REGULATION OF GENE EXPRESSION BY THE CD44- INTRACYTOPLASMIC DOMAIN (CD44-ICD)

BY

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A THESIS

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ABSTRACT

CD44 is a major cell membrane hyaluronan receptor and cell adhesion glycoprotein. It is involved in multiple cellular processes including cell migration and invasion, apoptosis, stemness and cell proliferation. It exists in a variety of isoforms mainly due to the alternative splicing of ten of its 20 exons and posttranslational modifications. CD44 also undergoes proteolytic cleavages that generate the CD44 intracytoplasmic domain (CD44-ICD). The CD44-ICD is translocated to the nucleus where it can regulate gene expression. The CD44-ICD-dependent regulation of gene expression is likely to be mediated by protein-protein interactions (PPI) with factors of the transcriptional machinery, which makes of the CD44-ICD a transcriptional co-regulator. However, the existence of the CD44-ICD response element (CIRE) and its reported binding to it makes of the CD44-ICD a transcription factor. We hypothesize that the CD44-ICD is a novel transcriptional regulator with properties of a co-regulator and a transcription factor. To test this hypothesis we carried out PPI assays such as proximity ligation assays (PLA) and plate array PPI assays. We also analyzed the expression of genes related with CD44-associated cellular events such as apoptosis, oxidative stress and stemness using proteome profiler assays and transcription factor activation plate arrays. PLA data showed that the CD44-ICD interacts with the transcription factor Runx2 in the nucleus as well as in the cytoplasm. We also found that the CD44-ICD interacts with more transcription factors. The expression of CD44 in MCF-7 cells, which is expected to generate the CD44-ICD, inhibited the expression of Nrf2, caspase 3 and E-cadherin, important genes in oxidative stress, apoptosis and stemness, respectively. The inhibition of Nrf2 in MCF-7/CD44 cells, promoted a higher sensitivity to oxidative stress caused by hydrogen peroxide compared to MCF-7 cells. Altogether, these data

supports that CD44 can act as a regulator of gene expression and that this regulation is in part via its intracytoplasmic domain once released into the cytoplasm and nucleus. The CD44-ICD appears to be able to act as a co-activator as well as a co-repressor. Because the CD44-ICD size of only 72 residues, the CIRE sequence to which it can bind is unusual. This type of protein-DNA interaction is usually associated with larger DNA-binding proteins such as bona fide transcription factors. The dual capacity of the CD44-ICD to interact with proteins of the transcriptional machinery as well as to interact with its own response element on promoter sequences, might place the CD44-ICD in a novel category of transcriptional regulators

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LIST OF ABBREVIATIONS

A/A .....Antibiotic/Antimycotic
ARE.....Antioxidant Response Element
BRET.....Bioluminescence Resonance Energy Transfer
CaMKII.....Ca2+/Calmodulin-Dependent Protein Kinase II
CIRE.....CD44 ICD Response Element
CSC..... Cancer Stem Cells
CTF.....C-Terminal Fragment
DNA.....Deoxyribose Nucleic Acid
ECD.....Extracellular Domain
ERM.....Ezrin, Radixin, Moesin
ES.....Embryonic Stem Cells
FBS.....Fetal Bovine Serum
G.....Earths Gravitational Force
HA.....Hyaluronic Acid
HCAM.....Homing Cell Adhesion Molecule
ICD.....IntraCytoplasmic Domain
IPS.....Induced Pluripotent Stem Cells
kDa.....Kilo Daltons
TF.....Transcription Factor
TPA 12-O-Tetradecanoylphorbol 13-Acetate
TMR.....Transmembrane Region
MMP.....Matrix Metalloproteinases
mRNA.....Messenger Ribose Nucleic Acid
MTS.....MTS Cell Proliferation Assay Kit
NRF2..... Nuclear Factor Erythroid 2-Related Factor 2
PKA.....Protein Kinase A
PKC.....Protein Kinase C
PCR.....Polymerase Chain Reaction
RIP.....Regulated Intramembrane Proteolysis
ROS.....Reactive Oxygen Species
RPM.....Rotations Per Minute
RT.....Room Temperature
SOC.....Super Optimal Broth with Catabolite Repression
SREBP.....Sterol Regulatory Element Binding Proteins
TSS.....Transcriptional Start Site
WT.....Wild Type

CHAPTER 1: INTRODUCTION

The focus of this thesis is the proposed function of CD44 as a transcriptional regulator of gene expression. We hypothesize that this CD44-mediated regulation of gene expression is in part via the CD44 intracytoplasmic domain (CD44-ICD). Herein we present molecular, biochemical and bioinformatics data that supports our hypothesis as well as suggest that the CD44-ICD is a novel type of transcriptional regulator.

1.1 Transcription and transcriptional regulators

The process of transcription is one of the first steps in accessing and decoding the genetic information found in the genome (O'Connor *et. al*., 2010). It is the process where the information in a strand of DNA is converted into a molecule of messenger RNA (mRNA) transcript. Broadly described it is composed of three steps: initiation, elongation and termination. Playing vital roles in this process are a large number of proteins including general and specific transcription factors (TFs) and RNA polymerases. TFs bind to promoter sequences within the DNA to recruit RNA polymerase to the appropriate transcription site (Lee and Young, 2000; O'Connor *et. al*., 2010). The interaction between the RNA polymerase and TFs forms the transcription initiation complex, which is a key step in the regulation of gene expression. Initiation is then started and the mRNA synthesis begins, the mRNA molecule is then elongated and once the strand is completely synthesized, transcription is terminated. (O'Connor *et. al*., 2010).

1.2 CD44 as a transcriptional regulator

The Cluster of Differentiation 44 (CD44) gene encodes a type I transmembrane receptor and adhesion molecule (Underhill, 1992). In addition of mediating cell to cell and cell to extracellular matrix (ECM) interactions, CD44 is involved in transducing cellular signals (Miletti-Gonzalez et al., 2012; Orian-Rousseau 2015). The protein-protein interaction of CD44 with other cell membrane receptors with kinase activity can mediate the typical signal transduction pathway via phosphorous cascades (Ponta et al., 2003). Another signaling activity in which CD44 is involved and the focus of this thesis project, is the gamma-secretase complex-mediated signaling pathway (Murakami et al., 2003). The intracytoplasmic domain of CD44 (i.e., CD44-ICD) is cleaved within the cell membrane by presenilin 1, the active proteolytic enzyme in the gamma-secretase complex (Lammich et al., 2002). We and others have shown that the CD44-ICD can act as a signal transduction molecule (Pelletier et al., 2006; Miletti-Gonzalez et al., 2012). For example, Okamoto et al. (2001) reported that the CD44-ICD activates transcription through the 12-O-tetradecanoylphorbol 13-acetate–response element (TPA) resulting in the upregulation of genes containing the TPA-responsive element (TRE), including CD44 itself. The CD44-ICD can also interact with the transcriptional coactivator CBP/p300 to promote transcription (Okamoto et al., 2001). In addition, the nuclear translocation of the CD44-ICD has been shown to be essential for the signaling proteins Notch and SREBPs (sterol regulatory element-binding proteins) that upon translocating to the nucleus can regulate transcription (Sakai et al., 1996; Schroeter et al., 1998; Struhl and Adachi, 1998; Brown et al., 2000). We have also shown that the CD44-ICD can interact with the transcription factor Runx2/AML3 at the matrix metalloproteinase 9 (MMP-9) promoter to regulate its expression (Miletti-Gonzalez et al., 2012).

1.3 CD44 in cellular responses and phenotypes

The expression and function of CD44 have been associated with numerous cellular phenotypes and responses to cellular environments. Three of them oxidative stress, apoptosis and stem cell characteristics or stemness (Echiburú-Chau et al., 2011; Fedorchenko et al., 2013; Chanmee et al., 2015) are discussed below as they were studied in this thesis project.

Oxidative Stress

Oxidative stress is an imbalance between the production of free radicals and the ability of the antioxidant systems of cells to detoxify their effects through neutralization. (Yoshikawa and Naito, 2002). A free radical (i.e., an endogenous form of oxidants within the body) is an oxygen-containing molecule that has an unpaired electron. This in turn makes it highly reactive with other molecules. As a result of normal cellular metabolism, organisms produce reactive oxygen species (ROS). (Birben *et al*., 2012). When found at low concentrations ROS function normally in physiological processes of the cell. At higher concentrations however, they can cause disruptions in the mechanisms of cellular physiology by damaging proteins, lipids and DNA (Birben, *et al*., 2012). The shift in the balance between oxidant and antioxidant where oxidants are highly favored is termed "oxidative stress." (Birben *et al*., 2012). Oxidative stress is known to be involved in many different cancers, neurodegenerative diseases (e.g., Parkinson's disease and Alzheimer's disease), atherosclerosis, hypertension and diabetes mellitus. (Yoshikawa and Naito, 2002). Exogenous forms of oxidants that may affect the human body are cigarette smoke, ozone exposure and radiation.

In response to oxidative stress, cells normally activate transcription factor nuclear factor erythroid 2-related factor 2 also known as Nrf2. (Nguyen *et al*., 2009). Nrf2 is a regulator

involved in protecting cells via the antioxidant response element (ARE). This protein is latent within the cells cytoplasm until there is an increase of cellular oxidative stress. (Vomhof-DeKrey *et al*., 2012). Once there is an increase in oxidative stress Nrf2 migrates into the nucleus and binds to the DNA. (Vomhof-DeKrey *et al*., 2012). Nrf2 protects against oxidative damage usually triggered by injury and inflammation. It controls the induced expression of ARE dependent genes to regulate the normal functions of living organisms outcome of oxidant exposure. Nrf2 senses oxidants and regulates the necessary antioxidant defense. (Ma, 2013). It has been shown that subsets of CD44 high T cells increase the levels of Nrf2 and its target gene transcripts. (Kim and Nel, 2005). It is also suggested that APAP-induced hepatotoxicity and immunological changes involving CD44 may activate Nrf2 as an adaptive response. (Kay *et. al*., 2011).

Apoptosis

Apoptosis is a normal process that cells undergo in order to regulate their own rate of cell death. When cells are no longer needed they activate an intracellular death program to commit suicide (Alberts *et al*., 2002). This is a controlled part of the growth and development of organisms. Apoptosis is also known as programmed cell death. Apoptosis is healthy for the organism because cell death helps regulates the sustainability of normal cells vs abnormal ones. In adult tissues, cell death balances out cell division, which otherwise tissues would continue to grow or shrink (Alberts *et al*., 2002). When a cell undergoes apoptosis it shrinks and condenses. The cell surface is completely altered. The nuclear envelope breaks apart and the DNA breaks up into fragments (Alberts *et al*., 2002). Proteolytic enzymes called caspases mediate apoptosis. Caspases trigger cell death by the cleavage of proteins in the cytoplasm and nucleus. (Alberts *et*

al., 2002). This process is irreversible. CD44 can induce or protect the cell from apoptosis. (Artus *et al*., 2006; Maquarre *et al*., 2005; Fujita *et al*., 2002; Gore *et al*., 2008). It has been shown that CD44 engagement protects CLL cells from spontaneous and fludarabine-induced apoptosis (Herishanu et al., 2011). Higher expression of CD44 and a stronger anti-apoptotic effect of CD44 activation in UCLL cells was found (Herishanu et al., 2011).

Stemness

Stemness is the ability of a cell to self-renew and differentiate. It is the ability of a cell to immortalize its lineage, produce differentiated cells, and to interact with its environment. Stemness is the ability to maintain a balance between inactivity, proliferation, and regeneration. (Aponte and Caicedo, 2017). Embryonic stem cells (ES) are pluripotent stem cells derived from the inner cell mass of embryos. Induced pluripotent stem cells (iPS) are generated by the reprogramming of somatic cells. Somatic cells express the transcription factors Oct-3/4, KLF4, SOX2, and c-Myc, which help in reprogramming the cells into ES-like cells. (Aponte and Caicedo, 2017). Cells normally referred to as induced pluripotent stem (iPS) cells resemble embryonic stem cells in genomic, cell biologic and phenotypic characteristics (Aponte and Caicedo, 2017). Pluripotent stem cells have potential for treatments of heart disease, diabetes and neurodegenerative disorders. (Aponte and Caicedo, 2017).

Recently, Cho et al. (2015) reported that the intracellular domain of CD44 (CD44-ICD) activates as well as regulates stemness factors such as Nanog, Sox2 and Oct4, which contributes to the tumorigenesis of breast cancer (Cho, *et al*., 2015). Stem cell transcription factors, such as Nanog, Sox2 and Oct4 have been studied in ES cell pluripotency and cancer stem cell formation. These coordinated networks of stemness factors are the regulatory mechanisms of pluripotency

and differentiation in stem cells (Cho, *et al*., 2015). It has been observed that there is an interaction between the C-terminal region of CD44-ICD and these stemness factors, and of the coordination of their nuclear translocation (Cho, *et al*., 2015). It has been suggested that hyaluronic acid (HA) mediates the activation of the interaction between CD44 and stemness factors.

Hypothesis:

We hypothesize that CD44 is a novel transcriptional regulator via its CD44-ICD protein-protein interaction (PPI) with transcription factors and its DNA binding functions.

CHAPTER 2: LITERATURE REVIEW

CD44 is a gene that encodes a cell membrane receptor involved in multiple cellular functions including cell migration and invasion, apoptosis and cell proliferation. It exists as a variety of isoforms. The most common isoform is known as CD44 standard (CD44s) and others CD44 variants. As shown in figure 1, this diversity of isoforms is due to the alternative splicing of 10 of the CD44 gene 20 exons. In the CD44 standard isoform all variable exons are alternatively spliced out (Ponta *et al*., 2003). In humans CD44 is encoded by the CD44 gene on chromosome 11 and has been referred to as the homing cell adhesion molecule (HCAM), HUTCH-1, and Hermes antigen. (Dzwonek and Wilczynski, 2015). All isoforms retain the hyaluronic (HA)-binding region and a common transmembrane and cytoplasmic domain. This is highly conserved between species. (Thorne *et al*., 2004).

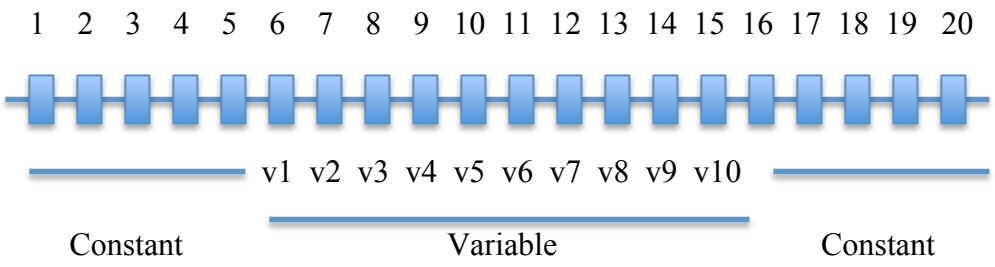


Figure 1. CD44 Schematic Map. v1-v10 represents the exons that are alternatively spliced (makes up the isoforms). Number 1-20 represents all the exons within the gene.

The CD44 protein undergoes a proteolytic process in which the intracytoplasmic domain (ICD) is generated. (Okamoto *et. al*., 2001). There is the binding of the ligand, for example a HA, which induces matrix metalloproteinases (MMP-9) transcription to degrade the extracellular matrix. MMPs are a group of endopeptidases that degrade extracellular matrix. (Visse and

Nagase, 2003). MMP-9, as well as other MMPs, bind to CD44 and can be regulated by the CD44s-HA interaction. Hyaluronan also increases MMP-9 activity and gene expression. (Alaniz et al., 2004). The proteolytic processing of CD44 by presenilin-1/ γ-secretase complex within the transmembrane region then follows this degradation of the extracellular matrix by MMP-9 and other MMPs. The CD44-ICD will translocate into the nucleus via a nuclear pore where there it binds to its response element within the DNA and regulates transcription and gene expression. (Miletti-Gonzalez *et al*., 2012). Figure 2 shows a description of this proposed process.

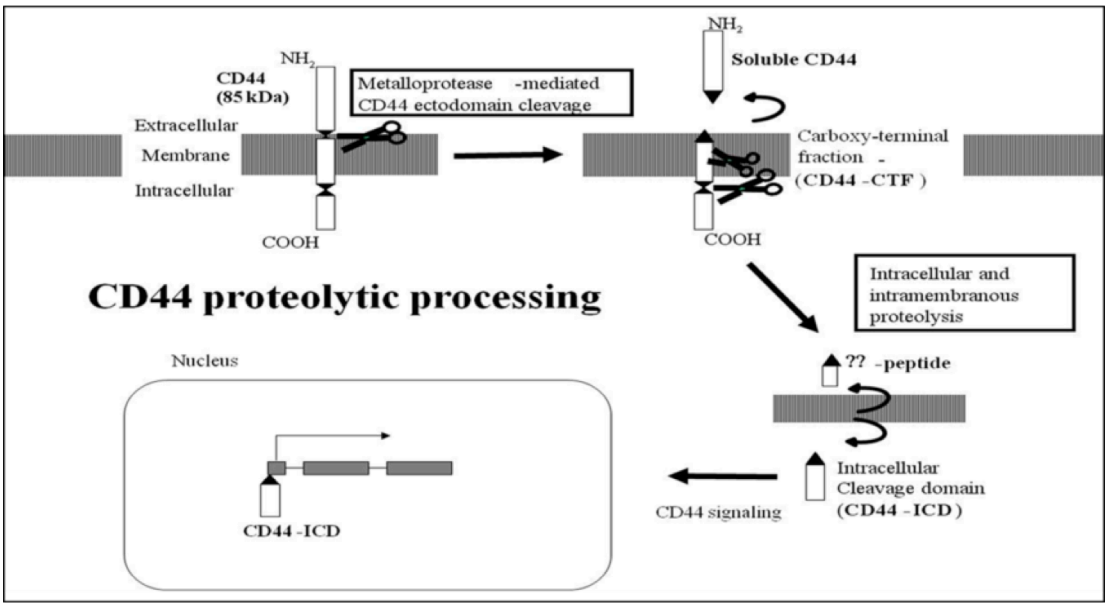


Figure 2 CD44 Undergoes Proteolytic Cleavages to generate the CD44-ICD

CD44 ICD may be involved in signal transduction pathways and can possibly interact with many transcription factors. It is able to bind to the CD44-ICD response element (CIRE), which appears to inhibit or induce gene expression (Miletti-Gonzalez et al., 2012). The ICD has also been known to interact with transcription factors that do not contain CIREs via protein-protein interaction.

The function of this glycoprotein is regulated by its post-translational modification. Due to these post-translational modifications that produce the many different isoforms of CD44, some may promote tumor formation (Naor *et. al*., 2008). The normal functions of CD44 are necessary for the physiological activities of normal cells but some of these functions are associated with the activities of cancer cells. CD44 is a marker for breast cancer stem cells and has been seen as an indicator of survival time in epithelial ovarian cancer patients (Lin *et. al*., 2017). The isoforms have a role in the progression of head and neck squamous cell carcinoma (Lin *et. al*., 2017). In cancer a high level of CD44 is not always associated with an unfavorable outcome. CD44 upregulation in neoplasia is sometimes very favorable. All CD44 expression is not negative. (Naor *et. al*. 2008).

2.1 The Post-Translational Modification of CD44

CD44 undergoes post-translational modification including phosphorylation a several serines at the intracytoplasmic domain (e.g., serine 291 and serine 325) (Neame and Isacke, 1992; Peck and Isacke, 1998; Pure *et al*., 1995). Phosphorylation of Ser325 occurs in approximately 25-40% of CD44 molecules. This modification is mediated by Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Lewis *et al*., 2001). Mutations at the phosphorylated Ser325 impair the hyaluronan mediated cell migration (Peck and Isacke, 1996; Peck and Isacke, 1998). CD44 phosphorylation is regulated by the activation of protein kinase C (PKC). Following PKC activation there is an almost complete dephosphorylation at Ser325 resulting in phosphorylation at alternative serine(s). But the total levels of CD44 phosphorylation are not significantly altered (Legg *et al*., 2002). Ser316 residue (which is highly conserved) lies in a

protein kinase A (PKA) consensus site. This site does not get phosphorylated in resting cells, but can be phosphorylated after cell stimulation (Neame and Isacke, 1992).

Cys286 and/or Cys295; membrane proteins of CD44 are palmitoylated. Palmitoylation is the covalent attachment of fatty acids, to cysteine or to serine and threonine residues of proteins, which are typically membrane proteins. This leads to the impairment of anti-CD3-mediated signal transduction in lymphocytes and influences the connection of CD44 with ankyrin. (Bourguignon *et al*., 1991; Guo *et al*., 1994). The palmitoylation of these cysteines in the CD44 sequence might be essential in regulating its association with ERM proteins; which are a family of proteins that consists of ezrin, radixin and moesin. The three closely related proteins are genes related by duplication within a genome and are highly conserved.

CD44 cleavage can result in two cell-associated CD44 species (25 kDa and 12 kDa) in addition to the extracellular domain fragment that is released. (Murakami et al., 2003; Okamoto et al., 1999a; Okamoto et al., 2001; Okamoto et al., 1999b). The 25 kDa species corresponds to the membrane-bound C-terminal fragment (CTF), where the major product isolated from the 12 kDa band is a CD44 intracellular domain (CD44-ICD) fragment that is a result from a cleavage just inside the CD44 transmembrane region (TMR) (Okamoto *et al*., 2001). Presenilin-1/γ-secretase mediates the cleavage within the TMR. It cleaves after Ala280 and Ile287 in this region (Lammich et al., 2002; Murakami et al., 2003).

The production of the ICD fragment has been termed regulated intramembrane proteolysis (RIP) (Brown *et al*., 2000). It has been shown that the CD44 intracytoplasmic domain translocates to the nucleus and stimulates transcription via a phorbol ester response element and that one of its target genes is the gene encoding CD44 itself (Okamoto *et al*., 2001).

2.2 Gene Expression

Genes encode proteins and those proteins regulate cell function. The genes expressed in a cell will determine what that cell can do. The steps in this process go from DNA to RNA to proteins. This series of events start with transcription and continues with translation thus, providing the cell with a control point for self-regulating its functions by controlling the amount and what type of proteins it produces. (Alberts *et. al*., 2002). The amount of protein that is produced in a cell reflects the balance between that protein's synthetic and degradative biochemical pathways. Protein levels are influenced by the way a cell processes its RNA transcripts and newly made proteins.

Gene expression is regulated at the beginning of the protein production process also known as the initiation of transcription. The amounts and types of mRNA molecules in a cell reflect its function. Transcripts are produced every second in every cell. RNA transcription makes an efficient control point because many proteins can be made from a single mRNA molecule (O'Connor *et. al*., 2010). Transcription begins when an RNA polymerase binds to a promoter sequence on the DNA molecule. Enhancer sequences also play a part in transcription because they provide binding sites for regulatory proteins that affect RNA polymerase activity. Binding of regulatory proteins to an enhancer sequence causes a shift in chromatin structure that either promotes or inhibits RNA polymerase and transcription factor binding (O'Connor *et. al*., 2010). Some regulatory proteins affect the transcription of genes. Regulatory proteins can have different roles for different genes, and this is how cells can coordinate the regulation of many genes at once (O'Connor *et. al*., 2010).

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell Culture

MCF-7 cell line generated from a human breast adenocarcinoma was used to derive the MCF-7/CD44s cell line. They are CD44 negative and CD44 positive, respectfully. The cells were cultured in Opti-MEM (Gibco) medium with 5% fetal bovine serum (FBS), and 1% antibiotic/antimycotic (A/A). Cells were incubated in 5% CO_2 at 37° C.

3.2 H_2O_2 Cell Viability Assay (MTS Assay)

Cells were plated in a 96-well plate (10,000-20,000 cells per well) in approximately 100 uL of cell culture medium and grown for 24 hours. Following this incubation we treated the cells with diferrent concentrations of hydrogen peroxide (H_2O_2). We ten incubated the plates for approximately 21 hours and then a MTS analysis was performed to measure cell viability. 20 µL of the combined MTS/PMS reagent was added to each well of the 96-well assay plate and then incubated for 1-4 hours at 37° C, 5% CO_2. The absorbance of the plate was recorded using Synergy HTK (Biotek), a multi-mode plate reader at a wavelength of 490 nm.

The MTS assay is used to quantify the viability of cells undergoing cytotoxicity. This assay is a colorimetric sensitive assay based on the reduction of the MTS tetrazolium by viable cells to produce a colored formazan product that is cell culture media soluble.

3. 3 Reverse Transfection of Antioxidant Response Element (ARE) Reporter Constructs. (BPS Bioscience)

Cells were cultured in 96-well white plates with a clear bottom (Corning). The transfection reagent used was siPORT NeoFX (Invitrogen). 6 wells were transfected in triplicates for a total of 18 wells with a mock transfection (MT) containing no DNA, positive

ARE reporter (IV), and the negative ARE reporter (NIV). In 0.5 mL microcentrifuge tubes the NeoFx agent plus serum free Opti-MEM medium was prepared and incubated for 10 minutes as well as the diluted ARE plasmids with serum free Opti-MEM. Both dilutions were then combined in a microcentrifuge tube and incubated for an additional 10 minutes. Following the incubation period the NeoFX/ARE plasmid solution was added to each well according to their contents, whether it was the MT, IV, or NIV transfectant. 20.5 uL was added to each MT well, and 21.5 uL was added to the NIV and IV wells. The plates were then incubated for 24 hours at 37° C, 5% CO_2 and read using a dual luciferase assay (Promega).

Reverse Transfection is a process in which DNA is placed in the wells for the transfection process to occur before the addition of transfection reagents and diluted plasmids. The order of the addition of DNA and diluted plasmids or reagents is reverse that of conventional transfection.

3.4 Luciferase Assay

To measure the gene expression of the transfection mentioned above, the Dual-Glo Lucifearse assay system was used. To the 96-well plate mentioned above, 75 uL of Dual-Glo reagent was added to each well. Following a 10-minute incubation to allow for lysis of the cells to occur luminescence from firefly luciferase was measured. Following this reading 75 uL of the Dual-Glo Stop & Glo reagent was added to each well. After an additional 10-minute incubation luminescence from renilla luciferase was measured. From these readings a ratio was calculated to quantify the normalized expression of the reporter plasmid.

A luciferase assay is used to study gene expression at the transcriptional level. It provides the opportunity to explore the activity of the promoter by measuring the output of light from a luciferase enzyme that is expressed under the control of the promoter of interest.

3.5 Promega Flexi Vector Construct System

Assemble the reaction based on whether not it is the C-terminal or N-terminal fusion proteins being used. For the N-terminal reaction in a microcentrifuge tube we mixed the digest buffer, acceptor vector, donor vector, flexi enzyme blend, and nuclease free water to complete a mixture of 20 uL total. For the C-terminal reaction we took two different microcentrifuge tubes and in one mix the digest buffer, donor vector, flexi enzyme blend, and nuclease free water to a total of 10 uL. In the other tube we mixed the digest buffer, the acceptor vector, the carboxy flexi enzyme blend, and nuclease free water to complete 10 uL total. Incubate all reactions at 37°C for 30 minutes. Next we heated the reactions at 65°C for 20 minutes to inactive the restriction enzymes then place on ice to prepare for ligation reaction. In a separate tube for the N-terminal reaction 10 uL was taken from the previous step and mixed with ligase buffer, T4 DNA ligase, and nuclease free water to complete to 21 uL total. For C-Terminal reactions 5 uL was taken from both of the previous tubes and mixed well. Then ligase buffer and T4 DNA ligase was added. All tubes were incubated at room temperature for an hour and proceeded to the cell transformation.

3.6 Cell Transformation

Prepare LB plates with either 15 ug/mL kanamycin or 50 ug/mL ampicillin. The bacterial competent cells used were HB101 cells. The cells were thawed on ice before use and mixed gently to ensure an even distribution. In a microcentrifuge tube 2.5 uL of the ligation was added

to 50 uL of HB101 cells. We placed the tubes on ice for 30 minutes, then heat shocked the cells at 42°C for exactly 45 seconds then place the tubes on ice again for 1-2 minutes. To the tubes pre-warmed SOC medium was added to bring the final volume to 200 uL total. We incubated the tubes by shaking for 1 hour at 37°C. Following the incubation period, all of the culture was placed on the selective medium (kanamycin or ampicillin) and we incubated the LB plates overnight at 37°C.

3.7 High Speed Plasmid Mini Kit

To prepare mini preps of the competent cell transformation a high-speed plasmid mini kit (MIDSCI) was used. In microcentrifuge tubes, 1.5 mL of cultured bacterial cells were transferred and centrifuged at 14,000-16,000 x g for 1 minute. The supernatant was discarded and the following reagents were added to the cell pellet: 200 uL of PD1 buffer, (and mixed by vortexing), and 200 uL of PD2 buffer and inverted 10 times. A incubation at room temperature followed. Next we added 300 uL of PD3 buffer mixing immediately and centrifuging at 14-16,000 x g for 3 minutes. While that centrifuge process is taking place, a PD column provided by the company was placed into a 2 mL collection tube. The supernatant was added from the previous step into the column and centrifuged again at 14-16,000 x g for 30 seconds. We discarded the supernatant and placed the column back inside the collection tube. Next the 600 uL of the wash buffer was added and centrifuged at 14-16,000 x g for 30 seconds and the flow through was discarded then the column was added back to the collection tube and centrifuged for an additional 3 minutes to ensure that the column matrix was dry. Once the matrix was dry we added 50 uL of the elution buffer to elute the DNA out of the column matrix. Let it stand for

approximately 2 minutes and centrifuge for 2 minutes at 14,000-16,000 x g. Finally we are left with the purified DNA.

3.8 Human Pluripotent Stem Cell Array Kit

This array kit was purchased from R&D systems. All reagents were at RT before starting the assay. To begin this array first 1 mL of array buffer 1 (blocking buffer) was pipetted into each 8-well multi-dish. Then using flat tip tweezers, each membrane was removed from the protective sheets and placed into the 8-well multi-dish with the number of the membrane facing upward and then incubated for an hour on a rocking shaker. During the blocking of the arrays, the samples were prepared: 167 uL of lysate to 833 uL of array buffer 1 and adjust to 1 mL with lysis buffer 16. Once the blocking incubation was done, array buffer 1 was removed from the wells and 167 uL of the prepared sample was added and incubated overnight on a rocking shaker at 2-8°C. Next each membrane was removed and placed in containers for a washing with 20 mL of 1X wash buffer for 10 minutes on a rocking shaker. The washing phase was done 2 additional times for a total of 3 washes while the 8-well multi-dish was rinsed with diH_20. After rinsing and drying of the 8-well multi-dish, 1 mL of diluted detection antibody cocktail was added to each well. The arrays were then placed back into the multi-dish and covered with a lid. Following this there was an incubation period for 2 hours on a rocking shaker then another wash of the array was done as described before. 1 mL of streptavidin-HRP was pipetted into each well and the arrays were placed back into the multi-dish and incubated for 30 minutes on rocking shaker. Another wash step as described before followed the incubation. Each membrane was placed on the bottom sheet of the plastic sheet protector with the numbers facing up and 500 uL of the chemi luminescent reagent mix with placed onto the entire membrane. The top sheet of the

plastic sheet protector was placed on top avoiding any air bubbles and incubated for 1 minute. Before moving to the next step any excess chemi luminescent reagent mix was carefully squeezed out. Then the entire membrane with the bottom plastic sheet protector is wrapped in plastic wrap then placed on a C-Digit scanner (Li-Cor). The scanner read the membrane for approximately 12 minutes.

3.9 Apoptosis Array Kit

This array kit was purchased from R&D systems. All reagents were at RT before starting the assay. First, 1 mL of array buffer 1 (blocking buffer) was pipetted into each 4-well multi-dish. Then using flat tip tweezers, each membrane being used was removed from the protective sheets and placed into the 4-well multi-dish with the number of the membrane facing upward and then incubated for an hour on a rocking shaker. During the blocking of the arrays, the samples were prepared. The desired amount of lysate was added to 1.25 mL of array buffer 1 and the volume was adjusted to 1.5 mL as necessary with lysis buffer 17. Once the blocking incubation was done, array buffer 1 was aspirated from the wells and 250 uL of the prepared sample was added and incubated overnight on a rocking shaker at 2-8°C. Next each membrane was removed and placed in containers for a washing with 20 mL of 1X wash buffer for 10 minutes on a rocking shaker. The washing phase was done 2 additional times while the 4-well multi-dish was rinsed with diH_20. After rinsing and drying of the 4-well multi-dish, 1.5 mL of diluted detection antibody cocktail was added to each well. The arrays were then placed back into the multi-dish and covered with a lid. Following this there was an incubation period for 1 hour on a rocking shaker then another wash of the array was done as described before. Then 2 mL of streptavidin-HRP was pipetted into each well and the arrays were placed back into the multi-dish and

incubated for 30 minutes on rocking shaker. Another wash step as described before followed the incubation. Each membrane was placed on the bottom sheet of the plastic sheet protector with the numbers facing up and 1mL of the chemi luminescent reagent mix was placed onto the entire membrane. The top sheet of the plastic sheet protector was placed on top avoiding any air bubbles and incubated for 1 minute. Before moving to the next step any excess chemi luminescent reagent mix was carefully squeezed out. Then the entire membrane with the bottom plastic sheet protector was wrapped in plastic wrap then placed on a C-Digit scanner (Li-Cor). The scanner read the membrane for approximately 12 minutes.

3.10 Plasmid Maxiprep

ZymoPURE Maxiprep kit (centrifuge protocol) was used to prepare maxipreps for transfections. First in a 50 mL centrifuge tube a spin down at 3,400 x g for 10 minutes of the bacterial culture was done to separate the bacterial cells from the supernatant. The supernatant was discarded. 14 mL ZymoPURE P1 solution was added to break up the bacterial cell pellet from the previous step. Following this step, 14 mL of ZymoPURE P2 and P3 was added to the tube and gently mixing by inverting the tubes. Next the mixture was placed in a syringe filter. Once in the filter, there was approximately a 5-8 minute wait for the precipitate to float to the top of the syringe filter. Once the precipitate floated to the top the syringe filter was placed into a new 50 mL conical tube and the mixture was pushed through the syringe filter and into the new 50 mL conical tube. To the new 50 mL tube, 14 mL of ZymoPURE Binding Buffer was added and mixed by inverting the capped tube 10 times. Next the Zymo-Spin V-P Column Assembly, the 15 mL Conical Reservoir and the Zymo-Spin V-P column were placed in another 50 mL tube where 14 mL of the syringe-filtered mixture was added. This assembly was centrifuged at 500 x

g for 2 min and the supernatant was discarded. This step was completed until the entire mixture has been passed through the column. Once the entire mixture has been passed through the column 5 mL of ZymoPURE Wash 1 was added to the column and centrifuged at 500 x g for 2 minutes. The supernatant was discarded and then 5 mL of wash 2 was added and centrifuged at 500 x g for 2 minutes. This step was done twice. To ensure that the entire residual wash buffer was removed, the 15 mL Conical Reservoir was removed and placed into a collection tube. Centrifugation of the column at 10,000 x g for 1 minute was completed. The column was then transferred into a clean 1.5 mL tube. 400 uL of ZymoPURE Elution Buffer was added directly to the column matrix. Following an incubation period of two minutes, the column within the 1.5 mL tube was centrifuged at 10,000 x g for 1 minute. The remaining was the eluted DNA and stored at -20°C.

3.11 DNA Precipitation

To precipitate the DNA in solution, 1/10 volume of 3M sodium acetate, pH 5.2 and 2 volumes of 100% ethanol was added to each tube, mixed and incubated overnight at -20°C. Then the tubes were centrifuged at 14,000 rpm at 4°C for 30 minutes. The resulting supernatant was discarded and the lids were left open for approximately 15 minutes to dry the DNA pellets. After drying, 100 uL of water was added to each tube and mixed well by vortex.

3.12 TF Activation Profiling Plate Array II

This array kit was purchased from Signosis. The purpose of this experiment was to monitor the expression of multiple transcription factors (TF) in nuclear extracts from MCF-7/ vector and MCF-7/CD44s cells. To begin this procedure all the components for the reaction in which the TFs, if present in the nuclear extracts, would bind a double stranded DNA probe with

a particular response element sequence, were mixed into a microcentrifuge tube. These components consisted of 15 uL of TF binding buffer mix, 5 uL of TF probe mix II, 3 uL of nuclear extract (5ug-15ug) and 7 uL of ddH_20 to complete the final volume to 30 uL total. The reaction mix was then incubated at room temperature (RT) for 30 minutes. To separate the TF/DNA probe complex from the free probes, the reaction tubes were centrifuged at 6,000 rpm for 1 minute at room temperature and pipetted 30 uL of the reaction mix directly onto a filter in an isolation column trying to avoid bubbles and incubated for no more than 30 minutes on ice. After completion of the incubation, 500 uL of pre-chilled filter wash buffer was added to the isolation column and incubated another 3 minutes on ice. The isolation column was then centrifuged at 6,000 rpm for 1 minute at 4°C and the flow through was discarded. The previous step was continued for 3 additional washes. The next step was to elute the bound probes. First I added 100 uL of elution buffer to the center of the isolation column and incubated at RT for 5 minutes. Next I placed the column into a new 1.5 microcentrifuge tube and centrifuge the tube at 10,000 rpm for 2 minutes at RT. Once the centrifugation is complete, the eluted probe was transferred to a PCR tube and denatured at 98°C for 5 minutes. Immediately following the denaturation period the denatured probes were transferred to chilled ddH_20 and placed on ice. The hybridization of eluted probes with a hybridization plate was next. 10 mL of pre-warmed TF plate hybridization buffer was pipetted to a dispensing reservoir and 600 uL of denatured probes were added. The two were mixed by gently by shaking. With a pipette I immediately pipetted 100 uL of the previous mixture to the corresponding wells in the 96-well plate then sealed the wells with aluminum adhesive to secure the well contents. The hybridization plate was placed on an incubator set to 42°C overnight. Finally the last step of the array was the detection of bound

probes. I removed the plate from the incubator and removed the aluminum adhesive keeping the unused wells sealed. The plate was inverted and the contents were forcibly expelled. The plate was then washed by adding 200 uL of pre-warmed 1X plate hybridization wash buffer to each well and incubated on a plate shaker for 5 minutes. After the 5 minutes of incubation, I removed the wash buffer by tapping the plate against clean paper towels. This step was completed two additional times. 200 uL of blocking buffer was added to each well and again incubated by shaking for 5 minutes. The plates were inverted over the appropriate container and I forcibly remove the blocking buffer from each well. Following this step 95 uL of diluted Streptavidin-HRP Conjugate was added to each well and incubated for 45 minutes on a plate shaker at RT. After the 45 minutes was completed, the contents were removed from the well plate by tapping the plate against clean paper towels then another wash was performed. To wash, 200 uL of 1X detection wash buffer was added to each well and incubated on a plate shaker at RT for 5 minutes. This step was repeated for 2 additional washes. 95 uL of substrate solution was then added to each well. I incubated the wells at RT for 1 minute. Lastly, within 5-20 minutes after incubation, the luminescence was detected in the Synergy HTX (Biotek) plate reader.

3.13 Transcriptional Interaction TF Plate Array I

The purpose of this array is to monitor the interaction of TF's with other TF and/or co-regulators. Before staring the assay the following components for the reaction were prepared in a microcentrifuge tube; 10 uL of nuclear extract, 15 uL of 5x binding buffer, 10 uL of probe mix and 40 uL of ddH_20 to make a total volume of 75 uL. The reaction mixture was incubated at RT for 30 minutes, added 200 uL of 1X binding buffer, and incubated for an additional hour at 4°C on a shaker. This reaction was considered the TF-Antibody mixture. The next step of the assay is

to separate the TF and antibody complex from the free probes. 20 uL of the agarose resin was then resuspended and transferred to a filter column. Both the column and resin were washed with 200 uL of 1X binding buffer by centrifugation at 1500 rpm for 1 minute. During this step it is very important not to let the beads dry out. Next I transferred the TF-Antibody mixture that was prepared at the beginning of the array to the filter column and incubated on a shaker for an hour at 4°C. Once this incubation period was complete the TF-Antibody mixture and beads were washed with 200 uL of 1X binding buffer and centrifuging at 1500 rpm for 1 minute. The wash step was done an additional 2 times. Next is the elution of the bound probes. The column was placed in a 1.5 microcentrifuge tube. 100 uL of the elution buffer was added to the center of the column and incubated for 5 minutes then centrifuged at 10,000 rpm for 2 minutes. The eluted probes were then transferred to a PCR tubes and denatured at 98°C for 10 minutes then placed the tubes on ice. Following the elution step was the hybridization of denatured, eluted probes. I pipetted 100 uL of the denatured probes and added it to 5.5 mL of warmed hybridization buffer to a dispensing reservoir and mixed gently by shaking the reservoir. Immediately after the shaking I dispensed 100 uL of the mixture to the corresponding wells, then sealed the well with film and let hybridize overnight at 42°C. Finally the last step of the array is the detection of bound probes. The plate was removed from the incubator and the film from the experimental wells was removed as well. The plate was inverted and the wells were emptied forcibly into the appropriate containers. The plate was then washed 3 times by adding 200 uL of pre-warmed 1x plate hybridization wash buffer and incubated for 5 minutes by shaking. After washing the plate, all the liquid was emptied by tapping the plate against clean paper towels then 200 uL of blocking buffer was added and another incubation was done by shaking for 5 additional minutes.

The plate was then inverted over the corresponding container and emptied. Following this step 95 uL of diluted Streptavidin-HRP Conjugate was added to each well and incubated for 45 minutes on a plate shaker at RT. After the 45 minutes is completed the contents were removed from the well plate by tapping the plate against clean paper towels and another wash was done. To wash, 200 uL of 1X detection wash buffer was added to each well and incubated on at plate shaker at RT for 5 minutes. 95 uL of substrate solution was added to each well. The wells were incubated at RT for 1 minute. Lastly, within 5-20 minuets after incubation, the plate was placed into a plate reader and a reading was done.

3.14 Data Analysis

All data were analyzed and graphed using Microsoft Excel. . For data obtained from MTS assays, the absorbance at 490 nm was determined from three repetitions (n=3) and the standard deviation used as error bars. The control well (no hydrogen peroxide) was assigned a numeric value of 1 (raw reading divided by itself) and experimentals were assigned a value of its raw reading divided by control's raw reading. Two-tailed T-test was applied to determine significance ($P < 0.05$).

CHAPTER 4: RESULTS

4.1 CD44-mediated gene expression regulation: Cell Morphology

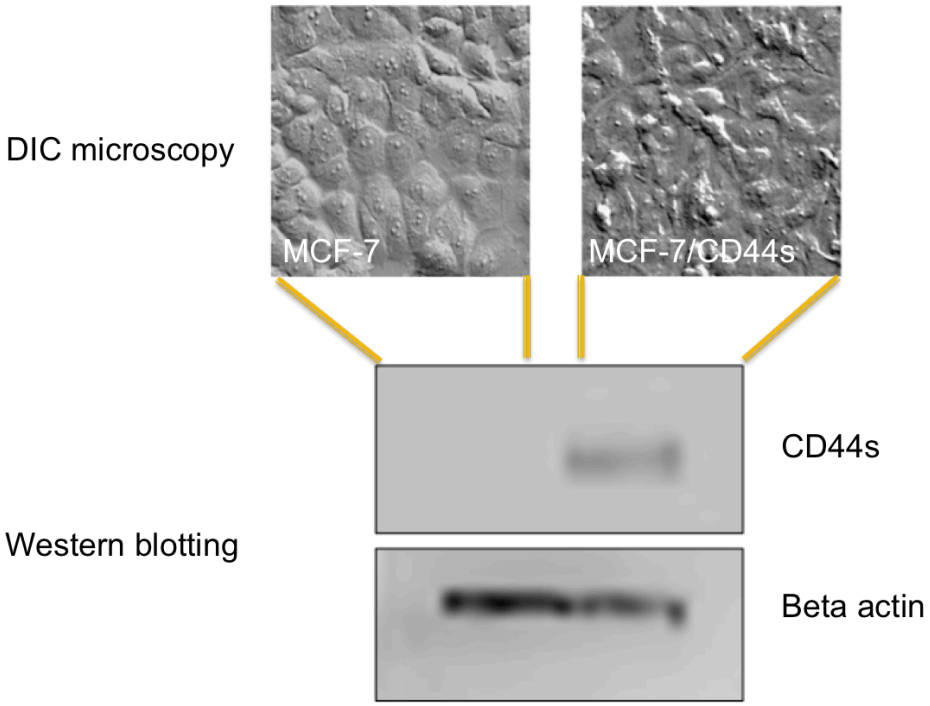


Figure 3 Change in cell morphology associated with the expression of CD44. MCF-7 cells and MCF7/CD44s show a change in cell morphology. A western blot validates the stably transfected CD44 expression in the cell line.

The MCF-7/CD44s cell line was derived from the stable transfection of CD44s in MCF-7 cells, a breast cancer cell line (Miletti-Gonzalez et. al., 2005). The morphology of these cell lines varies when observed under a microscope. MCF-7 cells, which are CD44 negative, show higher contact inhibition compared to MCF-7/CD44s cells, which exhibit a constant growth (figure 3). The formation of each MCF-7 cell in culture is clear and concise. MCF-7/CD44s where CD44 is expressed show a differential morphology. A western blot analysis suggests that CD44 is associated with this change in cell morphology (Figure 3). Beta actin, a commonly housekeeping was used as a loading control.

4.2 Genes encoding transcription factors that can be regulated by CD44

TF	MCF-7	MCF-7/ CD44	fold change	CIRE sequence
HOX4C	142.09	7078	49.8	positive
Stat6	606.75	24000	39.6	positive
HNF-1	940.85	30000	31.9	positive
NRF2	188.17	7.55	-24.9	positive
PPAR	4435.5	43000	9.7	positive
Pax2	126.73	15.1	-8.4	positive
MyoD	195.85	26.425	-7.4	positive
PIT1	215.05	1555.3	7.2	positive
SMAD	57.6	347.3	6.0	positive
Nkx2-5	195.85	33.975	-5.8	positive
PLAG1	16000	90000	5.6	positive

TF	MCF-7	MCF-7/ CD44	fold change	CIRE sequence
COUP-TF	24000	90000	3.8	positive
Pax3	126.73	37.75	-3.4	positive
Pax-5	65.28	215.17	3.3	positive
FREAC-2	72.964	218.95	3.0	positive
Gli-1	49.923	147.22	2.9	positive
ATF2	115.21	298.22	2.6	positive
CBF	672.04	268.02	-2.5	positive
ER	552.99	241.6	-2.3	positive
Nkx3-2	195.85	90.599	-2.2	positive
CDP	414.74	200.07	-2.1	positive
KLF4	69.124	33.975	-2.0	positive

TF	MCF-7	MCF-7/ CD44s	fold change	CIRE sequence
SF-1	1524.6	40000	26.2	negative
PXR	491.55	11000	22.4	negative
NF-1	460.83	10000	21.7	negative
XBP-1	69.12	698.37	10.1	negative
Oct-1	199.69	30.2	-6.6	negative
SMUC	18000	86000	4.8	negative
TFE3	65.284	241.6	3.7	negative
MEF2	88.33	271.8	3.1	negative
Myb	99.85	305.77	3.1	negative
TCF/LEF	61.44	181.2	2.9	negative
Snail	368.66	128.35	-2.9	negative
AR	280.34	788.97	2.8	negative
NRF1	261.13	98.149	-2.7	negative

Table 1 TF Activation Profiling Plate Array II suggest a CD44 mediated regulation of genes and a potential underlying cause for the differential change in cell morphology between MCF-7 and MCF-7/CD44s.

The differential expression of these genes in MCF-7 vs MCF-7/CD44 cells suggests a connection of changes in cell morphology with changes in gene expression. The plate contained a total of 96 different single stranded DNA complementary sequences with response elements associated with 96 different transcription factors. We only are showing the ones that are 2-fold and higher in the differential expression between MCF-7 and MCF-7/CD44 cell lines. The ones lower than 2-fold are not in this table because we did not consider them significantly different. In the MCF-7/CD44 cell line there is an increase in the expression in most of the genes encoding these specific transcription factors. The CIRE sequence column on the far right of the table, identified the genes that contain one or more CIRE sequences (positive) or none (negative) in their promoter regions. Interestingly, the entire group of genes that did not have any CIRE sequences in their promoter regions did show the presence of the response element for the

transcription factor Runx2 (i.e., 5'-tg(c/t)ggt-3'). Runx2 is the transcription factor that we have shown that interacts with the CD44-ICD on the promoter region of the MMP-9 gene (Miletti-Gonzalez et al., 2012).

4.3 H_2O_2 Viability Assay

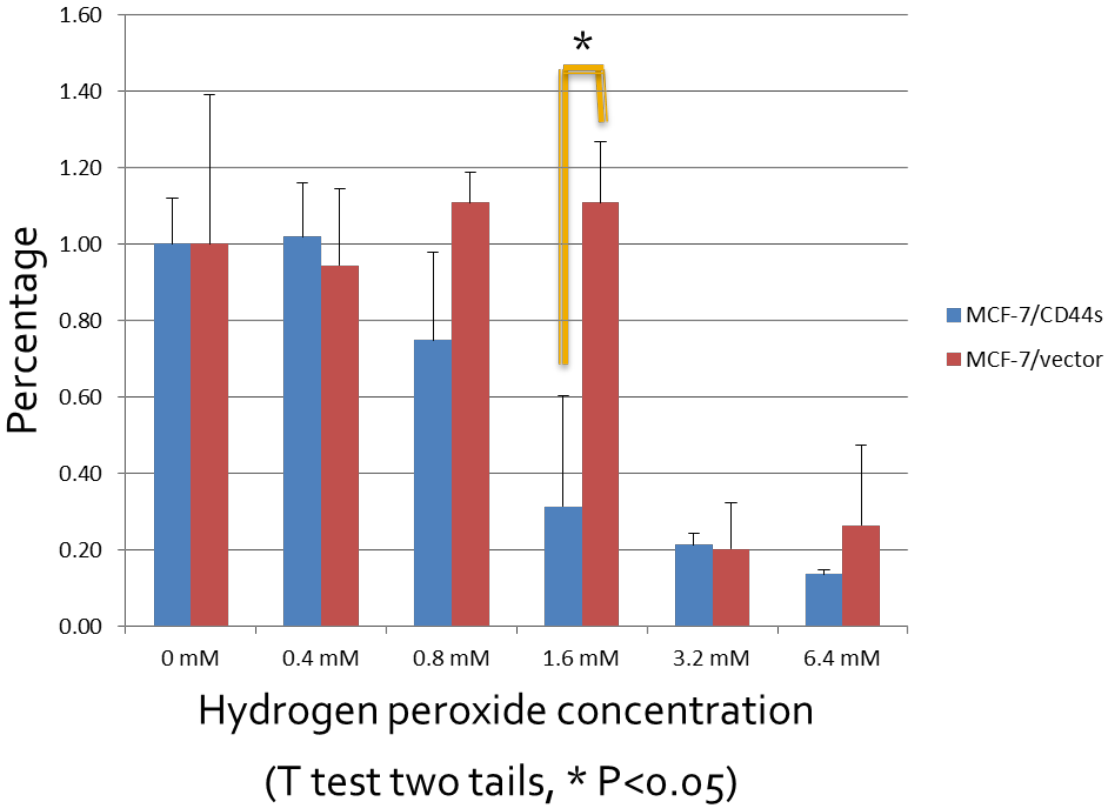


Figure 4 Hydrogen Peroxide Assay. The X-Axis represents the varying concentrations of hydrogen peroxide treatment performed on each cell line. The Y-Axis represents the absorbance in which the viability was read. The red bars represent the MCF-7/vector cell line and the blue bars represent the MCF-7 stably transfected with CD44s.

With this data we wanted to show that there is differential effect between the cell lines when hydrogen peroxide was added. The MCF-7/CD44 cell line is more resistant to hydrogen peroxide treatment. The MCF-7/vector is becomes more resistant when there are higher concentrations of H_2O_2 were added to this cell line. The MCF-7/ CD44 cells sensitivity to H_2O_2

is consistent to the detected reduction in Nrf2 expression in these cells compared to MCF-7/vector cells.

4.4 ARE-mediated Luciferase Reporter

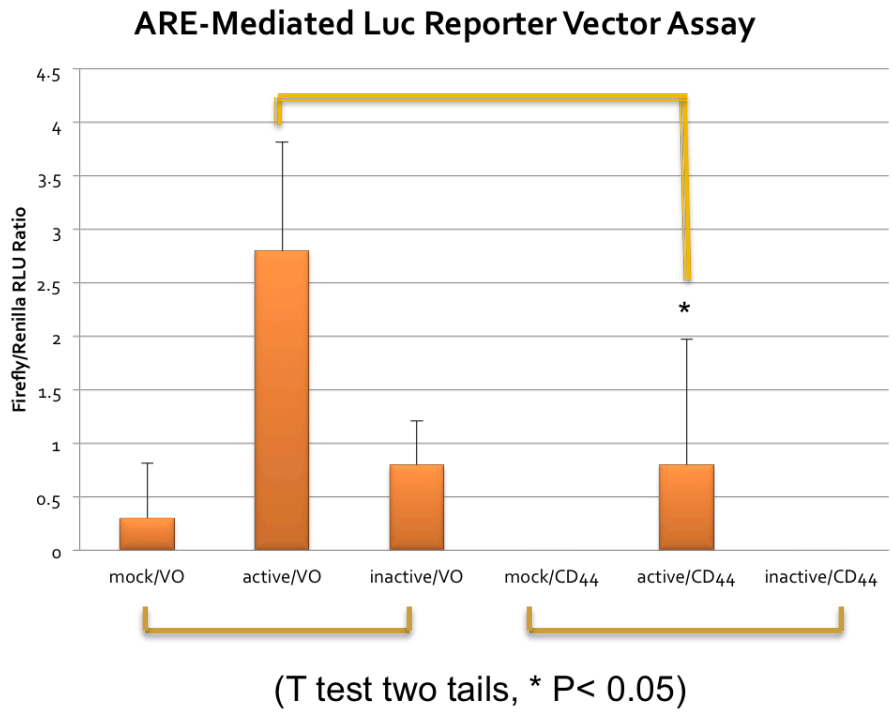


Figure 5 The affect of CD44 on the ARE luciferase reporter. Mock represents no ARE reporter transfected into the cell, active is the positive ARE control and inactive is the promoter-less ARE plasmid control. VO represents MCF-7/vector and CD44 represents MCF-7/CD44. The absorbance was measured at 490 nm.

Nrf2 is a transcription factor involved in the protection of cells against oxidative stress insults. The response element mediating this response is called ARE, or the antioxidant response element. In the MCF-7/CD44 cell line the levels of Nrf2 are about 25-fold less than in the MCF-7/vector cell line as shown in table 1. This difference in Nrf2 expression is reflected in the result of the ARE-mediated luciferase reporter assay. A significant increase in the expression of the reporter luciferase gene in MCF-7/vector cells compared to MCF-7/CD44 cell was measured.

4.5 CD44- associated mediated differential gene expression

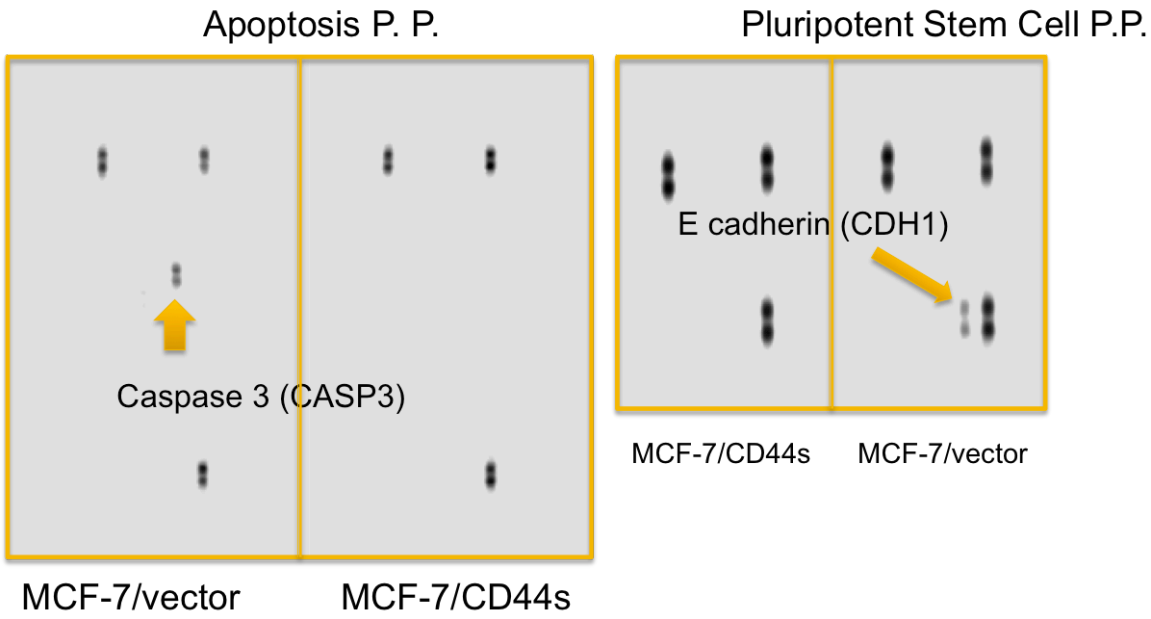


Figure 6 differential expressions of apoptosis and pluripotent stem cell markers between MCF-7 and MCF-7/CD44s. Caspase 3 (CASP3) and E cadherin (CDH1) are present MCF-7/vector cells (CD44 negative cells) but absent in the CD44 positive MCF-7/CD44 cell line.

Caspase 3, an important effector molecule in apoptosis, is inhibited in MCF-7/CD44s cells but not in MCF-7/vector cells. E-Cadherin, a cellular marker in pluripotent stem cells, is inhibited in MCF-7/CD44s cells but not in MCF-7/vector cells. These two membranes are testing the presence of specific biomarkers for these two events in which there is a potential regulatory mechanism associated with the expression of CD44.

4.6 Possible CD44-ICD involvement: E cadherin and Caspase 3 promoter region

>FP019021 CDH1_1 :+U EU:NC; range -1000 to 300.
ATTAGGCCGCTCGAGGCAGAGTGCAGTGGCTCACGCCTGTAATCCAACACTTCAGGAGGC
TGAAGAGGGTGGATCACCTGAGGTCAGGAGTTCCAGACCAGCCTGGCCAACATGGTGAAA
CCCCGTCTGTACTAAAAATACAAAATTAGCCGGTGTGGTGGCACACGCCTGTAGTCCCAG
CTACTCAATAGGCTGAGACAGGAGAGTCTCTTGAACCCGGCAGGCGGAGGTTGCAGTGAG
CCGAGATCGTGCCACTGCACTCCAGCCTGGGCAAGACAGAGCGAGACTCCGTCTCAAAAA
ATACAAACAAAACAAACAAACAAAAAATTAGGCTGCTAGCTCAGTGGCTCATGGCTCACA
CCTGAAATCCTAGCACTTTGGGAGGCCAAGGCAGGAGGATCGCTTCAGCCCAGGAGTTCG
AGACCAGGCTGGGCAATACAGGGAGACACAGCGCCCCCACTGCCCCTGTCCGCCCCGACT
TGTCTCTCTACAAAAAGGCAAAAGAAAAAAAAAATTAGCCTGGCGTGGTGGTGTGCACCTG
TACTCCCAGCTACTAGAGAGGCTGGGGCCAGAGGACCGCTTGAGCCCAGGAGTTCGAGGC
TGCAGTGAGCTGTGATCGCACCACTGCACTCCAGCTTGGGTGAAAGAGTGAGACCCCATC
TCCAAAAACGAACAAACAAAAAATCCCAAAAAACAAAAGAACTCAGCCAAGTGTAAAAGCC
CTTTCTGATCCCAGGTCTTAGTGAGCCACCGGCGGGGCTGGGATTCGAACCCAGTGGAAT
CAGAACCGTGCAGGTCCCATAACCCACCTAGACCCTAGCAACTCCAGGCTAGAGGGTCAC
CGCGTCTATGCGAGGCCGGGTGGGCGGGCCGTCAGCTCCGCCCTGGGGAGGGGTCCGCG
CTGCTGATTGGCTGTGGCCGGCAGGTGAACCCTCAGCCAATCAGCGGTACGGGGGGCGGTG
CCTCCGGGGCTCACCTGGCTGCAGCCACGCACCCCCTCTCAGTGGCGTCGGAACTGCAAA
GCACCTGTGAGCTTGCGGAAGTCAGTTCAGACTCCAGCCCGCTCCAGCCCGGCCCGACCC
GACCGCACCCGGCGCCTGCCCTCGCTCGGCGTCCCCGGCCAGCCATGGGCCCTTGGAGCC
GCAGCCTCTCGGCGCTGCTGCTGCTGCTGCAGGTACCCCGGATCCCCTGACTTGCGAGGG
ACGCATTCGGGCCGCAAGCTCCGCGCCCCAGCCCTGCGCCCCTTCCTCTCCCGTCGTCAC
CGCTTCCCTTCTTCCAAGAAAGTTCGGGTCCTGAGGAGCGG

>FP006683 CASP3_1 :+U EU:NC; range -1000 to 300.
CCATTACCAGTTCTCACCACAGGACTCTGTGACTATAAAAGATGGGGCCTTCGGCAGCTC
CCAACAGCCGGCTTAATTGACGCCGTCTTTGCAGAAGGACCTCTCGGCCCAAACCAGGCT
TCTTTTCCTCTTCTTCACTCCCTCTGGATTGGTTCGTTAATCTCTTTGCCCCATCTCTTT
TTCCTCATGATGTTAAATGCTACTTTGTTGTGGGATGCTTAACTTGTAACATTTATATGT
GGATTAAGTACACTACTATGATGCGTGAGCGGCTTGAGCCTGTGTGTCAGGCTCTGACTA
CCCAGTGACCAGCAAGTGCTGAGAACTGCCTCCTTGGGAACTCCACGCAGCTCGTGGCTT
TTGTGATCGAAAGAGCGTCCACAGCAGCCTGACATGGTGGAAAACACAGCATGCGTGGAC
CTGGTTATTTCTAGCCTGGCACCCTGATGACAGGAGGTTAAGGCTTTTGATTTAGGGAGA
GAGACCTGAGGGTAACCCAAAGAAAGGCCTCAGGTCATCGCAGAGGAGACAGAAATTACC
GGTGGGCGTCCCGGCCTGCTCCTCTTCTTCCCAGAGCTCAAACTGGGCAGCAGGCCTGGC
TTTGTCTCCCTTCTAGGTACCCGTTGGGCCTGGCGCGGTTTCCAAGTCTCCCTCAATTTC
CCTCCAGAATCCCGCGCTGGGAGCGGAAGCCCGCGGACTCTCAGGAAGACCTAGATTTCA
AATCCGGGGCGGGGGAGCCGGGCCGAGCCTCAGGGCGGGACCAAAGCGGGAGGACTGGCG
CGGGACGCGAGGGCGGGACCAGGGCGTGAGGACGCGGGGCGCGGGGGCGGGGCGCGCTGC
GGTGGGCGGGCGCCTCCTGGGGCCCAGCCTGGGGCTGGGTTCTCGGGCGGGCGGGAGCTA
GGGGACGGGCCGCAGCGGAGGCGGCGCGAGGGGCCGACAGGGGCGGGCGGGAGGCGGG
GCTGGCGCTGGAGCACATCTCCCGGCGGCGGGCCGCGGAAGCAGTGCAGACGCGGCTCCTAG
CGGATGGGTGCTATTGTGAGGCGGTTGTAGAAGGTATGAGGAGGCTGTGCGGTGACTGCC
GCCCTCGCTCCGGGCCGTCAGCCCTGCCCCCGCTCCCTGCGTTCTCCCAGGCCCTGCGCC
CGCCCGGGTACCTGCCCGGTCTGGAAGGCGGAGTGTTTGTTTTGCCACCTCGCCCGGGCG
GGGGCGAGCAGTGAAGCCCGCGGAGCGAGAGACTTGGCCCGGAGGCGGGAGCCTGGGCCG
GGGCCGGGGAAGCCAGGCAGCGGGTTAAGGCTTTTGATTTA

Figure 7 E cadherin (CDH1) and Caspase 3 (CASP 3) promoter region. Green T = +1 (TSS), red nucleotides = CIRE sequence (5'-cctgcg-3').

As shown in Figure 7 the promoter regions of E cadherin (CDH1) and caspase 3 (CASP 3) contains CIRE sequences downstream of the transcriptional start site (+1). The E cadherin (CDH1) promoters CIRE sequence is 232 base pairs down stream. The Caspase 3 (Casp 3) promoters CIRE sequences are 113 and 130 base pairs down stream

4.7 The CD44-ICD involvement in gene expression regulation

TF	MCF-7	MCF-7 CD44	Fold Change
C/EBP	51.922	1005.1	19.4
Pit *	122.39	789.95	6.5
AP2	92.717	407.96	4.4
Stat6 *	315.24	1185.8	3.8
Stat4	229.94	845.58	3.7
Stat3	307.82	1034.7	3.4
PPAR *	211.4	686.11	3.2
SMAD *	274.44	864.13	3.2
Stat5	289.28	916.05	3.1
TCF/LEF *	348.62	1045.9	3.0
PXR *	311.53	715.78	2.3
Sp1	367.16	819.62	2.2
SRE	452.46	960.55	2.1
Pbx1	370.87	775.12	2.1
NFAT	448.75	912.34	2.0

Table 2. Protein-protein interactions of the CD44-ICD with multiple transcription factors in MCF-7and MCF-7/CD44 cells.

As shown in Table 2, the CD44-ICD can interact with various transcription factors MCF-7/CD44 cells. However, these interactions do not appears to be completely specific since they were also detected in MCF-7 cells, which are normally CD44 negative and thus the presence of the CD44-ICD is not expected. Despite this apparent discrepancy, the ratio between the assay value indicating the CD44-ICD/Runx2 interaction in MCF-7/CD44 cells versus the same one in MCF-7 cells showed a difference of up to 19.4 fold.

4.8 Proximity ligation assay (PLA)

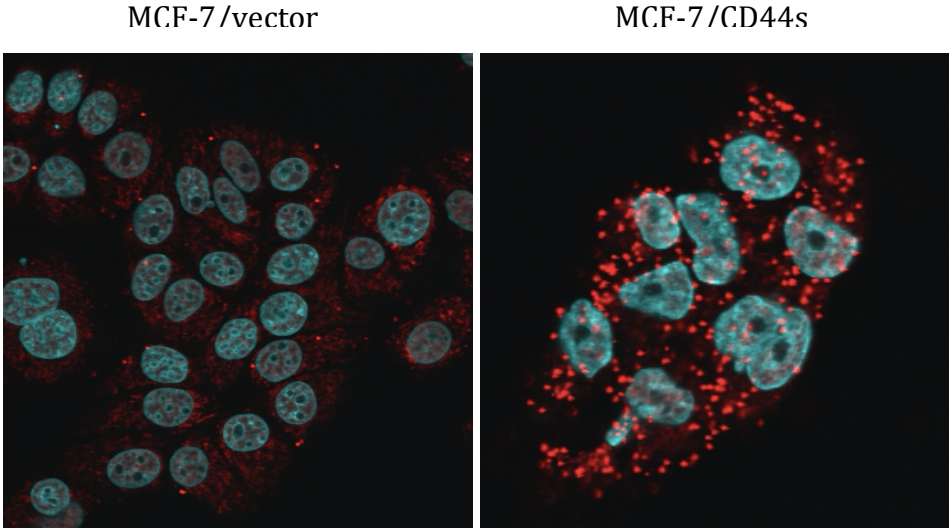


Figure 8 Protein-protein interaction between the CD44-ICD and RunX2 and its cellular localization. (blue= DAPI, red=CD44-ICD/Runx2 interaction)

The proximity ligation assay performed detected a protein-protein interaction in the nucleus between the CD44-ICD and RunX2 in MCF-7/CD44 cells but not in MCF-7/vector cells. These result indicate that the CD44-ICD and Runx2 were in close proximity, which suggest a possible direct protein-protein interaction. Unexpectedly, this interaction between the CD44-ICD and Runx2 was not only detected in the nucleus, as we have hypothesized, but also in the cytoplasm.

CHAPTER 5: DISCUSSION

The collected data for this thesis project in combination with previous reports supports our hypothesis: CD44 is a novel transcriptional regulator via its CD44-ICD protein-protein interaction (PPI) with transcription factors and its DNA promoter binding activity.

Supportive data related to the hypothesized PPI of the CD44-ICD with transcription factors include the Transcriptional Interaction TF Plate Array I and the PLA results. Both of these experimental approaches indicate a PPI between the CD44-ICD and numerous transcriptions factors. For the PPI between the CD44-ICD and the transcription factor Runx2 we also identified the cellular location of such interaction by PLA. Interestingly, we observed not only a PPI nuclear interaction, as previously proposed, but also a PPI cytoplasmic interaction. This data suggest of the existence of an unidentified mechanism in which the CD44-ICD may regulate the expression of Runx2-target genes by interacting with Runx2 in the cytoplasm. Many questions remain to be considered and answered in this regard. What would be the function of a CD44-ICD/Runx2 cytoplasmic PPI when both of these factors are known to be involved in transcriptional regulation? Is this PPI interaction direct or indirect depending where is taking place? Is this cytoplasmic interaction required to translocate the CD44-ICD into the nucleus for the regulation of CD44-mediated Runx2-target genes? Additional experimentation of this PPI and of other transcription factors interacting with the CD44-ICD will start clarifying these questions. It will be important to cross-validate these two experimental approaches.

Supportive data related to the hypothesized CD44-ICD interaction with promoter regions of CD44-mediated regulated genes includes the apoptosis and human pluripotent stem cell array

data and the TF Activation Profiling Plate Array II results. Even though this data is not as conclusive as the CD44-ICD/Runx2 PPI data, it shows a consistent trend. For both of the genes (CDH1 and CASP3) that their expressions were inhibited in the CD44-expressing cell line MCF-7/CD44 (as assayed in the proteome profiler array kits), a CD44-ICD response element (CIRE) sequence was found downstream of their transcriptional start site. This result suggests a CD44-ICD-mediated transcriptional inhibitory effect. Indicating a similar trend, the results from the TF Activation plate array (Table 1) showed the presence of CIRE sequences in differentially expressed genes when comparing MCF-7 vs MCF-7/CD44 cells. In genes that also showed a differential expression of genes but did not contain CIRE sequences in their promoter regions, a Runx2 response element was present. This is a key observation since we have already shown that the CD44-ICD has the ability to interact with Runx2 to regulate gene expression (Miletti-Gonzalez et al., 2012).

Noteworthy, the molecular evidence presented that supports our hypothesis also showed a phenotype associated with it. For example, the decreased of the Nrf2 transcription factor in MCF-7/CD44 compared to MCF-7 cells (Table 1) was functionally validated by the hydrogen peroxide cell viability assay (Figure 4). This assay demonstrated that MCF-7/CD44 cells are more sensitive to hydrogen peroxide than MCF-7 cells. Likewise, the Antioxidant Response Element (ARE) reporter constructs assay, which functionally measures the use the ARE-dependent luciferase reporter construct by Nrf2, showed a higher luciferase activity in MCF-7 cells compared to MCF-7/CD44 cells. This shows a direct correlation with the Nrf2 differential expression in these two cell lines.

Altogether, these data supports that CD44 can regulate gene expression and that this regulation is in part via its intracytoplasmic domain. The CD44-ICD appears to be able to act as a co-activator as well as a co-repressor. Because the CD44-ICD size of only 72 residues, the specific CD44-ICD response element (CIRE) sequence to which it can bind is unusual. This type of protein-DNA interaction is usually associated with larger DNA-binding proteins such as transcription factors. The dual capacity of the CD44-ICD to interact with proteins of the transcriptional machinery as well as to interact with its own response element on promoter sequences might place the CD44-ICD in a novel category of transcriptional regulators. Below is a proposed model for such transcriptional activities.

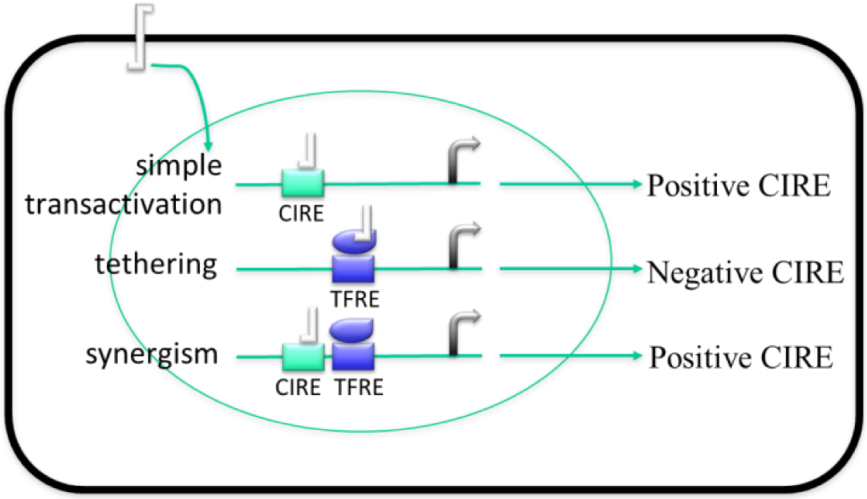


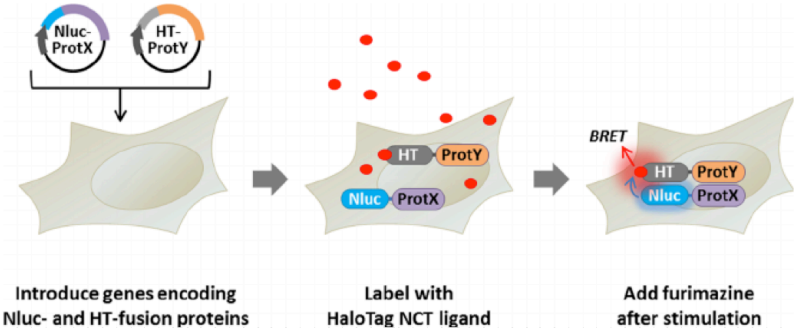
Figure 9 Proposed model of CD44-ICD gene regulation

The CD44-ICD may act as a simple transactivator where it binds to its response element and regulates transcription and gene expression. It may act in a tethering manner where it binds to a transcription factor to regulate gene expression. And it may act in a synergistic manner where the CD44-ICD can interact with both elements of transcriptional regulation.

Future Directions

Bioluminescence Resonance Energy Transfer (BRET) assay

To quantitatively test protein-protein interactions between the CD44-ICD and Runx2, p53 and other transcription factors. Plasmid constructs expressing fusion proteins such as the CD44-ICD fused to NanoLuc (Nluc) and p53 or Runx2 fused to HaloTag (HT) are completed.



https://www.researchgate.net/profile/Thomas_Machleidt/publication/277252735/figure/fig2/AS:293892811046913@1447080914205/Figure-2-Schematic-of-NanoBRET-for-detecting-protein-protein-interactions.png

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