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

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology

THE ROLE OF CYTOPLASMIC POLYADENYLATION ELEMENT BINDING PROTEIN-2 (CPEB2) IN BREAST CANCER

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by

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Abstract

Over-expression of inflammation associated enzyme cyclo-oxygenase (COX)-2 promotes breast cancer progression, metastasis and sustains cancer stem-like cells (SLCs) by activating prostaglandin E_2 receptor EP4. Two COX-2 induced oncogenic miRNAs, miR-655 and miR-526b, target and down-regulate the cytoplasmic polyadenylation element binding protein ***(CPEB)-2***. **Hypothesis**: Down-regulation of *CPEB2* promotes an aggressive breast cancer phenotype through SLC induction and epithelial to mesenchymal transition (EMT). We found that high COX-2/miRNA expressing cell lines MDAMB231 and MCF7-COX-2 had significantly lower expression of *CPEB2* than MCF7 cells (low COX-2/miRNA). CPEB2 knockdown (KD) in CPEB2-high MCF7 cell line resulted in increased migratory and invasive capacity *in vitro*. CPEB2 KD increased spheroid forming ability (SLC surrogate), expression of SLC markers (Nanog, ALDH1, SOX-2), and mesenchymal marker (Twist1), and decreased epithelial marker (E-Cadherin). Furthermore, treatment with COX-2 inhibitor and EP4 antagonist increased CPEB2 expression. Collectively, CPEB2 demonstrates anti-oncogenic functions and CPEB2 inhibition promotes an aggressive breast cancer phenotype.

Keywords

Cyclo-oxygenase (COX)-2, cytoplasmic polyadenylation element binding protein (CPEB)-2, breast cancer, stem like cells (SLCs), epithelial to mesenchymal transition (EMT), Prostaglandin E receptor 4 (EP4)

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Table of Contents

List of Figures

List of Appendices

Abbreviations

ALDH	Aldehyde dehydrogenase
ATCC	American Type Culture Collection
BrdU	bromo-deoxyuridine
BSA	bovine serum albumin
cAMP	cyclic 3,5-adenosine monophosphate
CDH1	E-Cadherin
CDH2	N-Cadherin
CMV	cytomegalovirus
COX-2	cyclo-oxygenase 2
CPE	cytoplasmic polyadenylation elements
CPEB-2	cytoplasmic polyadenylation element binding protein-2
CPSF	cleavage and polyadenylation specificity factor
DCIS	ductal carcinoma in situ
eEF	eukaryotic elongation factor
EGF	epidermal growth factor

EIF	eukaryotic initiation factor
EMEM	eagle’s minimum essential media
EMT	epithelial to mesenchymal transition
ER	estrogen receptor
Erβ1	estrogen receptor β1
ESR1	Estrogen receptor 1
FBS	fetal bovine serum
FGFb	fibroblast growth factor basic
GFP	green fluorescence protein
GLd2	germ-line development factor
HER2	human epidermal receptor 2
HIF-1α	hypoxia inducible factor-1α
IL-1β	interleukin-1β
LCIS	lobular carcinoma in situ
MET	mesenchymal to epithelial transition
miRNA	microRNA

MMP	matrix metalloproteinase
NSAIDs	non-steroidal anti-inflammatory drugs
PABP	poly(A)-binding protein
PARN	poly(A) ribonuclease
Pen/strep	Penicillin/streptomycin
PG	prostaglandin
PGI_2	prostacyclin
PI3K	phosphatidylinositol 3-kinase
PR	progesterone receptor
qRT-PCR	quantitative real-time polymerase chain reaction
RISC	RNA-induced silencing complex
RRM	RNA recognition motifs
siRNA	small-interfering RNA
SLCs	stem like cells
UniProt	universal Protein Resource
UTR	untranslated region

VIM	Vimentin
αCaMKII	α-Ca^{2+}/calmodulin-dependent protein kinase II

1 INTRODUCTION

1.1 Overview of Cancer

Cancer is a disease that starts in the cells of various tissues in the body. Cancer cells acquire mutations that progressively transform them into a malignant state. Reaching this state is a multi-step process. Mutations leading to activation of growth promoting genes (oncogenes) or deactivation of growth suppressor (tumour suppressor) genes typically lead to cancer. Cancer cells develop mechanisms to avoid senescence, cell death signals and expression of tumour suppressors (Hanahan & Weinberg, 2011).. They often break out of normal tissue confines by increased migration and invasiveness. Cancer cells can induce the formation of blood vessels (angiogenesis, to derive their own nourishment) and sometimes new lymphatic vessels (lymphangiogenesis, which promotes lymphatic metastasis). These processes allow them to metastasize to other parts of the body (Hanahan & Weinberg, 2011). According to current statistics, one in nine Canadian women are expected to develop breast cancer over their lifetime and one in twenty-nine Canadian women will die from the disease (Canadian Cancer Society 2013). Breast cancer accounts for the second highest cancer-related mortality in females (Canadian Cancer Statistics).

Normal breast architecture consists of multiple milk-making glands that form a cluster of lobules, which are connected to the nipple through interlobular ducts. The lobules and ducts are surrounded by a layer of luminal and myoepithelial cells, and are separated from surrounding tissue by a basement membrane. Breast cancer can be broken down into categories based on histology. The two common types of non-metastatic breast cancer are ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS), which refer to cancers located in the milk ducts or milk-making glands. The tumour becomes

invasive when the cells break down the basement membrane and from there spread to surrounding breast tissue, and ultimately metastasize to other parts of the body.

Breast cancer can also be classified based on the presence or absence of specific immunopathological markers. Treatment options and prognosis are often based on these markers. The markers are comprised of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal receptor 2 (HER2) (Bertos & Park, 2011). The usual course of treatment for ER+ tumours is anti-estrogen endocrine therapy, while HER2+ tumours are linked with poorer prognosis and are usually treated with the HER2 inhibitor Trastuzumab (Herceptin) (Vuong, Simpson, Green, Cummings, & Lakhani, 2014).

Breast cancer can be further classified according to mRNA transcription categories: luminal A, luminal B, basal, HER2 and claudin low. Luminal A subtypes have higher levels of Estrogen receptor 1 (*ESR1*) and ER regulated genes, lower ki-67 expression, a marker for cell proliferation, and better survival rates compared to the luminal B subtype. Claudin-low tumours are linked to poor outcomes, while the basal subtype is associated with ER-/PR-/HER2- or the triple negative cohort, the most severe form of breast cancer (Bertos & Park, 2011; Vuong et al., 2014). Despite these classifications, breast cancer tumours contain a heterogeneous population of cells, which makes them harder to treat. This heterogeneity might arise from a combination of mutations within cells and differentiation of mutated stem like cells (SLCs) during cancer (Reya, Morrison, Clarke, & Weissman, 2001). Traditional therapies are often unable to completely eradicate all cancer cells, leaving behind the SLC tumour cell subset, which leads to tumour reoccurrence. As such, there is a pressing need to identify novel drug targets and biomarkers for therapeutic monitoring.

1.2 Stem Like Cells

SLCs are a rare population of cells within the tumour that possess certain stem cell like properties that drive the initiation and growth of tumours, and promote reoccurrence of cancer (Campbell & Polyak, 2014; Reya et al., 2001). Normal stem cells persist in the body through self-renewal properties, and are able to generate mature cells through differentiation (Reya et al., 2001). The cancer stem cell model suggests that SLCs could be derived from transformed stem cells, progenitor cells or differentiated cells (Reya et al., 2001). The genetic makeup of SLCs is continuously evolving, giving them treatment-resistant properties that are passed down to daughter generations (Valent et al., 2012). Quiescent SLCs can evade traditional therapies such as radiation and chemotherapy, which target rapidly dividing cells that make up the bulk of the tumour population. This causes a reduction in tumour size but is not a complete cure due to SLCs that exist in a quiescent or dormant state (Kai, Arima, Kamiya, & Saya, 2010; Tysnes, 2010; Wicha, Liu, & Dontu, 2006). Recent evidence suggests that SLCs adopt a functional state dependent on extrinsic micro-environmental factors in the "SLC-niche" (Visvader & Lindeman, 2012). For instance, in colorectal cancer, myofibroblasts secrete factors which are able to confer the stem cell phenotype in more differentiated tumour cells (Vermeulen et al., 2010).

With advancements in technology, we are now able to address SLC properties *in vitro*. One such assay cultures undifferentiated cells in spherical colonies referred to as tumourspheres. These are grown under low-attachment (anchorage independent) conditions in serum free medium supplemented with growth factors (Dontu et al., 2003).

SLCs are also characterized by the presence or absence of cell surface markers and enzyme activities. Tumourspheres in breast cancer were found to be enriched primarily for undifferentiated cells and cells expressing surface markers CD44+/CD24- (Dontu et al., 2003; Ponti et al., 2005). Cancer cells sorted for CD44+/CD24- phenotype displayed increased tumourigenicity after injection into immune-compromised mice (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). Aldehyde dehydrogenase (ALDH) activity has also been associated with SLCs. ALDH is a cytoplasmic detoxification enzyme and is highly expressed in multiple progenitor cell lineages (Vasiliou & Nebert, 2005). CD44+/CD24-/ALDH^{high} and CD44+/CD133+/ALDH^{high} cells demonstrated increased tumoursphere forming ability and lung metastasis following tail vein injection in immune-compromised mice (Croker et al., 2009). Interestingly, another study also found that high ALDH1 expression in DCIC breast biopsies is associated with increased risk of breast cancer development (Kunju et al., 2011). In addition, expression of pluripotency genes such as SOX-2, Nanog, OCT4 has also been associated with SLCs in various cancers including the breast (Chiou et al., 2010; Ezeh, Turek, Reijo, & Clark, 2005; Leis et al., 2012). Thus, SLCs may be prospectively identified in breast cancer by the CD44+/CD24-/CD133+/ALDH^{high} phenotype (Al-Hajj et al., 2003; Croker et al., 2009). Targeting these SLCs in breast cancer may reduce reoccurrence of cancer and improve patient survival.

1.3 Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is an important component of cancer metastasis. Cancer metastasis is a complex process which involves the generation of

motile tumour cells, breakdown and migration through the basement membrane and migration into blood or lymphatic vessels, extravasation to a secondary site and establishment of secondary tumours (Tsuji, Ibaragi, & Hu, 2009). At the sub-cellular level, loss of tight junctions, adherens junctions and desmosomes, is observed as well as cytoskeletal changes and differential expression of adhesion molecules and transcription factors. During EMT, decreased expression of *CDH1* (E-Cadherin) is associated with loss of cell-cell contacts and epithelial cell polarity (Adams & Nelson, 1998; Vincent-Salomon & Thiery, 2003). There is also a concurrent increase in expression of mesenchymal markers *Twist1*, *Snail1* (Snail) and *Zeb1* which are translational repressors of E-Cadherin (Peinado, Olmeda, & Cano, 2007). Changes in cells from highly organized epithelial like cells to a more motile mesenchymal cell are characteristics of cancer cell plasticity (Thiery, 2002). Identifying cells prone to EMT will help us target cells that are more likely to metastasize.

1.4 Cyclo-oxygenase Enzymes and Prostanoids

Over the past decade, our laboratory has pioneered the investigation into the role of cyclo-oxygenase (COX) system in cancer promotion (Dunn, Majumder, & Lala, 2014; Landman, Majumder, Liu, & Lala, 2014; Majumder, Xin, Liu, Girish, & Lala, 2014; Rozic, Chakraborty, & Lala, 2001; Timoshenko, Lala, & Chakraborty, 2004; Xin et al., 2012). Elevated COX-2 expression was observed in approximately 85% of human colorectal cancers and in 37.4% of breast cancer tumours compared to normal tissue (Eberhart et al., 1994; Ristimaki et al., 2002). Furthermore, high expression of COX-2 has been demonstrated in hepatocellular carcinoma and lung cancer (Soslow et al., 2000;

Tang, 2005). Aberrant cyclo-oxygenase (COX)-2 up-regulation during chronic inflammation is believed to play a major role in carcinogenesis (FitzGerald, 2003).

COX enzymes catalyze the rate limiting step in the formation of prostanoids. There are three isoforms of the enzyme, COX-1 and COX-2, and the lesser studied COX-3, which is a variant of *COX-1* (Chandrasekharan et al., 2002). COX-1 is constitutively expressed in most tissues including the lungs, small and large intestine, liver, and kidney. It generates steady and low levels of prostanoids needed for physiological functions such as vaso-relaxation, platelet aggregation and protection of gastro-intestinal lining epithelia. In contrast, COX-2 expression is induced locally in multiple tissues by inflammatory cytokines and mitogens, producing high prostanoid levels required to combat pathogens through mobilization of leukocytes into interstitial space (FitzGerald, 2003).

Production of prostanoids, in particular prostaglandin (PG)E_2, depends mainly on COX-1 and COX-2 expression. When stimulated, arachidonate is cleaved from the lipid bilayer by phospholipase-A_2 (PLA_2). Liberated arachidonic acid is then converted to prostaglandin (PG)H_2 in a two step reaction via the COX enzymes. Through different cell-specific synthases, PGH_2 is converted to signaling molecules, which include Prostacyclin (PGI_2), Thromboxane A_2, Prostaglandin D_2, Prostaglandin $F_{2\alpha}$ and Prostaglandin E_2. These prostanoids subsequently mediate pleiotropic effects through multiple receptors. Notably, PGE_2 was determined to be the most abundant prostaglandin released from breast cancer cells (Timoshenko, Xu, Chakrabarti, Lala, & Chakraborty, 2003). PGE_2 mediates its effects through four different G-protein-coupled receptors, EP1-4.

EP1 activation is coupled with G_q and leads to increased intracellular calcium mobilization. In contrast, PGE_2 signaling is mediated primarily by EP2 and EP4 receptors coupled with G_s, resulting in increased cyclic 3,5-adenosine monophosphate (cAMP), whereas EP3 stimulation is coupled with G protein G_i and inhibits adenylate cyclase activity resulting in decreased (cAMP) levels (Bos, Richel, Ritsema, Peppelenbosch, & Versteeg, 2004). More recently, EP4 was also demonstrated to mediate its effects through the phosphatidylinositol 3-kinase (PI3K) pathway which phosphorylates extracellular signal-regulated kinases (ERKs) (Fujino & Regan, 2006; Fujino, Xu, & Regan, 2003). This pathway is unique to EP4 and not utilized by EP2. Prostanoids mediate a complex array of signaling pathways essential to homeostatic function, on the other hand uncontrolled signaling has been implicated in cancer.

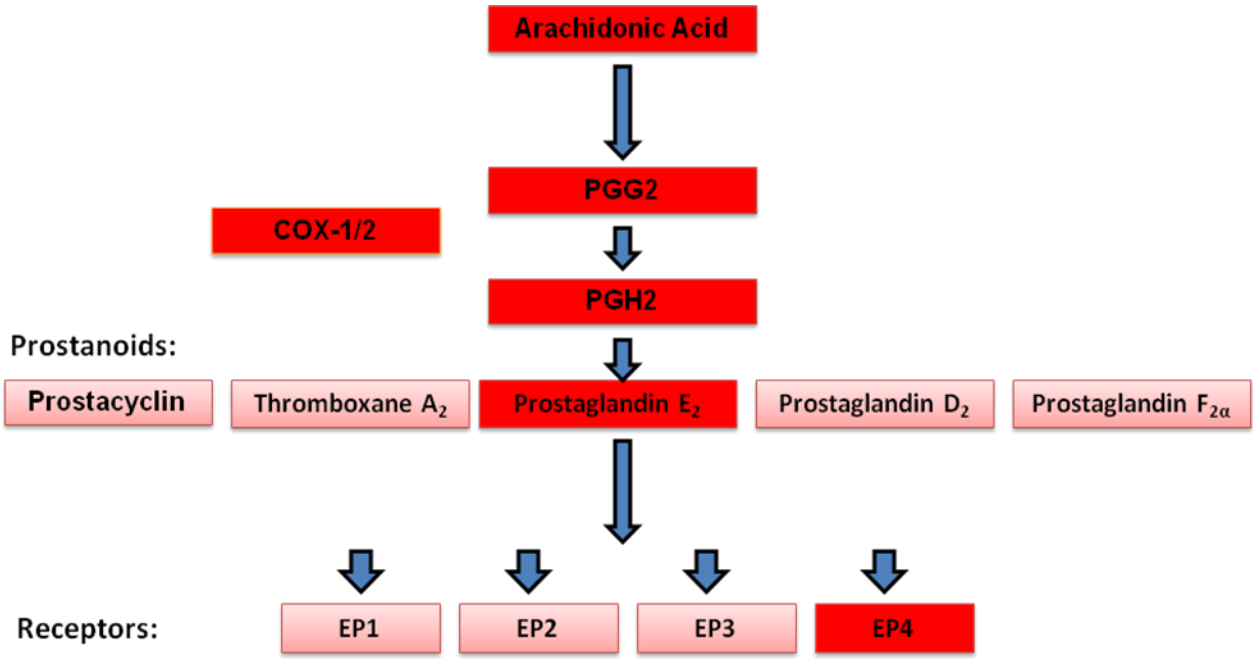


Figure 1. Cyclo-oxygenase enzymes, prostanoids and EP receptors

COX-1 and COX-2 convert arachidonic acid into prostaglandin precursors PGG2 and PGH2 through a two step rate limiting reaction. PGH2 is converted to various prostanoids through different prostanoid synthases. Most notably, COX enzymes mediate the formation of PGE_2, which has been associated with breast cancer aggressiveness. PGE_2 acts on four different EP receptors. Our lab is investigating COX-2 mediated breast cancer progression, focusing on the pathway highlighted in red.

1.5 COX Inhibitors

Non-selective COX-1/COX-2 inhibitors are non-steroidal anti-inflammatory drugs (NSAIDs) with Aspirin-like properties, and primarily utilized for analgesic, anti-inflammatory and fever reducing activities (Flower, 2003). In the 1970s, Aspirin and Aspirin-like-drugs were shown to reduce prostaglandin levels in humans (Smith & Willis, 1971). Interestingly, these drugs also showed promise as anti-cancer agents. For instance, patients treated with low-dose Aspirin had a decrease in fatal colon cancer (Thun, Namboodiri, & Heath, 1991). However, chronic use of NSAIDs is associated with adverse effects such as gastric intolerance and delay of blood clotting. Selective COX-2 inhibitors, such as Celecoxib (Celebrex, Pfizer), have shown promising therapeutic effects and chemoprotective effects in a variety of cancers (Harris, 2009). The use of COX-2 inhibitors has exhibited increased apoptosis in head and neck carcinoma, reduced tumour formation in colon cancer and breast cancer (Harris, Alshafie, Abou-Issa, & Seibert, 2000; Kim et al., 2010; Sheng et al., 1997). However, prolonged use of COX-1 and COX-2 inhibitors has been associated with adverse cardiovascular events such as myocardial infarction, cardiac arrest, stroke and pulmonary embolisms in a small subset of patients (Fitzgerald, 2004; Graham, 2006; Nussmeier et al., 2005). The increased incidence of cardiovascular events is believed to result from the inhibition of cardio-protective prostanoids such as $PGI_{2;}$ which blocks platelet aggregation and promotes vaso-dilation (Fitzgerald, 2004). COX-2 inhibitors disrupt the balance of PGI_2 and thromboxane A_2 present in the body (Fitzgerald, 2004). Thromboxane A_2 plays a role in vasoconstriction and platelet aggregation, and is mainly produced by COX-1 thus generally unaffected by selective COX-2 inhibitors. Because PGE_2 signals through EP4

via a unique pathway not shared by other EPs, our lab is investigating the receptor as more selective down-stream target of COX-2 activity that avoids cardiovascular side-effects observed with prolonged use of COX-2 inhibitors.

1.6 COX-2 and Breast Cancer

COX-2 has an important role in breast cancer progression. In tumours, COX-2 expression has been correlated with negative hormone receptor (ER and PR) status and HER2 amplification (Ristimaki et al., 2002) (Majumder et al. submitted Oncogene). Furthermore, studies in our laboratory have demonstrated that ectopically expressed COX-2 in breast cancer cell lines increase cell migration, invasion and proliferation (Majumder et al. submitted Oncogene). Increased proliferation is also a characteristic of COX-2 high tumours (Ristimaki et al., 2002). In breast cancer tissue, high COX-2 has been associated with matrix metalloproteinase (MMP)-2 activation, which degrade components in the extracellular matrix and the basement membrane (Sivula et al., 2005). Furthermore, high COX-2 expression is associated with high VEGF-C and LYVE-1, markers for endothelial lymphatic cells (Timoshenko, Chakraborty, Wagner, & Lala, 2006), and COX-2 over-expression demonstrated up-regulated production of angiogenic (VEGF-A) and lymphangiogenic (VEGF-C and –D) factors (Timoshenko et al., 2006) (Majumder et al. submitted Oncogene). Furthermore, COX-2 transfection markedly increased the size and frequency of clonogenic spheroid formation (a surrogate of SLC function *in vitro*) (Majumder et al. submitted Oncogene). Tumourspheres formed from COX-2 over-expressing cells had increased co-expression of the breast cancer stem cell markers CD44 and ALDH, as well as embryonic stem cell pluripotency markers SOX-2

and OCT-3/4 (Majumder, Xin, Liu, Girish, et al., 2014) (Majumder et al. submitted Oncogene). EMT phenotype was observed in COX-2 over-expressing breast cancer cells, shown by decreased expression of epithelial marker *CDH1* and increased expression of mesenchymal markers *VIM* (Vimentin), *Twist1* and *CDH2* (N-Cadherin) (Majumder, Landman, Liu, Hess, & Lala, 2015) (Majumder et al. submitted Oncogene). Finally, COX-2 over-expressing cells demonstrated a marked increase in lung colony forming ability and orthotopic tumourigenicity after serial transplantation into immunodeficient murine recipients (Majumder et al. 2014). Collectively, these observations are consistent with increased metastasis and reduced survival in breast cancer patients with high COX-2 expression within tumours (Ristimaki et al., 2002).

1.7 PGE_2 and Breast Cancer

As mentioned previously, PGE_2 is the main prostainoid resulting from increased COX-2 activity in breast cancer cells (Timoshenko et al., 2003). Our lab has shown that PGE_2 promotes tumour progression and metastasis by multiple mechanisms; (1) including the inactivation of cancer-fighting immune cells (Lala, Santer, Libenson, & Parhar, 1985; Parhar & Lala, 1985), (2) stimulation of cancer cell migration (Rozic et al., 2001; Timoshenko et al., 2003) and invasion (Timoshenko et al., 2004), (3) cancer-associated angiogenesis (Rozic et al., 2001), and (4) lymphangiogenesis by up-regulation of VEGF-C or VEGF-D (Timoshenko et al., 2006; Xin et al., 2012). Therefore, COX-2 mediated production of PGE_2 promotes cancer associated functions.

1.8 COX-2 induced miRNAs in breast cancer

MicroRNAs (miRNAs) are small regulatory RNA molecules (19-24 nucleotides) that are transcribed from DNA but not translated into protein. miRNAs act to down-regulate gene expression at the post-transcriptional level by either degrading mRNA of their target gene or by blocking translation of the target gene into protein. They recognize their target gene through sequence complementarity, usually located on the target gene's 3'untranslated region (UTR) (Sassen, Miska, & Caldas, 2008). MiRNA biogenesis starts with RNA polymerase II or III driven generation of pri-miRNA strands. Drosha RNase III endonuclease performs nuclear cleavage of the the pri-miRNA strand, generating a stem loop structure intermediate or the miRNA precursor (pre-miRNA). The pre-miRNA is then transported to the cytoplasm by Ran-GTP and Exportin-5. Next, Dicer cleaves the pre-miRNA, removing the loop and terminal base pairs. This produces the mature miRNA and a similar sized complementary fragment referred to as the miRNA*. The miRNA:miRNA* duplex is short lived and the miRNA* is subsequently degraded. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which then suppresses target gene expression (Bartel, 2004). MiRNAs have been associated with oncogenic and anti-oncogenic (tumour-suppressor-like) functions. Interestingly, miRNAs can be found in body fluids within micro-vesicles (Kosaka, Iguchi, & Ochiya, 2010). Therefore, expression of certain miRNAs can be utilized as potential cancer biomarkers (Calin & Croce, 2006).

Affymetrix microRNA micro-array comparison of the MCF7-COX-2 cells and MCF7-Mock cells, identified two miRNAs, miR-526b and miR-655, that are up-regulated in the COX-2 over-expressing cell lines. COX-2 low breast cancer cells treated with PGE_2 or

EP4 agonists had higher expression of miR-526b and miR-655 (Majumder et al., 2015) Furthermore, COX-2 over-expressing tumourspheres demonstrated higher expression of these miRNAs, suggesting that these miRNAs are COX-2 and EP4 induced (Dunn et al., 2014; Majumder, Postovit, et al., 2014). MiR-526b and miR-655 over-expressing cells also showed increased migration, invasion, proliferation and tumoursphere formation (Dunn et al., 2014; Majumder et al., 2015). When these miRNA over-expressing cells were treated with EP4 antagonists, a reduction in spheroid formation was observed. *In vivo* studies of these miRNA over-expressing breast cancer cells revealed an increase in proliferative lung colonies in mice compared to MCF7-Mock cells (Majumder et al., 2015; Majumder, Xin, Liu, Bell, et al., 2014). *In situ* studies demonstrate that both miR-526b and miR-655 are higher in breast cancer tissue and are negatively correlated with patient survival (Majumder et al., 2015; Majumder, Postovit, et al., 2014). Therefore, miR-526b and miR-655 are considered oncogenic with SLC-inducing properties which are mediated through COX-2 and EP4. Interestingly, cytoplasmic polyadenylation element binding protein (CPEB)-2 is the common gene target for both miRNAs. By extension, we propose that CPEB2 may demonstrate anti-oncogenic properties, and down-regulation of CPEB-2 by miR-526b and miR-655 promotes breast cancer aggressiveness.

1.9 The CPEB Family of Proteins

Translation of eukaryotic mRNAs can be regulated through the length of their poly(A) tails (Macdonald, 2001). CPEB1, the founding member of the CPEB family, was first described to play a role in oocyte maturation (Hake & Richter, 1994). CPEB1 regulates

translation by associating with specific sequences in the 3' UTR of their target mRNA called cytoplasmic polyadenylation elements (CPEs, sequence: UUUUUAU) (D'Ambrogio, Nagaoka, & Richter, 2013; Hake, Mendez, & Richter, 1998). All CPEBs have some common structural elements including an N-terminal regulatory domain and a C-terminal RNA-binding domain. The RNA-binding domain contains two RNA recognition motifs (RRMs) and a cysteine-histidine zinc finger region, which when deleted impair the ability of CPEBs to bind to target mRNAs (Hake et al., 1998). Based on amino acid homologies in the coding regions and RNA binding regions (RRMs and zing finger regions), human CPEBs can be divided into two subfamilies: CPEB1 and CPEB2-4 (Huang, Kan, Lin, & Richter, 2006; Kurihara et al., 2003). The RNA binding domains of CPEB2, CPEB3 and CPEB4 are very similar, suggesting that they might share similar recognition sequences (Kurihara et al., 2003).

Unlike CPEB1, CPEB3 and CPEB4 do not interact with CPEs and bind other RNA binding sequences (Huang et al., 2006). Furthermore, unlike CPEB1, CPEB3 does not bind the cleavage and polyadenylation specificity factor (CPSF) (Huang et al., 2006). This suggests that CPEB3 and CPEB4 do not regulate translation through polyadenylation and do so through an independent mechanism. Although little is known on CPEB2 function, it is postulated that since CPEB2 is more similar to CPEB3-4 than CPEB1, then CPEB2 RNA binding sequences should be similar to CPEB3-4 and not CPEB1. However, a more recent paper highlights that both CPEB1 and CPEB2 bind to the CPE in HIF-1α RNA, to regulate its protein expression (Hägele, Kühn, Böning, & Katschinski, 2009). This suggests that CPEB2 regulates translation through polyadenylation and possibly another mechanism similar to CPEB3-4.

CPEB1 has been associated with cellular senescence, loss of polarity and suppression of malignancy (D'Ambrogio et al., 2013). CPEB1 knock-out mouse embryonic fibroblast cells avoid senescence and continue to divide (Groisman et al., 2006). When CPEB1 is re-introduced into CPEB1 low cells, cell stop dividing and express senescence associated marker β-galactosidase (Burns & Richter, 2008; Groisman et al., 2006). CPEB1 has also been shown to play a role in synaptic plasticity by regulating the translation α-Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) mRNA (Huang, Jung, Sarkissian, & Richter, 2002; Wu et al., 1998). Meta-analysis of global gene expression in cancers compared to normal tissues has shown that CPEB1 mRNA is lower in brain and reproductive system cancer, and CPEB3 mRNA is lower in digestive tract cancer, brain tumours and, head and neck tumour (D'Ambrogio et al., 2013). On the other hand, CPEB4 mRNA is higher in pancreatic glioblastomas (D'Ambrogio et al., 2013).

1.9.1 CPEB2

In mouse organs, *CPEB2* RNA has been detected in large quantities in the testis, and is also found at lower levels in the brain, liver, thymus, salivary glands, spleen, kidney, intestines and ovaries (Chen & Huang, 2012; Kurihara et al., 2003). The amount of CPEB2 in breast tissue has not been investigated. HeLa cells transfected with a CPEB2-GFP construct demonstrated that the protein is localized in the cytoplasm in cells (Huang et al., 2002; Kurihara et al., 2003). The role of CPEB2 in breast cancer has not been studied, but it may represent an important therapeutic target for cancer therapy due to its known targets.

1.9.2 CPEB2 Targets

Genes involved in antioxidant defence systems, such as hypoxia inducible factor (HIF)-1α, are up-regulated in breast cancer stem cells (Kai et al., 2010). HIF-1α over-expression in cancer has been associated with increased patient mortality in cancers of the brain, breast, cervix, oropharynx, ovary, and uterus (Semenza, 2003). HIF-1α has been shown to up-regulate genes involved in apoptosis resistance, angiogenesis and metastasis (Semenza, 2003). Under normoxic conditions, CPEB2 interacts with eukaryotic elongation factor (eEF)-2 and binds to the 3'UTR of HIF-1α, reducing HIF-1α peptide elongation (Chen & Huang, 2012). Under arsenite induced stress, CPEB2 is released from HIF-1α RNA and no longer interacts with eEF2. This allows eEF2 to resume maximum GTPase activity and increases the translation of HIF-1α (Chen & Huang, 2012; Hägele et al., 2009), leading to up-regulation of hypoxia induced factors such as VEGF.

Nairismägi et al. (2012) have shown that CPEB2 also has a role in the post-transcriptional regulation of Twist1, a gene involved in the EMT transition. Using immuno-precipitation, the authors determined that CPEB2 associates with Twist1 mRNA and over-expressi*ng* CPEB2 leads to decreased Twist1 protein levels (Nairismägi et al., 2012). Recently, β-catenin, CaMKIIα, and ephrin receptor A4 (EphA4) have been identified as CPEB2 targets in mouse brain (Turimella et al., 2015). β-catenin and CaMKIIα are also established CPEB1 targets (Hägele et al., 2009; Wu et al., 1998). This shows that there is an overlap of target molecules between CPEB1 and CPEB2.

1.9.3 MiRNAs and CPEB2

CPEB2, along with CPEB3-4, have previously been shown to be negatively regulated by miR-92 and miR-26. Both these miRNAs bind to the predicted recognition motif in the 3' UTR of CPEB2-4 and reduce transcript levels (Morgan, Iaconcig, & Muro, 2010). MiR-92 is up-regulated in certain human cancers, is associated with increased proliferation and reduced apoptosis, and is inversely correlated with estrogen receptor β1 (Erβ1) in breast cancer cell lines (Al-Nakhle et al., 2010; Manni et al., 2009; Shigoka et al., 2010; Tsuchida et al., 2011; Ventura et al., 2008). On the other hand, miR-26 has both oncogenic and tumour suppressive roles, where it has high expression in high grade glioma cells and reduced expression in breast cancer tumour tissues (Huse et al., 2009; Zhang et al., 2011).

1.10 Rationale

Our laboratory has previously shown that COX-2 promotes aggressive properties and induces a SLC phenotype in breast cancer cells; and that these functions are at least in part mediated by PGE_2, EP4 and COX-2-induced miR-526b and miR-655. Stable transfection of *COX-2* into human breast cancer cell lines MCF7 (COX-2 low, ER+, HER2–) and SKBR3 (COX-2 low, ER–, HER2+), induced all the phenotypic properties of aggressive breast cancer *in vitro* and *in vivo* (Majumder, Postovit, et al., 2014). Therefore, a combined Affymetrix gene expression and microRNA analysis of the COX-2 transfected cells compared to the mock transfected cells was performed. Out of the 848 microRNAs tested, only two were up-regulated by COX-2 transfection, miR-526b and miR-655. At the same time, out of 28,870 genes tested only 26 were down-regulated with COX-2 transfection. Interestingly, out of the 26 down-regulated genes, either miR-655 or miR-526b targeted 13 genes identified through the micro-array. The only common gene target of both miR-655 and miR-526b was CPEB2. Since both oncogenic miR-655 and miR-526b have target sites of the 3' untranslated region (UTR) of CPEB2, and miRNAs down-regulate specific gene expression, we postulate that CPEB2 might have an anti-oncogenic role in breast cancer.

Since microRNAs are stable in patient's serum, these oncogenic miRNAs can serve as potential markers for screening of SLCs and therapeutic responses in the clinic. Furthermore, gene expression profiles can be important prognostic markers in cancer. Thus, establishing a tumour-suppressor-like role for the CPEB2 will allow us to identify CPEB2 as a novel therapeutic target for tumour suppression in breast cancer.

1.11 Central Hypothesis:

Down-regulation of CPEB2 promotes an aggressive breast cancer phenotype through SLC induction and EMT.

Specific Aims

1) To investigate if there is an inverse relationship between expression levels of a) *CPEB2* and *COX-2* and b) *CPEB2* and miR-655 or miR-526b in human breast cancer cell lines.

2) To elucidate the effects of CPEB2 knock-down on MCF7 cell migration, invasion, proliferation, SLC function, and EMT *in vitro*

3) To investigate the effects of COX-2 inhibitor, and PGE_2 (EP1-4 ligand), EP4 agonist and antagonist treatments on CPEB2 levels.

2 EXPERIMENTAL METHODS

2.1 Cell Culture

The breast cancer cell lines used in this project are summarized in Table 1. All cells were purchased from the American Type Culture Collection (ATCC). MCF7 cells were extensively used in this thesis. MCF7 cells originated from the mammary gland of a patient with adenocarcinoma (ATCC, 2015). These cells are ER and PR positive, and HER2 negative (Kao et al., 2009). Furthermore, MCF7 cells are low in *COX-2* (Majumder, Postovit, et al., 2014) and high in *CPEB2*, making them ideal candidates for CPEB2 knockdown in this study.

All breast cancer cell lines were maintained as monolayers in T-75 cm^2 flasks (Falcon) in a humidified incubator with 5% CO_2 at 37°C. MCF7 cells were grown in Eagle's Minimum Essential Media (EMEM, ATCC) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), 100 µg/mL of penicillin/streptomycin (pen/strep, Gibco) and 10 µg/mL of insulin (Sigma). SKBR3 cells were grown in McCoy's 5A Modified Media (Gibco), and MDAMB231, HS578T and T47D cells were grown in RPMI-1640 Media (Gibco) supplemented with 10% FBS and 100 µg/mL pen/strep. Cells were washed with DPBS (Gibco) and media was changed every 48 hours. Cells were detached with 0.25% Trypsin (Gibco) and re-plated, as required.

2.2 COX-2, CPEB2, miR-655 and miR-526b status in breast cancer cell lines

To determine the status of *COX-2* and *CPEB2* expression, miR-655 and miR-526b in different breast cancer cell lines, real-time PCR was performed using the TaqMan gene expression assay as described in '2.5'.

2.3 MCF7 Cell Transfection

Over-expression of COX-2 was achieved using a pCMV-IRES2-EGFP-COX-2 vector which contained a cytomegalovirus (CMV) promoter. The plasmid also contains an enhanced green fluorescence protein (EGFP) and neomycin resistance genes which can be used to sort transfected cells.

Dr. Mousumi Majumder previously transfected MCF7 cells with 2 µg of either pCMV-IRES-EGFP (control) plasmid or pCMV-IRES2-EGFP-COX-2 over-expression plasmid (Dr. Michael Archer, University of Toronto). The resulting cell line was named MCF7-COX-2. COX-2 over-expression was validated with real-time PCR as described in '2.5'. Media was supplemented with 500 µg/mL of Geneticin® (Gibco) to maintain selective pressure.

2.4 Nucleofection

Electroporation is a transfection method that involves the application of short electronic pulses to cells, which increase their permeability to macromolecules (Iversen, Birkenes, Torsdalen, & Djurovic, 2005). To increase transfection efficiency, nucleofection was employed. Nucleofection involves cell-specific delivery systems (nucleofector solution) and electronic pulses to optimize delivery of DNA, small-interfering RNA (siRNA) oligonucleotides to the nucleus of cells (Han et al., 2008). Nucleofection was performed using the Amaxa Biosystems Nucleofector® 2 system, the Cell Line Nucleofector® Kit V (Lonza) and associated protocol. The nucleofection protocol was validated by

transfecting MCF7 cells with the supplied pmaxGFP Vector® (Lonza). Expression of GFP was confirmed by fluorescence microscopy in MCF7 cells.

2.4.1 *CPEB2* knockdown with siRNAs

In vitro CPEB2 knockdown was achieved using siRNAs. Similar to miRNAs, siRNAs bind and degrade target genes with complementary mRNA sequences through RISC (Carthew & Sontheimer, 2009). For this project, siRNAs were transfected into MCF7 cells to down-regulate *CPEB2* expression. Parental MCF7 (COX-2 low) human breast cancer cell line expressed high levels of *CPEB2*. MCF7 cells were grown until 80% confluent in T-75 cm^2 flasks (Falcon). Cells were trypsinized and pelleted. Two million cells were transferred into certified cuvettes along with supplemented nucleofector® solution (Lonza) and 1 µM of either *CPEB2* siRNA (OriGene) or Universal Scrambled Control siRNA ([Control siRNA], OriGene). The program used for transfection was P-020. After nucleofection, cells were incubated at 37°C and 5% CO_2 in appropriate antibiotic free media. After 24 hours media was changed and experiments were conducted after 48 hours. The resulting cell lines were named MCF7-Scrambled and MCF7-CPEB2 KD. CPEB2 KD was validated using RT-PCR. To look at the functional role of *CPEB2*, the following assays were tested: migration (Transwell assay), invasion (Matrigel Transwell Assay), proliferation (BrdU uptake), EMT phenotype (real-time PCR/IF) and *in vitro* SLC content (clonogenic tumoursphere formation on ultra-low attachment plates/IF).

2.5 Quantification of CPEB2 knockdown with polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction (real-time PCR) was used to quantify the amount of specific mRNA transcripts present in a sample using fluorescent technology. We used a multi-step protocol that involves: (1) RNA purification, (2) conversion of RNA into cDNA, and (3) detection of PCR product (Fraga, Meulia, & Fenster, 2008). In general, the less time it takes for an amplified target sequence to pass the detection threshold, the greater the amount of target sequence there is in the starting material (Fraga et al., 2008).

Cells were grown until 80-90% confluent, after which they were trypsinized and pelleted. Total RNA and miRNA was extracted using RNeasy Minikit and RNeasy MiniElute Cleanup Kit (Qiagen) by following manufacture's protocol (Qiagen, 2010a, 2010b). Total RNA and miRNA concentration was quantified using the Epoch Microplate Spectrophotometer (BioTek®).

RNA and miRNA was converted into cDNA using a RNA-dependent DNA polymerase (Reverse Transcriptase). cDNA was synthesized using reagents from the High Capacity cDNA Reverse Transcription Kit and TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). In the case of RNA synthesis, random primers were used, while for miRNA synthesis 3 µL of specific primers for miR-526b, miR-655, RNU44 and RNU48 (Life Technologies) were used. For each sample, 1 µg of RNA per 20 µL reaction or 0.5 µg of miRNA per 28 µL reaction were used to synthesize cDNA using the C1000™ Thermal Cycler (BioRad). The following parameters were used to perform reverse transcription from RNA: hold for 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, then

hold at 4°C; and from miRNA: hold for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, then hold at 4°C.

For accurate detection of PCR product, PCR mixture was made using reagents from the TaqMan Universal PCR Master Mix (RNA) and TaqMan Universal PCR Master Mix, no AmpErase UNG kit (miRNA, Life Technologies). Each qPCR reaction tube was prepared to a volume of 20 µL with 1 µL of appropriate TaqMan Probe, and 2 µL of cDNA. The probes that were used in this experiment are listed in Table 2. House-keeping genes β-actin and RPL5 (for RNA), and RNU44 and RNU48 (for miRNA) were used as controls. The real-time PCR was performed on the Rotor-Gene 3000 (Corbett Research), using the profile: hold for 2 min at 50°C, 10 min at 95°C followed by 45x cycling with denaturing 15 sec at 95°C, and anneal/extension cycles for 1 min at 58°C.

Data was analysed using the relative quantification method ($2^{-\Delta\Delta CT}$), where change in expression of a target gene from a treated or transfected group was compared to a control group (Livak & Schmittgen, 2001).

2.6 Transwell Assay

2.6.1 Migration Assay

MCF7-Parental cells, MCF7-Scrambled, and MCF7-CPEB2 KD were grown until 80% confluent. Cells were serum starved in basal EMEM media overnight. A 24-well cell culture plate fitted with 8 µm microporous polycarbonate cell culture inserts (Fisher Scientific) was used to measure migration. The upper chamber contained 300 µL of cell suspension (2×10^5 serum starved cells/mL of basal EMEM), while the bottom contained

700 µL of EMEM media with no FBS or 5% FBS. Both sides of the insert membranes were cell culture pre-treated to promote membrane cell-adherence and limit cell-growth on the walls of the insert. Cells that adhered to the bottom surface of the membrane inserts were counted as migratory cells.

Previous work in the laboratory has shown that peak migration occurs at 24 hours (Rozic et al., 2001). Therefore, plates were incubated at 37°C and 5% CO_2 for 24 hours after which membrane inserts were removed and the top of the membrane inserts were wiped carefully with a cotton swab to remove non-migratory cells. The membranes were fixed with cold 100% methanol and stained with Eosin and Thiasine (VWR), which stains the cytoplasm pink and nucleus purple. The membrane was then carefully removed from the inserts and mounted onto microscope slides. Cells from the whole insert were counted under a light microscope (Leica-DFC295).

2.6.2 Invasion Assay (Matrigel)

Another characteristic of the tumour cell metastatic phenotype is proteolytic activity that degrades the extracellular matrix (ECM) barrier (L. M. Shaw, 2005). Once cells have breached this barrier they can access the vasculature and lymphatic system (L. M. Shaw, 2005). The protocol for this experiment is similar to "2.6.1 Transwell Migration Assay", except that the upper section of the micro-porous polycarbonate membranes were coated with a Matrigel gelatinous protein mixture, which mimics the basement membrane. Matrigel is isolated from Englebreth-Holm-Swarm mouse sarcoma, and contains a mixture of basement membrane ECM proteins such as laminins, collagen IV, and

enactins (Hughes, Postovit, & Lajoie, 2010). The mixture was prepared by mixing Growth Factor Reduced Matrigel® (VWR) in 1:100 dilution with cold EMEM. Then, 100 µL of diluted Matrigel was transferred onto the microporous polycarbonate membranes and left overnight to polymerize at room temperature. Before plating the cells, the matrigel layer was re-constituted with warm media for 30 mins at 37°C. Three hundred microliters of cell suspension (2×10^5 serum starved cells/mL of basal EMEM) was transferred to the upper well. As previously described, 700 µL of media (no FBS or 5% FBS) was transferred to the bottom well. Cells were incubated for 48 hours, which was time period that showed the highest invasion as determined by previous work in the laboratory (Rozic et al., 2001). Membranes were fixed, stained and invading cells were counted.

2.7 Bromodeoxyuridine (BrdU) Assay and Enzyme-Linked Immunosorbent Assay (ELISA)

Cellular proliferation requires the synthesis of new DNA. The Bromo-deoxyuridine (BrdU) assay measures the incorporation of BrdU into newly synthesizing DNA. BrdU is a pyrimidine analogue and is able to base pair with adenine during DNA replication. The incorporation of BrdU is used as a measure of cell proliferation (Porstmann, Ternynck, & Avrameas, 1985).

BrdU incorporation was detected using the Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche) following manufacture's protocol (Roche, 2010). Cells (100 µL at 10^4 cells/mL) were transferred into a 96-well tissue culture microplate and incubated at 5%

CO_2 and 37°C for 24 hours. Ten microliters of 100 µM BrdU was added to the cells and the cells were re-incubated at 37°C for 6 h. The labeling medium was removed and 200 µL of FixDenat solution was added to fix cells for 30 min at room temperature. The solution was removed and 100 µL of anti-BrdU POD working solution was added to each well for 90 min. The anti-BrdU-POD binds to the BrdU in the denatured newly synthesized DNA. Following incubation, wells were washed three times with 200 µL of washing solution (1x PBS), after which 100 uL of substrate solution was added per well for 30 min. BrdU incorporation was quantified using an Epoch Microplate Spectrophotometer at a wavelength of 370 nm with a reference wavelength of 492 nm.

2.8 Tumoursphere Formation Assays

Non-adherent tumourspheres can be clonally derived from cells with stem cell properties (Dontu et al., 2003). MCF7 cells were grown until 80% confluent, trypsinized and re-suspended in HuMEC media (Life Technologies) supplemented with epidermal growth factor (EGF, 20 ng/mL), fibroblast growth factor basic (FGFb, 20 ng/mL) and B-27® Supplement (1X, Invitrogen). To ensure that cells are plated as single cells, the solution was passed through a 27 ½ G needle (BD) and a 40 µM strainer (Falcon). Five cells per well were plated into 96-well ultra-low attachment plates (Corning) for 7 days or until tumourspheres reached a size of at least 60 µm in diameter (F. L. Shaw et al., 2012). Spheroids were counted and photomicrographs were taken using a light microscope (Leica-DFC295). Spheroid formation efficiency $\left(\frac{Number\ of\ spheroids\ formed}{Total\ number\ of\ cells\ plated}\right)$ was also calculated.

2.9 Cell Staining for Immuno-fluorescence Microscopy

2.9.1 Adherent cells

MCF7-Parental, MCF7-Scrambled, and MCF7-CPEB2 KD cells were grown until 80%, harvested with trypsin, and 4 x 10^4 cells were plated on cover-slips. After 24 hours cells were rinsed with PBS. To fix the cells, covers-slips were immersed in 4% para-formaldehyde solution (Electron Microscopy Sciences) at room temperature for 30 min, and rinsed with PBS three times. For cell permeabilization, 0.5% Triton-X100 (J T Baker Chemical Co.) in PBS was added for 10 min at room temperature, followed by three times PBS wash. Cells were blocked in 4% BSA in PBS with 0.01% Tween® 20 (Sigma) for 30 min. After rinsing with PBS, cells were incubated with primary antibody (Table 3) in 4% BSA in PBS at 4°C overnight. If the primary antibody was conjugated to a fluorochrome, incubation with secondary antibody was not necessary. If not, the slides were rinsed with PBS and incubated with secondary antibody in 4% BSA in PBS for an hour. Twenty microliters of VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories) was added on to the cover-slip and transferred onto a slide. The following primary antibodies were tested: E-Cadherin, Twist1, Nanog and ALDH1.

Fluorescence intensities for Twist1 and E-Cadherin were calculated using ImageJ software. The raw integrated density was calculated for each cell and normalized to the cell area. Data were presented as an average of all the cells.

2.9.2 Non-adherent cells (Tumourspheres)

Cells were grown until 80% confluent, trypsinized and re-suspended in supplemented HuMEC media following protocol outlined in tumoursphere formation assay. Ten thousand cells per mL cells were plated in 6-well non adherent cell culture plates for 5 days or until the tumourspheres reach a size of 60 µm in diameter. After which, the tumourspheres and media were transferred into a 1.5 mL Eppendorf tube and left on the bench for 15 min to allow the tumourspheres to settle to the bottom. Media was carefully removed by manual pipetting and the tip of the pipette was cut to maintain spheroid structure. The fixation, permeabilization, and anti-body staining for non-adherent cells were identical to the protocol described previously for adherent cells. The only exception is that tumourspheres were allowed to settle for 1-2 min between PBS rinses to minimize loss. The following primary antibodies were tested: ALDH1, SOX-2, and Nanog.

Quantification of fluorescence for ALDH1, Nanog, and SOX-2 were presented as ratio of cells with positive staining to the total number of cells marked by DAPI.

2.10 COX-2 inhibitors, PGE_2, EP4 Antagonists and Agonist Experiments

MCF7-COX-2 ($5x10^4$) cells were plated onto 6-well plates. Cells were serum starved overnight, after which they were washed with PBS and incubated for 24 hours with Celecoxib (10 or 20 µM), ONO-AE3-208 (10 or 20 µm) or vehicle control (0.003% DMSO). Celecoxib is a selective COX-2 inhibitor while ONO-AE3-208 is a selective EP4 antagonist.

To address the effect of PGE2 activity and EP4 signaling, MCF7 ($5x10^4$) cells were plated onto 6-well plates. Cells were serum starved overnight, washed with PBS and incubated for 24 hours with PGE_2 (10 µM) or PGE_1OH (10 µm) or vehicle control (EtOH). PGE_2 is a natural ligand for all EP receptors, while PGE_1OH acts as a selective agonist for EP4.

For all treated cells, RNA and miRNA was extracted as previously described, converted to cDNA and real-time PCR was performed to quantify the expression of *CPEB2*.

2.11 Statistical Analysis:

Statistical analyses were performed using GraphPad Prism Software Version 5.01 (GraphPad Software Inc 2007). Data compared non-transfected, Scrambled and CPEB2-KD cell lines using one-way ANOVA followed by Tukey's post hoc test or Scrambled and CPEB2-KD cells using unpaired students t-test. Results were considered statistically significant if $p<0.05$.

3 RESULTS

3.1 Inverse relationship of CPEB2 with COX-2, miR-655 and miR-526b expression

Differential gene and miRNA microarray analysis of MCF7 breast cancer cells stably transfected with COX-2 over-expressing plasmid revealed down-regulation of 26 genes and up-regulation of two miRNAs. These two miRNAs, miR-655 and miR-526b, have been found to be oncogenic by our lab (Dunn et al., 2014; Majumder et al., 2015). Furthermore, their expression has been positively correlated with COX-2 expression. Out of the 26 genes suppressed by COX-2 expression, thirteen are targeted by either miR-655 or miR-526b, with CPEB2 the only putative gene target of both miRNAs. Expression of *COX-2*, miR-655, miR-526b and *CPEB2* was quantified in different breast cancer cell lines. Cell lines with high *COX-2* and miRNAs expression, showed low expression of *CPEB2* (Figure 2), reinforcing the inverse relationship suggested by the differential gene and miRNA microarray analyses previously performed (Majumder et al., 2015).

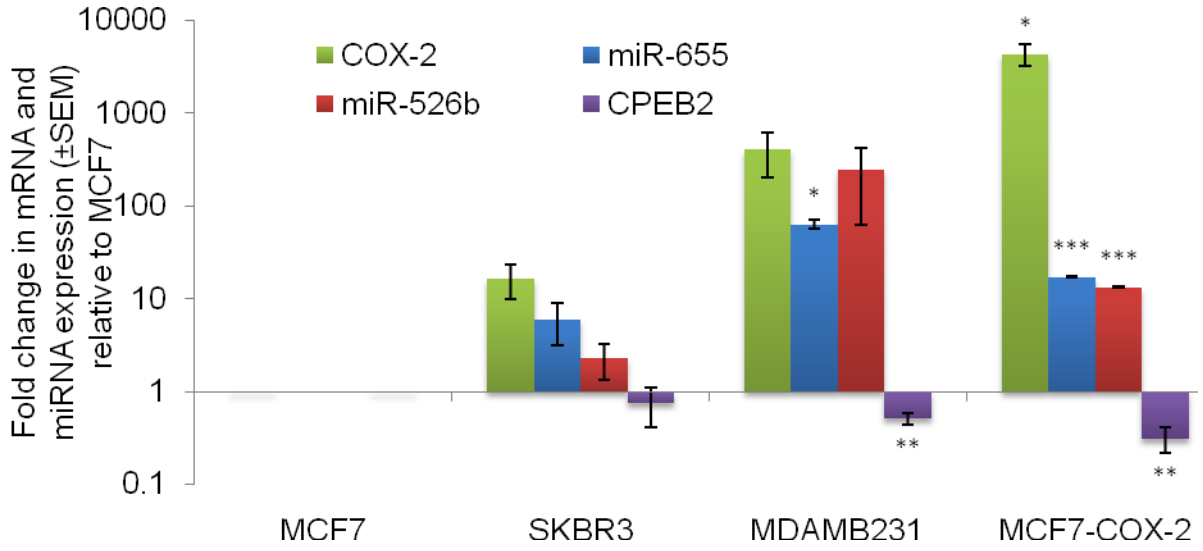
Figure 2. Breast cancer cell lines with high *COX-2*, and miR-655 and miR-526b expression demonstrate low *CPEB2* expression

A. We hypothesized that cells with high COX-2 have increased expression of oncogenic miR-655 and miR-526b, and low expression of CPEB2. **B.** RNA and miRNA was extracted from 4 distinct breast cancer cell lines with disparate COX-2 expression. Data are presented as average fold change normalized to MCF7-Parental ($2^{-\Delta\Delta CT}$) ± SEM. β-Actin and RNU-44 were used as internal controls for RNA and miRNA respectively. High *COX-2* expressing cell lines, MCF7-COX-2 and MDAMB231, showed high expression of miR-526b and miR-655 and low expression of *CPEB2*. (*: $p<0.05$, **: $p<0.005$, ***: $p<0.0005$) indicates a significant difference compared to MCF7 cells (one-way ANOVA, Tukey's post hoc test, n=3).

A.


COX-2
miR-655
miR-526b
CPEB2

B.


Fold change in mRNA and miRNA expression (±SEM) relative to MCF7
10000
1000
100
10
1
0.1
COX-2
miR-655
miR-526b
CPEB2
*
*
***
***
**
**
MCF7
SKBR3
MDAMB231
MCF7-COX-2

3.2 Validation of CPEB2 knockdown (KD) with small interfering RNA (siRNA)

MCF7 cells (ER+/PR+/HER2-) are low in *COX-2*, miR-655, miR-526b and high in *CPEB2* mRNA expression. To investigate the functional role of CPEB2 *in vitro*, *CPEB2* expression was suppressed in MCF7 cells using siRNAs targeting CPEB2 and a universal scrambled siRNA control at 1μM concentrations. The resulting cell lines were labeled as MCF7-CPEB2 KD and MCF7-Scrambled. CPEB2 expression was quantified in non-transfected (MCF7-Parental), MCF7-Scrambled and MCF7-CPEB2 KD using real-time PCR ($2^{-\Delta\Delta CT}$). MCF7-CPEB2 KD cells demonstrated significant down-regulation of CPEB2 compared to Parental cells (Figure 3).

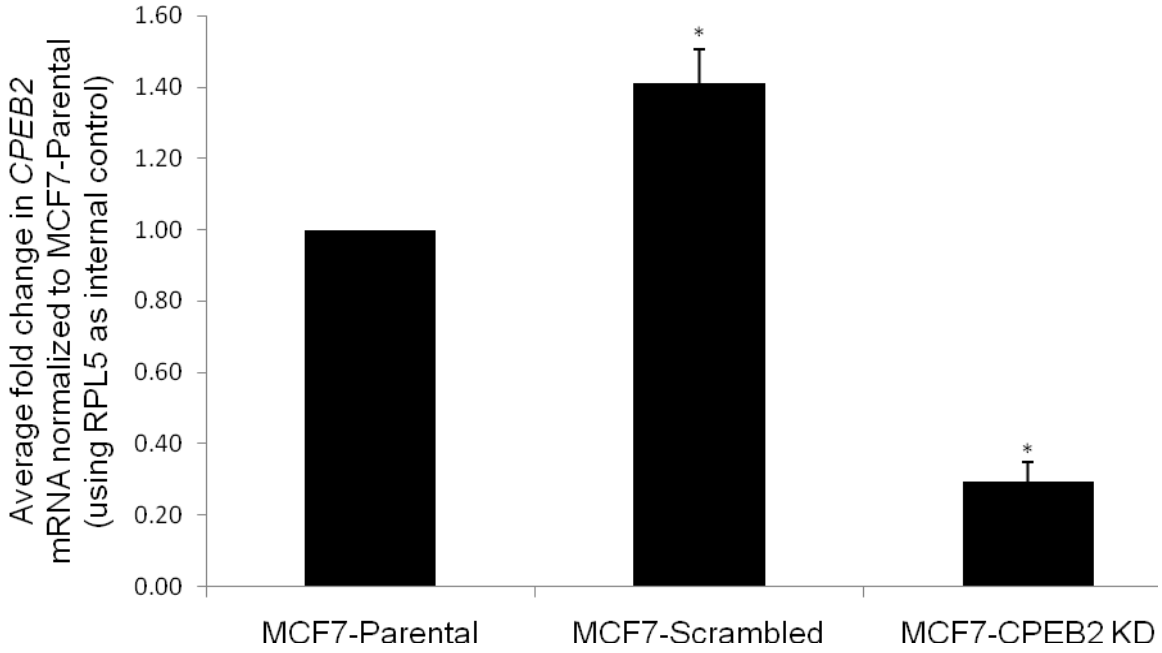


Figure 3. MCF7 cells transfected with CPEB2 siRNA have significantly lower expression of CPEB2

MCF7 cells were transfected with human CPEB2 siRNA (1 μM) and universal scrambled negative control siRNA. Resulting cell line, MCF7-CPEB2 KD showed 70% down-regulation of CPEB2 compared to non-transfected MCF7-Parental cell line using relative quantification of real-time PCR data ($2^{-\Delta\Delta CT}$). The data are presented as average fold change normalized to MCF7-Parental ± SEM for 4 independent experiments. RPL5 was used as an internal control. (*) indicates a significant difference ($p<0.0001$) compared to parental cells (one-way ANOVA, Tukey's post hoc test).

3.3 Knockdown of CPEB2 increased breast cancer cell migration and invasion

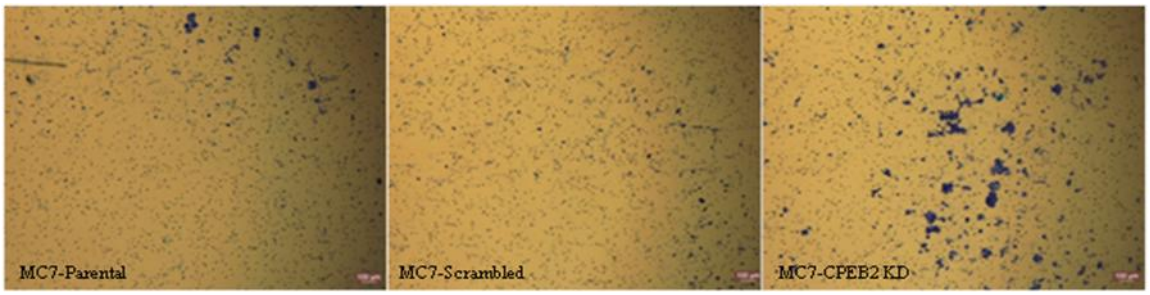
MCF7-COX-2, MCF7-526b and MCF7-655 cells showed increased migration and invasion compared to control MCF7 cells (Dunn et al., 2014; Majumder et al., 2015; Majumder, Postovit, et al., 2014). Therefore, our putative target CPEB2 might also play a role in cell migration and invasion. To assess the migratory ability of breast cancer cells with reduced CPEB2, MCF7-CPEB2 KD cells were used in transwell migration assays. The transwell assay is a method of measuring cell migration in a 2-D environment, through a semi-permeable membrane. MCF7-CPEB2 KD cells had significantly increased migratory capacity compared to MCF7-Parental or Scrambled controls (Figure 4).

Furthermore, MCF7-CPEB2 KD cells had significantly increased invasion capacity (Figure 5) using a complementary transwell invasion method through matrigel coated transwell inserts. Here, the ability of cells to degrade this ‘basement membrane’ layer and pass through the membrane was evaluated. Taking these results together, CPEB2 knockdown promotes migration and invasion *in vitro*.

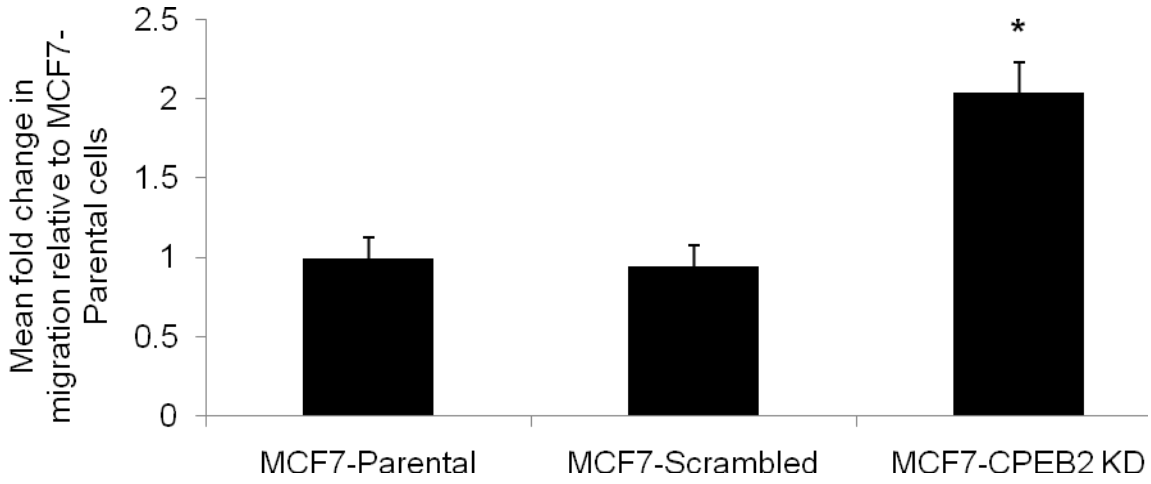
Figure 4. Knockdown of CPEB2 increased migration in MCF7 cells

MCF7-Parental, MCF7-Scrambled or MCF7-CPEB2 KD cells were plated in the upper chamber of transwell inserts and incubated for 24 hours. The cells that passed through the insert membrane were stained with Eosin and Thiasine, and counted by microscopy. A. Images of migration transwell inserts showing number of migratory cells (scale bar: 100 μm). B. MCF7-CPEB2 KD cells had a 2-fold increase in migration compared to MCF7-Parental cells. There was no difference in migration between MCF7-Parental and MCF7-Scrambled cells. Data are represented as average fold change normalized to MCF7-Parental ± SEM for 3 experiments. (*) indicates a significant difference ($p=0.0038$) compared to parental cells (one-way ANOVA, Tukey's post hoc test).

A.


MC7-Parental
MC7-Scrambled
MC7-CPEB2 KD

B.


Mean fold change in migration relative to MCF7-Parental cells
2.5
2
1.5
1
0.5
0
*
MCF7-Parental
MCF7-Scrambled
MCF7-CPEB2 KD

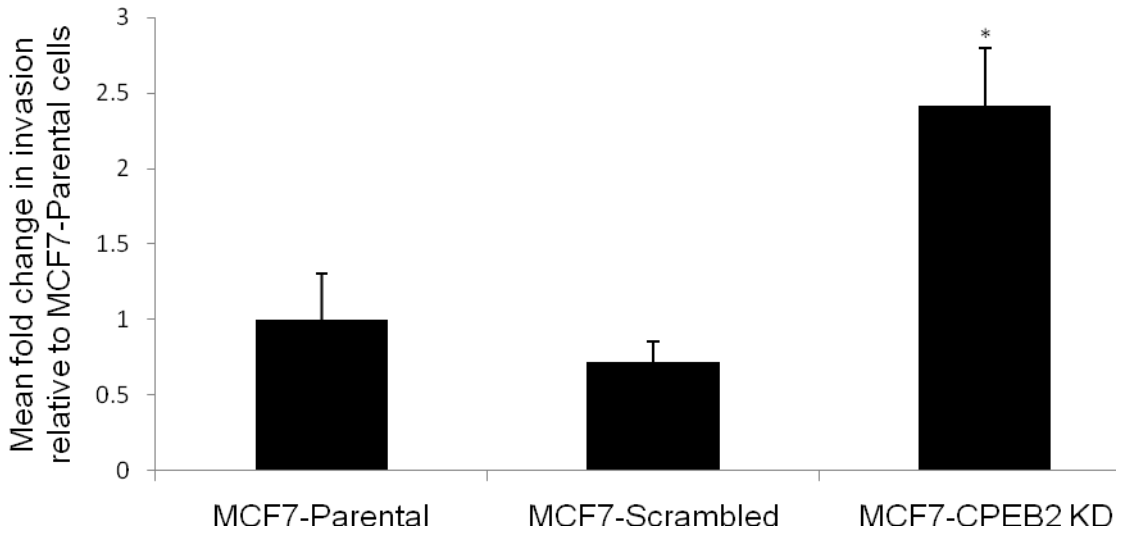


Figure 5. Knockdown of CPEB2 increased invasion in MCF7 cells

MCF7-Parental, MCF7-Scrambled and MCF7-CPEB2 KD cells were plated in the upper chamber of transwell inserts coated with matrigel. After 48 hours, the cells that degraded the layer of matrigel and passed through the insert membrane were stained with Eosin and Thiasine, and counted. MCF7-CPEB2 KD had a 2.4-fold increase in invasion compared to MCF7-Parental cells. There was no significant difference in invasion between MCF7-Parental and Scrambled cells. Data are represented as average fold change normalized to MCF7-Parental ± SEM for 3 experiments. (*) indicates a significant difference ($p=0.0127$) compared to parental cells (one-way ANOVA, Tukey's post hoc test).

3.4 Knockdown of CPEB2 has no effect on breast cancer cell proliferation

To delineate the role of CPEB2 in cell proliferation, a BrdU incorporation assay was performed. BrdU is integrated into newly synthesizing DNA during replication. The amount of BrdU present in the cells is then used as a measure of cellular proliferation. There was no significant difference in the amount of BrdU incorporated between Parental and CPEB2 KD cells (Figure 6). Therefore, we concluded that CPEB2 did not play a major role in cellular proliferation.

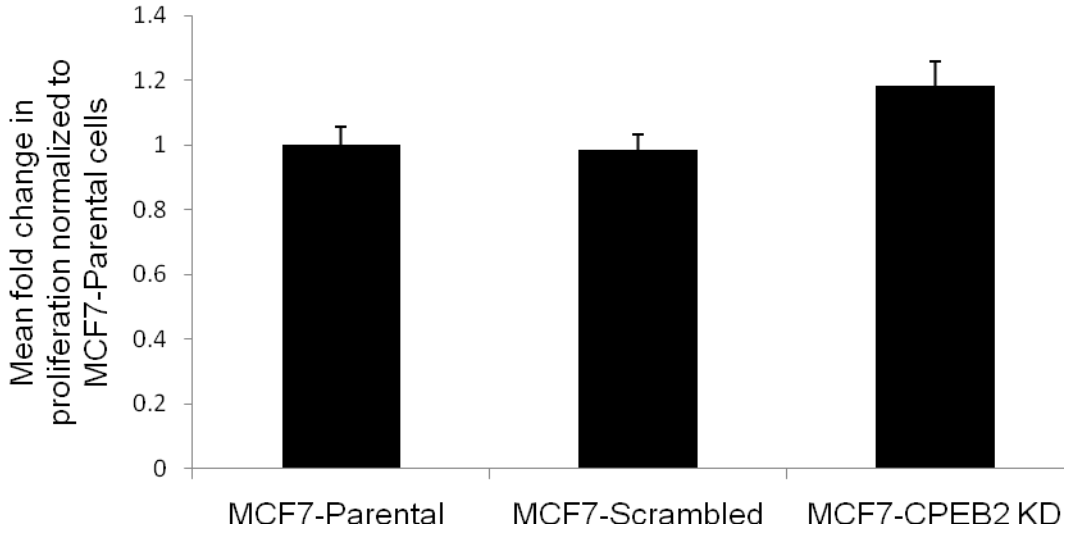


Figure 6. MCF7-CPEB2 KD cells have no change in cell proliferation

MCF7-Parental, MCF7-Scrambled and MCF7-CPEB2 KD cells were plated in 96 well plates. BrdU incorporation was measured after 6 hours of incubation. MCF7-CPEB2 KD cells showed no significant change (1.18 fold increase) in the average BrdU incorporated into cells compared to MCF7-Parental cells. The data are represented as average fold change normalized to MCF7-Parental ± SEM for 3 experiments (One-way ANOVA, $p=0.11$).

3.5 Down-regulation of CPEB2 promoted an EMT phenotype

Previous work done in this laboratory has determined that over-expressing miR-526b *in vitro* decreased expression of *CDH1* and up-regulated mesenchymal markers *Twist1*, *VIM*, and *Snail1* (Majumder et al., 2015). Furthermore, as shown in Figure 4, down-regulating *CPEB2* resulted in increased migration ability. To test whether CPEB2 KD was associated with an EMT switch, different epithelial and mesenchymal markers were tested (mRNA and IF). CPEB2 KD resulted in an increase in *Twist1* and *ZEB1*, however there was no change in *Snai1* and *VIM* (Figure 7). Figure 8 validated that Twist1 expression increased at the protein level as well. At the same time, E-Cadherin levels decreased with CPEB2 KD (Figure 9). Collectively, we determined that CPEB2 KD decreases E-Cadherin protein and increases Twist1 and Zeb1 in MCF7 cells, thus has an important role in EMT.

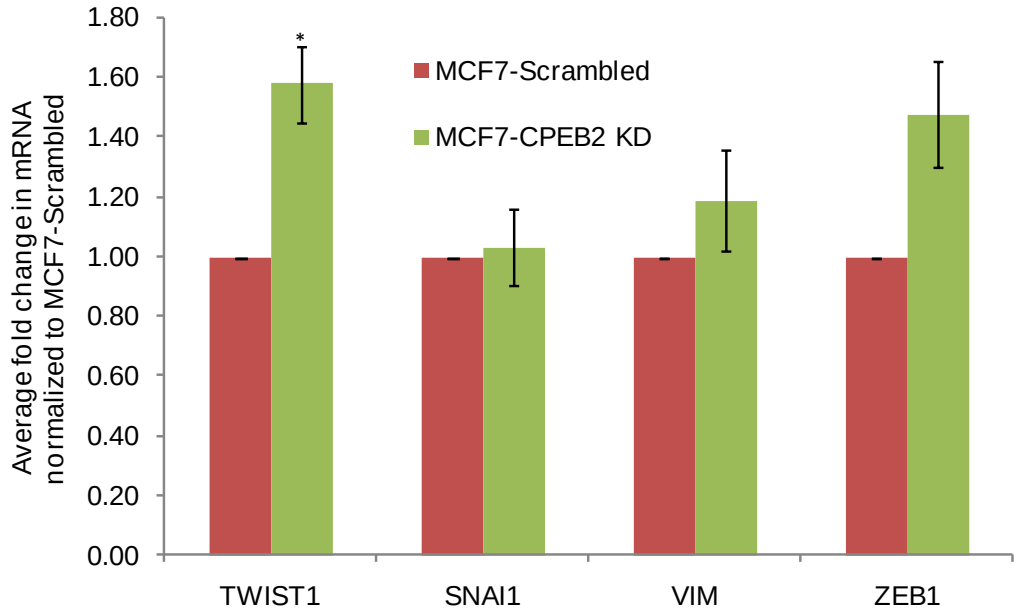


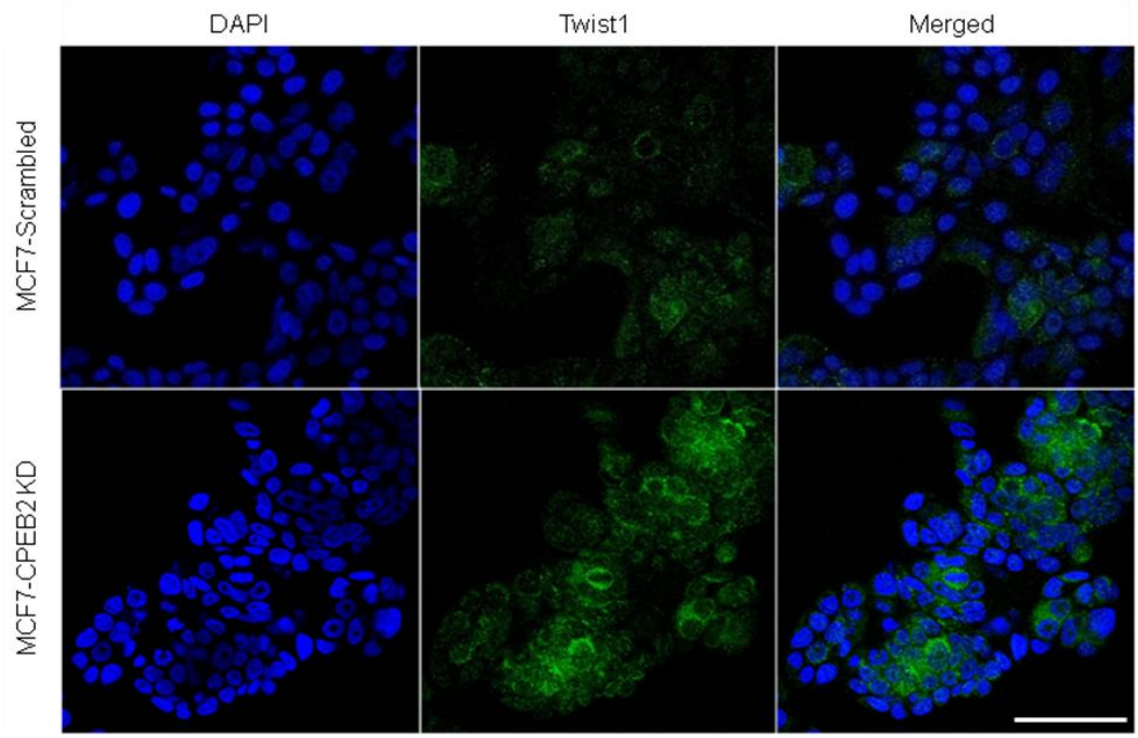
Figure 7. MCF7-CPEB2 KD cells have increased expression of mesenchymal marker Twist1

MCF7 cells transfected with human CPEB2 siRNA were compared to scrambled control transfected cells for mesenchymal markers using relative quantification by real-time PCR ($2^{-\Delta\Delta CT}$). MCF7-CPEB2 KD cells had a 1.6 fold increase in *Twist1* expression compared to MCF7-Scrambled cells (p=0.0104). Furthermore there was a trend towards increased *ZEB1* in CPEB2 KD cells (p=0.0556). There was no change in *SNAI1* and *VIM* between CPEB2 KD and control groups. The data are presented as average fold change normalized to MCF7-Scrambled ± SEM for 3 experiments. RPL5 was used as an internal control. (*) indicates a significant difference (unpaired t-test).

Figure 8. MCF7-CPEB2 KD cells have increased expression of mesenchymal marker Twist1

A. Twist1 antibody (1:250 in 4% BSA in PBS) was added onto MCF7-Scrambled and CPEB2 KD cells fixed onto coverslips. Cells were imaged using Olympus FV1000. Representative images are shown at the same magnification (scale bar: 100 µm, bottom right panel). DAPI is shown as blue nuclei staining and Twist1 is shown as green cytoplasmic staining, and merged shows both. **B.** Quantification of representative immuno-fluorescence images was performed using ImageJ. Data are presented as the average of raw integrated density divided by cell area. MCF7-CPEB2 KD cells had a 2.6 fold increase in Twist1 intensity compared to MCF7-Scrambled. The data are presented as average fold change normalized to MCF7-Scrambled ± SEM. (*) indicates a significant difference compared to Scrambled (unpaired t-test $p<0.0001$).

A.


DAPI
Twist1
Merged
MCF7-Scrambled
MCF7-CPEB2 KD

B.

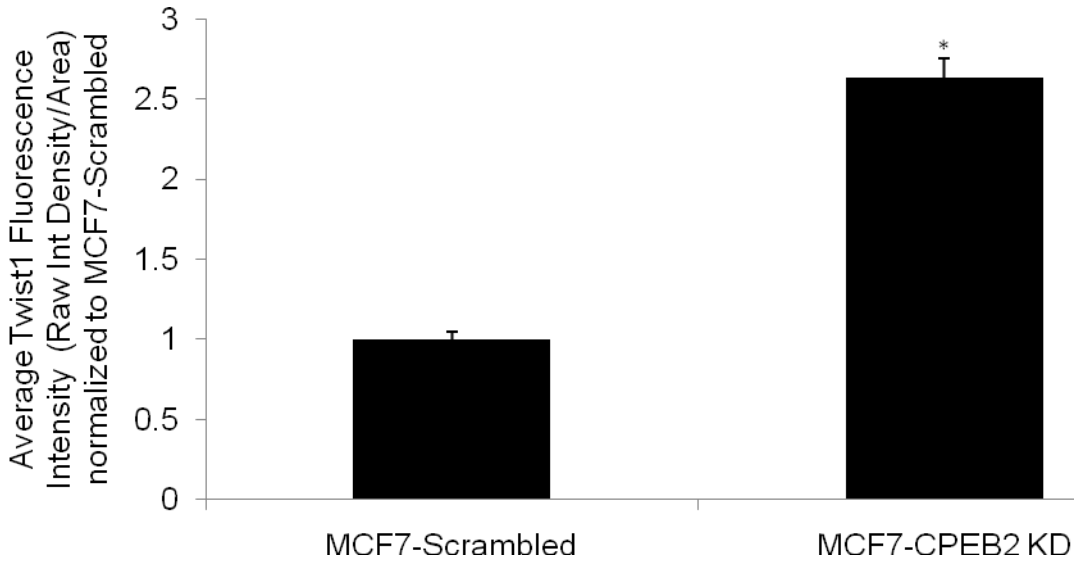
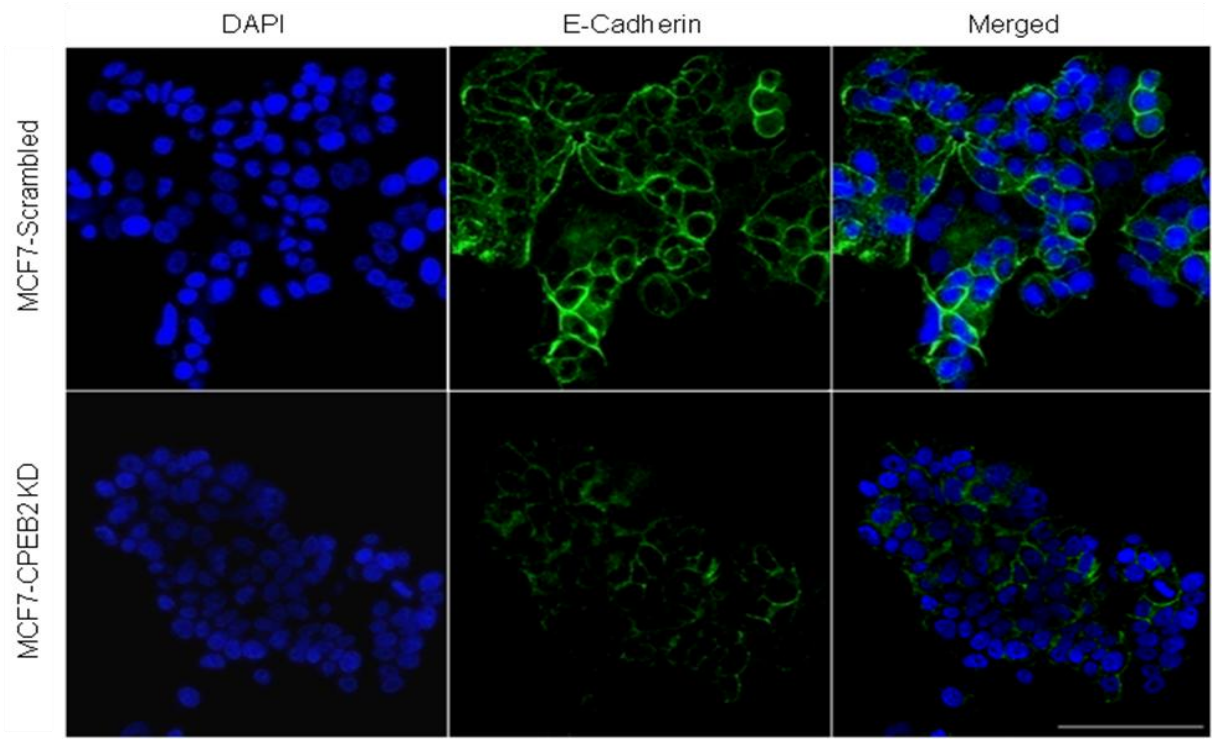

Average Twist1 Fluorescence Intensity (Raw Int Density/Area) normalized to MCF7-Scrambled
3
2.5
2
1.5
1
0.5
0
*
MCF7-Scrambled
MCF7-CPEB2 KD

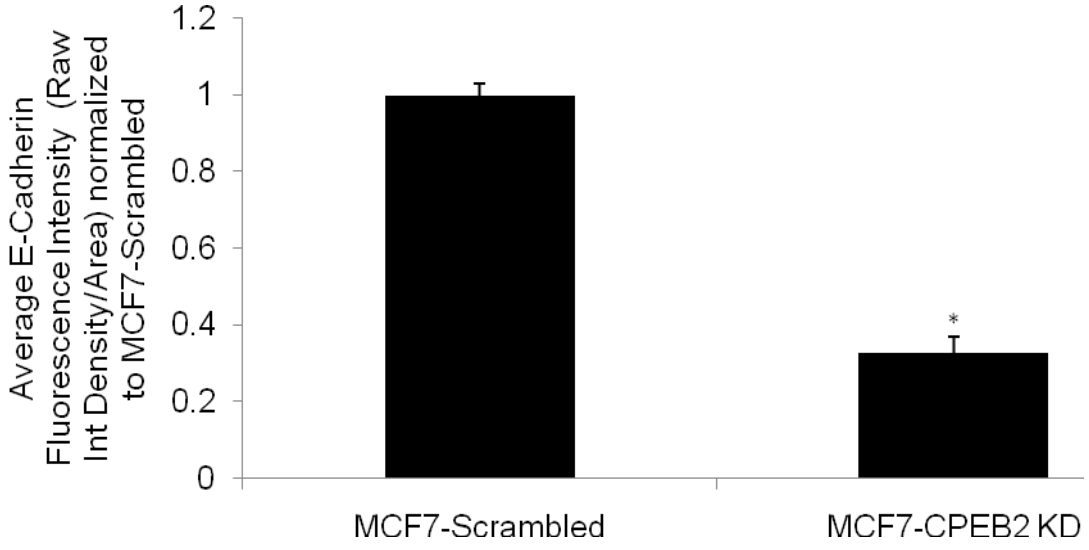
Figure 9. MCF7-CPEB2 KD cells have decreased expression of epithelial marker E-Cadherin

A. E-Cadherin antibody (1:250 in 4% BSA in PBS) was added onto MCF7-Scrambled and CPEB2 KD cells fixed onto coverslips. Cells were imaged using Olympus FV1000. Representative images are shown at the same magnification (scale bar: 100 µm, bottom right panel). DAPI is shown as blue nuclei staining and E-Cadherin is shown as green cell membrane staining, and merged shows both. **B.** Quantification of immuno-fluorescence pictures using ImageJ. Data are represented as the average of raw integrated density divided by cell area. MCF7-CPEB2 KD cells had 67% reduced E-Cadherin staining intensity compared to MCF7-Scrambled. Data are presented as average fold change normalized to MCF7-Scrambled ± SEM. (*) indicates a significant difference (unpaired t-test $p<0.0001$).

A.


DAPI
E-Cadherin
Merged
MCF7-Scrambled
MCF7-CPEB2 KD

B.


Average E-Cadherin Fluorescence Intensity (Raw Int Density/Area) normalized to MCF7-Scrambled
1.2
1
0.8
0.6
0.4
0.2
0
*
MCF7-Scrambled
MCF7-CPEB2 KD

3.6 Down-regulation of CPEB2 stimulated a SLC phenotype

MCF7-COX-2 cells demonstrated higher proportion of $ALDH^{high}CD44^{+}$ cells, increased tumoursphere forming ability when cultured under ultra-low attachment conditions and higher expression of miR-655 and miR-526b compared to MCF7-Mock transfected cell (Majumder, Postovit, et al., 2014). Furthermore, MCF7-526b cells cultured in 6 well ultra-low attachment plates demonstrated higher spheroid formation efficiency than MCF7-Mock cells (Majumder et al., 2015). Because these results suggest increased SLC phenotype in MCF7-COX2 and MCF7-526b cells, we next investigated the role of CPEB2 KD on SLC phenotype.

CPEB2 siRNA transfected cells were maintained in non-adherent conditions for 8 days. Real-time PCR demonstrated that MCF7-CPEB2 KD cells retained reduced CPEB2 expression compared to MCF7-Scrambled cells for the duration of the spheroid formation assay (8 days, Figure 10). MCF7-CPEB2 KD cells demonstrated higher spheroid formation efficiency compared to scrambled control cells (Figure 11). Different markers for SLC were also tested for MCF7-CPEB2 KD and MCF7-Scrambled cells cultured in monolayer and tumoursphere conditions. MCF7-CPEB2 KD cells showed a higher proportion of ALDH1 positive cells in monolayers compared to scrambled controls (Figure 12). In addition, Figure 13 demonstrates that CPEB2 KD tumourspheres also retained higher expression of ALDH1. CPEB2 KD cells also showed higher Nanog expression in monolayer and tumourspheres (Figure 14 and 15). Furthermore, SOX-2 expression was demonstrated to be higher in CPEB2 KD tumourspheres compared to scrambled control tumourspheres (Figure 16). Collectively, we demonstrated that CPEB2 KD promotes a SLC phenotype, with increased spheroid formation efficiency and

expression of pluripotency markers Nanog, SOX-2 and ALDH1.

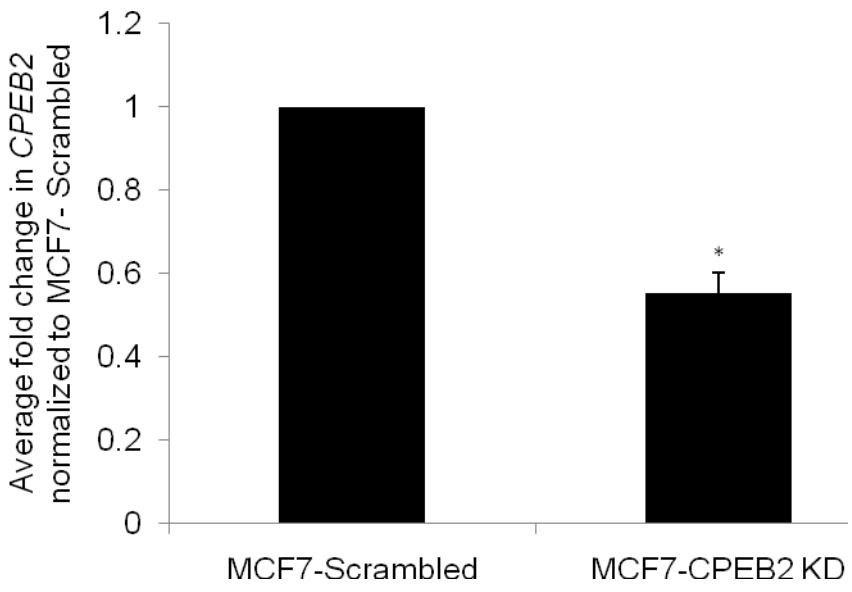


Figure 10. Eight days after siRNA transfection, MCF7-CPEB2 KD cells retained lower expression of *CPEB2*

MCF7 cells were transfected with human CPEB2 siRNA (1 µM) and universal scrambled control siRNA. Eight days after transfection, MCF7-CPEB2 KD demonstrated a 45% reduction in *CPEB2* compared to MCF7-Scrambled cells using relative quantification of real-time PCR ($2^{-\Delta\Delta CT}$). Data are presented as average fold change normalized to MCF7-Scrambled ± SEM for 3 experimental replicates. RPL5 was used as an internal control. (*) indicates a significant difference compared to control cells (unpaired t-test, p=0.0007).

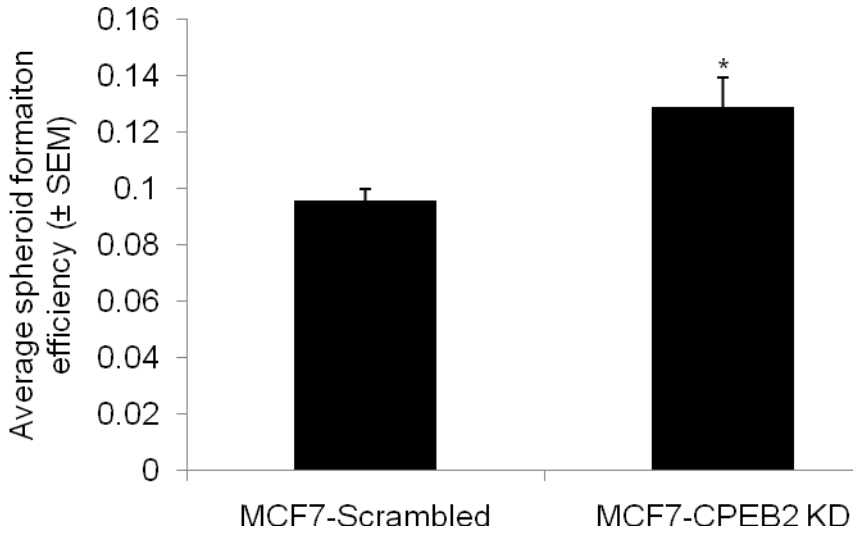


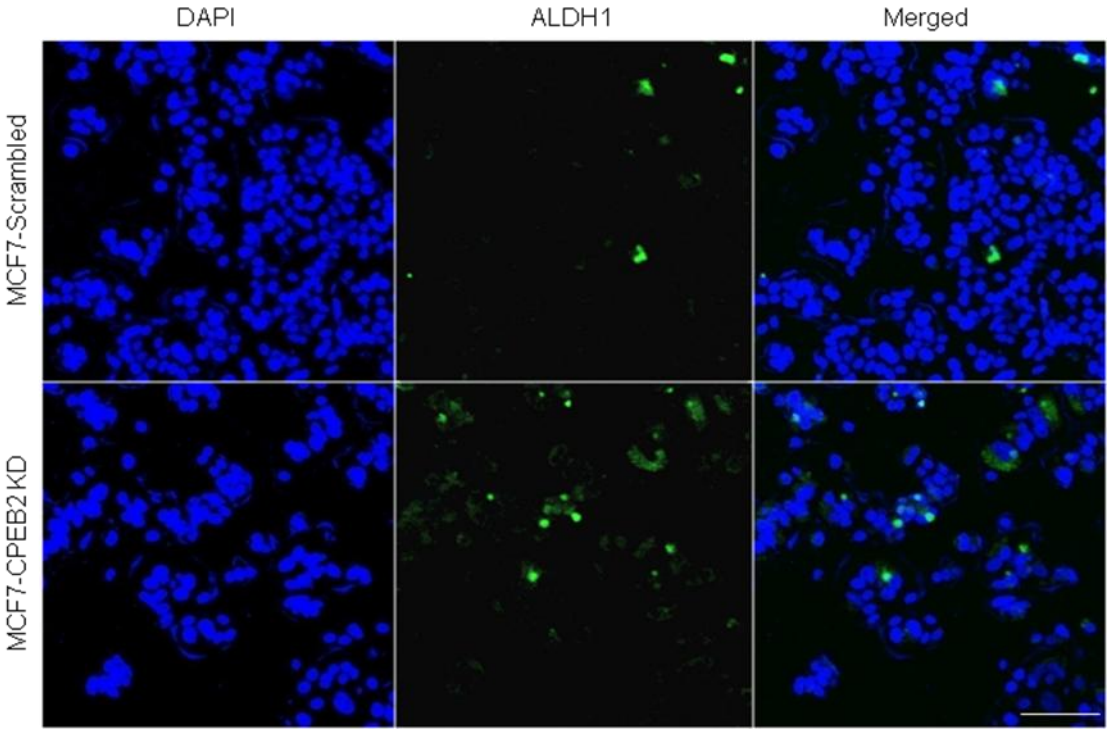
Figure 11. MCF7-CPEB2 KD cells demonstrated increased spheroid formation

MCF7-Scrambled and MCF7-CPEB2 KD cells were plated in 96 well ultra-low attachment plates at a concentration of 5 cells per well. After 7 days, spheroids greater than 60 µm were counted manually under bright-field microscopy. Spheroid formation efficiency was calculated as the ratio of number of spheroids formed to number of cells plated. MCF7-CPEB2 KD cells had a higher SFE (0.13) than MCF7-Scrambled cells (0.10). (*) indicates a significant difference (unpaired t-test, p=0.0411, n=3).

Figure 12. MCF7-CPEB2 KD cells grown in monolayer have increased expression of ALDH1

A. ALDH1 antibody (1:300 in 4% BSA in PBS) was added onto MCF7-Scrambled and CPEB2 KD cells grown in monolayers. Cells were imaged using an Olympus FV1000 fluorescent microscope. All image panels are at the same magnification (scale bar: 100 µm, bottom right panel). DAPI is shown as blue nuclei staining and ALDH1 is shown as green cytoplasmic staining, and merged shows both. **B.** Quantification of ALDH1 expression. Data are presented as ratio of positive cells to the total number of DAPI+ cells normalized to MCF7-Scrambled controls. MCF7-CPEB2 KD cells demonstrated a 3.8 fold increase in ALDH1 positive cells compared to MCF7-Scrambled cells. (*) indicates a significant difference (unpaired t-test, $p=0.0389$).

A.


DAPI
ALDH1
Merged
MCF7-Scrambled
MCF7-CPEB2 KD

B.

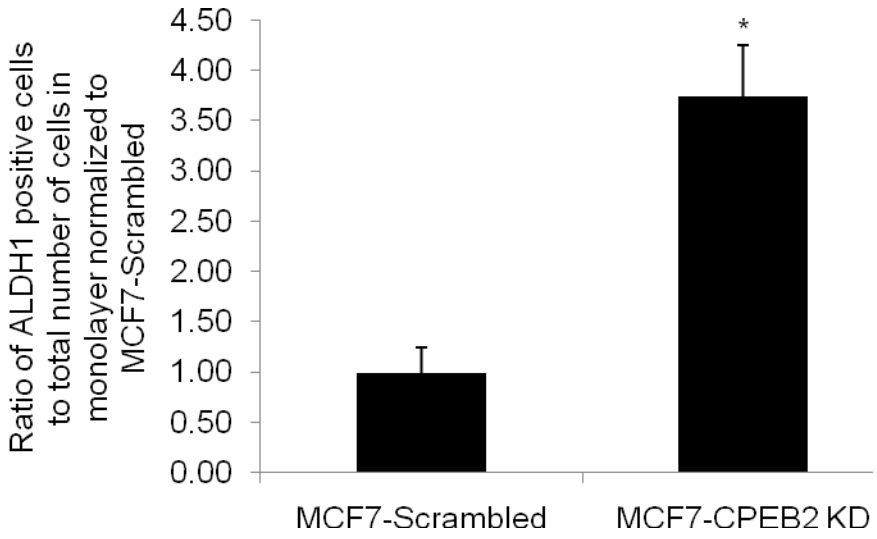
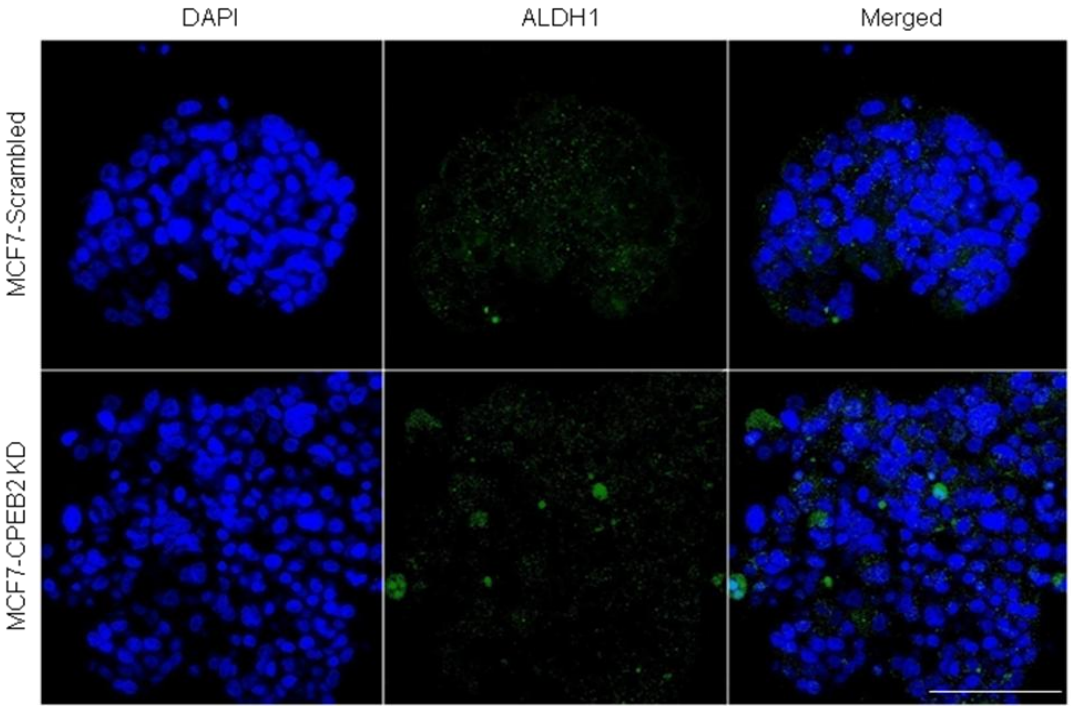

Ratio of ALDH1 positive cells to total number of cells in monolayer normalized to MCF7-Scrambled
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
MCF7-Scrambled
MCF7-CPEB2 KD

Figure 13. MCF7-CPEB2 KD tumourspheres have increased expression of ALDH1

A. ALDH1 antibody (1:300 in 4% BSA in PBS) was added onto MCF7-Scrambled and CPEB2 KD tumourspheres. Cells were imaged using an Olympus FV1000 fluorescent microscope. All image panels are at the same magnification (scale bar: 100 µm, bottom right panel). DAPI is shown as blue nuclei staining and ALDH1 is shown as green cytoplasmic staining, and merged shows both. **B.** Quantification of ALDH1 expression. Data are presented as ratio of positive cells to the total number of DAPI+ cells normalized to MCF7-Scrambled controls. MCF7-CPEB2 KD cells demonstrated a 2.6 fold increase in ALDH1 positive cells compared to MCF7-Scrambled. (*) indicates a significant difference (unpaired t-test, p=0.0428).

A.


DAPI
ALDH1
Merged
MCF7-Scrambled
MCF7-CPEB2 KD

B.

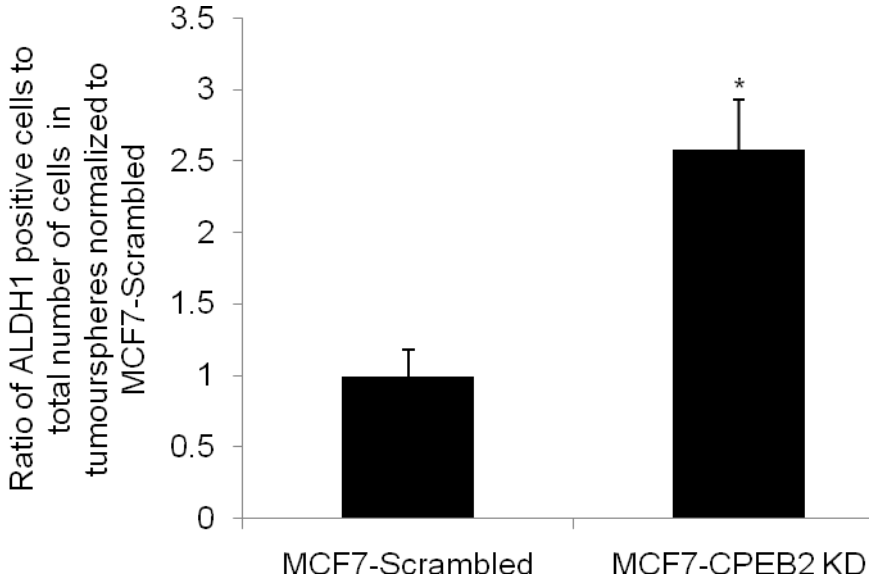
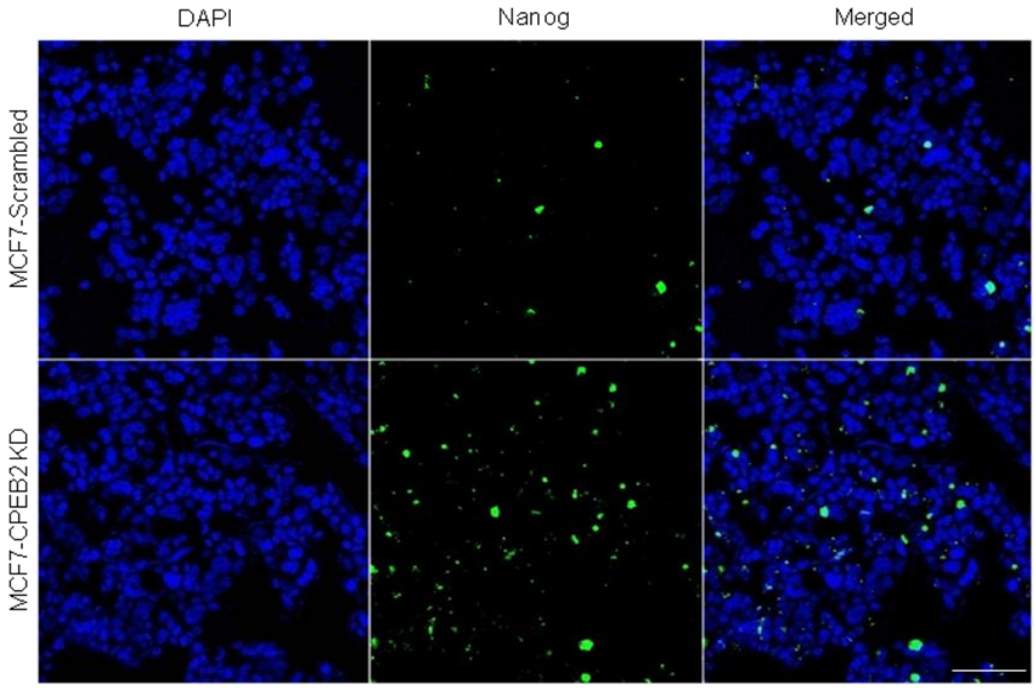

Ratio of ALDH1 positive cells to total number of cells in tumourspheres normalized to MCF7-Scrambled
3.5
3
2.5
2
1.5
1
0.5
0
*
MCF7-Scrambled
MCF7-CPEB2 KD

Figure 14. MCF7-CPEB2 KD cells grown in monolayer demonstrated increased Nanog expression

A. Nanog antibody (1:300 in 4% BSA in PBS) was added onto MCF7-Scrambled and CPEB2 KD cells grown in monolayers. Cells were imaged using an Olympus FV1000 fluorescent microscope. All image panels are at the same magnification (scale bar: 100 µm, bottom right panel). DAPI is shown as blue nuclei staining and Nanog is shown as green nuclear staining, and merged shows both. B. Quantification of Nanog expression. Data are presented as ratio of positive cells to the total number of DAPI+ cells normalized to MCF7-Scrambled controls. MCF7-CPEB2 KD cells demonstrated 6.5 fold increase in Nanog positive cells compared to MCF7-Scrambled. (*) indicates a significant difference (unpaired t-test, $p=0.0064$).

A.


DAPI
Nanog
Merged
MCF7-Scrambled
MCF7-CPEB2 KD

B.

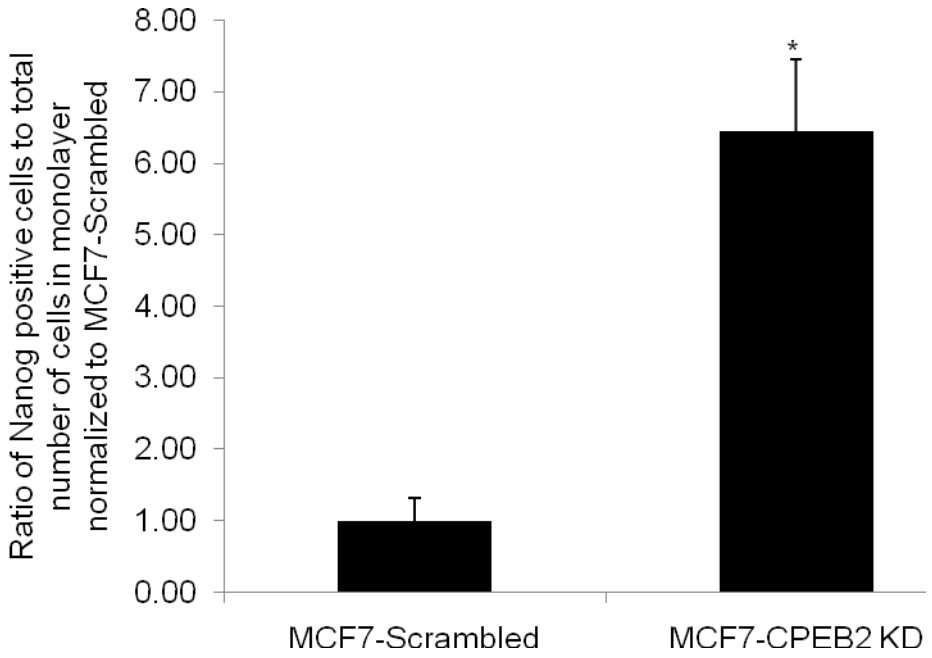
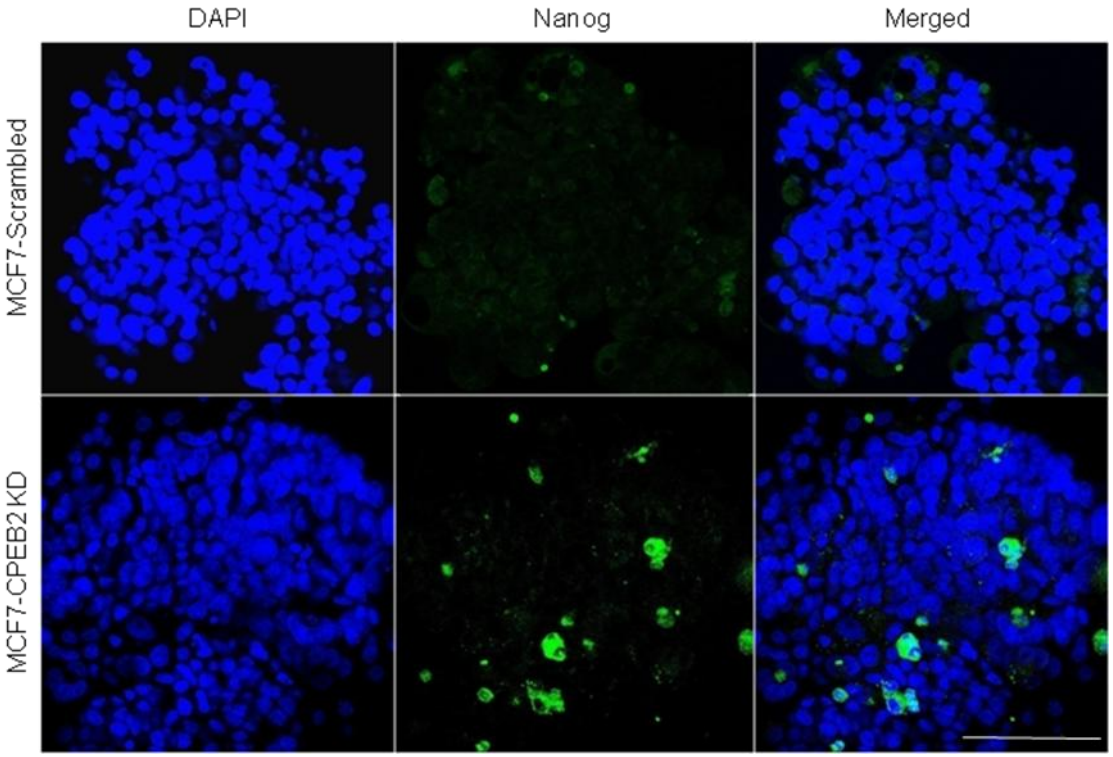

Ratio of Nanog positive cells to total number of cells in monolayer normalized to MCF7-Scrambled
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
*
MCF7-Scrambled
MCF7-CPEB2 KD

Figure 15. MCF7-CPEB2 KD tumourspheres demonstrated increased Nanog expression

A. Nanog antibody (1:300 in 4% BSA in PBS) was added onto MCF7-Scrambled and CPEB2 KD tumourspheres. Cells were imaged using an Olympus FV1000 fluorescent microscope. All image panels are at the same magnification (scale bar: 100 µm, bottom right panel). DAPI is shown as blue nuclei staining and Nanog is shown as green nuclear staining, and merged shows both. B. Quantification of Nanog expression. Data are presented as ratio of positive cells to the total number of DAPI+ cells normalized to MCF7-Scrambled controls. MCF7-CPEB2 KD cells demonstrated a 4.1 fold increase in Nanog positive cells compared to MCF7-Scrambled. (*) indicates a significant difference (unpaired t-test, $p < 0.0001$).

A.


DAPI
Nanog
Merged
MCF7-Scrambled
MCF7-CPEB2 KD

B.

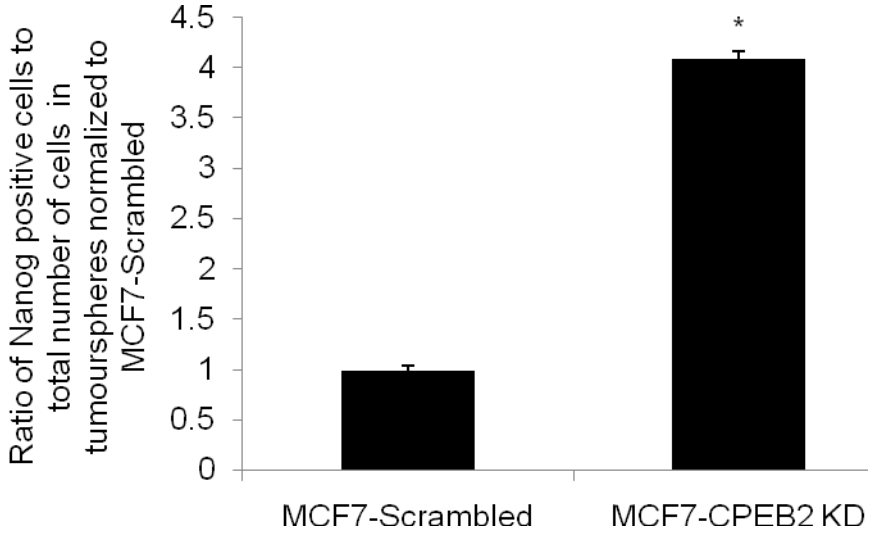
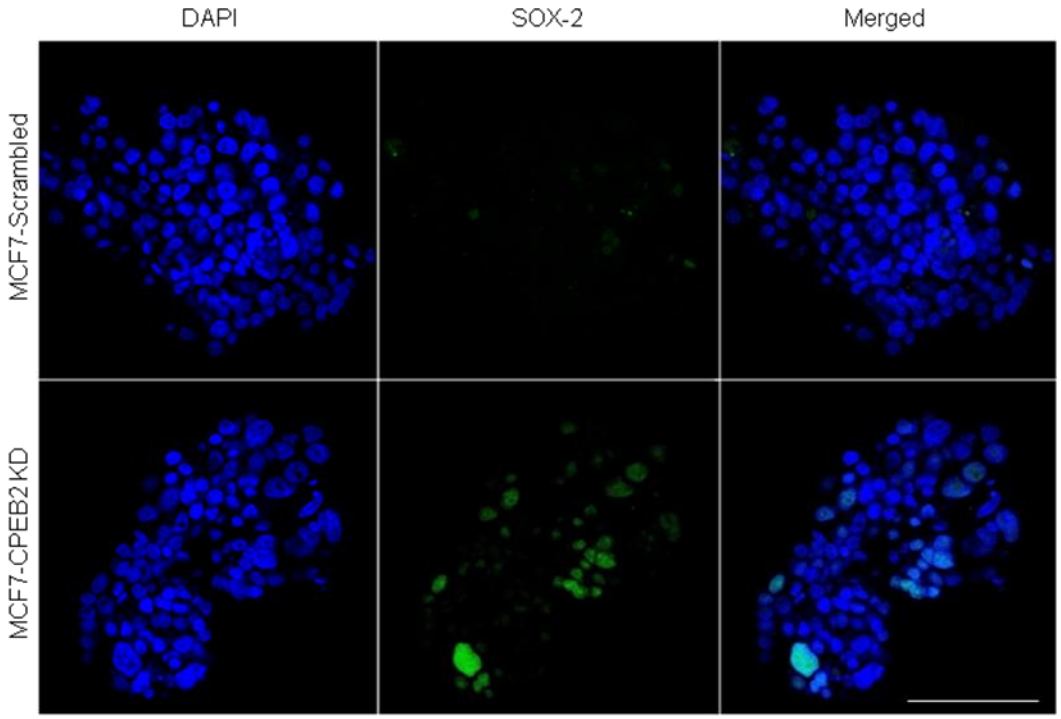

Ratio of Nanog positive cells to total number of cells in tumourspheres normalized to MCF7-Scrambled
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
MCF7-Scrambled
MCF7-CPEB2 KD

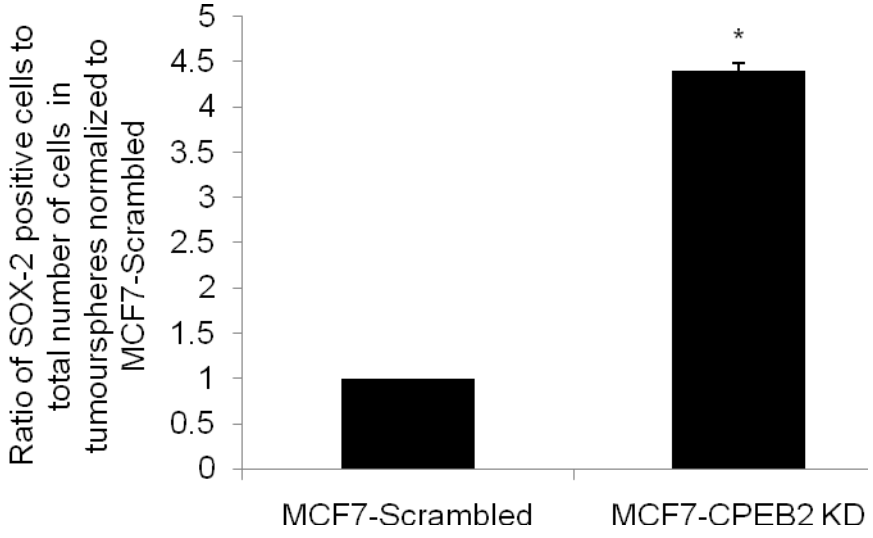
Figure 16. MCF7-CPEB2 KD tumourspheres have increased SOX-2 expression

A. SOX-2 antibody (1:300 in 4% BSA in PBS) was added onto MCF7-Scrambled and CPEB2 KD tumourspheres. Cells were imaged using an Olympus FV1000 fluorescent microscope. All image panels are at the same magnification (scale bar: 100 µm, bottom right panel). DAPI is shown as blue nuclei staining and SOX-2 is shown as green nuclear staining, and merged shows both. **B.** Quantification of SOX-2 staining. Data are presented as ratio of positive cells to the total number of DAPI+ cells normalized to MCF7-Scrambled controls. MCF7-CPEB2 KD cells demonstrated a 4.4 fold increase in Nanog positive cells compared to MCF7-Scrambled. (*) indicates a significant difference (unpaired t-test, p=0.002).

A.


DAPI
SOX-2
Merged
MCF7-Scrambled
MCF7-CPEB2 KD

B.


Ratio of SOX-2 positive cells to total number of cells in tumourspheres normalized to MCF7-Scrambled
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
MCF7-Scrambled
MCF7-CPEB2 KD

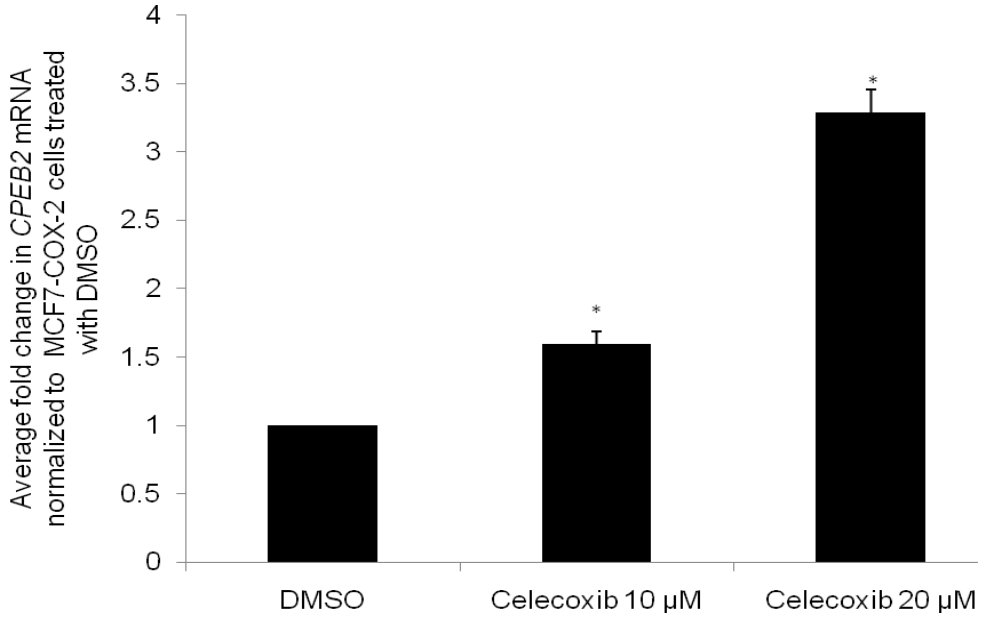
3.7 COX-2 and EP4 inhibition increased CPEB2 expression

We have previously shown that blocking COX-2 activity and EP4 signaling resulted in reduced angiogenesis, lymphangiogenesis, tumour growth and metastasis to the lungs (Xin et al., 2012). Furthermore, MCF7 cells treated with PGE_2 and selective EP4 agonist demonstrated an increase in miR-655 and miR-526b levels, while treatment with COX-2 inhibitor and EP4 antagonists resulted in decreased miRNA levels (Majumder et al., 2015; Majumder, Postovit, et al., 2014). To examine the ability of COX-2 activity and EP4 signaling to modulate CPEB2 expression, MCF7-COX-2 cells were treated with selective COX-2 inhibitor (Celecoxib) and EP4 antagonist (ONO-AE3-208, ONO), while MCF7 cells were treated with PGE_2 (EP1-4 ligand) and EP4 agonist (PGE_1OH). Figure 17 demonstrates that MCF7-COX-2 cells treated with Celecoxib had significantly higher *CPEB2* expressions at both 10 µM and 20 µM compared to DMSO treated cells. At the same time, MCF7-COX2 cells treated with ONO also had significantly higher expression of CPEB2 at 20 µM compared to DMSO treated cells (Figure 17). Surprisingly, MCF7 cells treated with PGE_2 and PGE_1OH demonstrated no change in CPEB2 expression compared to EtOH treated controls (Figure 18). Therefore, we were able to demonstrate that only blocking COX-2 activity and EP4 signaling could modulate *CPEB2* expression.

Figure 17. Cells treated with EP4 antagonist and COX-2 specific inhibitors have increased *CPEB2* expression

MCF7-COX-2 cells were treated with **A.** selective COX-2 inhibitor Celecoxib, or **B.** EP4 Antagonist ONO-AE3-208 at two different concentrations (10 μM and 20 μM), and compared to DMSO (0.0003%) control after for 24 hours culture. MCF7-COX-2 cells treated with Celecoxib at 10 μM and 20 μM showed a 1.6 and 3.3 fold increase in *CPEB2* expression compared to DMSO (one-way ANOVA, Tukey's post hoc test, $p<0.0001$). MCF7-COX-2 cells treated with ONO at 20 μM showed a 1.25 fold increase in *CPEB2* expression compared to DMSO treated cells (one-way ANOVA, Tukey's post hoc test, $p=0.0391$). The data are presented as average fold change normalized to DMSO treated cells ± SEM for 3 experiments. (*) indicates a significant difference.

A.


Average fold change in CPEB2 mRNA normalized to MCF7-COX-2 cells treated with DMSO
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
DMSO
Celecoxib 10 μM
Celecoxib 20 μM

B.

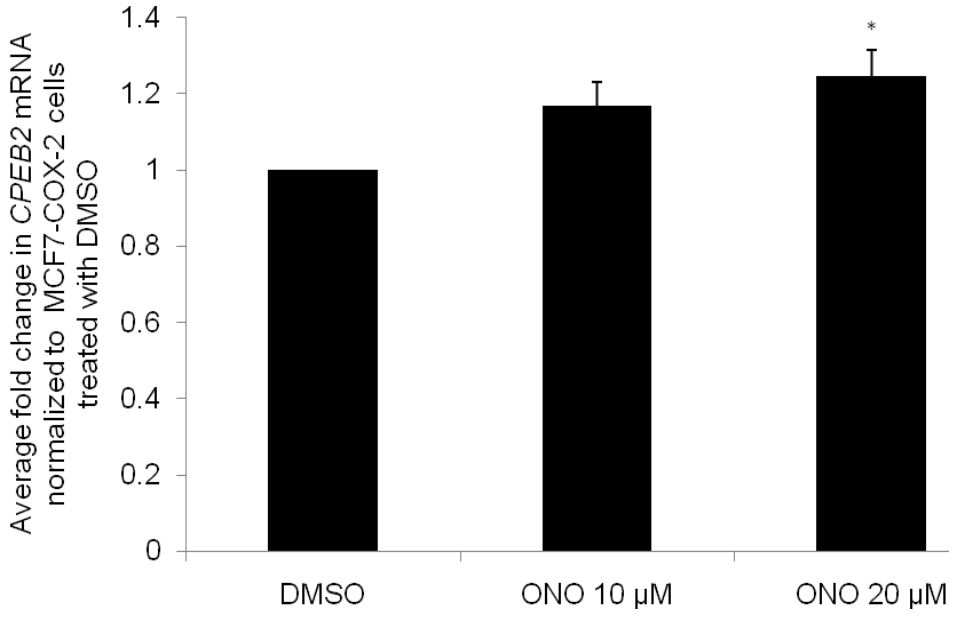
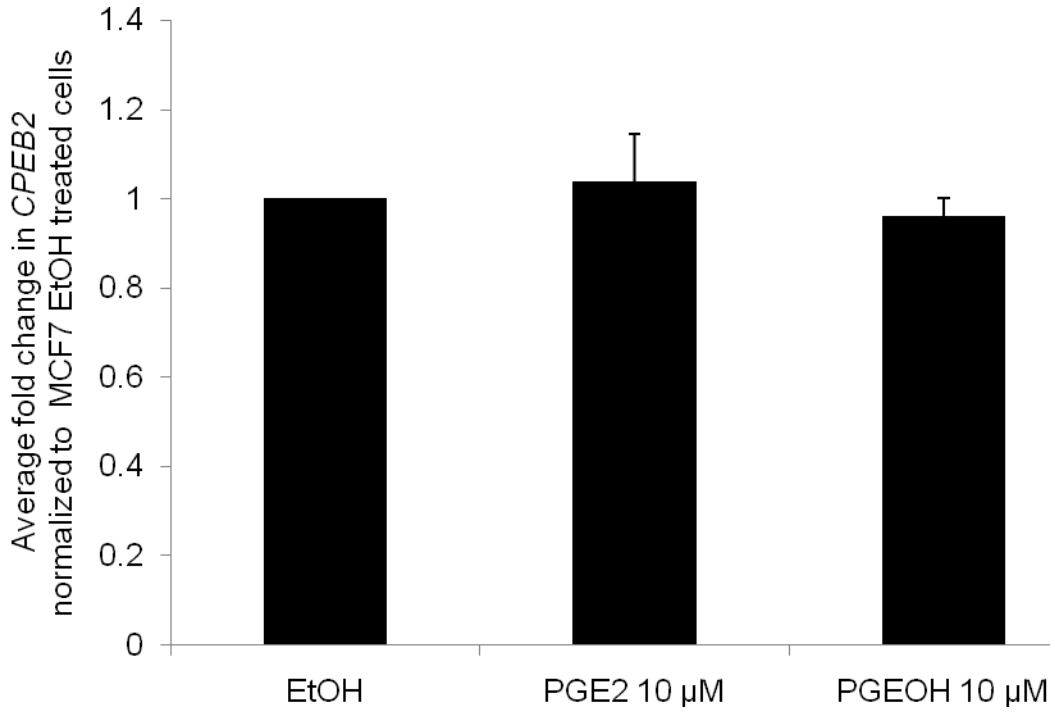

Average fold change in CPEB2 mRNA normalized to MCF7-COX-2 cells treated with DMSO
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
DMSO
ONO 10 μM
ONO 20 μM

Figure 18. Cells treated with PGE2 and EP4 Agonist demonstrated no change in CPEB2 expression

MCF7 cells were treated with 10 µM PGE_2 (EP1-4 ligand), 10 µM PGE_1OH (EP4 agonist), and compared to 0.13% EtOH control after 24 hours culture. There were no significant changes in *CPEB2* expression in either treatment. The data are presented as average fold change normalized to EtOH treated cells ± SEM for 3 experiments (one-way ANOVA, p=0.7065).



4 DISCUSSION AND CONCLUSIONS

4.1 Summary of Findings and Conclusions

Objective 1: To investigate if there is an inverse relationship between expression levels of a) CPEB2 and COX-2 and b) CPEB2 and miR-655 or miR-526b in human breast cancer cell lines.

Expression of COX-2, miR-655, miR-526b and CPEB2 were tested in MCF7, SKBR3, MDAMB231 and MCF7-COX2 cell lines. High COX-2 cell lines such as MCF7-COX-2 and MDA-MB-231 demonstrated high expression of miR-655 and miR-526b and low expression of CPEB-2 compared to low COX-2 cell lines such as MCF7 and SKBR3. The observed association between high COX-2, high miRNA and low CPEB2 expression, validates the inverse relationship demonstrated by the differential gene and miRNA microarray analysis (Majumder et al., 2015).

Objective 2: To investigate the functional role of CPEB2 in human breast cancer.

CPEB2 expression was down-regulated using siRNAs in MCF7 (low COX-2/miRNAs) cells. The resultant MCF7-CPEB2 KD cells demonstrated increased migration and invasion in transwell assays, with no change in cellular proliferation rates. Furthermore, CPEB2 down-regulation was also associated with an EMT phenotype where E-Cadherin protein decreased and Twist1 mRNA and protein increased. There was also an increase in ZEB1 mRNA. Additionally, MCF7-CPEB2 KD cells displayed increased tumoursphere forming ability in ultra-low attachment plates. When tested for different SLC-associated maker expression, CPEB2 KD cells had a higher frequency of ALDH1, Nanog and SOX-

2 expressing cells. These data suggest that CPEB2 is anti-migratory and anti-invasive in vitro, and down-regulation of CPEB2 results in a more aggressive breast cancer phenotype. Thus, CPEB2 expression can also be implicated in the metastatic cascade during COX-2 mediated breast cancer progression.

Objective 3: To investigate the effects of COX-2 inhibitor, and PGE_2 (EP1-4 ligand), EP4 agonist and antagonist treatments on CPEB2 levels.

MCF7-COX-2 cells were first treated with Celecoxib (selective COX-2 inhibitor) and ONO-AE3-208 (selective EP4 antagonist), and DMSO control. Both treatments resulted in an increase in CPEB2 expression compared to DMSO treated cells, as expected. Thus CPEB2 expression in high COX-2 cells is dependent on both COX-2 and EP4 activity. Surprisingly, activating the EP receptors (with PGE_2) and EP4 receptor (with selective agonist PGE_1OH) in low COX-2/high CPEB2 cells resulted in no significant change in CPEB2 expression. These data suggest that down-regulation of CPEB2 in high CPEB2 expressing cells is possibly regulated by alternate signaling pathways outside of EP activity. Therefore, it is likely that cells might have different mechanisms of up-regulating and down-regulating CPEB2.

4.2 Contribution to the Current Field of Research

The role of COX-2 in cancer progression is widely recognized. *In vivo* mouse studies have shown that deletion of *COX-2* results in reduced intestinal tumourigenesis (Oshima et al., 1996). At the same time, patients who were treated with COX-2 inhibitors or low-dose aspirin demonstrated a decrease in the number of polyps formed and fatal colon cancer (Thun et al., 1991). In breast cancer, high COX-2 has been associated with an aggressive breast cancer phenotype, with increased invasion, and decreased disease free survival rates (Barnes, Haywood, Flint, Knox, & Bundred, 2006; Larkins, Nowell, Singh, & Sanford, 2006; Majumder, Postovit, et al., 2014). Additionally, COX-2 inhibitors in breast cancer displayed chemo-preventive properties (Harris et al., 2000; Harris, 2009; Majumder, Xin, Liu, Girish, et al., 2014; Xin et al., 2012). Unfortunately, use of COX-2 inhibitors for a prolonged period revealed serious thromboembolic side-effects in a subset of patients (Fitzgerald, 2004; Nussmeier et al., 2005). Therefore, our lab is investigating downstream effectors of the COX-2 pathway as potential therapeutic targets in high COX-2 breast cancer.

Our laboratory has previously established that COX-2 exerts its effects through over-production of PGE_2 activating the prostanoid receptor EP4 (Majumder, Xin, Liu, Girish, et al., 2014; Timoshenko et al., 2004, 2003; Xin et al., 2012). Both increased COX-2 activity and stimulation of EP4 resulted in increased expression of miR-655 and miR-526b in breast cancer cells. Both miRNAs revealed similar oncogenic properties including SLC induction *in vitro* (Dunn et al., 2014; Majumder et al., 2015). MiRNAs play an important role in cancer. These small non-coding RNA molecules bind and down-regulate the expression of certain target genes. Since we have found both miR-

526b and miR-655 to be oncogenic in our lab, by extension we believe that their target gene, CPEB2, should have tumour-suppressor-like functions. The underlying purpose of this study was to identify the functional role of CPEB2 in breast cancer progression.

4.3 Role of CPEB2 in breast cancer cell migration, invasion, SLC induction and EMT

We observed that high COX2, high miRNA breast cancer cell lines have decreased CPEB2. Both COX-2 induced miR-655 and miR-526b have been shown to increase cellular migration and invasion. Therefore, we predicted that the gene target CPEB2 should be anti-migratory and anti-invasive. As expected CPEB2 KD cells showed increased migration and invasion in transwell assays, supporting our hypothesis that CPEB2 plays an anti-oncogenic role in breast cancer. Interestingly, a recent study has shown that miR-550a is pro-metastatic in hepatocellular carcinoma (Tian et al., 2012). MiR-550a binds to and down-regulates CPEB4. Gain-of function studies have demonstrated that CPEB4 is able to suppress miR-550a induced migration and invasion (Tian et al., 2012). Additionally, miR-92 and miR-26 both bind to the 3'UTR of CPEB2, suggesting that CPEB2 contains functional miRNA binding sites (Morgan et al., 2010).

In a previous study, CPEB1 was shown to be SLC suppressive. Ectopic CPEB1 expression induced differentiation of glioma stem cells and reduced spheroid formation (Yin et al., 2014). In addition, CPEB1 was shown to reduce the expression of SLC markers SOX-2 and Nestin and increase expression of differentiation marker GFAP (Yin et al., 2014). In the present project, we extensively explored the ability of CPEB2 KD to

increase SLC properties of breast cancer cells *in vitro*. We found that CPEB2 KD cells had increased ability to form spheroids, and displayed increased expression of SOX-2, Nanog and ALDH1. Thus, we demonstrate for the first time that CPEB2 suppresses SLC phenotype in human breast cancer.

EMT has also been implicated with increased invasion, metastasis and cancer progression (Thiery, 2002), and has more recently associated with SLCs (Mani et al., 2008). Cells transfected with Snail and Twist1 over-expressing plasmids had increased tumoursphere forming ability and were enriched for stem cell markers indicated by a $CD44^{high}/CD24^{low}$ cells. Conversely, mammary epithelial cells sorted for $CD44^{high}/CD24^{low}$ population showed high expression levels of EMT-associated genes, for instance low levels of *CDH1* and high levels of *CDH2* (Mani et al., 2008). In our studies, we found that CPEB2 KD decreased expression of E-Cadherin protein and increased expression of mesenchymal marker Twist1. Thus, CPEB2 KD cells were enriched in both SLCs and resembled an EMT-like phenotype. A previous study demonstrated that CPEB2 is a negative regulator of EMT progression and Twist1 expression (Nairismägi et al., 2012). EMT phenotype is considered to be a promoter of cell motility. Present observation of increased migration may be a consequence of EMT in CPEB2 KD cells. Taken together, CPEB2 stimulation might represent an important target for the prevention of cancer progression.

4.4 Role of COX-2 activity, EP4 Agonists and Antagonists in breast cancer

Multiple studies from other laboratories and ours have demonstrated the importance of EP4 signaling in COX-2 mediated breast cancer progression. EP4 is a downstream target in the COX-2 pathway that avoids the disruption of prostanoids such as PGI_2 and associated adverse cardiovascular events (Graham, 2006). As such EP4 represents a promising therapeutic target in breast cancer. EP4 has previously been shown to regulate proliferation, invasion and immune-suppression of natural killer cells (Ma et al., 2013; Robertson et al., 2008). Furthermore, the EP4 receptor has been linked to angiogenesis, lymphangiogenesis and metastasis (Timoshenko et al., 2006; Xin et al., 2012). COX-2 up-regulation of EP4 mediated signaling also increases the expression of oncogenic miR-526b and miR-655 (Majumder et al., 2015; Majumder, Postovit, et al., 2014). By extension, we expected that EP4 antagonists should increase CPEB2 levels in high COX-2 cells. MCF7-COX-2 cells were treated with Celecoxib (selective COX-2 inhibitor) and ONO-AE3-208 (EP4 antagonist). As expected, both treatments in high COX-2 cells displayed high *CPEB2* mRNA levels. Interestingly, Celecoxib induced a greater up-regulation of *CPEB2* than ONO-AE3-208 suggesting that *CPEB2* expression might be modulated through other EP receptors as well. Surprisingly, treatment with PGE_2 and EP4 agonist in high CPEB2 MCF7 cells could not suppress CPEB2 expression. Thus, it is likely that EP4 signaling is just one of the players regulating CPEB2 expression in CPEB2 high cells, suggesting that CPEB2 up-regulation and down-regulation have different mechanisms. Since we could demonstrated an increase in CPEB2 expression following EP4 antagonist treatment, low CPEB2 might be used as a marker for EP4

targeted therapy used in conjunction with traditional therapy in high COX-2 breast cancer.

4.5 Limitations of the Study

CPEB2 gene manipulations were only tested in one cell line, MCF7, which is a poorly metastatic breast cancer cell line. Ideally inclusion of a non-tumourgenic mammary epithelial cell line such as MCF10A would strengthen the present study. Furthermore, a naturally occurring CPEB2 low cell line, such as MDAMB231, could also have been manipulated to examine CPEB2 gain-of-function effects. Another limitation of the present study is that CPEB2 levels were measured at the RNA level and not protein level. Unfortunately, at this time there is no reliable and commercially available antibody for CPEB2, therefore changes at the protein level could not be addressed. There are currently six different predicted isoforms of *CPEB2* (*CPEB2* A-F, Table 4). Thus, deciphering the role of each isoform will be an interesting and important part of future studies to understand the role of CPEB2 in breast cancer.

Our proliferation assay using 6 h BrdU uptake did not reveal any change in proliferation in CPEB2 KD cells compared to control cells. The results would have been strengthened by an additional assay such as temporal changes in viable cell numbers for a longer period such as 72 hours using a MTT assay.

To examine the SLC phenotype, an *in vitro* assay for spheroid formation efficiency was conducted. This was supplemented with immuno-fluorescence images at both monolayer and tumoursphere for SLC markers ALDH1, Nanog and SOX-2. This section can be

further improved by an examination of SLC markers at the transcription level and measurements of ALDH activity by flow cytometry.

Another aspect of the project that can be further explored is the role of the miR-655 and miR-526b on CPEB2 gene manipulation. By looking at the different breast cancer cell lines, we can conclude that high COX-2, high miRNA cell lines have low CPEB2 expression. However, we cannot assume that the miRNAs directly target CPEB2. A luciferase reporter assay can properly validate that the miRNAs target and down-regulate CPEB2 expression.

4.6 Future Directions: Role of CPEB2 in Hypoxia

Cancer cells with a rapid proliferation rate expand into a tumour mass. Eventually the middle of the mass experiences cell death as the rate of oxygen diffusion is not able to keep up with the rapidly expanding mass (Brahimi-Horn, Chiche, & Pouysségur, 2007). One mechanism through which cancer cells adapt to low oxygen levels is by the up-regulation of (HIF)-1. HIF-1 is composed of two transcription factors, inducible HIF-1α and constitutively expressed HIF-1β. These transcription factors regulate a number of genes that promote cell-survival such as angiongenic factors like VEGF (Brahimi-Horn et al., 2007; Jung, Isaacs, Lee, Trepel, & Neckers, 2003).

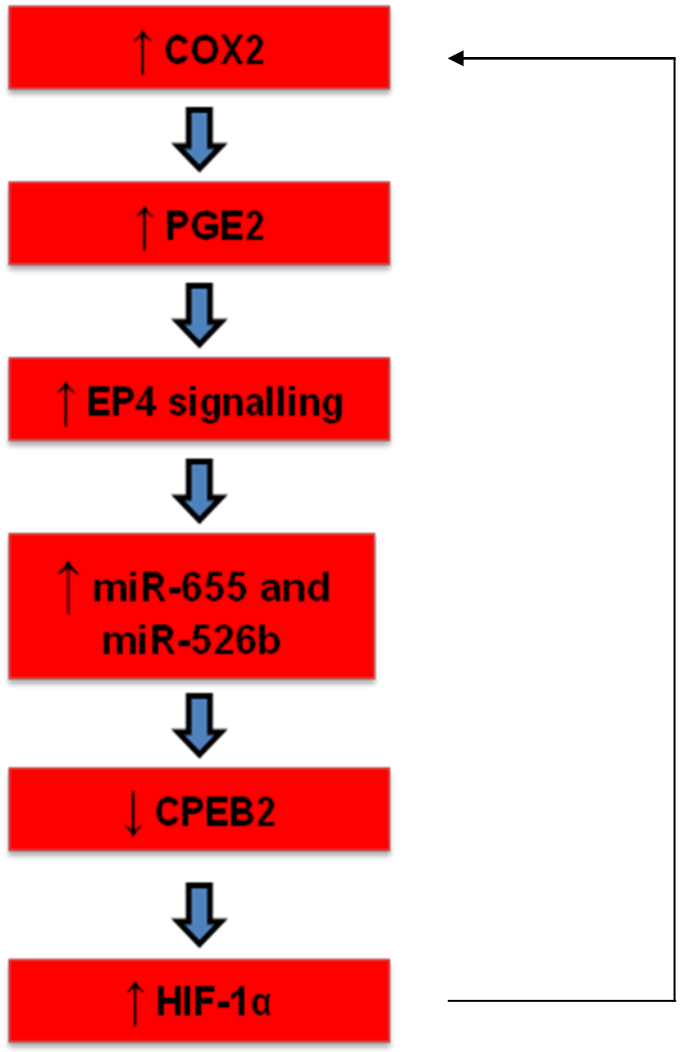
Colorectal carcinoma cell lines exposed to hypoxic condition demonstrated an expected increase HIF-1α (Kaidi, Qualtrough, Williams, & Paraskeva, 2006). Interestingly COX-2 protein was also up-regulated in these hypoxic conditions (Kaidi et al., 2006). A complementary study determined that COX-2 contains a functional binding site for HIF-1α (Csiki et al., 2006; Kaidi et al., 2006). Furthermore, stimulation with PGE_2 has been shown to increase HIF-1α protein (Csiki et al., 2006). These results establish a link between COX-2 and HIF-1α.

As mentioned previously, CPEB2 binds to the 3’ UTR of HIF-1α and modulates translation of the protein (Chen & Huang, 2012; Hägele et al., 2009). In normal conditions, CPEB2 interacts with eEF2 and the 3’UTR of HIF-1α, to suppress translation of the protein (Chen & Huang, 2012). In stressed conditions, CPEB2 dissociates from eEF2 and HIF-1α, allowing the translation of HIF-1α protein (Chen & Huang, 2012).

Taking the results from our study into account, it is possible that COX-2 up-regulation suppresses CPEB2, via PGE_2, EP4 and oncogenic miR-526b and miR-655, and by doing so increases HIF-1α production in breast cancer. HIF-1α has been implicated in a positive feedback loop, where it up-regulates COX-2. This in turn can suppress CPEB2 levels and maintain translation of HIF-1α protein. Therefore, it would be interesting to test if there is increased COX-2, decreased CPEB2 and increased HIF-1α in a hypoxic environment.

Figure 19. Possible mechanism of COX-2, CPEB2 and HIF-1α Pathway in Hypoxia

Up-regulation of COX-2 enzyme results in increased prostanoid production, more specifically increased PGE_2. PGE_2 can bind to four EP receptors, including EP4. Increased EP4 signaling results in the production of two oncogenic miRNAs in breast cancer, miR-655 and miR-526b. Both oncogenic miRNAs have target sites on CPEB2. It is predicted that increased miRNA production results in decreased CPEB2 expression. CPEB2 is a translational repressor of HIF-1α. With reduced CPEB2 levels, repression of HIF-1α is alleviated. HIF-1α has binding sites on COX-2, and participates in a feed forward loop which results in increased COX-2 production (Jung et al., 2003; Kaidi et al., 2006; Majumder et al., 2015).


↑ COX2
↑ PGE2
↑ EP4 signalling
↑ miR-655 and miR-526b
↓ CPEB2
↑ HIF-1α

4.7 Conclusions

In this study it was observed that high COX-2 and high miRNA (miR-655 and miR-526b) expressing breast cancer cells lines have low CPEB2 expression. As determined by *in vitro* assays, CPEB2 KD cells revealed increased cellular migration and invasion, without any effect on proliferation. For the first time, we showed that CPEB2 KD has a role in SLC induction, indicated by increased tumoursphere formation and expression of SLC markers ALDH1, Nanog and SOX-2. Also, CPEB2 KD cells exhibited EMT demonstrated by reduced E-Cadherin protein levels and increased Twist1 expression. Our results also reveal that CPEB2 expression could be reduced by blocking EP4 signaling with antagonists. These results suggest that CPEB2 is a tumour-suppressor-like gene and down-regulation of this gene results in an aggressive breast cancer phenotype.

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6 APPENDICES

Table 1: Summary of breast cancer cell lines used in this project (ATCC, 2015; Chavez, Garimella, & Lipkowitz, 2010; Hackett et al., 1977; Holliday & Speirs, 2011; Kao et al., 2009; Lehmann et al., 2011)

Cell Line	Classification	Estrogen receptor (ER)	Progesterone receptor (PR)	HER2	Cell Type	Disease	Tissue
HS578T	Basal B	—	—	—	epithelial	adenocarcinoma	mammary gland/breast
MCF7	Luminal A	+	+	—	epithelial	adenocarcinoma	mammary gland, breast; derived from metastatic site: pleural effusion
MDAMB231	Basal B	—	—	—	epithelial	adenocarcinoma	mammary gland/breast; derived from metastatic site: pleural effusion
SKBR3	HER2	—	—	+	epithelial	adenocarcinoma	mammary gland/breast; derived from metastatic site: pleural effusion
T47D	Luminal A	+	+	—	epithelial	ductal carcinoma	mammary gland; derived from metastatic site: pleural effusion

Table 2: Probes used for real-time PCR

Probe	Company	Catalog Number	Assay ID
CPEB2 TaqMan® Gene Expression Assay	Life Technologies	4331182	Hs01039673_m1
hsa-miR-526b TaqMan® MicroRNA Assay	Life Technologies	4427975	2382
hsa-miR-655 TaqMan® MicroRNA Assay	Life Technologies	4427975	1612
PTGS-2 (COX2) TaqMan® Gene Expression Assay	Life Technologies	4427975	Hs00153133_m1
RNU44 TaqMan® Gene Expression Assay	Life Technologies	4427975	1094
RNU48 TaqMan® MicroRNA Assay	Life Technologies	4427975	1006
RPL5 TaqMan® Gene Expression Assay	Life Technologies	4331182	Hs03044958_g1
SNAI1 TaqMan® Gene Expression Assay	Life Technologies	4331182	Hs00195591_m1
TWIST1 TaqMan® Gene Expression Assay	Life Technologies	4331182	Hs01675818_s1
VIM (Vimentin) TaqMan® Gene Expression Assay	Life Technologies	4331182	Hs00185584_m1
ZEB1TaqMan® Gene Expression Assay	Life Technologies	4331182	Hs00232783_m1

Table 3: Antibodies used for IF

Antibody	**Raised in**	**Dilution (1:x)**	**Company**	**Catalog Number**
E-Cadherin	Rabbit	1000	Cell Signaling Technology	31955
SOX-2	Mouse	300	BD Biosciences	561593
Twist1	Rabbit	500	Santa Cruz Biotechnology	SC_15393
Vimentin	Mouse	500	EMD Millipore	MAB3400
CF488A anti-Mouse IgG (H+L)	Goat	400	Biotium	20018-1
CF488A anti-Rabbit IgG (H+L)	Goat	400	Biotium	20019-1

Table 4: CPEB2 Isoforms based on BLAST and UniProt (Universal Protein Resource) search. All isoforms are compared to CPEB2 isoform D

Isoforms	**Nucleic acids**	**Amino acids**	**Difference from isoform D**	**Molecular Weight (kDa)**
isoform D	*6878 bp*	*1034 aa*	*The same*	*109.8 kDa*
isoform A	6764 bp	559 aa	Missing sequences 1950-2034	61.7 kDa
isoform B	6854 bp	589 aa	Missing sequences 2175-2199	64.9 kDa
isoform C	6797 bp	1007 aa	Missing sequences 1950-2034	106.9 kDa
isoform E	6788 bp	1007 aa	Missing sequences 1950-2034	106.9 kDa
isoform F	6773 bp	562 aa	Missing sequences 1950-2034	62.1 kDa

Table 5: Abstract of COX-2/EP4/Notch-Wnt Axis in Breast Cancer Cell Induction Paper (Majumder et al. submitted Oncogene)

Manuscript #	ONC-2015-01468
Current Revision #	0
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Current Stage	All Reviewers Assigned
Title	COX-2/EP4/NOTCH-WNT Axis in Breast Cancer Stem Cell Induction
Running Title	COX-2/EP4 induces breast cancer stem cells
Manuscript Type	Original Article
Special Section	N/A
Manuscript Comment	Yes.
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Abstract	Cancer stem like cells (SLC) resist conventional therapies, necessitating searches for SLC-specific targets. We established that cyclo-oxygenase(COX)-2 expression promotes human breast cancer progression via multiple mechanisms including up-regulation of an oncogenic microRNA by activation of the prostaglandin(PG)E-2 receptor EP4. Present study revealed that COX-2 additionally induces SLCs by EP4-mediated NOTCH and WNT up-regulation. Ectopic COX-2 over-expression in MCF-7 and SKBR-3 human breast cancer cell lines (named MCF-7-COX-2 and SKBR-3-COX-2) resulted in aggressive phenotypes: increased migration/invasion/proliferation, EMT, up-regulation of EP4/VEGF-A/C/D expression and increased SLCs, evidenced by spheroid formation for successive generations in vitro, ALDH activity and co-expression of COX-2/SLC markers. These changes were reversed with a COX-2 inhibitor or EP4 antagonist (EP4A), indicating their dependence on COX-2/EP4 activities. Treating COX-2-low cells with PGE2 or PGE1OH (EP4 agonist), stimulated SLC marker expression and up-regulated NOTCH and WNT pathway genes that could be blocked with PI3K/AKT inhibitors. Supporting above findings, micro-array analysis showed up-regulation of numerous SLC-regulatory and EMT-associated genes in MCF-7-COX-2 cells. MCF-7-COX-2 showed increased orthotopic tumorigenicity and spontaneous multi-organ metastases in NOD/SCID/IL-2Rγ-deficient mice on transplantation for successive generations with limiting cell inocula. They also showed increased lung colonization in NOD/SCID/GUSB-null mice, an effect reversed with EP4 knockdown or EP4A treatment of MCF-7-COX-2 cells. COX-2, EP4 and ALDH1A expression in breast cancer tissues were significantly higher in situ than in non-tumor tissues, and were also correlated with one another. High COX-2/EP4 expression was also associated with poor survival. Thus EP4 represents a novel therapeutic target to eradicate SLCs in breast cancer.
Receiving Editor	Assigned
Techniques	Life sciences techniques, Signal transduction techniques [Stem cells]; Life sciences techniques, Cellular imaging [Confocal microscopy]; Life sciences techniques [Gene expression analysis]; Life sciences techniques, Gene expression analysis [Microarray analysis]; Life sciences techniques [Gene transfer]; Life sciences techniques, Gene transfer [Transfection]; Life sciences techniques, Signal transduction techniques [Tissue culture]; Life sciences techniques, Genomic analysis [Reverse transcriptase polymerase chain reaction]; Life sciences techniques [High throughput screening]; Life sciences techniques, High throughput screening [Flow cytometry]; Life sciences techniques [Cellular imaging]; Life sciences techniques, Experimental organisms [Mouse]; Life sciences

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