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The Role of Cytoplasmic Polyadenylation Element Binding Protein -2 (CPEB-2) in Human Breast Cancer

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Abstract

Cyclooxygenase-2 (COX-2) is overexpressed in 40-50% of breast cancers, and promotes tumour progression through increased proliferation, migration, invasion, Epithelial-to-Mesenchymal Transition (EMT), and induction of therapy-resistant Stem-Like-Cells (SLCs). COX-2 stimulates expression of two oncogenic and SLC-promoting microRNAs (miR-526b, miR-655), which simultaneously target one gene, Cytoplasmic Polyadenylation Element Binding Protein-2 (CPEB-2). **Hypothesis:** CPEB-2 is a tumour- and SLC-suppressing gene in breast cancer. **Results:** CPEB-2 knockout in a non-tumourigenic mammary epithelial cell line MCF10A demonstrated increases in proliferation, migration, invasion, EMT markers, SLC content, and VEGF-D expression. CPEB-2, an mRNA-binding translation-regulating protein, was found to regulate the translation of tumour suppressor p53. When intravenously injected into NOD/SCID/IL2Rγ-null mice, CPEB2KO cells formed micrometastases in the lung, and after orthotopic injection into the mammary region, they formed tumours in 3/5 mice, including spontaneous lung metastases. Isoform A/E of CPEB-2 was decreased in HER2+ breast cancer samples. **Conclusion:** CPEB-2 is a tumour-suppressing gene in breast cancer.

Keywords

CPEB-2, COX-2, Breast Cancer, p53, EMT

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List of Abbreviations

Akt/PKB	Protein Kinase B
ALDH	Aldehyde Dehydrogenase
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BRCA-1	Breast Cancer 1
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CCND1	Cyclin D1
CD	Cluster of Differentiation
CDH1	E-cadherin
CDH2	N-cadherin
CDK4/6	Cyclin Dependent Kinase 4/6
CLIP	Crosslinking Immunoprecipitation
COX	Cyclo-oxygenase
CPE	Cytoplasmic Polyadenylation Element
CPEB	Cytoplasmic Polyadenylation Element Binding Protein
CPEB2KO	CPEB-2 Knockout
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Threshold Cycle

DAPI	4',6-Diamidino-2-Phenylindole
DCIS	Ductal Carcinoma *In Situ*
DMEM:F12	Dulbecco's Modified Eagle Medium: Nutrient Supplement F-12
DNA	Deoxyribonucleic Acid
E1A	Adenovirus Early Region 1A
EdU	5'-ethynyl-2'-deoxyuridine
eEF2	Eukaryotic Elongation Factor 2
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-to-Mesenchymal Transition
EP	Prostaglandin E Receptor Family
ER	Estrogen Receptor
ERK	Extracellular Signal Regulated Kinases, or MAPK
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H&E	Hematoxylin & Eosin
HER2	Human Epidermal growth factor Receptor 2
HIF-1α	Hypoxia Inducible Factor 1α
HLA	Human Leukocyte Antigen
HS	Horse Serum

HuMEC	Human Mammary Epithelial Cell
IL2Rϒ	Interleukin 2 Receptor ϒ chain
MAPK	Mitogen-Activated Protein Kinase
MCF-7/10A	Michigan Cancer Foundation - 7/10A
MMP	Matrix Metalloproteinase
M-PER	Mammalian Protein Extraction Reagent
MTX	Methotrexate
NK	Natural Killer
NOD	Non-Obese Diabetic
NPGPx	Non-selenocysteine-containing Phospholipid hydroperoxide Glutathione Peroxidase
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NSCLC	Non-Small Cell Lung Cancer
PBS	Phosphate Buffered Saline
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2 (Prostacyclin)
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PLC	Phospholipase C

PR	Progesterone Receptor
PTGS	Prostaglandin-endoperoxide Synthase
PVDF	Polyvinulidene Fluoride
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
Rb-1	Retinoblastoma 1
RIP	RNA Immunoprecipitaiton
RNA	Ribonucleic Acid
RRM	RNA Recognition Motif
SCID	Severe Combined Immune-Deficient
SEM	Standard Error of the Mean
sgRNA	Single Guide RNA
siRNA	Small Interfering RNA
SLC	Stem-Like Cells
TBST	Tris Buffered Saline with Tween 20
TN	Triple Negative
TXA_2	Thromboxane A_2
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
ZEB1/2	Zinc-finger E-Box-Binding homeobox 1/2

1 INTRODUCTION

1.1 Cancer

Cancer is a general term used to describe malignant or aggressive forms of tumours. “Tumour” is an abnormal growth of cells in any of the tissues in the body, caused by mutations in cells. A tumour is called “benign” when the growth is usually slow, and the mass of cells is highly localized and non-invasive. It is curable by surgical resection. It is called “cancer” upon acquiring malignant properties, typically associated with rapid cell division, invasion and metastasis to other parts of the body. Usually multiple mutations lead to the malignant or cancerous phenotype. Most tumours arise by multiple somatic mutations, whereas certain germ-line mutations can cause tumours in the newborn (such as retinoblastoma by mutation of the *Rb-1* gene) or later in life (such as hereditary breast cancer by mutation in the *BRCA-1* gene) (Lynch & Lynch, 1996; Steeg, 1992). Cancer-causing mutations are usually found in three types of genes: proto-oncogenes, tumour suppressor genes, or DNA repair genes (National Cancer Institute, 2015; Ponz de Leon, 1996). Proto-oncogenes are genes required for normal cell growth and differentiation. One mutation that increases activity of these genes triggers them to become oncogenes and can cause cancer. Tumour suppressor genes are protective genes, usually involved in controlling key cell division processes, and generally require mutations or dysregulation in both copies to cause cancer. DNA repair genes help repair DNA damage caused by the environmental insults (UV radiation, chemicals, etc.) and so if this mechanism is disrupted or altered, mutations will occur more frequently (National Cancer Institute, 2016). Cells that become cancerous acquire a number of characteristics termed the “hallmarks of cancer”: unlimited replicative ability, resistance to apoptosis, self-sufficiency in growth signals, reduced response to antigrowth signals, ability for

invasion and metastasis, and sustained angiogenesis (Hanahan & Weinberg, 2000). Two additional hallmarks were added later on: ability for reprogramming of energy metabolism and evading immune destruction (Hanahan & Weinberg, 2011).

Cancers can be broadly subdivided into several classes: “carcinomas” are those arising in epithelial tissues; “sarcomas” are those arising from connective tissues; “leukaemias” are cancers arising from white blood cells; and lymphomas are solid tumours of lymphocytes. Breast cancer, or mammary carcinoma, can arise in the ductal epithelium (ductal carcinoma) or lobular epithelium (lobular carcinoma). It can also be mixed ductal-lobular. Unfortunately, 1 in 9 women will develop breast cancer in their lifetime, with 1 in 30 women succumbing to the disease. Over 25% of cancers diagnosed in women are of the breast origin, making breast cancer the most diagnosed cancer in women (Canadian Cancer Society, 2016). During the development of breast cancer, breast epithelium usually undergoes a change into atypical breast hyperplasia, followed by a ductal carcinoma *in situ* (DCIS) in which cancer cells are still limited by the basement membrane (Rivenbark, O’Connor, & Coleman, 2013). Ductal carcinomas, as opposed to lobular carcinomas, are the most common type of breast cancer (Canadian Cancer Society, 2016). Low grade ductal carcinomas, when non-invasive (e.g., DCIS) are usually not life-threatening (Rivenbark et al., 2013). High grade ductal carcinomas are more invasive, faster growing and can subsequently lead to metastatic breast cancer (Allegra et al., 2010).

Breast cancers are typically classified by the protein receptor biomarkers that they express, such as Estrogen Receptor (ER+), Progesterone Receptor (PR+), or Human Epidermal Growth Factor Receptor 2 (HER2+) or triple negative (TN) for all three

receptors (Patani, Martin, & Dowsett, 2013). ER+ breast cancers represent approximately 70% of total breast tumours (Anderson, Chatterjee, Ershler, & Brawley, 2002), and are usually co-expressed with PR. HER2+ breast cancers represent approximately 12-22% of breast cancer cases (Prat & Perou, 2011), and are usually more aggressive than ER+/PR+ tumours (Slamon et al., 1987). The triple negative class represents the most aggressive type of breast cancer (Prat & Perou, 2011). Gene expression profiling using cDNA microarrays to correlate tumour characteristics with clinical outcome (Sorlie et al., 2001) led to a further refinement of the classification: Luminal A, Luminal B, HER2+, Basal-like, and Claudin-low (Rivenbark et al., 2013; Weigelt et al., 2010). Luminal A and Luminal B are both ER+ breast cancers, with Luminal B exhibiting co-expression of ER and HER2 (Sorlie et al., 2001). HER2+ breast cancers are generally ER and PR negative, and can be selectively targeted using the HER-2 blocking agent Trastuzumab (Herceptin) (Rivenbark et al., 2013). TN breast cancers are divided into Basal-like and Claudin-low subtypes. Basal-like tumours are highly aggressive, proliferative, and express genes from the breast myoepithelium, whereas Claudin-low tumours usually express Epithelial-to-Mesenchymal Transition markers and Stem-Like-Cell features (Rivenbark et al., 2013).

Genomic profiling for breast cancer patients is a common technique used to assess risk for metastasis and clinical outcomes in patients (Espinosa et al., 2011; Paik, 2011). Two current commercially available kits being used in the clinic are called the Oncotype Dx (Genomic Health, Inc, CA) and Mammaprint (Agendia, Irvine, CA), which both function through qRT-PCR analysis of tumour RNA expression of 21 or 70 genes, respectively (Turaga, Acs, & Laronga, 2010). These allow physicians to make personalized recommendations for the type of chemotherapy or a combination of

therapies. They also provide further prognostic information such as a 'recurrence score' that predicts risk of recurrence (Rivenbark et al., 2013; Turaga et al., 2010). Due to the heterogeneity of human breast cancers, determining personalized gene profiles should ultimately reduce amount of unnecessary chemotherapies, and long-term side effects (Turaga et al., 2010). Compiling a list of genes that need to be included in examination of genetic signatures will be an important step in this process (Espinosa et al., 2011). However, currently these approaches are costly and have yet to live up to expectations (Arango, Rivera, & Glück, 2013).

1.1.1 Stem-Like-Cells

A subset of tumour cells called Stem-Like Cells (SLCs) are believed to perpetuate tumour growth and contribute to resistance to traditional chemotherapeutic drugs and radiation therapies (Reya, Morrison, Clarke, & Weissman, 2001). SLCs were first discovered in acute myeloid leukemia through serial transplantation of cells in limiting numbers into Severe Combined Immune-Deficient (SCID) mice. These cells (1 in 250,000) expressed CD34+ (stem/progenitor cell surface marker) CD38- (lineage commitment marker) and were able to successfully form multiple colonies in the bone marrow of these mice (Lapidot et al., 1994). In breast cancer, it was first discovered that cells bearing CD44+/CD24- surface markers were highly tumourigenic in immunodeficient mice, requiring as few as 100 cells isolated from patient tumours (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). More recently, the Aldehyde Dehydrogenase 1 (ALDH1) enzyme was used to identify stem cell properties within

heterogeneous tumour cell populations, correlating with ability for self-renewal, poor prognosis, and ability to recapitulate the original tumour heterogeneity (Ginestier et al., 2007). A combination of ALDH-high expressing cells and CD44+/CD24- markers, although representing less than 1% of total tumour cell population, were able to form tumours with as few as 20 cells (Ginestier et al., 2007). Croker et al. demonstrated that ALDH-high/CD44+/CD24- cells as well as ALDH-high/CD44+/CD133+ cells, isolated from a number of human breast cancer cell lines had SLC properties and enhanced tumourigenic and metastatic ability in NOD/SCID/IL2Rϒ-null mice (Croker et al., 2009). Using immunohistology, the CD44+/CD24- phenotype in patients' primary tumours was found to be more associated with therapy resistance and poor outcome compared to the ALDH-1 phenotype (Horimoto et al., 2016).

Amongst many regulatory pathways of SLC induction and maintenance, the Wnt/β-catenin signaling pathway was shown to govern the transcription of genes involved in proliferation, self-renewal and motility (Jang et al., 2015; Paul & Dey, 2008; Polakis, 2000). β-catenin is the key effector molecule in this pathway, with the ability to induce transcription of *CCND1*, *AXIN1*, *AXIN2*, and *Myc* genes, among others (Clevers, 2006; Polakis, 2000). ALDH positive cells showed significantly higher expression of β-catenin signaling (Jang et al., 2015). Blockage of this pathway has been shown to reduce tumour cell proliferation, migration, invasion, and tumour growth and metastasis *in vivo* (Jang et al., 2015).

HER2+ tumours were reported to be rich in SLCs. Clinically, these tumours are usually responsive to the specific HER-2 blocking drug Herceptin, but when

CD44+/CD24- HER2+ cells are enriched, they exhibited significant resistance to this agent (Oliveras-Ferraros et al., 2012). In an examination of different breast cancer cell lines, treatment with paclitaxel or 5'fluorouracil demonstrated a significant increase in enrichment of SLCs in the surviving population (Fillmore & Kuperwasser, 2008). Due to the persistence of SLCs after traditional chemotherapies or radiation therapies, mechanisms that increase the formation of these cells are important to investigate.

1.1.2 Epithelial-to-Mesenchymal Transition

Epithelial-to-Mesenchymal Transition (EMT) is a biological process that causes polar epithelial cells to undergo a change into an elongated nonpolar mesenchymal phenotype (Kalluri & Weinberg, 2009). This phenotype is usually associated with an acquisition of mesenchymal cell markers, increase in migratory ability, cell survival, and invasiveness (Kalluri & Weinberg, 2009). E-cadherin, an epithelial cell junction protein, is one of the main epithelial markers that is lost during EMT (Kourtidis, Lu, Pence, & Anastasiadis, 2017). E-cadherin is responsible for mediating cell-cell adhesions, allowing for cell contact inhibition and control of cell proliferation (N.-G. Kim, Koh, Chen, & Gumbiner, 2011). Although reduced E-cadherin is an essential marker for EMT, loss of E-cadherin alone is not adequate for EMT, and therefore other markers must be assessed (A. Chen et al., 2014). "Cadherin switching" is a process by which expression of E-cadherin is exchanged for other cadherins, such as N-cadherin or P-cadherin (Wheelock, Shintani, Maeda, Fukumoto, & Johnson, 2008). N-cadherin, a mesenchymal cell-adhesion molecule, when ectopically overexpressed in breast cancer cell line MCF-7,

increased migratory ability, invasiveness through upregulation of MMP-9, and metastasis (Hazan, Phillips, Qiao, Norton, & Aaronson, 2000). Vimentin, another mesenchymal marker, is an intermediate filament responsible for increasing a cell's ability to directionally migrate, maintains homeostasis within transitioning cells, and has the ability to regulate EMT-associated transcription factors such as SNAI2 (Liu, Lin, Tang, & Wang, 2015). E-cadherin expression is downregulated by multiple mesenchymal transcription factors, including SNAI1, SNAI2, ZEB1, ZEB2 and Twist (Oliveras-Ferraros et al., 2012; Smit & Peeper, 2010; Zhou et al., 2017). These transcription factors, individually or in combination with each other, have been shown to drive EMT. Cells that have undergone EMT, like SLCs, are also resistant to traditional chemotherapeutic therapies (Oliveras-Ferraros et al., 2012). In epithelial cancers, the ability of the cancer cells to undergo this transition is crucial for their ability to metastasize (Kalluri & Weinberg, 2009).

1.2 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) or prostaglandin-endoperoxide synthase (PTGS) family of enzymes include three members COX-1, COX-2 and COX-3. COX-3 is an isoform of COX-1 produced by alternative splicing of the *PTGS-1* gene, not present in the human (Simmons, Botting, & Hla, 2004). COX-1 is constitutively expressed by most cells to mediate many physiological functions. COX-2 or PTGS-2 is a constitutive enzyme only in a small minority of cells such as macrophages and cells in reproductive organs such as decidual cells in the pregnant uterus (Williams, Mann, & DuBois, 1999) (Figure 1).

Typically, it is an inflammation-associated enzyme induced by inflammatory cytokines, mitogens and certain carcinogens. COX-1 and to a smaller extent COX-2 have physiological roles in hemostasis, gastric and renal function, development of T-cells, cardiovascular development, ovulation and implantation (Simmons et al., 2004). COX enzymes mediate the production of various prostanoids, most importantly prostaglandin E_2 (PGE_2) from arachidonic acid (Howe, 2007). Arachidonic acid (unsaturated fatty acid) is freed from the plasma membrane by phospholipase enzymes (Williams et al., 1999). Free arachidonic acid is then converted by COX-2 into Prostaglandin G_2, and subsequently into Prostaglandin H_2. Prostaglandin H_2 can then be converted into Eicosanoids, such as cardioprotective molecule prostacyclin (PGI_2), Thromboxane A_2 or PGE_2 (Howe, 2007; Williams et al., 1999) (Figure 2). PGE_2 production via COX-1 pathway is steady and at low concentrations. COX-2-mediated PGE_2 production during inflammation occurs at high local concentrations and stops after withdrawal of the inflammatory stimulus. However, aberrant COX-2 activity that occurs in some cancers leads to persistent PGE_2 production (Williams et al., 1999). PGE_2 can bind to the prostaglandin E receptor (EP) family, which is made up of 4 G protein coupled receptors (EP1-4) (Fujino, Xu, & Regan, 2003). EP1 couples with G_q, activating PLC and a rise in intracellular Ca^{2+}, while EP2 and EP4 couple with G_S, stimulating cAMP/PKA pathway (Breyer, Bagdassarian, Myers, & Breyer, 2001). Additionally, EP4 also stimulates non-canonical pathways PI3K/Akt and ERK, promoting cell survival and migration; and most EP3 isoforms couple with G_i, inhibiting cAMP (Fujino et al., 2003).

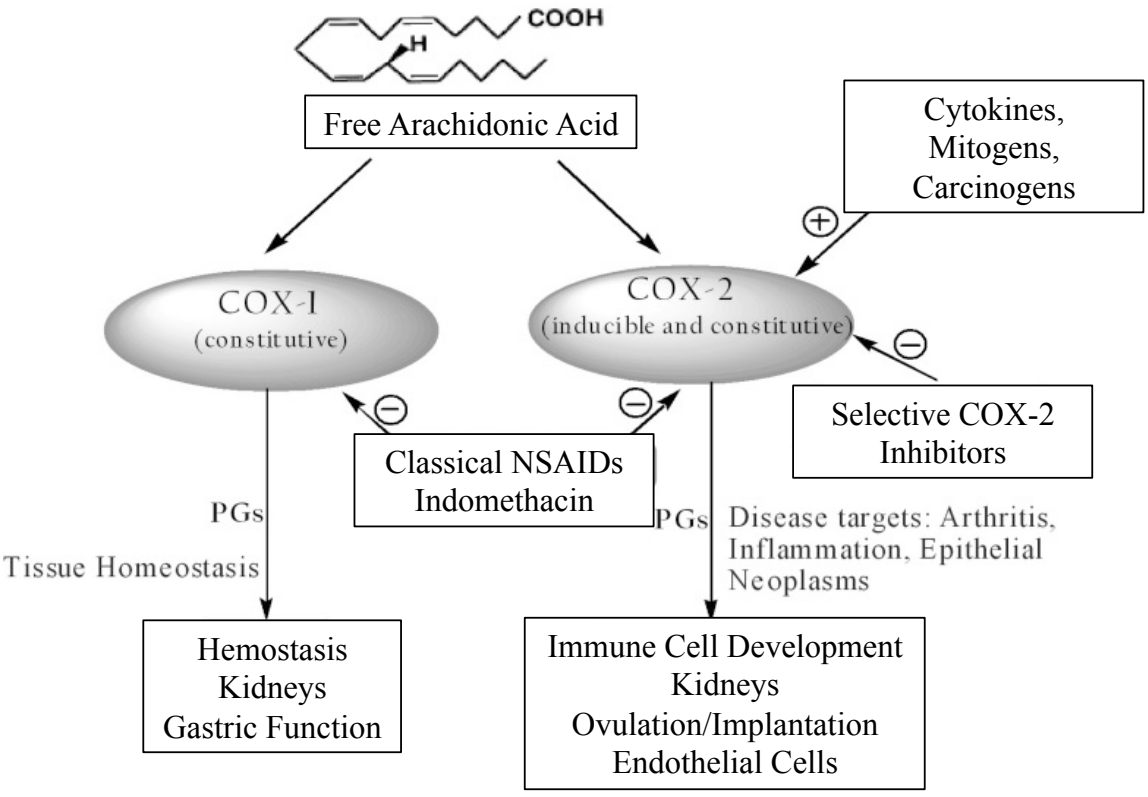

Figure 1. Simplified schematic of COX-mediated physiological and pathophysiological roles. COX-mediated prostaglandin (PG) synthesis from arachidonic acid, physiological and pathophysiological effects throughout the body, along with inhibition targets. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and Indomethacin non-selectively inhibit both COX-1 and COX-2. Adapted from (Zarghi & Arfaei, 2011).

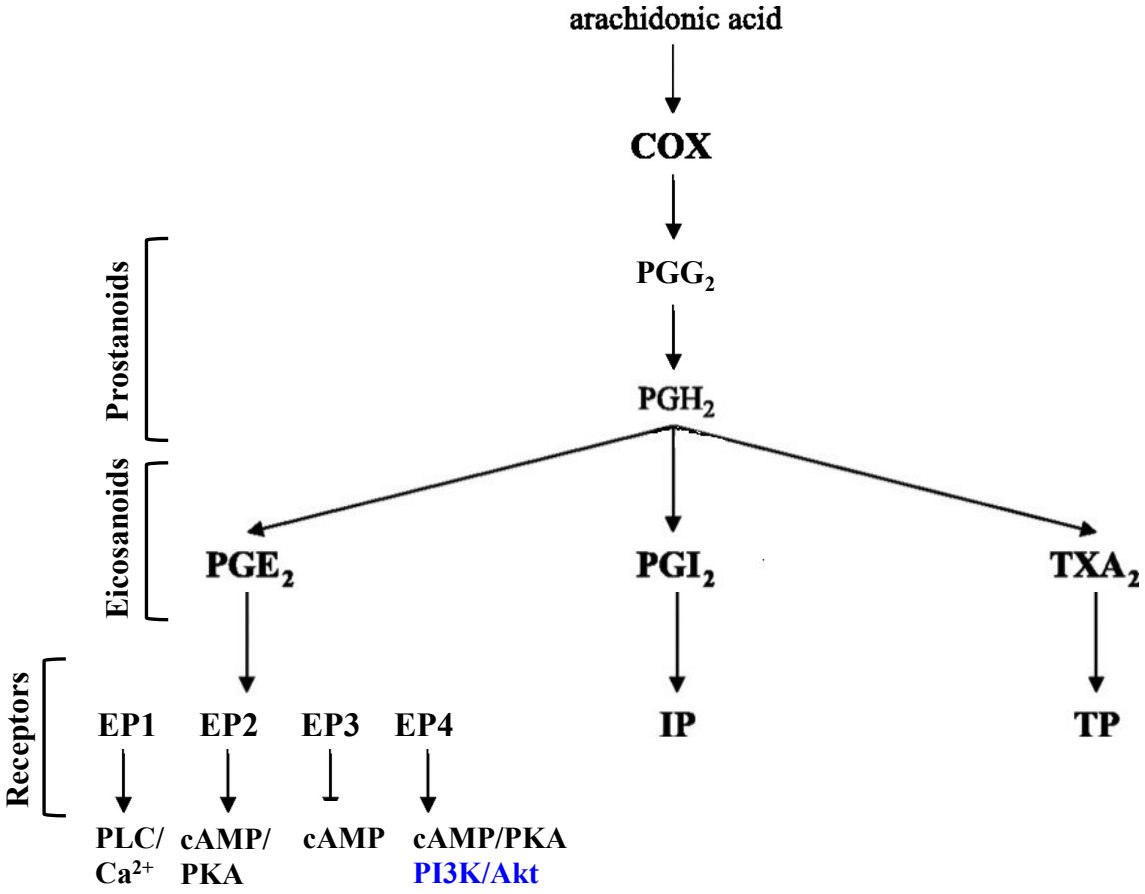

Figure 2. Simplified schematic of Cyclo-oxygenase (COX) production of Prostaglandins (PGG_2, PGH_2) and Eicosanoids (PGE_2, PGI_2, and TXA_2), as well as their respective receptors (EP, IP, TP). Adapted from (Hull, Ko, & Hawcroft, 2004).

1.2.1 COX-2 and Breast Cancer

Aberrant COX-2 expression has been found in many epithelial cancers including cancer of the colon, oropharynx, lungs, prostate, pancreas and the breast leading to disease progression (Harris, 2003). Elevated COX-2 expression, noted in 40-50% of breast cancer patients, is an indicator of poor prognosis (Ristimäki et al., 2002). In human breast cancer, COX-2 expression was shown to be localized primarily to the tumour epithelium in breast cancer samples, and extensively linked to tumour progression (Howe, 2007). A series of studies in our lab have established that COX-2 expression leading to high PGE_2 levels in the tumour milieu promotes breast cancer progression by multiple mechanisms: an inactivation of host anti-tumour innate immune cells (Lala, Parhar, & Singh, 1986), enhanced cancer cell migration and invasiveness (Rozic, Chakraborty, & Lala, 2001; Timoshenko, Xu, Chakrabarti, Lala, & Chakraborty, 2003), tumour-associated angiogenesis (Rozic et al., 2001; Xin et al., 2012), and tumour-associated lymphangiogenesis resulting from upregulation of VEGF-C and VEGF-D production (Timoshenko, Chakraborty, Wagner, & Lala, 2006) . These events resulted primarily by activation of the PGE_2 receptor EP4 on tumour and host cells. In a syngeneic mouse model, our lab discovered that inhibition of COX-2 or EP4 reduced tumour size, proliferation, migration, expression of VEGF-C and VEGF-D, and tumour-associated angiogenesis and lymphangiogenesis (Majumder, Xin, Liu, Girish, & Lala, 2014; Rozic et al., 2001; Timoshenko et al., 2003; Xin et al., 2012). Within the tumour are a group of immune cells called tumour-associated macrophages, and when treated with an EP4 antagonist, these immune cells significantly reduced the production of lymphangiogenic factors VEGF-C and VEGF-D (Majumder et al., 2014).

COX-2 expression in breast cancer has been correlated with increased size, grade, distant metastasis as well as poor survival (Ristimäki et al., 2002; Singh-Ranger, Salhab, & Mokbel, 2008). Furthermore, HER-2 expressing tumours significantly co-express COX-2, however COX-2 expression was not limited to HER-2+ tumours, as it was overexpressed in TN tumours as well (Bhattacharjee, Timoshenko, Cai, & Lala, 2010; Ristimäki et al., 2002). Our lab examined the consequences of COX-2 overexpression in the presence or absence of HER2 overexpression (Majumder et al., 2016). Through stable transfection of *COX-2* into non-metastatic human breast cancer cell lines MCF-7 (COX-2–, HER-2–) and SKBR3 (COX-2–, HER-2+), it was shown that irrespective of HER2 expression, COX-2 promotes proliferation, migration, invasiveness, an upregulation of VEGF-C and VEGF-D, as well as an EMT phenotype. Furthermore, it induces SLC phenotype tested *in vitro* and *in vivo* through upregulation of Notch/Wnt pathways (Majumder et al., 2016).

The effects of COX-1/COX-2 inhibition with a nonselective COX inhibitor, indomethacin, was previously examined by our lab in a spontaneous tumour-forming mouse model of retired breeder female C3H/HeJ mice. COX inhibition was shown to delay tumour formation, significantly increase life span of these mice, reduce lung metastases, increase tumour cell death and increase immune cell infiltration into the tumour (Lala, Al-Mutter, & Orucevic, 1997). Furthermore, COX inhibition succeeded in abrogating PGE_2-mediated inactivation of NK cells in tumour bearing mice, restoring NK cell activity (Lala et al., 1986). However, prolonged use of both COX-1 and COX-2 inhibitors were found to produce cardiovascular side effects (FitzGerald & Patrono, 2001). This has been attributed to COX-mediated inhibition of PGI_2 production, a

cardioprotective molecule (Cathcart et al., 2008), and therefore alternative downstream target molecules that can spare this side effect needed to be investigated.

Our lab identified EP4 as an ideal alternative therapeutic target for three reasons: (1) Most of the mechanisms underlying COX-2-mediated breast cancer progression resulted from EP4 activation (Majumder et al., 2014; Xin et al., 2012); (2) Most physiological functions of EP4 are shared by EP2 through PKA-mediated signaling pathway, whereas tumour cell survival and migration depend on EP4-mediated activation of PI3K/Akt and MAPK pathways not shared with EP2 (Fujino et al., 2003); (3) PGI_2 does not bind to EP4 and thus EP4 antagonists should spare the cardiovascular side effects of COX-2 inhibition. The potential of EP4 as therapeutic target was validated in murine syngeneic COX-2 over-expressing breast cancer, while allowing for blockade of COX-2 or PGE_2-mediated oncogenic effects (Majumder et al., 2014; Xin et al., 2012). Treating breast cancer cells with selective EP4 antagonists reduced *in vitro* oncogenic phenotypes (proliferation, migration, invasion, angiogenic and lymphangiogenic factor production) as well as treating syngeneic breast cancer-bearing mice with the same inhibitors abrogated tumour growth and metastases *in vivo* (Majumder et al., 2014; Xin et al., 2012; S. Xu et al., 2014). Currently, the use of EP4 antagonists as an adjunctive therapy remains to be explored in clinical trials.

1.3 COX-2-induced microRNAs

MicroRNAs are small RNA molecules, usually between 19 and 24 nucleotides, that can regulate translation of target mRNA molecules either by degrading the mRNA or

blocking translation through direct binding (Sassen, Miska, & Caldas, 2008). Over half of the microRNAs in the human genome are located in cancer-associated regions, and some of them can function either as tumour suppressors or oncogenes (Calin et al., 2004; Macfarlane & Murphy, 2010; Sassen et al., 2008). MicroRNAs can be secreted by cells into the blood stream or body fluids via packaging and exocytosis in vesicles called exosomes that protect them from RNase degradation (Mathivanan, Ji, & Simpson, 2010). Thus, some microRNAs may be promising cancer biomarkers. MicroRNA signature screening is a tool being investigated as an adjunct to the traditional mammography to diagnose breast cancer and metastases (Freres et al., 2016). Screening for 8 microRNAs in a cohort of 108 women proved to accurately identify metastatic cancer within these patients (Freres et al., 2016). These small molecules have been implicated in regulating many types of cancer including breast cancer, and can be used as novel cancer biomarkers in patient diagnosis (Sassen, Miska, and Caldas, 2008).

Our lab performed a combined gene and microRNA microarray analysis of COX-2 transfected MCF-7 cells (COX-2 negative) (Figure 3). Through this, they identified 26 genes that were downregulated and two microRNAs, microRNA-526b and microRNA-655, that were upregulated by COX-2 (Dunn & Lala, 2013; Majumder, Landman, Liu, Hess, & Lala, 2015). Both of these microRNAs have been shown by our lab to be oncogenic and SLC-promoting (Majumder et al., 2015). An overexpression of these microRNAs in poorly metastatic breast cancer cell lines MCF-7 and SKBR3 increased *in vitro* oncogenic and SLC phenotypes, as well as *in vivo* tumour formation (Dunn & Lala, 2013; Majumder et al., 2015). Of the 26 genes downregulated by COX-2, these microRNAs collectively targeted 13 of them, 12 of which were tumour-suppressor-like

genes (ex. *TP53*, *CDK6*, etc.). The last gene is the only common target of both microRNAs, named Cytoplasmic Polyadenylation Element Binding Protein (CPEB) -2.

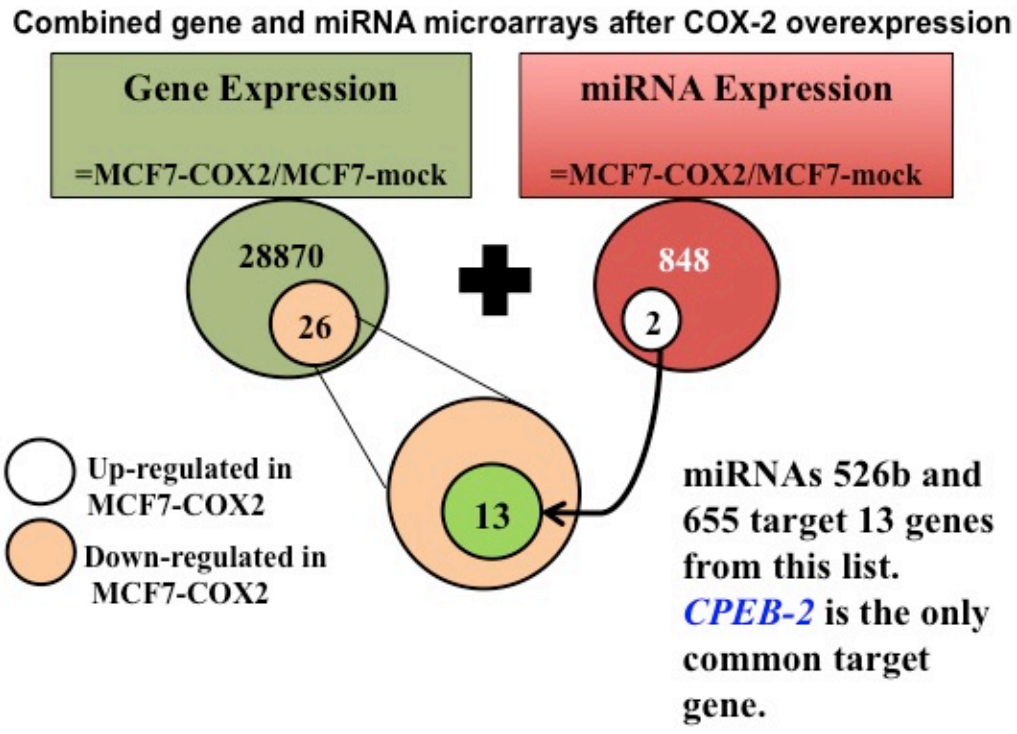

Figure 3. Combined gene and microRNA microarray analysis of MCF-7 cells (originally COX-2-negative) transfected to overexpress COX-2 compared to mock-transfected MCF-7 cells (control). Gene (left) and microRNA (right) microarray results, and in the middle are the collective 13 gene targets of the 2 microRNAs out of the 26 genes downregulated by COX-2. CPEB-2 was the only common gene target of both microRNAs.

1.4 CPEB Family

The CPEB family of proteins are made up of 4 members (CPEB1-4) (Fernández-Miranda & Méndez, 2012). This family harbours the ability to bind to the 3' UTR of mRNA molecules, specifically a domain called the Cytoplasmic Polyadenylation Element (CPE) (Fernández-Miranda & Méndez, 2012). Some mRNA targets require two CPE sequences, which must be less than 50 nucleotides apart in order to be translationally regulated by CPEB proteins (Piqué, López, Foissac, Guigó, & Méndez, 2008). To affect polyadenylation in their target mRNA molecules, there must be both a CPE sequence (UUUUUAAU) and a polyadenylation hexanucleotide signal (AAUAAA) (Paris & Richter, 1990). CPEB proteins can repress or activate translation of their target mRNA molecules by shortening or elongating the poly-A tail, respectively (Wakiyama, Imataka, & Sonenberg, 2000).

1.4.1 CPEB Family and Cancer

Because these proteins regulate the translation of a wide variety of genes, many groups have investigated their relationship with cancer (Y. Chen, Tsai, & Tseng, 2016; Fernández-Miranda & Méndez, 2012). CPEB-1, the most ubiquitously expressed member of the family, is known to suppress multiple cancer-promoting molecules, including TWIST1 (Grudzien-Nogalska, Reed, & Rhoads, 2014; Nairismägi et al., 2012), HIF1α (Hägele, Kühn, Böning, & Katschinski, 2009), and β-catenin (Jones et al., 2008), while increasing tumour-suppressor molecules such as p53 (Burns & Richter, 2008). CPEB-1 also suppresses EMT in mammary epithelial cells (Grudzien-Nogalska et al., 2014; Nagaoka et al., 2015), and when knocked down was reported to increase breast cancer

metastasis to the lung (Nagaoka et al., 2015). CPEB-1 has been reported to be decreased in many forms of cancer, including breast and ovarian cancers (Fernández-Miranda & Méndez, 2012; Giangarrà, Igea, Castellazzi, Bava, & Mendez, 2015).

As will be reviewed later and will be the focus of studies herein, the roles of CPEB-2 in breast or other cancers remain poorly defined (Chen et al., 2016). The Cancer Genome Atlas data on CPEB-2 in breast cancer reported conflicting results (D'Ambrogio, Nagaoka, & Richter, 2013), and therefore deserves further examination.

CPEB-3 expression was shown to be lower in different cancer types, including a report that CPEB-3 is targeted by microRNA-107 in hepatocellular carcinoma (Zou, Zhao, Wang, Li, & Huang, 2016). It was also shown that an isoform of CPEB-3, one that cannot be phosphorylated due to splicing out of the encoded phosphorylation region, was overexpressed in high-grade glioma samples and correlated with poor patient survival (Skubal et al., 2016).

Findings with CPEB-4 remain conflicting. CPEB-4 was recently reported to be increased in human breast cancer patients (Sun et al., 2015), among other cancers (Fernández-Miranda & Méndez, 2012). CPEB-4 has been found to be overexpressed in pancreatic adenocarcinomas and glioblastomas as well, promoting tumour growth, angiogenesis and invasion (Ortiz-Zapater et al., 2012). CPEB-4 is a direct target of microRNA-203, a tumour-suppressive microRNA, and when CPEB-4 was downregulated in colorectal cancer cell lines, apoptotic markers were increased (Zhong et al., 2015). On the other hand, another group reported that microRNA-1246 promotes migration, invasion, and metastasis in non-small cell lung cancer (NSCLC), by downregulating CPEB-4 expression (Huang, Li, & Luo, 2015). Furthermore, decreased CPEB-4

expression correlated with poor survival in NSCLC (Huang et al., 2015). Additionally, microRNA-550a was shown to promote migration and invasion in hepatocellular carcinoma by targeting the 3' UTR of CPEB-4 (Tian et al., 2012). CPEB-4 was significantly downregulated in hepatocellular carcinoma samples, and this was significantly associated with poor patient outcome (Tian et al., 2012). The contrasting results listed above may have been due to different CPEB-4 splice variants.

Due to the involvement of CPEB proteins in cancer, therapies using CPEB proteins or CPE sequences may be useful in certain cancer types. Viral therapies, including oncolytic viruses, are currently being designed to specifically target tumour cells, therefore an examination of tumour-specific regulation of protein expression has been crucial in developing potential targets (Miest & Cattaneo, 2014). Adenoviruses were designed to include CPE regulatory elements for a control gene E1A to be post-transcriptionally regulated. This inclusion in the oncolytic virus allowed for increased tumour specificity in CPEB4-overexpressing tumours and decreased non-specific toxicity *in vivo* (Villanueva et al., 2017). Similarly, since CPEB4 has been shown to be increased in certain tumours (Sun et al., 2015; H. Xu & Liu, 2013), attempts have been made to exploit the CPEB4 protein as a tumour antigen for immunotherapy (Peng et al., 2014). Dendritic cells transduced with an adenovirus expressing CPEB-4 were successfully able to reduce tumour size *in vivo* through stimulation of cytotoxic T cells and increase the release of interferon-gamma by T cells in mice (Peng et al., 2014).

1.5 CPEB-2

CPEB-2 was first sequenced after being discovered in the cytoplasm of mouse haploid germ cells (Kurihara, 2003). Like CPEB-1, within CPEB-2 are two RNA Recognition Motifs (RRMs), as well as a Zinc-finger motif that collectively allow CPEB-2 to bind to poly-U regions like the CPE (Kurihara, 2003). As reviewed below, CPEB-2 can have multiple functions, including a role in tumourigenesis.

A known target of the *CPEB-2* gene is Hypoxia Inducible Factor -1α (HIF1α) (Hägele et al., 2009), a tumour-promoting molecule. HIF1α is a transcription factor that has a fairly short half life under normoxic conditions, but is stabilized under hypoxic conditions to stimulate genes involved in angiogenesis, EMT, migration, SLC maintenance, metastasis and therapeutic resistance (Ajdukovic, 2016). Under normoxic conditions, *CPEB-2* is known to suppress the translation of HIF1α mRNA (Hägele et al., 2009). Specifically, the elongation phase of translation is interrupted by CPEB-2 interacting with eukaryotic elongation factor -2 (eEF2) (Chen & Huang, 2012). Subsequently, this group (Chen et al., 2015) identified an oxidative stress sensor molecule known as Non-selenocysteine-containing Phospholipid hydroperoxide Glutathione Peroxidase (NPGPx) that forms a disulfide bond with CPEB-2 in normoxic conditions, resulting in an inhibition of HIF1α translation. However, under hypoxic conditions, the disulfide bond is disrupted, leading to dissociation of CPEB-2 from HIF1α mRNA and subsequent translation.

Another reported target of CPEB-2 is the highly conserved transcription factor TWIST1. TWIST1 is known to promote EMT (Nairismägi et al., 2012). The TWIST1 3’

UTR contains a CPE domain, allowing CPEB-2 to interact and downregulate mRNA translation of this gene (Nairismägi et al., 2012), thus suggesting CPEB-2 as a suppressor of EMT. TWIST1 is also an important molecule in promoting the SLC phenotype, as it increases expression of the molecule β-catenin, a key effector in the Wnt signaling pathway. β-catenin has two major functions that it regulates via gene transcription: proliferation and motility (Paul & Dey, 2008), both of which are important in tumourigenesis.

Another report (Giangarrà et al., 2015) analyzed the family of CPEB proteins, and their functions in the mitotic cell cycle through knockdown of each family member. When CPEB-1 was knocked down, cell lines were arrested in prophase; CPEB-4-knockdown cells were found to have difficulty in cytokinesis during telophase. However, CPEB-3 was not found to be involved in mitotic cell cycling (Giangarrà et al., 2015). Importantly, when CPEB-2 was knocked down, cells were found to be arrested at metaphase, with unaligned metaphase plates and therefore could not progress past this metaphase-to-anaphase checkpoint (Giangarrà et al., 2015).

It was recently reported that CPEB-2 plays a vital role in respiratory function (Lai et al., 2016). CPEB-2 knockout mice were generated using a cre-loxP system injected into blastocysts by targeting exons 3-5 and achieving germline transmission. These mice were then crossed with mice bearing various specific cre promoters to achieve conditional or global knockouts. Although these CPEB2 knockout mice were initially born alive, most of them died within 3 days postnatally, suggesting an important physiological role (Lai et al., 2016). Respiratory function was significantly impaired in

these neonates due to hyperactive parasympathetic signaling from the brainstem, thereby creating severe bronchoconstriction in the pups (Lai et al., 2016). This paper demonstrated a significant role of CPEB-2 *in vivo* in postnatal survival.

1.5.1 CPEB-2 Isoforms

Splicing is a post-transcriptional modification where a premature form of the mRNA (heteronuclear RNA) becomes alternatively processed to produce the mature mRNA molecule (Modrek & Lee, 2002). The products of alternative splicing events are different isoforms/variants of the protein that may include or exclude different introns and exons, and therefore can significantly affect protein function (Modrek & Lee, 2002). NCBI genome data reported that CPEB-2 has 6 isoforms (A-F).

Johnson et al., 2015 reported that alternate splicing of CPEB-2 plays a role in breast cancer, specifically with anoikis-resistance. *In vitro*, anoikis is a form of cellular apoptosis whereby anchorage-dependent cells detach from the ECM to undergo cell death. Therefore, anoikis-resistance is the process where cells become desensitized to this anchorage-dependence, detach from the ECM and continue surviving (Johnson et al., 2015). The cells become more motile and can metastasize more easily. It was reported that an increase in the ratio of two CPEB-2 isoforms, B (inclusion of exon 4) to A (exclusion of exon 4), induced anoikis-resistance in breast cancer cells (Johnson et al., 2015). These authors overexpressed either isoform A or isoform B in MDA-MB-231 breast cancer cells and injected them into severely immunodeficient mice, demonstrating a decrease in tumour volume with CPEB2A overexpression and an increase in tumour

volume with CPEB2B overexpression. Therefore, isoform B of CPEB-2 appears to be oncogenic in nature, whereas isoform A appears to be tumour-suppressor-like in nature (Johnson et al., 2015). However, due to the high similarity between CPEB-2 isoforms, we examined the specific sequences that were used in this study. A closer examination of their primer sequences using NCBI Primer Blast matched their CPEB2 isoform A primers with the whole CPEB-2 gene sequence, and their CPEB-2 isoform B primer sequence matched with both isoform B and D (Table 2). Therefore, further isoform-specific examination is still needed to investigate the role of CPEB-2 in breast cancer.

1.6 CPEB-2 and MicroRNAs

Recently, several reports have shown a relationship between microRNAs, CPEB-2 and cancer. One paper demonstrated that overexpression of microRNA-885-5p mediates EMT, increased migration, invasion, stress fiber formation as well as *in vivo* tumour formation and metastasis in colon cancer (Lam et al., 2017). This microRNA was also found to bind to CPEB-2 and downregulate translation, leading to TWIST1 upregulation (Lam et al., 2017). Another group (C. Li, Gao, Li, & Ding, 2017) suggested that a long non-coding RNA TUG1 mediates methotrexate (MTX) resistance in colorectal cancer via microRNA-186/CPEB-2 axis. They reported that TUG-1 is overexpressed in methotrexate resistant colon cancer and that TUG-1 knockdown re-sensitized colorectal cancer cells to MTX. Bioinformatics analysis showed that miR-186, a CPEB2-targeting microRNA, could directly bind to TUG1, suggesting TUG1 might have worked by sponging microRNA-186. However, a direct role of microRNA-186 or

CPEB-2 in methotrexate resistance, was not established (C. Li et al., 2017). Similarly, it was reported that another long non-coding RNA, CCAT1, was overexpressed in Paclitaxel-resistant nasopharyngeal cancers (Wang, Zhang, & Hao, 2017). These authors also showed that knocking down CCAT1 increased sensitivity to Paclitaxel through sponging microRNA-181a in nasopharyngeal cancer cells. This microRNA also suppressed CPEB-2 expression, however again it was not shown whether overexpression of CPEB-2 mediated Paclitaxel resistance, or whether CPEB-2 expression was dysregulated by Paclitaxel treatment (Wang et al., 2017).

1.7 CPEB-2 Knockdown In Our Lab

Previous work in our lab (Asma Hasan MSc Thesis 2015) used a number of COX-2 disparate human breast cancer cell lines showing a positive correlation of COX-2 with the expression of microRNAs -526b and -655, and a negative correlation of CPEB-2 expression with COX-2 or either microRNA. These relationships indicated a tumour suppressor role of CPEB-2, knowing that COX-2 and both microRNAs were oncogenic. She examined the consequences of transient siRNA-mediated knockdown of CPEB-2 in poorly metastatic and high CPEB2 expressing breast cancer cell line MCF-7 on *in vitro* functions associated with a malignant phenotype. She found that MCF-7-CPEB2 knockdown cells had an increase in migration, invasion, tumoursphere forming efficiency (*in vitro* surrogate of SLC phenotype) and a partial acquisition of EMT phenotype, indicating a role of CPEB-2 as a tumour suppressor gene. Dr. Mousumi Majumder (unpublished), a post-doctoral fellow in our lab, showed that pharmacological inhibition

of COX-2 or EP4 in COX-2 overexpressing MCF-7 (MCF-7-COX-2) cells both significantly increased expression of CPEB-2, indicating an important role of COX-2/EP4 signaling pathway on CPEB-2 expression. However, to fully elucidate whether this gene is a tumour suppressor, a more robust approach was needed employing a non-tumourigenic mammary epithelial cell line, such as MCF10A.

1.8 Rationale

The functions of CPEB-2 still remain largely unclear in breast cancer. Our lab has previously demonstrated the oncogenic and SLC-inducing functions of COX-2, PGE_2, EP4, microRNA-526b and microRNA-655. These two microRNAs were discovered through overexpression of COX-2 in a poorly metastatic breast cancer cell line MCF-7 and performing a combined gene and microRNA microarray. These microRNAs simultaneously target CPEB-2 (Figure 4), collectively along with 12 other tumour-suppressor-like genes. Therefore, the potential tumour-suppressive role of CPEB-2 in breast cancer requires further evaluation.

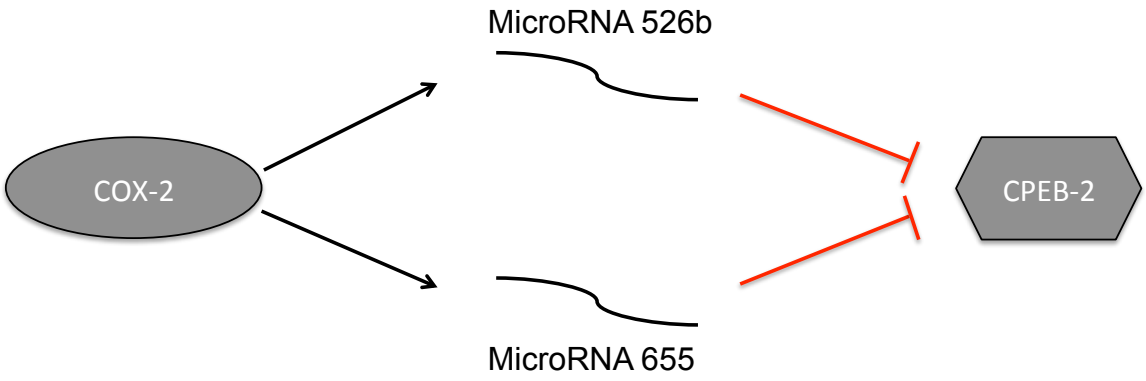

Figure 4. Simplified schematic of COX-2 - mediated upregulation of microRNAs 526b and 655, and collective suppression of CPEB-2 expression. COX-2 upregulates both microRNAs 526b and 655 to simultaneously suppress expression of CPEB-2 (Majumder et al., 2016, 2015).

1.9 Hypothesis

CPEB-2 is a tumour- and SLC-suppressing gene in human breast cancer.

1.10 Objectives

(1) *To define the consequences of CPEB-2 knockout in the MCF10A cell line on oncogenic functions tested in vitro.*

Tested functions: proliferation, migration, invasion, production of angiogenic factor VEGF-A, lymphangiogenic factors VEGF-C and –D, EMT phenotype and SLC contents.

(2) *To define the consequences of CPEB-2 knockout in the MCF10A cell line on oncogenic functions tested in vivo*.

Tested functions: Lung colony forming ability upon intravenous injection and orthotopic tumourigenicity and metastasis upon implantation at the mammary sites.

(3) *To evaluate the clinical significance of CPEB -2 expression in human breast cancer samples*.

Significance: CPEB-2 or its splice variants (isoforms) may be potential tumour and SLC-suppressing markers in breast cancer.

2 MATERIALS AND METHODS

2.1 Cell Culture

For our experiments, we used the non-tumourigenic immortalized mammary epithelial cell line MCF10A (ATCC) (Soule et al., 1990). Complete media for these cells is composed of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM:F12) (Gibco) supplemented with 5% Horse Serum (Invitrogen #16050-122), 20ng/ml EGF, 0.5 mg/ml hydrocortisone (Sigma #H-0888), 100ng/ml cholera toxin (Sigma #C-8052), 10 μg/ml insulin (Sigma #I-1882), and 1% penicillin/streptomycin (Invitrogen #15070-063).

2.1.1 MCF10A Cell Line

The MCF10A cell line is an immortalized epithelial non-tumourigenic breast epithelial cell line that is negative for both the estrogen receptor and the progesterone receptor (Subik et al., 2010). This cell line has been reported to be an excellent model for studying oncogenes and tumour suppressors through genetic manipulations in this cell line (Qu et al., 2015). MCF10A cells primarily express the CPEB2 isoform A, with little to no expression of isoform B (Johnson et al., 2015), which we confirmed in our lab.

2.1.2 CPEB-2 Knockout

We knocked out the CPEB-2 gene with a CRISPR/Cas9 double nickase plasmid (Santa Cruz, #sc-409367-NIC) targeting exon 1 of the gene (conserved across all isoforms). The first sgRNA used the sequence 5' GGCGTATGGTCCTTACGCCG 3',

while the second sgRNA used the sequence 5' GGACTGCTACTTCGGAGCG 3'. Using the double nickase CRISPR/Cas9 mutant enables us to ensure a high specificity with full knockout (Ran et al., 2013). Each double nickase mutant nicks the targeted gene as directed by a short guide RNA. Two simultaneous breaks in the gene mimics a double stranded break and successfully knocks out the portion of the targeted gene. Through electroporation, MCF10A cells were transfected with the double nickase CRISPR plasmid using the Amaxa Cell Line Nucleofector Kit IV (Lonza) according to the manufacturer's protocol. These cells were then seeded, subjected to 72 hours of puromycin selection and the surviving cell population was expanded.

2.2 RNA Extraction

Cells were grown to 80-90% confluency in 6 well plates, where total RNA was extracted using the RNeasy Mini Kit (Qiagen #74104) according to the manufacturer's protocol. RNA concentration was quantified using the Epoch Microplate Spectrophotometer (BioTek).

2.3 Protein Extraction

Cells were grown to 80-90% confluency in 6 well plates, where a mixture of M-PER (Mammalian Protein Extraction Reagent) (Thermo Fisher), 1x Halt protease inhibitor (Thermo Fisher) and 1x Phosphatase Inhibitor Cocktail (Sigma) are added for 5 minutes. The mixture was then pipetted up and down 3 times, added to a 1.5 mL

microcentrifuge tube and then sonicated 10 times for 30 seconds total. The microcentrifuge tube was then spun down at 14000 rpm at 4°C for 20 minutes. The resulting supernatant containing the protein was placed in a separate 1.5 mL microcentrifuge tube and stored at -80°C.

2.4 Quantitative RT-PCR

cDNA was synthesized from isolated RNA using the High Capacity Reverse Transcription Kit (Thermo Fisher #4368814) according to the manufacturer's protocol. Taqman Universal PCR Master Mix (Thermo Fisher #4304437) was then used to quantitatively analyze RNA expression (after cDNA synthesis) of the following: *CDH1* (E-cadherin), *VIM* (Vimentin), *SNAI1*, *ZEB1*, *TWIST1*, *PTGS2* (COX-2), *PTGER4* (EP4) and *TP53* (p53). PerfeCTa SyBR Green Fastmix (Quanta Biosciences) was used according to the manufacturer's protocol for qPCR analysis of VEGF-B, -C, and -D, using GAPDH as a loading control (Table 1).

Previous work with human breast cancer tissues was done by Dr. Mousumi Majumder in our lab. We used the same 105 breast cancer samples (Ontario Tumour Bank) along with 20 control breast tissues. Unfortunately, there are no isoform-specific probes, but rather ones that covered a couple of isoforms due to similarity among the isoforms. As highlighted earlier, primers used by Johnson et al., 2015 for CPEB2A covered all 6 isoforms, while primers for CPEB2B covered both isoforms B and D. Therefore, we examined human breast cancer tissues using probes with increased specificity towards isoforms than was previously done. We compared expression levels

of CPEB-2 isoforms, using a Taqman probe for isoform A/E and one for isoform B/D (Applied Biosystems) through qPCR (Rotor Gene 6) in a large panel of human breast cancer vs. non-cancerous tissues. We used a Taqman probe (Applied Biosystems) for GAPDH as our internal loading control. Delta Ct values were calculated by subtracting the average Ct values (triplicate) from the GAPDH loading control and analyzed as previously described (Majumder et al., 2015).

2.5 Western Blot

After protein extraction, protein was quantified using Pierce BCA Reagents A and B (Thermo Fisher) according to the manufacturer's protocol. 20µg of protein was diluted in 2x Laemmli Sample Buffer (Bio-Rad) and placed in a 95°C dry-bath system for 5 minutes for denaturation. Protein was then loaded into 10% polyacrylamide-SDS gels and electrophoresed for 1-1.5 hours at 100V. Gels were then transferred onto Immun-Blot® PVDF membranes (Bio-Rad) for 1.5 hours at 100V and blocked for 1 hour in 3% Bovine Serum Albumin (BSA) in 1x Tris Buffered Saline with Tween 20 (TBST). For CPEB-2 and p53 antibodies, membranes were blocked in 5% dry non-fat milk (Bio-Rad) in 1x TBST. Membranes were then incubated overnight on a shaker in primary antibody at the following dilutions: E-cadherin (Cell Signaling, Rabbit monoclonal, 1:1000), Vimentin (Cell Signaling, Rabbit monoclonal, 1:1000), N-Cadherin (Santa Cruz, Rabbit polyclonal, 1:200), β-actin (Santa Cruz, Mouse monoclonal, 1:4000), CPEB-2 (Origene, Rabbit polyclonal, 1:1000), p53 (Mouse monoclonal, Novus Biologicals, 1:200), p21 (Mouse monoclonal, Novus Biologicals, 1:200) and β-catenin (Sigma, Rabbit polyclonal,

1:4000). After primary antibody incubation, membranes are washed 3 times for 5 minutes each in 1x TBST, and then incubated for 1 hour in secondary antibody at the following dilutions: Goat Anti-Rabbit (1:10000, Li-COR) or Donkey Anti-Mouse (1:10000, Li-COR). Membranes were then washed twice in 1x TBST for 15 minutes each and scanned using the Odyssey Infrared Imaging System (Li-COR).

2.6 Immunofluorescence

Cells were grown on glass coverslips (Fisherbrand) to 70-80% confluency. The cells were then rinsed with PBS, and fixed for 30 minutes in 4% paraformaldehyde at room temperature. Once fixed, the cells were washed 3x with PBS and permeablized in 0.5% Triton-X-100 for 10 minutes. The cells were washed again 3x with PBS and blocked with 8% BSA with 0.01% Tween20 in PBS for 30 minutes. Cells were washed again 3x with PBS and then incubated in primary antibody at the following dilutions in 4% BSA overnight at 4°C: E-cadherin (Cell Signaling, 1:50), Vimentin (Cell Signaling, 1:50), and N-Cadherin (Santa Cruz, 1:100). The stained cells were then washed again 3x with PBS and stained for 1 hour with secondary antibody at the following dilutions: Goat Anti-Rabbit 594 (1:100, Biotium), Goat Anti-Rabbit 488 (1:100, Biotium), or Goat Anti-Mouse 488 (1:100, Biotium). Cells were incubated for 5 minutes in Vectashield anti-fade mounting medium with DAPI (Vector), which was also used to mount the slides. Immunofluorescent images were taken using the Zeiss LSM 510 Meta Multiphoton Confocal Microscope.

2.7 Scratch Assay for Cell Migration

Cells were grown to 90-100% confluency, and serum starved overnight (1% HS). Cells were treated with 0.5μM Mitomycin C 2 hours prior to scratching the plate in order to block proliferation. Mitomycin C was developed as an anti-tumour drug that has the ability to crosslink DNA, thus blocking proliferation (Tomasz, 1995). Each plate was then scratched down the middle with a P200 pipette tip, taking pictures at 0 hours, 24 hours, 48 hours, and 72 hours to measure rate of migration of each cell line (Justus, Leffler, Ruiz-Echevarria, & Yang, 2014). Media consisted of Basal DMEM:F12 media (Gibco), 1% HS and 0.5μM Mitomycin C. Cells were washed with PBS and media was replaced every 24 hours.

2.8 Migration and Invasion Assays using Transwell Chamber

Cells were grown to 70-80% confluency, and serum-starved overnight (1% HS) prior to seeding cells. Membrane inserts for these assays (Corning) have pores 8μm in diameter to allow for migration. Cells were seeded at a density of $2x10^4$ cells/insert. For the invasion assay, Matrigel (basement membrane analog) was placed on top of the membrane and allowed to dry overnight. Cells were trypsinized, counted and resuspended at a density of $2x10^3$ cells/700μl of basal media. Per insert, 300μl was placed on top of the membrane, and below the membrane 700μl of 5% HS media was placed in order to stimulate migration. The horse serum acted as a chemoattractant for these cells. After 24 hours and 48 hours respectively for migration and invasion assays (Justus et al., 2014), cells were washed off with PBS and the top layer of the membrane was carefully

wiped with a cotton swab to remove excess cells. The cells remaining were then fixed with cold methanol, stained with Eosin (cytoplasm) and Thiazine (nucleus), and mounted onto glass chamber slides. This was done in triplicate and cells were counted.

2.9 Tumoursphere Formation Assay

In order to assess SLC phenotype *in vitro*, we used the tumoursphere formation assay where single cell suspensions at low cell concentrations were plated in ultra-low attachment plates and allowed to form into spheroid aggregates called tumourspheres (Shaw et al., 2012). The ability to do so represents their ability to "self-renew", a fundamental property of stem-like cells. Cells are grown to 70-80% confluency, and then trypsinized and spun down. As previously done (Majumder et al., 2016), the cells were then resuspended in basal HuMEC media (Gibco) with added B-27 supplement (Gibco), EGF (20 ng/mL, Invitrogen) and FGF (20 ng/mL, Invitrogen), taken up by a 1 mL syringe and put through a 40-µm cell strainer (Falcon) to collect a single cell suspension. The cells were then counted and seeded at a density of 10 cells/well in an ultra-low attachment 96-well plate (Thermo Fisher) for spheroid forming efficiency, as well as $1x10^3$ cells/well in an ultra-low attachment 6-well plate (Corning) for immunofluorescence. After 4 days, spheroids (minimum 60µm in diameter) were counted and divided by total number of cells plated to calculate efficiency.

2.10 EdU Flow Cytometry

In order to measure proliferation *in vitro*, we used the Click-iT EdU Alexa Fluor 488 Imaging kit (Invitrogen) in collaboration with Dr. David Hess according to the manufacturer's protocol. Briefly, WT and CPEB2KO MCF10A cells were incubated for 24 hours with 5'-ethynyl-2'-deoxyuridine (EdU), allowing for incorporation during DNA synthesis. Cells that successfully incorporate EdU have progressed into or through S-phase, and were detected after fixation through a Click Chemistry reaction using a copper-catalyzed reaction with a fluorescent dye (AlexaFluor 488). Gates were set to include only cells, remove out doublets using side scatter and forward scatter, and remove debris. For each replication, cells were incubated in complete media with EdU or without EdU (negative control). The negative control was then used to set the final gate for EdU positive cells based on auto-fluorescence of the cells. This final gate provided a percentage of EdU-positive cells during the 24 hour period that was compared in each replication.

2.11 Experiments to Evaluate Oncogenicity in Mice

In collaboration with Dr. David Hess, 6-week old female immunodeficient NOD/SCID/IL2Rγ null mice (deficient in B-cells, T-cells or Natural Killer cells) were used to test lung colony forming capacity and orthotopic tumourigenicity of CPEB-2 knockout MCF10A cells compared to wildtype cells. CRISPR-mediated CPEB-2 knockout cells and wildtype cells were injected intravenously into the tail vein ($5x10^5$ cells per mouse into 6 mice for each cell type). We isolated lung, spleen and liver after 8

weeks to assess colony formation. We also orthotopically injected $5x10^5$ cells mixed 1:1 with Matrigel subcutaneously into both right and left inguinal mammary regions of 5 mice each for the CPEB2KO and parental cells (totaling 10 sites each). After 12 weeks, mice were sacrificed and one mammary fat pad was stored in O.C.T. for frozen sectioning while the other was stored in Bouin's solution for paraffin embedding. Lungs, liver and spleen were also harvested to assess metastasis from original mammary site. Lungs were inflated with PBS prior to isolation. The Human Leukocyte Antigen (HLA) antibody, provided by Dr. David Hess, was used to confirm that the tumours were of human origin. EdU was systemically injected 24 hours prior to sacrifice to examine proliferation of cells within tumours. Animals were maintained according to the Canadian Council of Animal Care guidelines with food and water ad libitum in the Robarts Research Institute ACVS barrier. All mice were weighed once per week.

2.12 Immunohistochemistry

Lungs, liver and spleen were extracted and stored either in O.C.T for frozen sectioning or Bouin's solution for formalin fixation. After 24 hours, organs were removed from Bouin's solution and placed in neutral buffered formalin (10%) for 48 hours. Tissues were then embedded in paraffin, sectioned at 5μm and stained with Hematoxylin and Eosin (H&E). Frozen organs were sectioned at 8μm for lungs, liver and spleen, while mammary fat pads were sectioned at 10μm. Sections were then fixed in 10% formalin, permeablized in 0.1% Triton-X-100, and blocked with M.O.M. according to the manufacturer's protocol. Mouse anti-Human HLA antibody (1:100, BD Pharmingen) was

used to detect human cells within all organs, with Horse anti-mouse FITC (1:200, Vector Labs) used to detect the HLA antibody. Sections were subsequently mounted using Vectashield (Vector Labs) with DAPI and viewed on a fluorescent microscope. Micrometastases were scored as single cells, clusters (2-8 cells) and colonies (>8 cells) as reported earlier (Majumder et al., 2016) and averaged within 3 sections, 5 images of $(400\mu m)^2$ each per section.

2.13 Statistical Analysis

Statistical analyses were performed using Graphpad Prism Software 5.0 (Graphpad Software Inc. 2007). Data comparing WT cells to CPEB2KO cells were analyzed using unpaired Student's t-test, and spheroid sizes were compared with the Mann-Whitney test. Human data was compared using a one-way ANOVA followed by a Dunnett's test. Statistical significance was considered if $p<0.05$ (1 asterisk) and highlighted if $p<0.01$ (2 asterisks).

3 RESULTS

3.1 CRISPR-Mediated CPEB-2 Knockout

In order to assess tumour-suppressing functions of CPEB-2, a robust approach was taken to knock out the whole gene (all isoforms) using a double nickase CRISPR/Cas9 plasmid in an immortalized mammary epithelial cell line MCF10A. To confirm knockout, both RT-PCR and Western blot were performed, demonstrating 79.2% knockout efficiency (Figure 4).

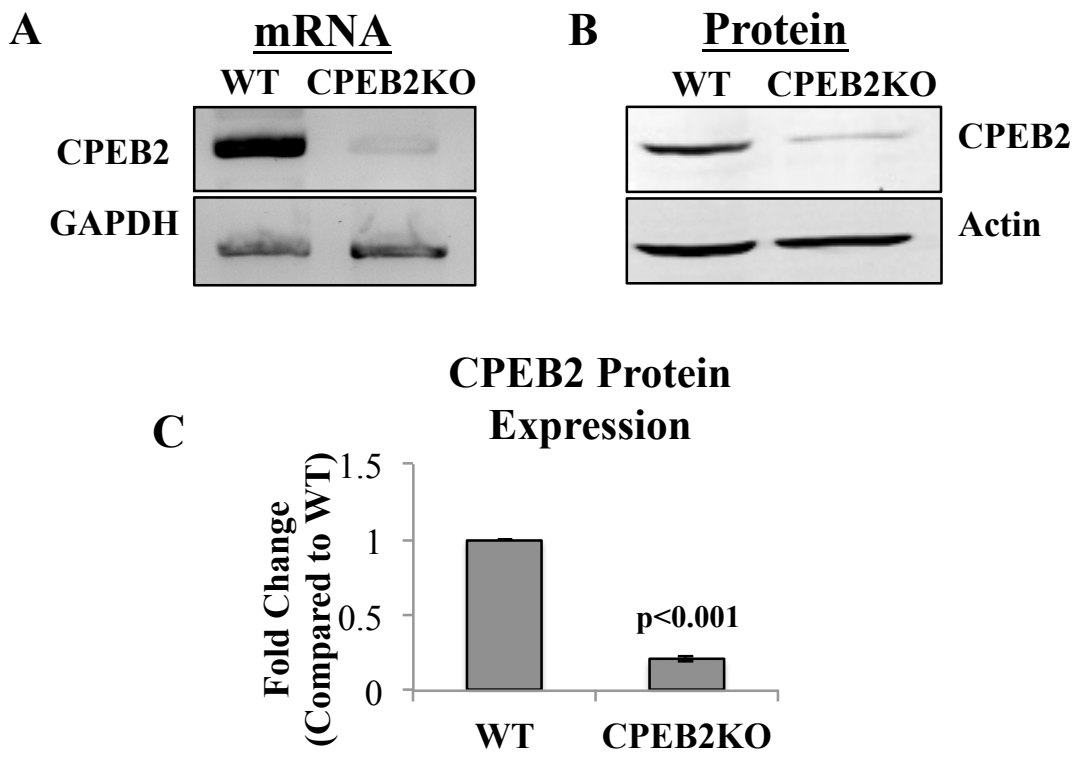

Figure 4. MCF10A CPEB-2 knockout through a double nickase CRISPR plasmid. (A) RT-PCR and (B) Western blot of total CPEB-2 expression with 79.2% knockout efficiency (C). Quantified Data in (C) represent the mean (n=3) ±SEM.

3.2 CPEB2KO cells undergo EMT

EMT is a change in cell phenotype that allows epithelial cancers to metastasize to distant parts of the body, and is usually associated with a change in morphology as well as EMT markers (Kalluri & Weinberg, 2009). Upon CPEB-2 knockout, a clear role for this gene was established in suppressing EMT. Morphologically, the original epithelial cells transformed into a more elongated mesenchymal-like shape due to CPEB-2 loss (Figure 5). This indicated a possible change through EMT, so we investigated markers associated with this alteration. Through qRT-PCR, epithelial marker *CDH1* (E-cadherin) (transmembrane protein) was found to be significantly decreased (0.116 fold change) in the CPEB2KO cells, with a significant concomitant increase in mesenchymal markers *VIM* (Vimentin) (intermediate filament) by 1.49-fold, *SNAI1* (transcription factor) by 4.38-fold, and *ZEB1* (transcription factor) by 4.06-fold (Figure 6). Western blots were performed to confirm this at the protein level, demonstrating almost complete abolishment of E-cadherin expression (0.078-fold change), with significant increases in mesenchymal markers N-cadherin (transmembrane protein) (6.93-fold change) and Vimentin (2.75-fold change) (Figure 7). Immunofluorescence was also used to confirm this phenotype using markers E-cadherin (0.20-fold change), N-cadherin (1.80-fold change), and Vimentin (3.50-fold change) (Figure 8). Cadherin switching, from E-cadherin to N-cadherin, was evident through the use of immunofluorescence (Figure 8).

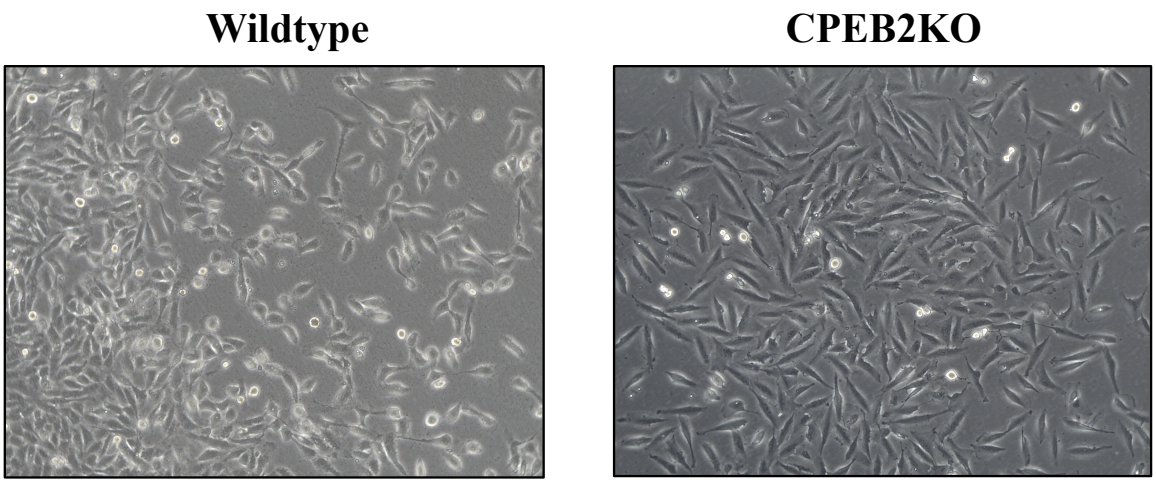

Figure 5. Mesenchymal morphology of CPEB2KO MCF10A cells. Wildtype MCF10A cell (left) exhibiting epithelial cell morphology. CPEB2KO cells (right), exhibiting elongated, mesenchymal (Fibroblast-like) morphology. (Magnification = 10x)

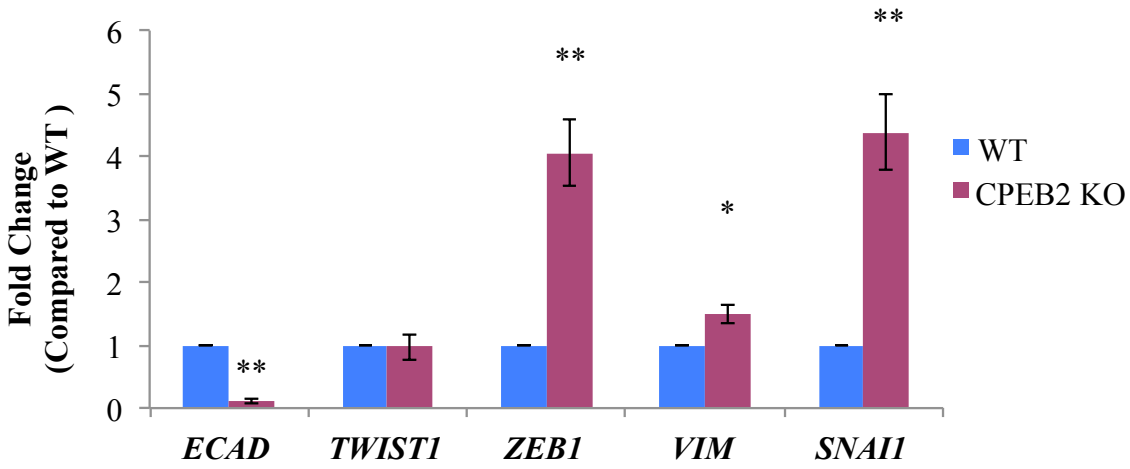

Figure 6. Quantitative RT-PCR for EMT markers showing EMT in CPEB2KO cells at the mRNA level. qRT-PCR (Mean ±SEM) for epithelial marker E-cadherin and mesenchymal markers TWIST1, ZEB1, Vimentin and SNAI1 using WT and CPEB2KO cell lines (n=3). E-cadherin was significantly decreased (0.116 fold change, p=0.00001), with significant increases in ZEB1 (4.06 fold change, p=0.002) Vimentin (1.49 fold change, p=0.01), and SNAI1 (4.38 fold change, p=0.002) in CPEB2KO cells. Student's T-test used for analysis. (*) indicates $p<0.05$. (**) indicates $p<0.01$.

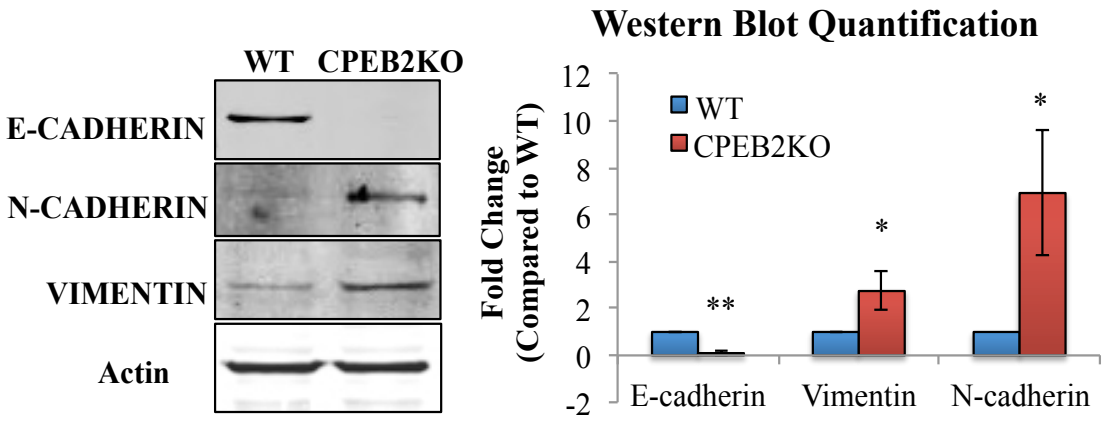

Figure 7. Western Blot showing induction of EMT in CPEB2KO cells at the protein level. Representative Western blots shown on the left. Quantification (Mean ±SEM) (ImageJ Software) of Western Blots (right) for E-cadherin, Vimentin and N-Cadherin in Wildtype (n=3) and CPEB2KO cell lines (n=3). E-cadherin protein expression was significantly decreased (0.078 fold change, p=0.00002), with a significant increase in Vimentin (2.75 fold change, p=0.010) and N-cadherin (6.93 fold change, p=0.046) in CPEB2KO cells. Student's T-test used for analysis. (*) indicates $p<0.05$, (**) indicates $p<0.01$.

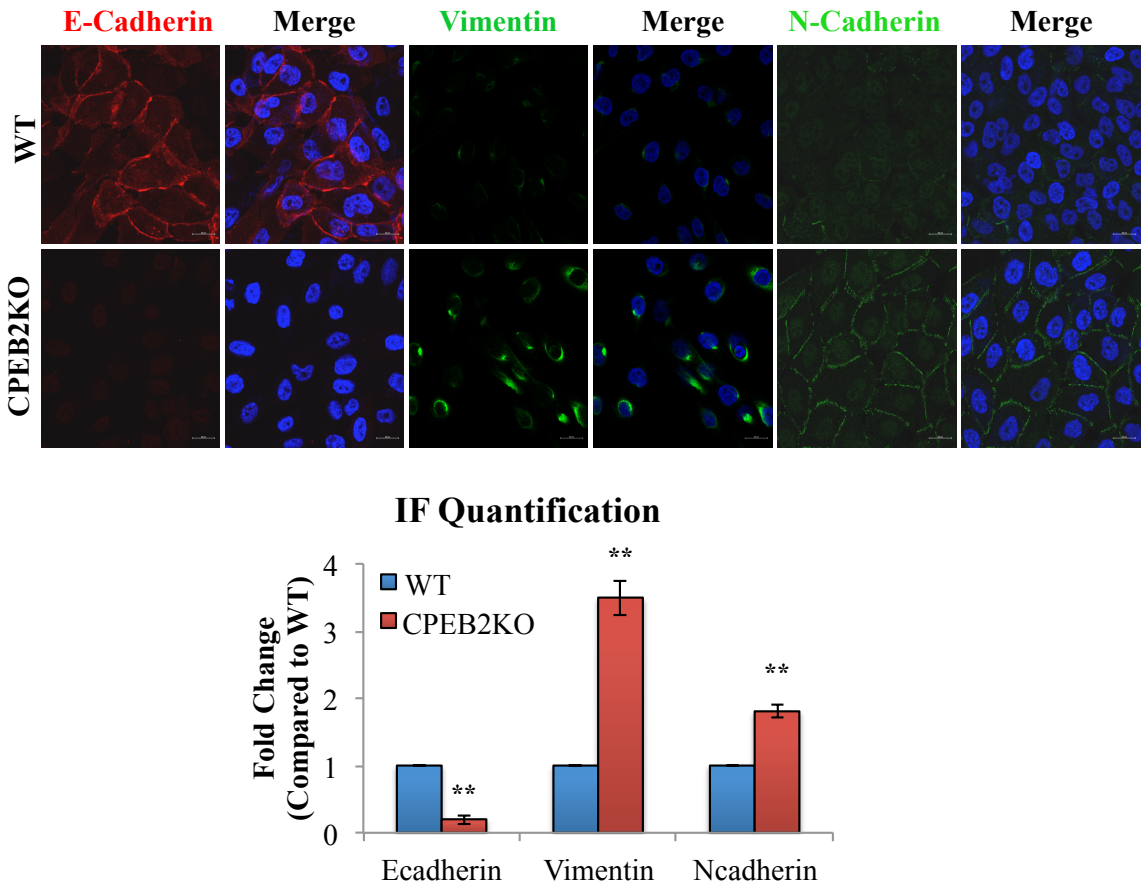

Figure 8. CPEB2KO induced EMT visualized through immunofluorescence of markers. Integrated Density was quantified (Mean ±SEM) (ImageJ Software) for immunofluorescence images and normalized to cell number for E-cadherin, Vimentin and N-Cadherin in WT (n=3) and CPEB2KO cell lines (n=3). E-cadherin protein expression was significantly decreased (0.20 fold change, p=0.0002), with a significant increase in Vimentin (3.50 fold change, p=0.00033) and N-cadherin (1.80 fold change, p=0.00046) in CPEB2KO cells. Scale = 20μm. Student's T-test used for analysis. (**) indicates $p<0.01$.

3.3 CPEB2KO increases chemokinesis, migratory rate and invasion

Transitioning from an epithelial cell type into a mesenchymal phenotype is usually associated with an increase in both migratory ability and invasiveness due to the changes in molecular expression. For example, a loss in E-cadherin allows the cells to detach from each other and an increase in Vimentin mediates cytoskeletal reorganization (N.-G. Kim et al., 2011; Kourtidis et al., 2017; Liu et al., 2015). To truly assess migration alone, we used a 72-hour scratch assay, but we serum-starved the cells overnight, while also blocking proliferation using Mitomycin C prior to scratching. Over 72 hours, the CPEB2KO cells migrated 35.82μm/24 hours, while their epithelial counterparts migrated 3.57μm/24 hours (Figure 9). Therefore, the CPEB2KO cells migrated 10.03-fold faster than the WT cells.

To further examine the migratory ability of these cell populations, we used a Transwell migration (chemokinesis) assay. Serum-starved CPEB2KO cells migrated through the membrane into the 5% Horse Serum chamber, in a similar fashion to the scratch or wound-healing assay with 10.89-fold as many cells on the bottom side of the membrane (Figure 10). Alongside migration, ability to invade distant sites is an important oncogenic phenotype used in metastasis of cancer cells. Using a similar transwell chamber, the microporous membrane was coated with basement membrane analog Matrigel to assess invasiveness. These CPEB2KO cells were able to degrade the Matrigel and invade 3.43-fold more than the WT cells over 48 hours (Figure 10).

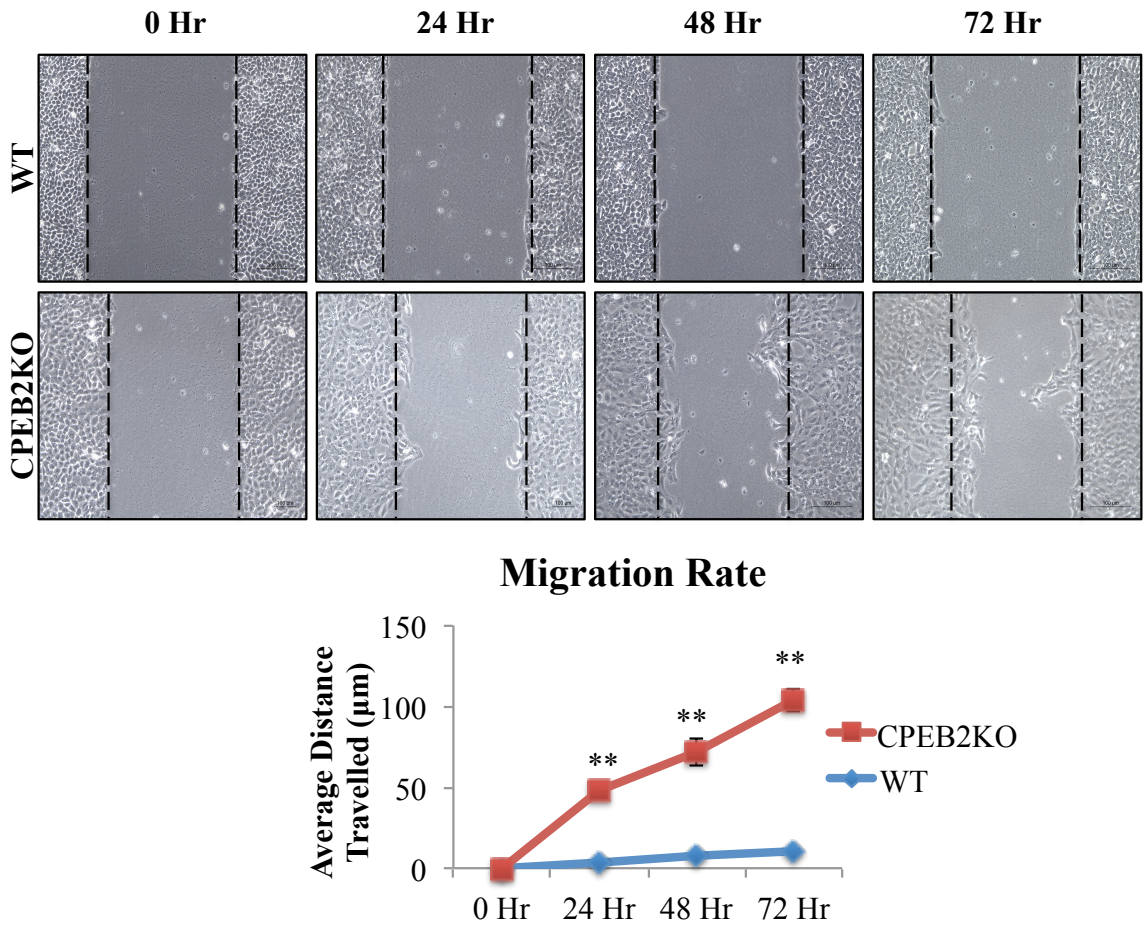

Figure 9. CPEB2KO cells migrated faster than WT cells in a Scratch Assay over 72 hours. Representative images of both wildtype MCF10A cells and CPEB2KO cells (n=3) that were scratched, allowed to migrate in 1% Horse Serum, treated with Mitomycin C to block proliferation. Average migratory rate (Mean ±SEM) was 35.82µm/24 hours in CPEB2KO cells (Total Distance - 24 hr: 43.94µm (p=0.0004), 48 hr: 64.495µm (p=0.001), 72 hr: 93.82µm (p=0.0001)) while Wildtype cells migrated 3.57µm/24 hours (Total Distance - 24 hr: 3.74µm, 48 hr: 7.097µm, 72 hr: 10.30µm). Scale = 100µm. Student's T-test used for analysis. (**) indicates $p<0.01$.

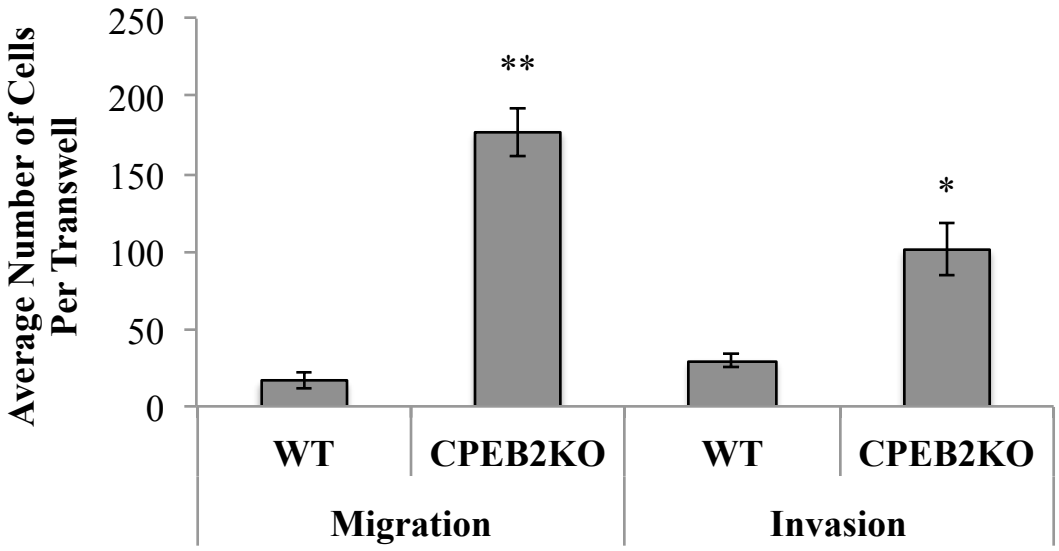

Figure 10. CPEB2KO cells exhibited increased migration and invasion in Transwell Assays. Both wildtype MCF10A cells and CPEB2KO cells were serum starved, plated in the top chamber of the transwell, which was covered with Matrigel for the invasion assays, and allowed to migrate/invade and into 5% Horse Serum in the bottom chamber. Cells were treated with Mitomycin C to block proliferation. Migration was 10.89x more in CPEB2KO cells, while invasion (Mean ±SEM) was 3.43x more in CPEB2KO cells as Wildtype. (*) indicates $p<0.05$. (**) indicates $p<0.01$.

3.4 Proliferation increases in CPEB2KO cells

Sustained proliferative ability is a hallmark of cancer cells (Hanahan & Weinberg, 2011) We therefore examined DNA synthesis, which is indicative of active proliferation of both CPEB2KO cells and wildtype MCF10A cells. We performed flow cytometry for 5'-ethynyl-2'-deoxyuridine (EdU) to measure DNA synthesis. Over 24 hours, EdU incorporates into the DNA of cells that are actively synthesizing DNA (cells that are preparing to divide), and can be measured through fluorescence. Across all three replicative measurements, CPEB2KO cells demonstrated an increase in proliferation compared to WT cells (Figure 11).

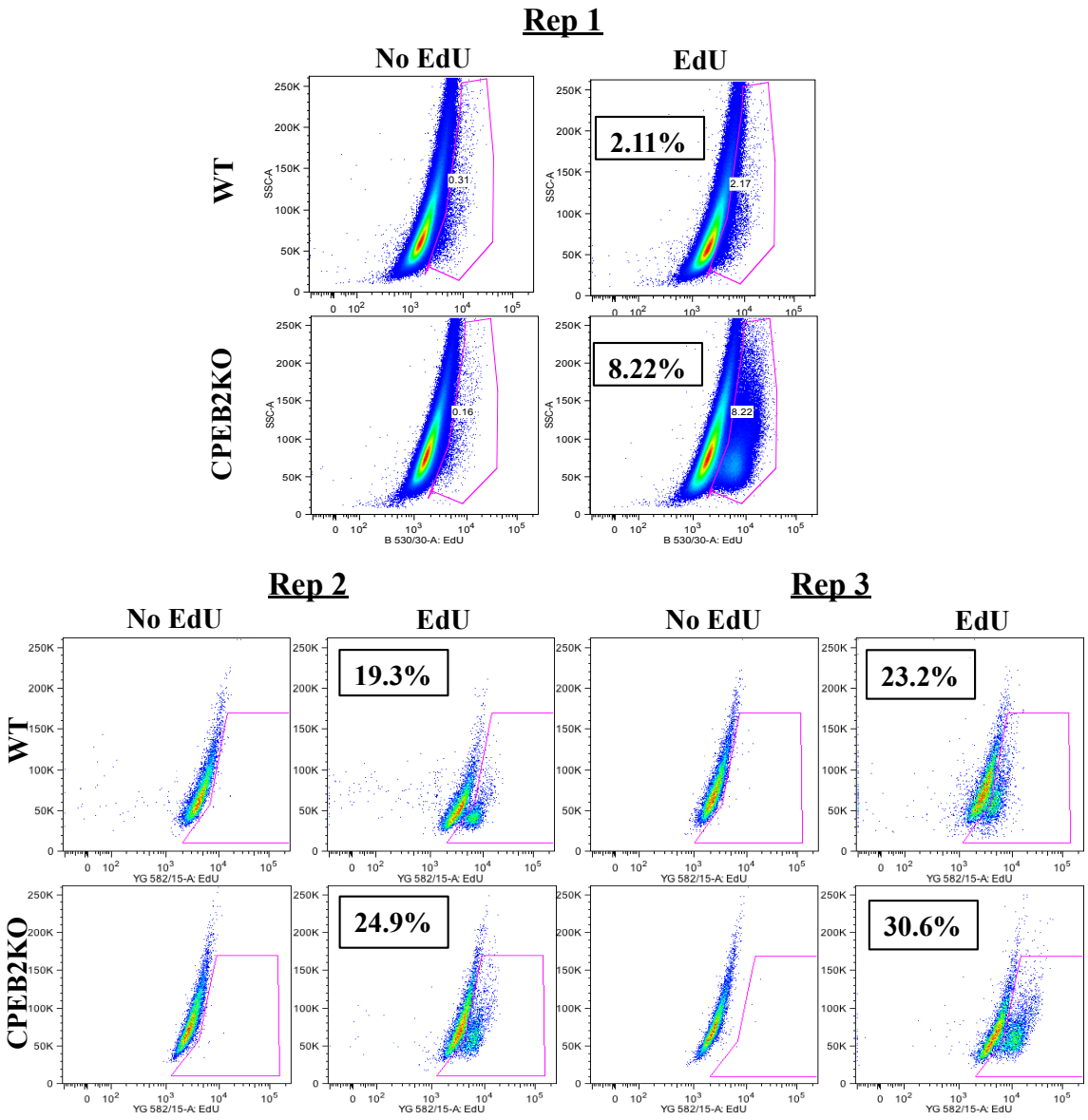

Figure 11. CPEB2KO cells exhibited increased proliferation after 24 hours measured using flow cytometry (Click-iT EdU Alexa Fluor 488 Imaging Kit). Wildtype MCF10A cells without EdU were used with the same protocol as a negative control. Replicate 1 used 1 million cells, while replicates 2 and 3 used 10,000 cells each. CPEB2KO MCF10A cells incubated with EdU for 24 hours showed increases in actively proliferating cells (S-phase) across all 3 replications.

3.5 CPEB2KO stimulates SLC Phenotype

We recently discovered that COX-2 plays a very important role in inducing the stem-cell state through Notch/Wnt pathways in breast cancer (Majumder et al., 2016). Therefore, we examined the role of CPEB-2 in SLC phenotype through the tumoursphere formation assay *in vitro*, which displays a cell's ability to self-renew in an ultra low attachment plate. The CPEB2KO cells had significantly larger sizes of tumourspheres (91.42μm) compared to WT cells (70.92μm) as well as an increase in tumoursphere formation efficiency (5.12-fold increase in CPEB2KO cells) (Figure 12). We have investigated the mechanism behind this phenotype further. CPEB-2 was reported in mouse neuronal cells to bind to β-catenin mRNA to repress translation (Turimella et al., 2015). Therefore, we compared expression of β-catenin at the protein level in wildtype and CPEB2KO cells, as well as downstream gene expression changes involved in the β-catenin/wnt pathway (AXIN2, AXIN1, CCND1, Myc). Using a western blot, β-catenin was upregulated in the CPEB2KO cell line (1.29-fold) compared to WT cells (Figure 13). Furthermore, this increase was associated with significant increases in CCND1 (3.49-fold) and AXIN1 (1.298-fold) at the mRNA level (Figure 13), suggesting that this pathway may be important in mediating the SLC phenotype noted earlier.

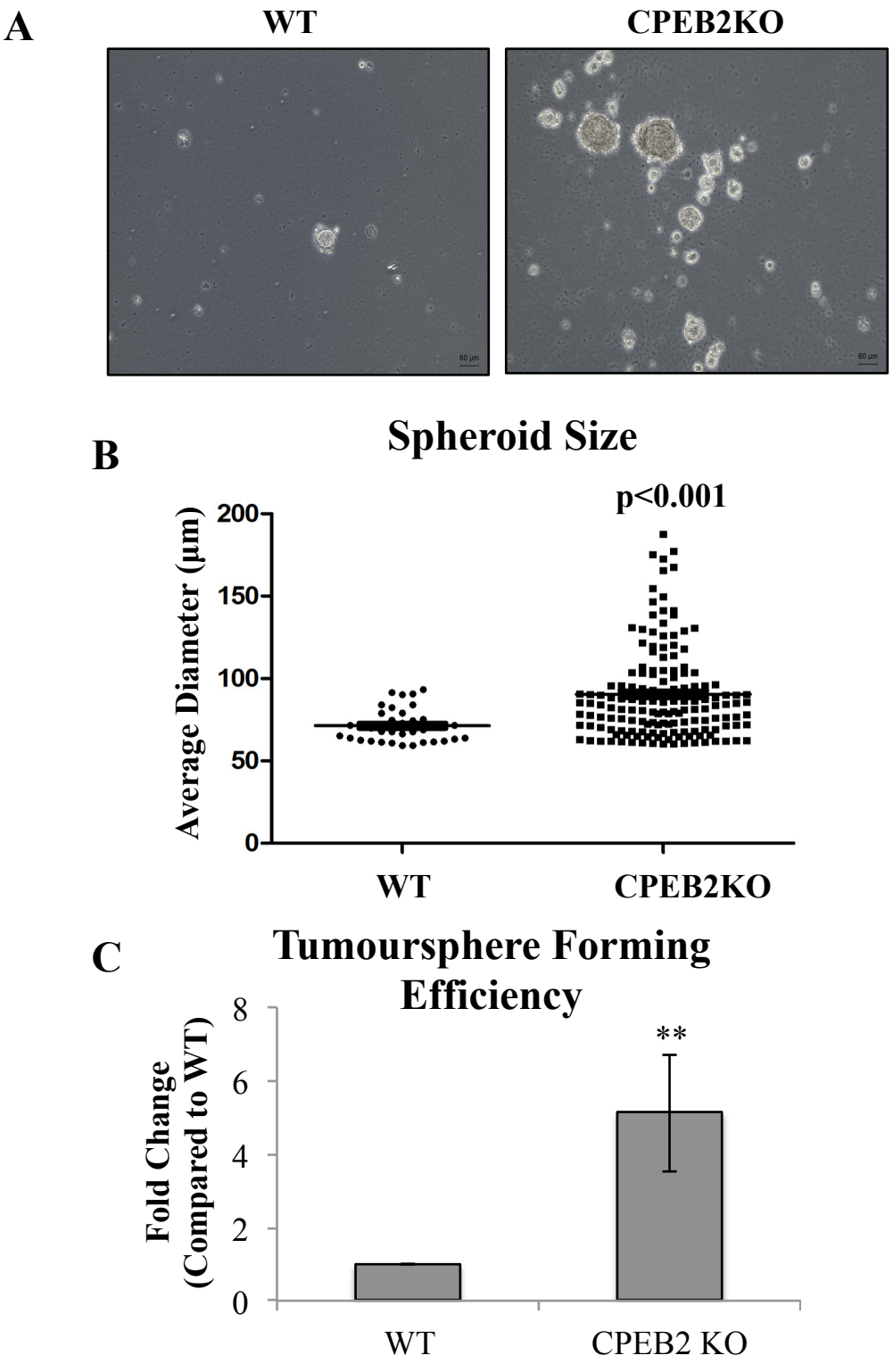
A
WT
CPEB2KO
B
Spheroid Size
p<0.001
Average Diameter (µm)
200
150
100
50
0
WT
CPEB2KO
C
Tumoursphere Forming Efficiency
Fold Change (Compared to WT)
8
6
4
2
0
**
WT
CPEB2 KO

Figure 12. Increased tumoursphere formation efficiency and size in CPEB2KO cells after 4 days. (A) Representative images of both WT MCF10A cells and CPEB2KO cells plated on ultra-low attachment plates. (B) Dot plot (Mean ±SEM) of spheroid size (Mann-Whitney Test for statistical significance) showing increased average diameter (WT=70.92μm, CPEB2KO=91.42μm, p=0.0017). (C) Efficiency of spheroid formation (Mean ±SEM) calculated as number of tumourspheres >60μm divided by total number of cells plated. CPEB2KO cells showed 5.12-fold increase (p=0.01) in tumoursphere forming efficiency. Student's T-test used for analysis. Scale = 60μm. (**) indicates $p<0.01$.

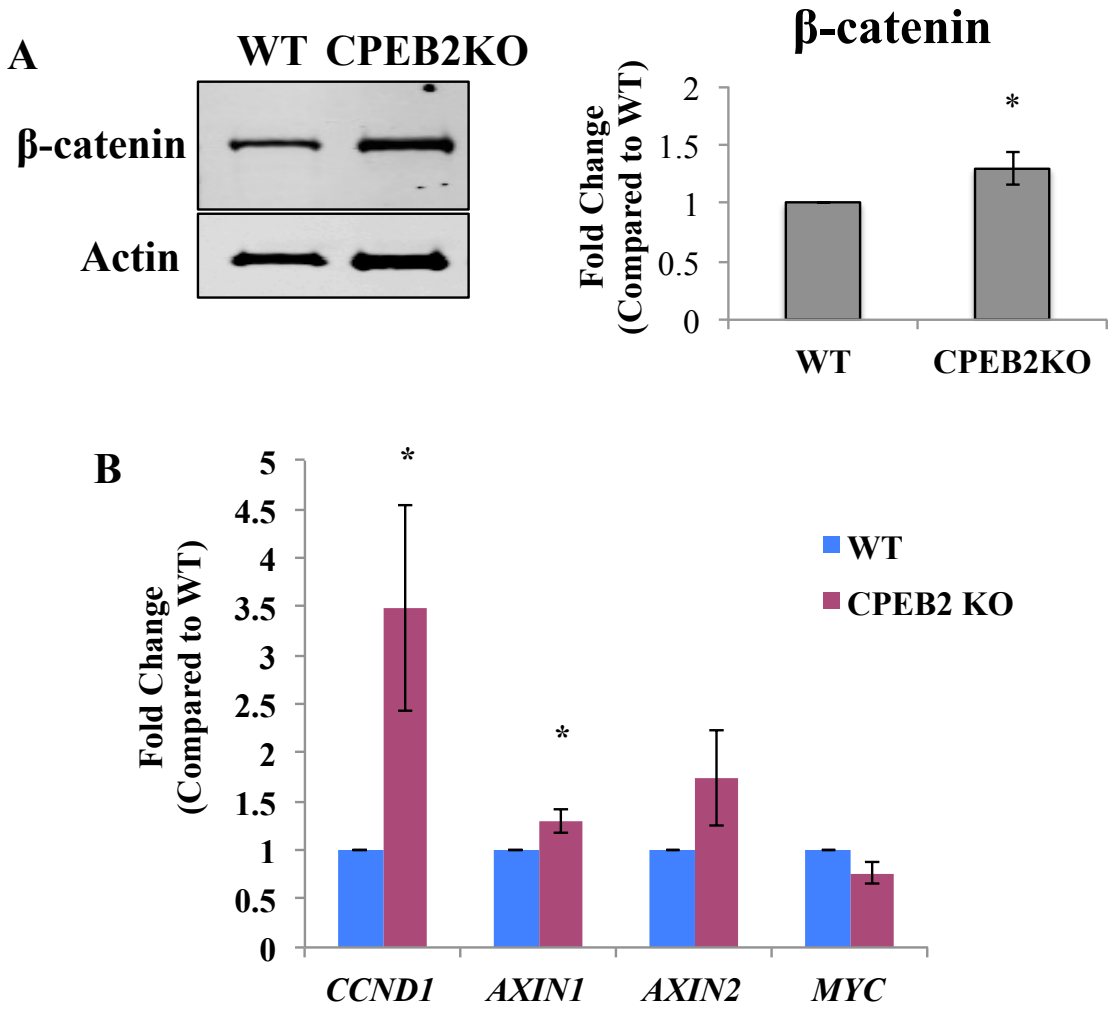

Figure 13. Increased β-catenin pathway signaling in CPEB2KO cells. (A) Representative Western blot and quantification (Mean ±SEM) (ImageJ Software) for β-catenin expression (n=3). (B) qRT-PCR (Mean ±SEM) for downstream genes of β-catenin pathway (n=3 for each). β-catenin was increased 1.29 fold (p=0.048) in CPEB2KO cells, with increases in downstream target genes CCND1 (3.49 fold increase, p=0.039), and AXIN1 (1.298 fold increase, p=0.034). No significant change was observed in AXIN2 (1.73 fold change, p=0.10) or Myc (0.76 fold change, p=0.051). Student's T-test used for analysis. (*) indicates $p<0.05$.

3.6 VEGF-D expression is increased in CPEB2KO cells

Our lab has shown a role of COX-2 in promoting lymphangiogenic factor production, specifically VEGF-C and VEGF-D, through the EP4 receptor activation (Xin et al., 2012). Overexpression of these factors allows tumour cells to recruit lymphatic vessel growth to ultimately be able to metastasize through the lymphatic system (Alitalo & Carmeliet, 2002). Through qRT-PCR, no change was seen in the production of VEGF-B or VEGF-C, however a 4.68-fold upregulation of VEGF-D expression was seen in the CPEB2KO cells compared to WT cells (Figure 14).

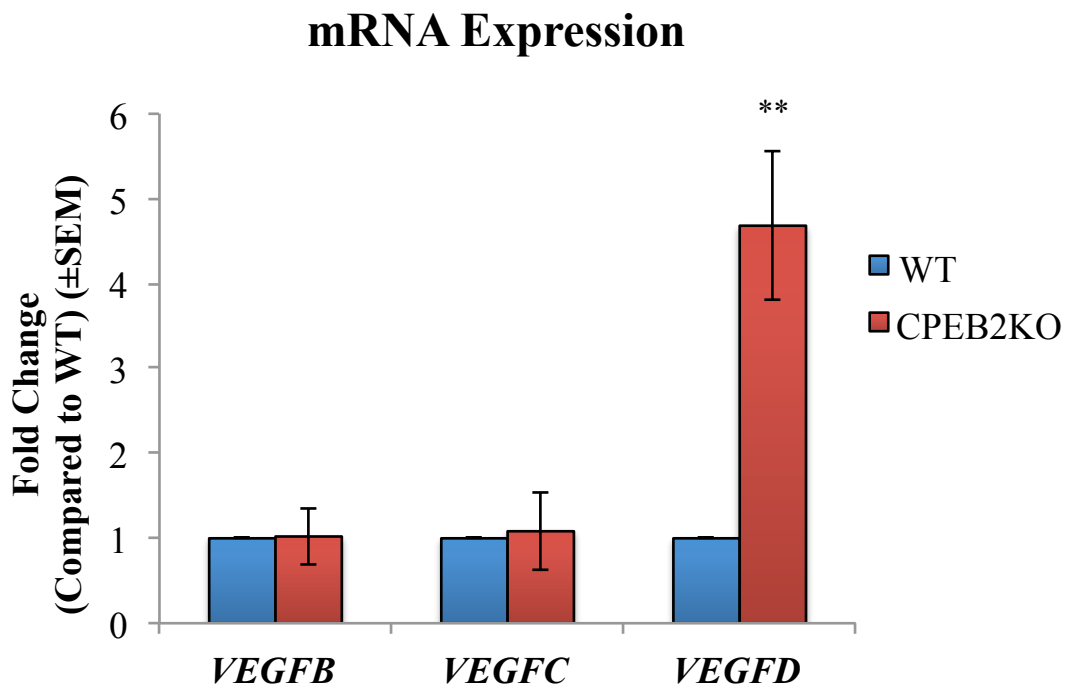

Figure 14. Increased VEGF-D expression at the mRNA level in CPEB2KO cells. qRT-PCR (Mean ±SEM) for VEGF-B, -C and -D using WT and CPEB2KO cell lines (n=3). *VEGF-D* mRNA expression was increased an average of 4.68 fold over WT cells (Student's T Test, $p=0.00016$). (**) indicates $p<0.01$.

3.7 COX-2 and EP4 are upregulated in CPEB2KO cells

Previous work in our lab demonstrated that pharmacological inhibition of COX-2 or EP4 increased expression of CPEB-2. Furthermore, overexpression of microRNAs -526b and -655 decreased expression of CPEB-2, while increasing expression of COX-2 and EP4, thereby showing a feedback loop, the underlying molecular mechanisms remaining to be identified. This prompted us to examine if COX-2 or EP4 expression were altered when CPEB-2 was knocked out to elucidate this mechanism. Through qRT-PCR, we discovered a 4.31-fold increase in COX-2 mRNA expression and a 3.45-fold increase in EP4 mRNA expression (Figure 15).

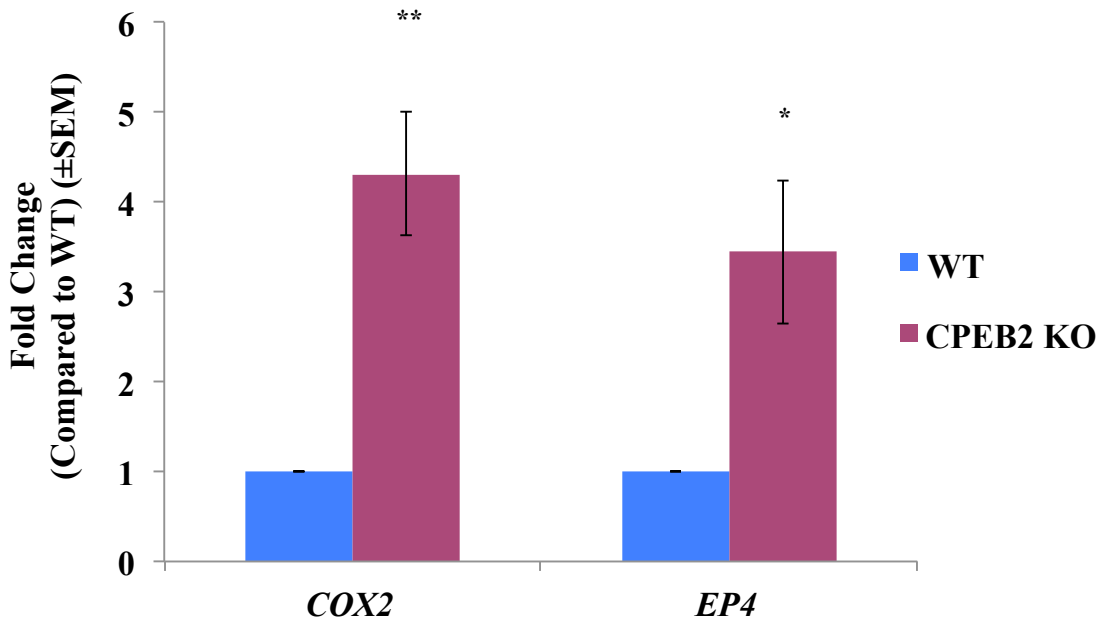

Figure 15. COX-2 (*PTGS2*) and EP4 (*PTGER4*) expression is increased in CPEB2KO cells. qRT-PCR (Mean ±SEM) for COX-2 and EP4 using WT and CPEB2KO cell lines (n=3). *COX-2* mRNA expression was increased an average of 4.31-fold compared to WT cells ($p=0.0024$). EP4 mRNA expression was increased an average of 3.45-fold compared to WT cells ($p=0.011$). Student's T-test used for analysis. (*) indicates $p<0.05$. (**) indicates $p<0.01$.

3.8 p53 Translational Regulation

CPEB-1 was reported to regulate the translation of a powerful tumour suppressor, p53, which had 2 CPE domains in its 3' UTR (Burns & Richter, 2008). CPEB-1 knockdown cells were reported to have reduced p53 expression with shorter poly-A tails, which led to reduced translation efficiency (Burns & Richter, 2008). CPEB-1 and CPEB-2 were reported to co-regulate many molecules, such as HIF1α and TWIST1 (Hägele et al., 2009; Nairismägi et al., 2012), and so we investigated p53 as another candidate using the CPEB2KO MCF10A cell line. Through qRT-PCR, we discovered that p53 mRNA expression in the CPEB2KO cells was not significantly different from the WT cells (Figure 16). However, through a western blot, we discovered about 60% lower expression of p53 at the protein level in CPEB2KO cells, suggesting that p53 is differentially regulated at the translational level (Figure 16). We also examined p21 expression, a downstream effector of p53, through a western blot and discovered a similar downregulation in the CPEB2KO cells, indicating that the p53 pathway is negatively affected in these cells (Figure 16).

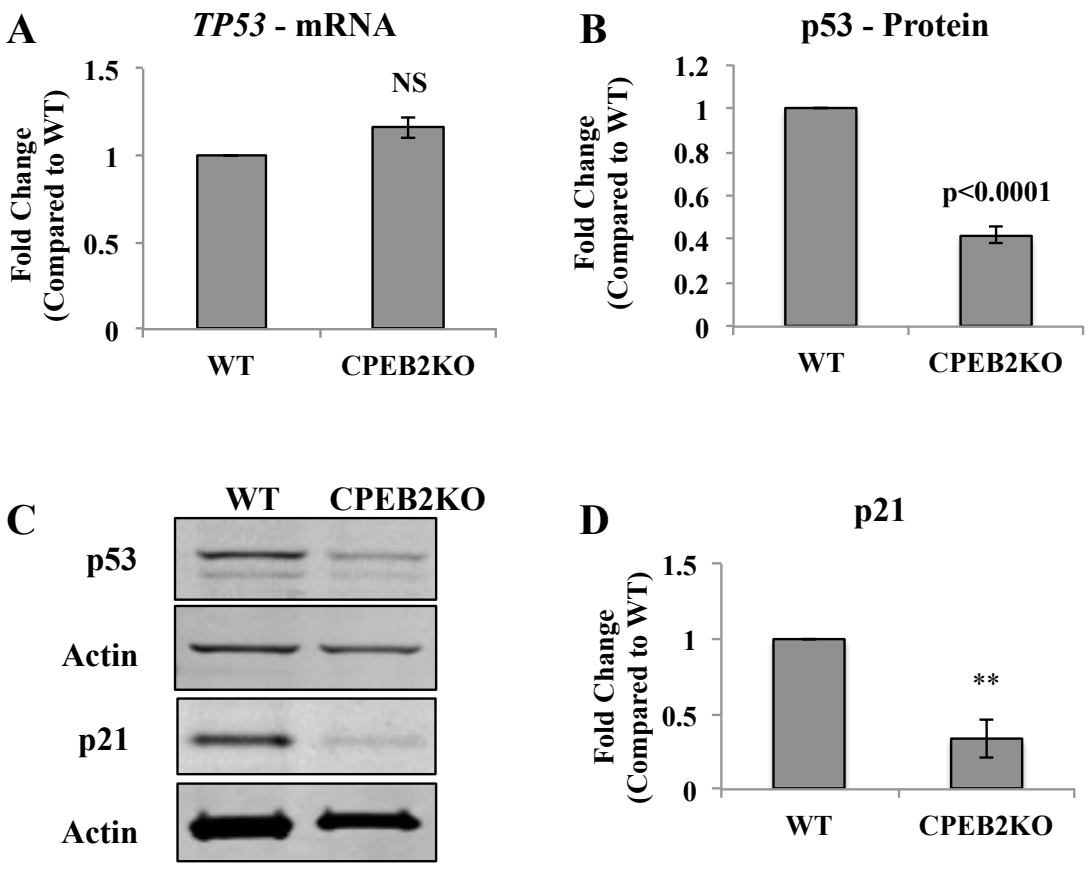

Figure 16. p53 translational regulation in CPEB2KO cells. (A) qRT-PCR (Mean ±SEM) for p53 expression showing no difference (n=3). (B) Quantification (Mean ±SEM) (ImageJ Software) of Western Blots for p53 (C) in Wildtype and CPEB2KO cell lines (n=3). p53 protein expression was significantly reduced (0.41 fold change, p=0.000006) in CPEB2KO cells. (D) Quantification of p21 (downstream effector of p53) protein expression (Mean ±SEM) (C) in WT and CPEB2KO cell lines (n=3). p21 was significantly reduced (0.34 fold change, p=0.0004) in CPEB2KO cells. Student's T-test used for analysis. (**) indicates p<0.01.

3.9 Tumour-Forming Ability and Metastatic Capacity

Wildtype MCF10A cells, although immortalized, are epithelial in nature and non-tumourigenic *in vivo* (Hurst, Xie, Edmonds, & Welch, 2009). We confirmed this finding in our study (Figure 18, 20). However, upon CPEB-2 knockout, these cells revealed a possible oncogenic phenotype. They formed micrometastasis-like lesions in the lungs, but not in the liver or spleen, of all 6 mice, 8 weeks after intravenous injection as seen through H&E staining (Figure 18A, 20), whereas the wildtype cells formed no micrometastases in lungs as expected (Figure 18,20). Through immunostaining for human marker HLA, we confirmed these were of human origin, discovering more undetectable human cells than could be visualized through H&E staining (Figure 18B). Additionally, macroscopic tumours were formed after orthotopic injection of the CPEB2KO cells in 3 out of the 5 mice (3 out of 10 total injection sites) (Figure 20). Furthermore, after 12 weeks, these cells were also able to spontaneously metastasize to the lungs of 2 out of the 5 mice (Figure 20D). However, weights of these mice were not significantly different between wildtype- versus CPEB2KO-injected recipients (Figure 17,19). As the micrometastases and tumours formed in these mice were not necessarily large, they may not have affected the morbidity of these mice and therefore did not affect the weights. During these time periods, there were no observations of any morbidity issues (lethargy, piloerection) in these mice.

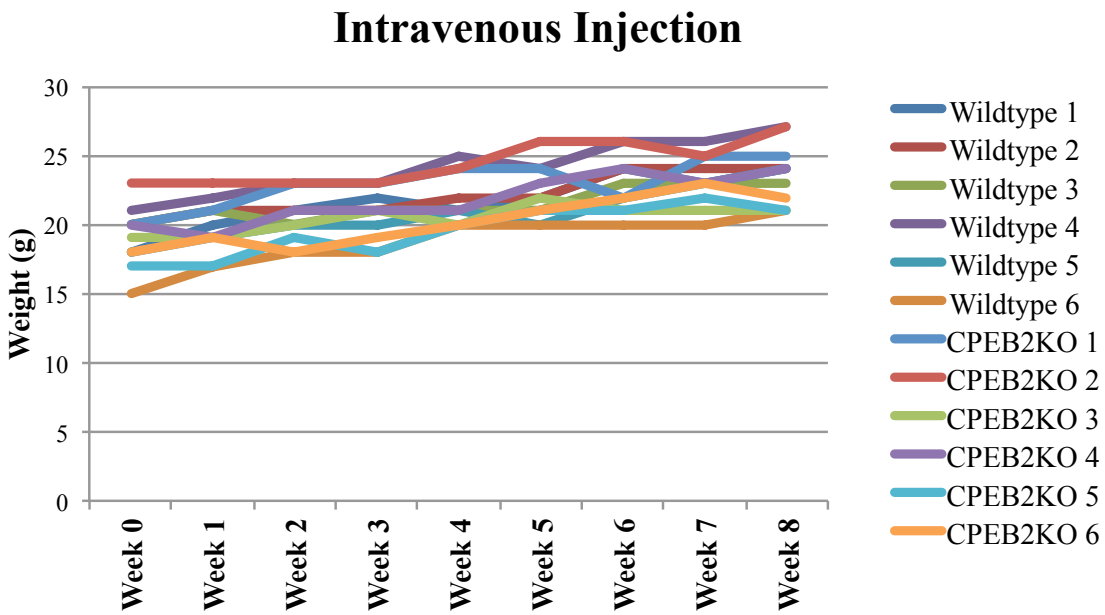

Figure 17. Weights of NOD/SCID/IL2Rγ null mice injected intravenously with WT and CPEB2KO cells. Changes in weight (g) per week in CPEB2KO mice did not differ from those in Wildtype mice over the 8-week period.

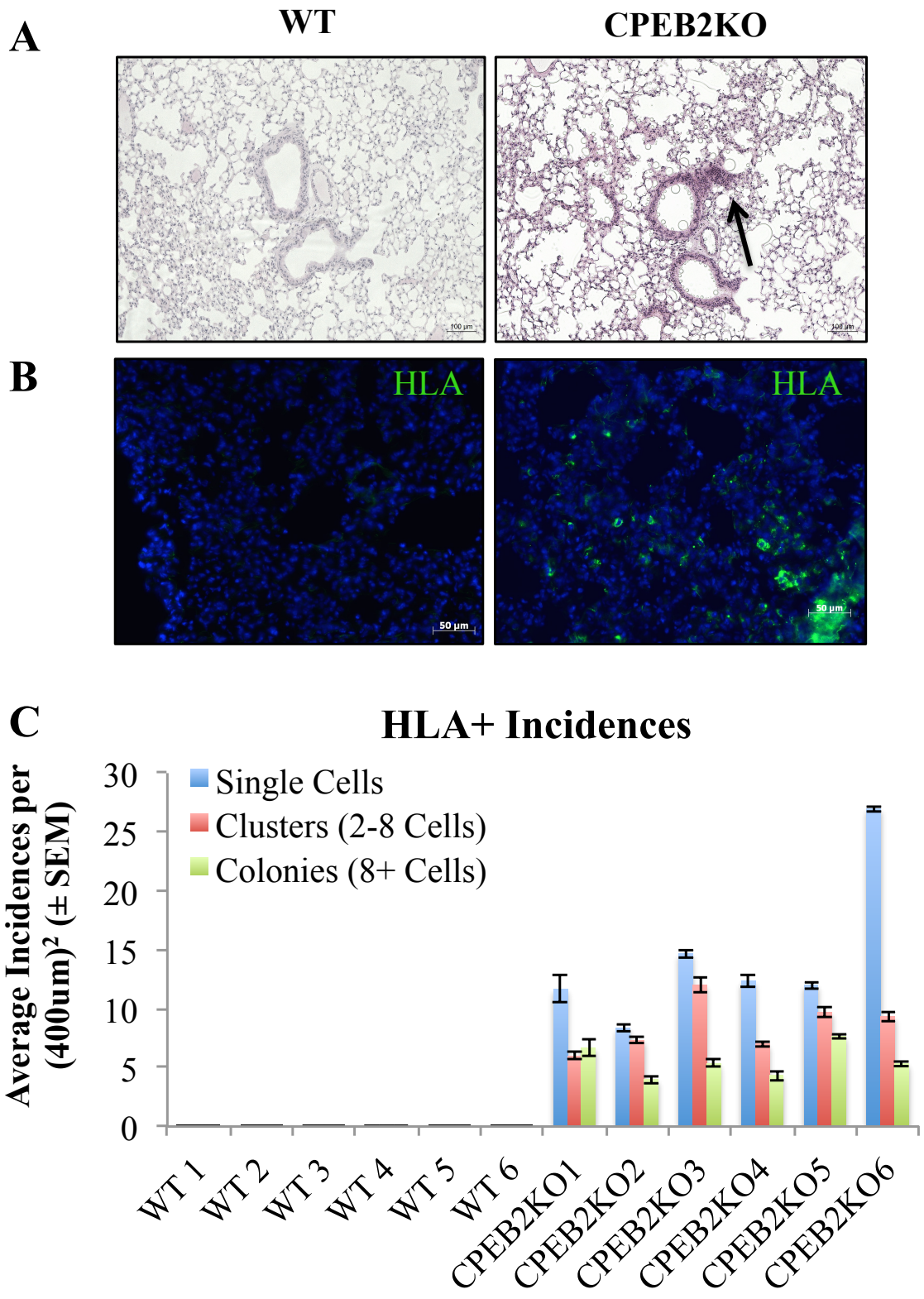
A
WT
CPEB2KO
B
HLA
HLA
50 µm
C
HLA+ Incidences
Average Incidences per (400um)² (± SEM)
Single Cells
Clusters (2-8 Cells)
Colonies (8+ Cells)
30
25
20
15
10
5
0
WT 1
WT 2
WT 3
WT 4
WT 5
WT 6
CPEB2KO1
CPEB2KO2
CPEB2KO3
CPEB2KO4
CPEB2KO5
CPEB2KO6

Figure 18. CPEB2KO cells injected intravenously into NOD/SCID/IL2Rγ null mice form micrometastases in the lung. (A) Representative images of mice lungs that received $5x10^5$ WT and CPEB2KO cells intravenously, and stained with H&E that showed possible micrometastases in the CPEB2KO recipient. Arrow pointing at micrometastatic lesion. (B) Lungs of WT and CPEB2KO cell recipient mice lungs stained with HLA (green) and DAPI (blue) showed human cells unidentifiable using H&E. (C) Quantification (5 images per section, 3 non-serial sections per mouse) (Mean ±SEM) of single cells, clusters and colonies within lung sections stained with HLA antibody. Scale = 50μm (IF images) and 100μm (H&E images).

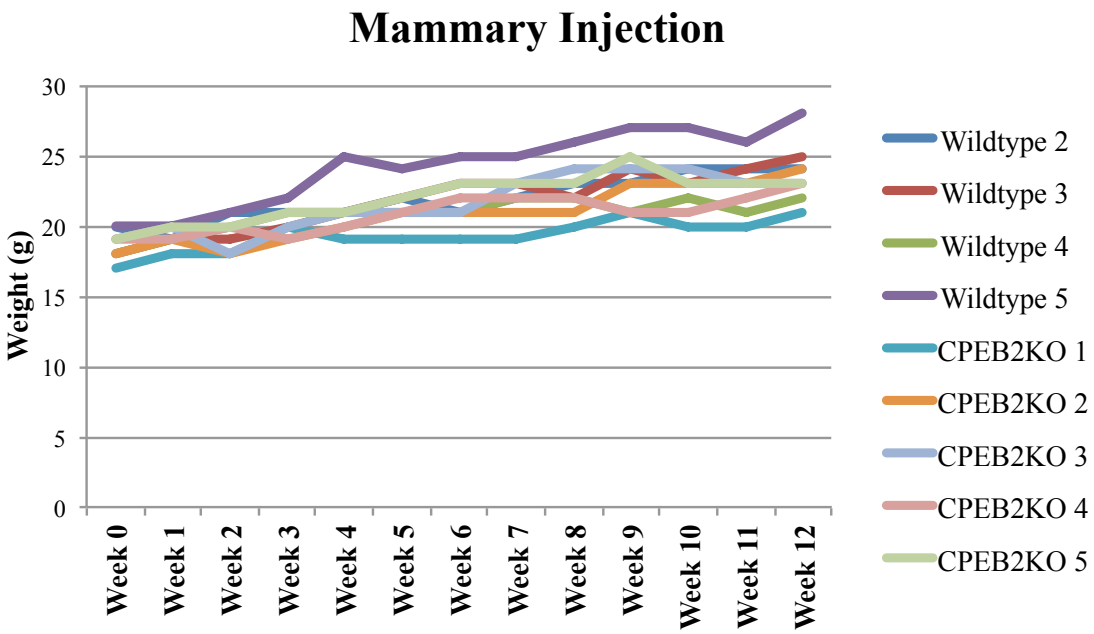

Figure 19. Weights of NOD/SCID/IL2Rγ-null mice injected orthotopically with WT and CPEB2KO cells. Changes in weight (g) per week in CPEB2KO mice did not differ from those in Wildtype mice over the 12-week period.

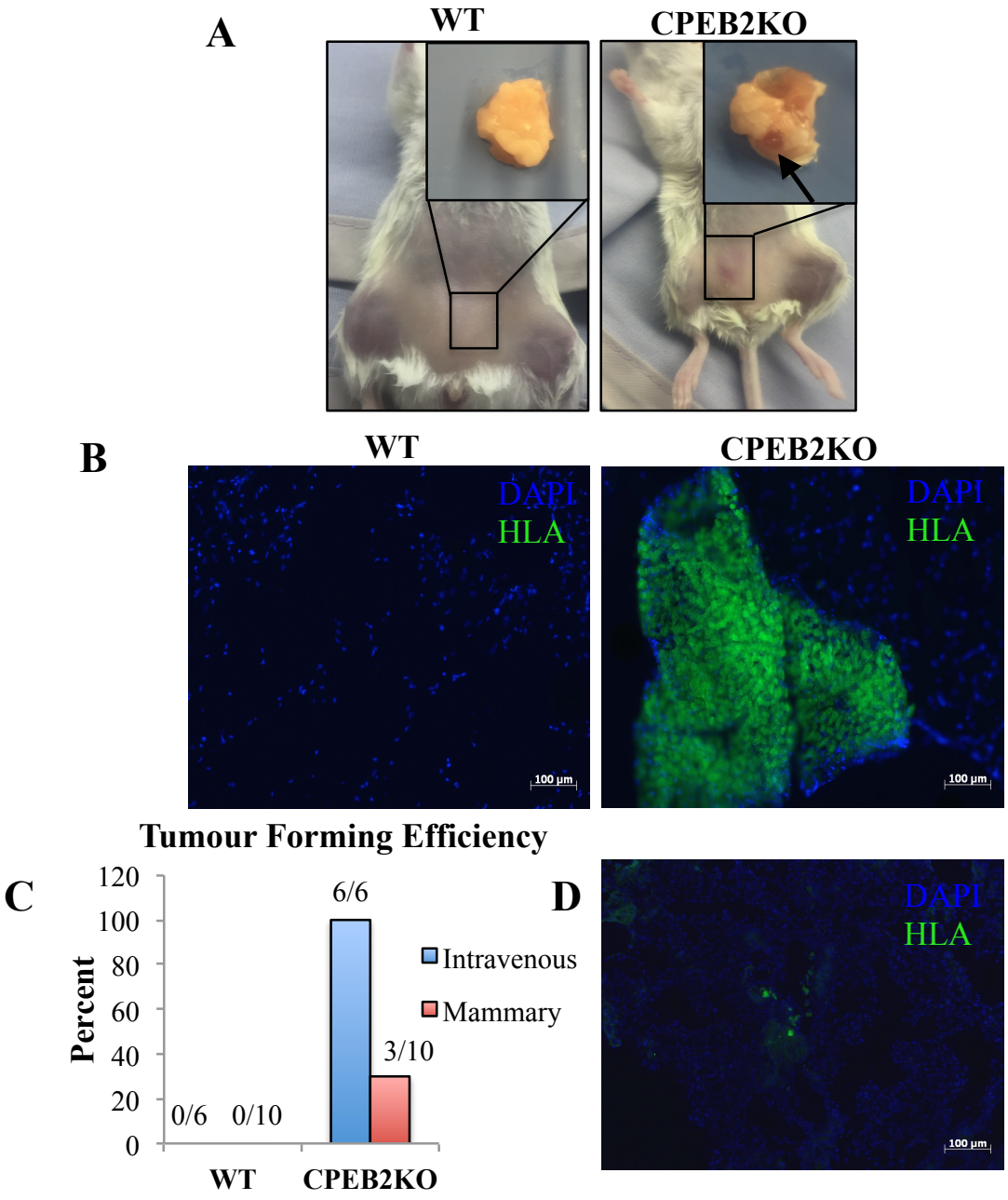
A
WT
CPEB2KO
B
WT
CPEB2KO
DAPI
HLA
100 µm
C
Tumour Forming Efficiency
Percent
120
100
80
60
40
20
0
0/6
0/10
6/6
3/10
WT
CPEB2KO
Intravenous
Mammary
D
DAPI
HLA
100 µm

Figure 20. CPEB2KO cells injected orthotopically into NOD/SCID/IL2Rγ-null mice form tumours and spontaneously metastasize to the lung. (A) Representative images of mice and mammary regions 12 weeks after $5x10^5$ cells were injected and organs were harvested. Arrow pointing to tumour. (B) Representative image of mammary region sectioned and stained with HLA antibody. (C) Tumour-forming efficiency calculated by number of mice identified with micro/macroscopic tumour lesions divided by total number of injection sites. (D) HLA positive cells found in the lungs of 2 mice. Scale=100μm.

3.10 Expression of CPEB-2 in Human Breast Cancer Tissues

In order to examine if CPEB-2 expression is altered in breast cancer, we examined expression of CPEB-2 isoforms in 105 tumour and 20 histologically identified non-tumour breast tissues. As seen in Figure 21, we have completed the analysis of isoform A/E in 105 breast cancer samples. Six of the samples failed to amplify our control gene (GAPDH) or CPEB2 isoform A/E and so they were excluded from analysis. The failure to amplify this isoform could have indicated much lower expression of isoform A/E, as most of these samples successfully amplified the control gene GAPDH. While comparing tumour samples to control, there was no significant difference in isoform A/E expression (Figure 21A). However, when we subcategorized the tumour samples into breast cancer subtype (ER/PR+, HER2+, Triple Negative), the delta Ct for the CPEB2A/E expression within the HER2+ subtype was significantly higher than the control tissues, indicating lower expression of this isoform (Figure 21B). We also examined isoforms CPEB2B/D (data not shown), however the CPEB2B/D probe showed no significant difference among these groups. There were only 15 HER2+ samples used in this analysis that amplified this isoform expression, but this significant difference warrants further investigation into this phenomenon.

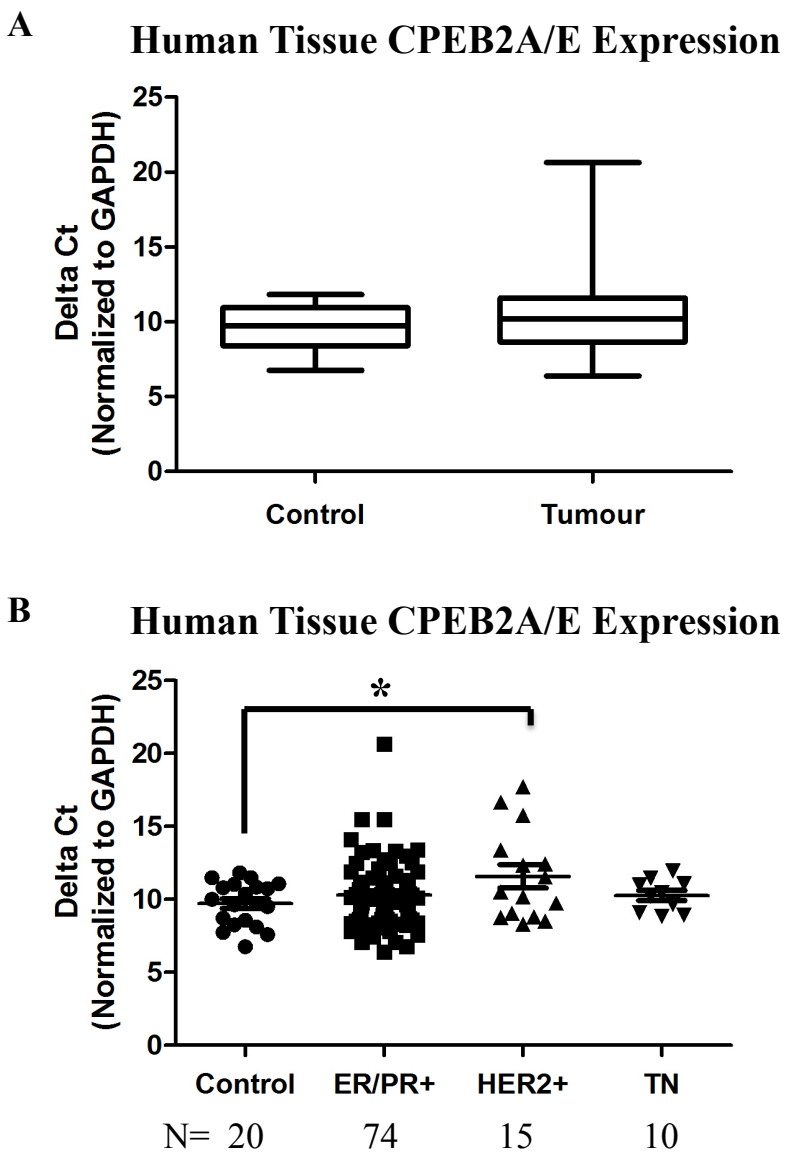

Figure 21. Human breast cancer tissue expression of CPEB-2 isoform A/E normalized to GAPDH control gene showed decreased expression in HER2+ subset. 105 breast cancer cDNA samples analyzed by qPCR, with 6 samples failing to amplify expression of GAPDH or CPEB2A (removed) and 20 control breast tissues used. (A) Total CPEB2A/E expression in tumour tissue compared to control. (B) CPEB2A/E expression (Mean ±SEM) broken down into breast cancer subtype. One-way ANOVA, Dunnett's post test for significance. (*) indicates $p<0.05$.

4 DISCUSSION AND CONCLUSIONS

4.1 Summary of Findings

Objective 1: To define the consequences of CPEB-2 knockout in the MCF10A cell line on oncogenic functions tested in vitro.

CPEB-2 was knocked out in a non-tumourigenic mammary epithelial MCF10A cell line using a double nickase CRISPR/Cas9 plasmid. These CPEB2KO cells demonstrated an increase in proliferation, migration, invasiveness, EMT markers and a mesenchymal morphology. Furthermore, these CPEB2KO cells increased most markers of SLC phenotype, with increased tumoursphere formation efficiency, and an increased in expression of SLC-linked protein β-catenin and downstream genes CCND1 and AXIN1. VEGF-D expression at the mRNA level was also increased in CPEB2KO cells. Upstream regulators of CPEB-2, COX-2 and EP4, were also increased at the mRNA level, demonstrating a possible feedback loop. Lastly, we demonstrate here that CPEB-2 is a possible novel translational regulator of tumour suppressor p53.

Objective 2: To define the consequences of CPEB-2 knockout in the MCF10A cell line on oncogenic functions tested in vivo.

Wildtype and CPEB-2 knockout cells were both injected into immunodeficient NOD/SCID/IL2Rγ-null mice intravenously into the tail vein and orthotopically into the mammary region. WT cells were not expected to form tumours *in vivo*, and this was confirmed with these experiments. However, upon knockout of CPEB-2, these cells were able to form micrometastases in the lungs of all 6 injected mice, while forming

macroscopic tumours in the mammary region of 3/5 mice (3/10 injection sites). In two of the tumour-bearing mice, cells spontaneously metastasized to the lungs.

Objective 3: To evaluate the clinical significance of CPEB -2 expression in human breast cancer samples.

CPEB-2 is alternatively spliced into 6 isoforms at the mRNA level, and the ratio of isoform B:A was reported to differ in expression in tumour vs control tissue (Johnson et al., 2015). We therefore wanted to examine tumour expression of CPEB2 isoforms A and B in our tumour bank of 105 samples. Examining expression of CPEB-2 isoforms A/E vs. B/D (isoforms could not be separated due to similarity among these isoforms) in human breast cancer samples compared to control tissues, we found no difference CPEB2A/E or CPEB2B/D expression in total tumour tissues compared to control non-tumour tissues. However, when breast cancer tissues were broken down into subtypes, CPEB-2A/E was significantly downregulated in HER2+ breast cancer samples, whereas no difference was found in ER+/PR+ or Triple Negative samples compared to control.

4.2 Contributions to Current Field of Research

COX-2 is overexpressed in 40-50% of breast cancer cases, demonstrating a necessity to examine mechanism of COX-2-mediated tumourigenicity. COX-2 inhibitors and low dose Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have been proven to reduce risk of breast cancer and growth of cancer cells (Singh-Ranger et al., 2008; Williams et al., 1999). However, due to cardiovascular side effects of using COX-2 inhibitors (associated with reduction in cardioprotective molecule prostacyclin) (Williams et al., 1999), our lab is examining downstream effectors of COX-2 in order to discover new therapeutic targets.

MicroRNAs -526b (Majumder et al., 2015) and -655 (data unpublished) are both upregulated by COX-2 overexpression, and have been shown by our lab to mediate oncogenic and SLC phenotypes when overexpressed and reduce these phenotypes when knocked down. COX-2 also induces the SLC phenotype through both Notch and Wnt pathways to perpetuate tumour growth (Majumder et al., 2016). In addition, overexpression or stimulation of COX-2, EP4 and overexpression of these microRNAs all reduced CPEB-2 expression, showing that CPEB-2 is regulated by the COX-2/EP4/microRNA axis. Therefore, our identification of CPEB-2 as a tumour-suppressor in breast cancer unveils a newer mechanism in COX-2/EP4-mediated tumour progression via CPEB2 as a downstream tumour suppressor gene.

4.3 Role of CPEB-2 in proliferation, migration, invasion, EMT, SLC phenotype, VEGF-D expression, COX-2/EP4 expression and p53 regulation

Here, we have demonstrated a robust role of CPEB-2 in suppressing oncogenic functions *in vitro*. CPEB-2 was demonstrated to suppress proliferation, with all three experiments showing increased DNA synthesis, thus higher proliferative capacity in CPEB2KO cells. Through the *in vitro* tumoursphere formation assay, the spheroids formed much larger sizes, showing that perhaps the SLC population proliferated much faster as well. Increased β-catenin noted in CPEB2KO cells may be responsible for a higher self-renewal capacity of stem-like cells and also increased proliferative ability of non-stem cells. Increased β-catenin signaling led to increased expression of *CCND1*, which is a nuclear protein that forms a complex with CDK4 and CDK6, and ultimately leads to progression through G1 into S-phase during proliferation (Baldin, Lukas, Marcote, Pagano, & Draetta, 1993).

Migration and invasiveness are two important phenotypes in the process of metastasis in breast cancer that can be assessed *in vitro*. COX-2 and EP4 inhibition was shown to reduce migration and invasiveness in murine and human breast cancer cells (Majumder et al., 2014; Timoshenko et al., 2003), while stimulation and overexpression of microRNA-526b was shown to increase migration and invasion (Majumder et al., 2015). Cells that have undergone EMT are usually also associated with an increased ability to migrate and invade (Kalluri & Weinberg, 2009). Here, by both scratch assay and Transwell migration assay, increased motility was seen in the CPEB2KO cells. While the signaling pathways responsible for migration stimulation on CPEB2KO cells remain

to be identified, we speculate this may be due to increased EP4 activity, since EP4 was shown to be upregulated in CPEB2KO cells. β-arrestin/c-Src signaling has been identified as one of the downstream mediators of EP4 activity responsible for migration stimulation in lung cancer cells (J. Il Kim, Lakshmikanthan, Frilot, & Daaka, 2010). In addition, CPEB2KO cells degraded the basement membrane analog Matrigel to invade more than the WT cells. The mechanisms underlying this function remain to be identified.

TWIST1 is a oncogenic transcription factor known to mediate EMT in breast cancer, as well as inhibit apoptosis through evasion of p53-induced cell death (J. Li & Zhou, 2011; Maestro et al., 1999). Due to CPEB-2 translational repression of TWIST1 expression, this could be a mechanism by which EMT is suppressed (Nairismägi et al., 2012). This confirms why we saw no effect on *TWIST1* mRNA expression, as it is regulated post transcriptionally by CPEB-2. Other transcription factors that were increased at the mRNA level (SNAI1, ZEB1) (Figure 4) after CPEB-2 knockout could have contributed to EMT regulation as well. Both SNAI1 and ZEB1 are known to suppress transcription of E-cadherin (*CDH1*) through binding the promoter, while playing important roles in invasion and metastasis as well (Batlle et al., 2000; Smit & Peeper, 2010). Not much is known about N-cadherin transcriptional regulation during cadherin switching, however Twist1 was reported to modulate N-cadherin expression by binding to the E-box on *CDH2* (N-cadherin) (Alexander et al., 2006; Wheelock et al., 2008). N-cadherin overexpression in breast cancer cells has been shown to promote motility, invasion, and metastasis, independent of E-cadherin expression (Nieman,

Prudoff, Johnson, & Wheelock, 1999). Therefore, an increase in N-cadherin expression could have contributed to these phenotypes in the CPEB2KO MCF10A cells.

CPEB-2 suppresses the translation of 2 oncogenic molecules, TWIST1 (Nairismägi et al., 2012) and HIF1α (P.-J. Chen et al., 2015; P.-J. Chen & Huang, 2012; Hägele et al., 2009). In a similar fashion, p53 is a very well known tumour-suppressing molecule, responsible for many functions including suppressing EMT, migration, and invasion through the transcriptional regulation of key molecules (Bieging, Mello, & Attardi, 2014). We have shown here that CPEB-2 is a possible novel regulator of p53. This key finding of p53 translational regulation by CPEB-2 demonstrates an even larger role in suppressing tumour formation. Furthermore, p53 has been shown to reduce self-renewal, a key function of stem-like cells (Bieging et al., 2014). This aligns with our findings that CPEB2KO cells show increased spheroid formation in the tumoursphere formation assay. We have also demonstrated a downstream effector of p53, called p21, to be significantly downregulated in CPEB2KO cells. There still remains the possibility of CPEB-2 regulating another molecule that regulates p53 translation, however because CPEB-1 has been found to regulate p53 translation through lengthening the poly-A tail, combined with 2 CPE sequences in the 3'UTR of p53, this remains unlikely (Burns & Richter, 2008).

Previous studies have indicated that an increased ratio of CPEB2 isoform B to A expression confers anoikis-resistance, a metastatic phenotype, to triple negative breast cancer cells. In the present study, we have used the MCF10A cell line, which primarily expresses isoform A, and our results of CPEB2 knockout indicate a tumour suppressive role. We also demonstrate a significant difference in just isoform A/E expression within

HER2+ breast cancer samples. Our lab showed that COX-2 and EP4 were both significantly upregulated in these 105 breast cancer samples (Majumder et al., 2016). It remains to be investigated whether this is due to COX-2 upregulation by HER-2, and whether CPEB-2 is downregulated in the COX-2 overexpressing subset. Further isoform-selective *in situ* studies are also required with larger number of samples.

COX-2 overexpressing MCF-7 cells have recently been shown to induce the formation of SLCs in this cell population, exhibiting increased expression of Notch and Wnt stem cell pathways (Majumder et al., 2016). Not only did these MCF-7-COX2 cells demonstrate increased tumoursphere formation efficiency, but Wnt pathway protein β-catenin was shown to be upregulated in these MCF-7-COX2 spheroids and downstream genes *CCND1*, *AXIN1* and *AXIN2* were all significantly increased, with no change in *c-Myc* (Majumder et al., 2016). These results are in concordance with the data we have collected from the CPEB-2 knockout cells.

Our lab demonstrated that overexpression of microRNAs-526b and -655 increased expression of COX-2 and EP4 (unpublished). COX-2 and EP4 were previously shown by our lab to increase expression of these microRNAs (Majumder et al., 2015), indicating a possible feedback mechanism here. It was therefore intriguing to examine if CPEB2KO cells demonstrated this expression pattern as well. Through qRT-PCR, mRNA expression showed significant increases in both COX-2 and EP4 due to loss of CPEB-2, thereby showing that CPEB-2 may also be part of this feedback loop. The underlying molecular mechanisms remain to be investigated.

Lymphangiogenesis is a process that tumour cells hijack to metastasize through lymphatic vessels to distant parts of the body. Growth factors, like VEGF-C and VEGF-D are known to stimulate lymphangiogenesis (Alitalo & Carmeliet, 2002). COX-2 promotes production and secretion of these factors through EP4 activation and stimulates lymphangiogenesis in breast cancer (Majumder et al., 2014; Nandi et al., 2017; A Timoshenko et al., 2006; Xin et al., 2012). Here, we show increased expression at the mRNA level of VEGF-D, but not VEGF-C in CPEB2KO cells over WT cells. Examination at the protein level (through Western blot) and secretion (through Enzyme-Linked Immunosorbent Assay (ELISA)) of this protein is necessary to confirm this phenotype.

4.4 Role of CPEB-2 in Tumour Suppression *in vivo* and in human breast cancer

The most rigorous test of a potential tumour-suppressing gene is to remove this gene from a cell line that is not capable of forming tumours *in vivo*, and examine whether this process converts it into an oncogenic cell line. After knocking out CPEB-2, we injected these cells and their WT predecessors into immunodeficient NOD/SCID/ IL2Rγ-null mice intravenously and orthotopically into the mammary region. As expected, the WT MCF10A cells did not form tumours *in vivo*. In contrast, CPEB2KO cells were able to form micrometastases in the lungs of all 6 mice after 8 weeks, and in the mammary region they were able to form macroscopic tumours in 3 of the 5 mice, while metastasizing spontaneously in 2 of those mice to the lungs after 12 weeks. This finding convincingly demonstrates a tumour suppressor role of CPEB-2.

MCF10A cells have been extensively used to examine oncogene and tumour suppressor function in breast cancer due to their epithelial heritage (Bessette et al., 2015; Nairismägi et al., 2012; Qu et al., 2015; Sung et al., 2009). The first model of an oncogenic transformation using MCF10A cells was with transfection with c-Ha-Ras, an oncogene (Basolo et al., 1991). After overexpression, these cells implanted into irradiated nude mice where they were able to form oncogenic lesions that progressed into breast carcinomas (Basolo et al., 1991; Dawson, Wolman, Tait, Heppner, & Miller, 1996). Cells were extracted from these tumours and an aggressive oncogenic cell line, MCF10AT1, was created (Dawson et al., 1996). Similarly, knocking out a single gene, CPEB-2, was able to promote tumour formation in a non-tumourigenic cell line.

CPEB-2 data from the Cancer Genome Atlas on breast cancer showed conflicting data, demonstrating poor expression, no change and overexpression in different types of breast cancer (D'Ambrogio et al., 2013). Along with the report of CPEB-2 isoform ratio B:A conferring anoikis resistance and a metastatic phenotype (Johnson et al., 2015), we hypothesize that this could be due to isoform-specific expression. Due to similarity of the isoforms, separation was only possible to examine both isoform A and E together, and B and D together. Whole breast tumour data showed no significant difference from control tissues in either isoform analysis, as expected from previous reports (D'Ambrogio et al., 2013). However, once separated into subtypes, HER2+ tumour samples showed a significant decrease in expression from control tissues. These results were very intriguing because COX-2 expression is known to be increased in HER2+ tumour samples as well (Ristimäki et al., 2002). Because there were only 15 HER2+ tumour samples in our tumour bank, even though the data was statistically significant, these results should be further examined in a larger population.

4.5 Limitations

Here, we examined 2 isoforms at a time (A/E, B/D) of CPEB-2 in human breast cancer samples. Ideally, if single isoform expression of CPEB-2 could have been analyzed in all human breast cancer samples, we may discern which isoform is significant in this phenotype. Due to the similarity of isoforms, this would not be possible, and therefore examination of two isoforms at a time was the closest we could get isoform-specific expression.

Experiments in this project have been limited to one human mammary epithelial cell line, MCF10A, and so it remains a possibility that some of these results are cell line dependent. Further studies could examine these phenotypes in the human luminal epithelial cell line MCF-12A, or through specific tissue ablation *in vivo* in mice mammary glands. However, some EMT and SLC experiments previously performed by Asma Hasan (MSc) have demonstrated a role of CPEB-2 in suppressing these phenotypes in a non-metastatic human breast cancer cell line MCF-7. Furthermore, other investigators have shown a role of CPEB-2 in suppressing oncogenic transcription factor TWIST1 (Nairismägi et al., 2012), angiogenic molecule HIF1α (P.-J. Chen et al., 2015; P.-J. Chen & Huang, 2012; Hägele et al., 2009), and SLC-linked protein β-catenin (Turimella et al., 2015). In addition, CPEB-2 was reported by Johnson et al., 2015 to mediate a metastatic phenotype, anoikis resistance, through isoform-selective expression.

Unfortunately, commercially available antibodies for CPEB-2 are extremely limited. No monoclonal antibodies exist for CPEB-2, and the polyclonal ones that do exist rarely work as expected/instructed by the manufacturing company. In my project,

we tried multiple antibodies, taking months until one worked. No CPEB-2 antibody has been made for immunoprecipitation experiments, and thus specificity would be very difficult to examine mRNA-binding targets of CPEB-2. Since CPEB-2 functions as an mRNA binding protein, it would be critical to identify specific targets relevant to our findings as a tumour suppressor. Once the antibodies become more specific and are verified for immunoprecipitation experiments, we could use them to confirm p53 translational regulation, as described below.

Our population of cells used in these experiments demonstrated a 79.2% knockout efficiency of CPEB-2. Because there is a mixed population of cells and thus still detectable expression of CPEB-2, our results could have been altered slightly. However, we confirmed that the knockout percentage didn't change at multiple points throughout these experiments through Western blot.

Furthermore, as previously stated, in our human breast cancer tissue examination of CPEB-2 isoforms, the number of HER2 positive human breast cancer tumours is limited to 15 samples. COX-2 is known to be co-expressed with HER2+ tumours (Bhattacharjee et al., 2010; Ristimäki et al., 2002), and because COX-2 suppresses CPEB-2 expression, these results can be explained as a consequence of COX-2 expression. Although promising, conclusions on this data should be reserved for a larger population sample including COX-2 expression.

4.6 Future Directions

In these experiments, we discovered that CPEB-2 is a novel translational regulator of tumour suppressor p53. To confirm this finding, upon better antibodies being made, CPEB-2 can be analyzed through RNA Immunoprecipitation (RIP) with p53, or by Cross Linking Immunoprecipitation (CLIP) to examine binding of CPEB-2 to p53 mRNA (Zhang, Xie, Xu, & Qu, 2015). Another way to examine this without a better antibody is through a method called polysomal profiling (Chassé, Boulben, Costache, Cormier, & Morales, 2016). This method consists of isolating polysomes from cell populations, fractionating them using a sucrose gradient, and examining mRNA through qRT-PCR of each fraction. The lower, thus heavier, fractions when examined indicate increased translation of the target mRNA, whereas higher and lighter fractions indicate less translated mRNA. In the WT and CPEB2KO populations, reduced translation in the CPEB2KO cell line (higher fraction) would be expected with our results.

Our results demonstrated a robust role of CPEB-2 in suppressing EMT and migration. The CPEB2KO cells were also able to break down the basement membrane analog Matrigel, in order to invade through into the bottom chamber of the Transwell more than three times faster than the WT cells. Although these results were promising, we could examine the underlying mechanisms through zymographic analysis of Matrix Metalloproteinases (MMPs). Zymography is an electrophoresis technique used to assess activity of ECM degradation enzymes, such as MMPs (Kupai et al., 2010). Through renaturation of the protein structures, the enzymes become active again after being run through a gelatin-based gel, and quantification of gel breakdown after staining can be examined.

We discovered that VEGF-D expression was significantly increased in the CPEB2KO cells. This was only shown at the mRNA level, and so future studies should focus on examination at the protein level through both western blot (production) and ELISA (secreted) to confirm this phenotype. Furthermore, once analyzed through ELISA, cell media can be taken from both cell populations and used to examine lymphatic vessel growth using lymphatic endothelial cells as previously done in our lab (Nandi et al., 2017).

COX-2 expression is increased in almost half of all breast cancer cases, and has a robust role in perpetuating tumour growth. One of these mechanisms was through the increase of microRNAs -526b and -655, which when overexpressed, increased expression of COX-2 and EP4. Because CPEB2KO cells showed significantly increased expression of both COX-2 and EP4 as well, future experiments should be focused on confirming this expression change at the protein level, as well as uncovering the mechanism by which this possible feedback loop may function.

Present experiments focused on knocking out CPEB-2 in a non-tumourigenic cell line and examining changes in oncogenic phenotypes *in vitro* and *in vivo*. Results showed a role of CPEB-2 in suppressing tumour formation. To follow these studies, CPEB-2 should be overexpressed in a highly aggressive breast cancer cell line, such as MDA-MB-231 through an inducible system. Induction of CPEB-2 overexpression can be controlled while examining *in vitro* oncogenic phenotypes (proliferation, migration, invasion, EMT, SLC, VEGF expression). In concordance with these results, decreases in *in vitro* oncogenic functions would be expected when CPEB-2 is overexpressed. Furthermore CPEB-2 overexpressing MDA-MB-231 breast cancer cells should be examined for

oncogenicity in immunodeficient mice. If CPEB-2 acted in a tumour-suppressing manner, we would expect a decrease in ability to form lung colonies, as well as decreased orthotopic tumourigenicity.

Lastly, our human breast cancer expression data indicated a significant decrease in CPEB2 isoform A/E expression in HER2+ tumours. Due to this population being limited to 15 human samples, future studies should examine this phenomenon in a larger group of samples, including COX-2 expression. Furthermore, through data mining in The Cancer Genome Atlas, for example, as previously done by our lab, we should examine isoform-selective expression in breast cancer subtypes including HER2.

4.6 Conclusions

In conclusion, CPEB2 knockout MCF10A cells exhibited oncogenic phenotypes *in vitro*: increased proliferation, migration, invasion, EMT phenotype (mesenchymal morphology, decreased E-Cadherin, increased Vimentin, N-cadherin, SNAI1, and ZEB1), increased COX-2/EP4 expression, increased lymphangiogenic factor VEGF-D expression and SLC properties (increased spheroid formation, expression SLC-linked molecule β-catenin and downstream gene expression). Knocking out CPEB-2 transformed the epithelial MCF10A cells into an oncogenic cell line capable of forming lung colonies after intravenous injection *in vivo*, as well as *in vivo* orthotopic tumours in the mammary region. This phenotype could be caused by CPEB-2 regulating the translation of the novel target p53, a powerful tumour-suppressing molecule.

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6 APPENDICES

Table 1: Primer sequences and Taqman Probes used in qRT-PCR analysis of mRNA expression.

Gene	Taqman Probe Cat#	Forward Primer (5'-3')	Reverse Primer (5'-3')
CPEB2		ACACTCTTACCCTTACAGGT	CGCCCATAACTCCTTGCATT
CPEB2A/E	Hs01039669_m1		
CPEB2B/D	Hs00699300_m1		

E-CADHERIN (*CDH1*)	Hs01023895_m1		
VIMENTIN	Hs00185584_m1		
SNAI1	Hs00195591_m1		
ZEB1	Hs00232783_m1		
TWIST1	Hs01675818_s1		
GAPDH	Hs99999905_m1	GAGAGAAACCCGGGAGGCTA	CAAATGAGCCCCAGCCTTCT
CCND1	Hs00765553_m1		
AXIN1	Hs00959587_m1		
AXIN2	Hs00610344_m1		
Myc	Hs00153408_m1		
VEGFB		CACCAAGTCCGGATGCAGAT	GGAGTGGGATGGGTGATGTC
VEGFC		GCCACGGGAGGTGTGTATAG	TATTGCAGCAACCCCCACAT
VEGFD		ATCGGTCCACTAGGTTTGCG	GCTGCACTGAGTTCTTTGCC
***PTGS2* (COX-2)**	Hs01573472_g1		
***PTGER-4* (EP4)**	Hs00168761_m1		

*For probes with catalog numbers, the sequences are proprietary and therefore could not be disclosed to us. Other sequences were designed by us and used with the SYBR Green PCR method.

Table 2: NCBI Primer-BLAST search for primers used in Johnson et al., 2015.

Sequences	Result

CPEB2 Isoform A For: 5'- GTGT TCAGAACAG ACAACAATA G-3' Rev: 5'-AATA TCGATAAGG GAATTTTCC-3'	>NM_001177384.1 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant F, mRNA product length = 117 Forward primer 1 GTGTTCAGAACAGACAACAATAG 23 Template 1900 ..... 1922 Reverse primer 1 AATATCGATAAGGGAATTTTCC 22 Template 2016 ..... 1995 **CPEB2 Isoform F** >NM_001177383.1 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant E, mRNA product length = 108 Forward primer 1 GTGTTCAGAACAGACAACAATAG 23 Template 1900 ..... 1922 Reverse primer 1 AATATCGATAAGGGAATTTTCC 22 Template 2007 ..... 1986 **CPEB2 Isoform E** >NM_001177382.1 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant D, mRNA product length = 198 Forward primer 1 GTGTTCAGAACAGACAACAATAG 23 Template 1900 ..... 1922 Reverse primer 1 AATATCGATAAGGGAATTTTCC 22 Template 2097 ..... 2076 **CPEB2 Isoform D** >NM_001177381.1 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant C, mRNA product length = 117 Forward primer 1 GTGTTCAGAACAGACAACAATAG 23 Template 1900 ..... 1922 Reverse primer 1 AATATCGATAAGGGAATTTTCC 22 Template 2016 ..... 1995 **CPEB2 Isoform C** >NM_182646.2 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant A, mRNA product length = 108 Forward primer 1 GTGTTCAGAACAGACAACAATAG 23 Template 1900 ..... 1922 Reverse primer 1 AATATCGATAAGGGAATTTTCC 22 Template 2007 ..... 1986 **CPEB2 Isoform A** >NM_182485.2 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant B, mRNA product length = 198 Forward primer 1 GTGTTCAGAACAGACAACAATAG 23 Template 1900 ..... 1922 Reverse primer 1 AATATCGATAAGGGAATTTTCC 22 Template 2097 ..... 2076 **CPEB2 Isoform B**
CPEB2 Isoform B For: 5'- CCTG GTCTATTCTG GATGTTCC-3' Rev: 5'- ACCC TTACAGGTG AGATCTAGT-3'	>NM_001177382.1 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant D, mRNA product length = 101 Forward primer 1 CCTGGTCTATTCTGGATGTTCC 22 Template 2035 ..... 2014 Reverse primer 1 ACCCTTACAGGTGAGATCTAGT 22 Template 1935 ..... 1956 **CPEB2 Isoform D** >NM_182485.2 Homo sapiens cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant B, mRNA product length = 101 Forward primer 1 CCTGGTCTATTCTGGATGTTCC 22 Template 2035 ..... 2014 Reverse primer 1 ACCCTTACAGGTGAGATCTAGT 22 Template 1935 ..... 1956 **CPEB2 Isoform B**

NCBI Primer-BLAST provided by (Ye et al., 2012).

Curriculum Vitae

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Publications

Dr. Helen Senderovich, Mary Lou Ip, Lynda Dunal, Helen Kuttner, Anna Berall, Jurgis Karuza, Michael Gordon, **Joshua Tordjman**, and Daphna Grossman.“Therapeutic Touch in a Geriatric Palliative Care Unit: A Retrospective Review”. Canadian Virtual Hospice. Nov 4, 2014

Dr. Helen Senderovich, Mary Lou Ip, Lynda Dunal, Helen Kuttner, Anna Berall, Jurgis Karuza, Michael Gordon, **Joshua Tordjman**, and Daphna Grossman. “Therapeutic Touch in a Geriatric Palliative Care Unit: A Retrospective Review.” F1000 Research. Sept 14, 2015

Joshua Tordjman, Asma Hasan, Mehdi Amiri, Mousumi Majumder, and Peeyush Lala. “The role of cytoplasmic polyadenylation element binding protein 2 (CPEB2) in breast cancer”. Anatomy and Cell Biology Research Day. London, ON, Canada. Oct 23, 2015

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