsistent with prior research. Differential roles of various NFAT isoforms (including NFATc2) have been observed numerous times. In some cell types, like T-cells, loss of NFATc2 has been linked to tumor formation, while in breast cancer cells NFATc2 has been shown to promote tumor formation.[23,24,43] If NFATc2 does have such different roles in these cell types, it is not entirely unexpected that cadmium would exhibit opposing results in breast cancer cells and T cells. However, PMA and ionomycin exposure was capable of inducing cytokine production in both Jurkat and MDA-MB-231 cells, although there were significant differences in how much this expression increased. This result could indicate that something more specific to cadmium is occurring that causes this differential expression.

In the future, other than repeating the knockdown of NFATc2 in breast cancer cells, chromatin immunoprecipitation (ChIP) and luciferase assays could be utilized to show NFATc2 dependence of the cadmium induction of transcription. ChIP of NFATc2 would allow us to determine where NFATc2 is binding to the DNA and which promoter sites are newly occupied by cadmium exposure. Luciferase assays would also indicate if cadmium is activating NFAT. By transfecting cells with luciferase plasmids containing NFATc2 promoter elements, an increase in

luminescence after cadmium treatment would suggest that cadmium is activating NFATc2 transcription of genes.

Should cadmium exposure be proven to induce NFATc2 nuclear localization and activate genes promoting breast cancer proliferation and invasiveness, this would provide both a potential therapeutic target and another means to lower risk of developing breast cancer. Already it appears that cadmium is capable of inducing some genes related to breast cancer migration, namely COX-2, suggesting that limiting exposure to cadmium could at the very least help minimize the aggressiveness of breast cancer if not lower the risk of developing it altogether. NFATc2 knockdowns would greatly aid the ability to see if this expression is entirely NFATc2 dependent. If so, NFAT inhibitors could be useful in treating breast cancer. Drugs inhibiting NFAT already exist on the market, such as cyclosporin A and FK506 used for immunosuppression. These drugs work by binding calcineurin, which prevents the activation of NFAT proteins.[44] It's possible that these drugs, or ones with a similar mechanism, could then inhibit breast cancer growth by inhibiting NFATc2 via the same pathway by which cadmium is hypothesized to activate it.

Given the current prevalence of breast cancer in the United States, it is worthwhile to pursue models that do not rely heavily on tumor suppressor mutations or hormonal therapies. It appears that a wide variety of factors contribute to breast cancer development, some of which may be environmental. Until these factors are elucidated and treatments developed to stop them, breast cancer will remain a burden of society.

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