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The Role of MicroRNAs in Cyclooxygenase-2 Mediated Breast Cancer Progression

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Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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**THE ROLE OF MICRORNAS IN CYCLOOXYGENASE-2
MEDIATED BREAST CANCER PROGRESSION**

(Thesis format: Monograph)

By

Leanna R. Dunn

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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Entitled:

**The Role of MicroRNAs in Cyclooxygenase-2
Mediated Breast Cancer Progression**

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Chair of the Thesis Examination Board

ABSTRACT

We had shown that overexpression of cyclooxygenase (COX)-2 in human, as well as murine breast cancer cells promotes tumor progression and metastasis by multiple mechanisms: host immune cell inactivation and stimulation of cancer cell migration, invasion, tumor-associated angiogenesis and lymphangiogenesis, which support blood-borne and lymph-borne metastasis. Most of these events resulted from the activation of the prostanoid receptor EP4 by endogenous PGE2. Recently, by stable transfection of COX-2 cDNA into a non-metastatic COX-2 negative human breast cancer cell line, MCF-7, we showed that COX-2 induces phenotypic properties of stem-like or “tumor initiating cells” (TICs) in MCF-7-COX-2 cells, as defined by *in vitro* studies and validated *in vivo*. Through combined gene expression and microRNA (miRNA) micro array analysis, we identified two miRNAs (miR-526b and miR-655) that are up-regulated in MCF-7-COX-2 cells that are associated with a down-regulation of 14 target genes linked to tumor-suppressor functions. We hypothesize that these miRNAs are important for COX-2 mediated TIC associated functions in human breast cancer. As a first step, we validated their expression in several COX-2 disparate human breast cancer cell lines: MCF-7, MCF-7-COX-2, SKBR-3 (HER-2 over-expressing but COX-2 negative) and SKBR-3-COX-2. The expression levels of miR-655 were strongly correlated with COX-2 mRNA expression in these cell lines. Furthermore, the migratory and invasive capacities of the cell lines went hand in hand with miR-655 expression. Expression of miR-655 was markedly inhibited by treating MCF-7-COX-2 cells with a COX-2 inhibitor (NS398) or an EP4 antagonist (ONO-AE3-208), indicating that the expression depended on both COX-2 and EP4 activity. Finally, we discovered that cells derived from tumorspheres exhibited a dramatic increase in COX-2 expression in comparison to the cells grown as monolayer. We also found that the tumorspheres derived from MCF-7, MCF-7-COX-2 and SKBR-3 overexpressed miR-655. These findings, taken together, fortify the notion that COX-2, EP4 and COX-2 induced miR-655 expression play important roles in promoting and maintaining the TIC phenotype in breast cancer cells.

KEYWORDS: Cyclooxygenase-2 (COX-2), EP4 receptor, prostaglandin E2 (PGE2), microRNA, miR-655, cancer stem cells (CSCs), tumor initiating cells (TICs), breast cancer.

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CO-AUTHORSHIP STATEMENT

Dr. Mousumi Majumder produced the MCF-7-COX-2 and SKBR-3-COX-2 cell lines.

She also did the RT-PCR and Western blots in Figure 5 (A) and Figure 6 (A).

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
COX	Cyclooxygenase
CSC	Cancer Stem Cell
DCIS	Ductal Carcinoma <i>In Situ</i>
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DPBS	Dulbecco's Phosphate-Buffered Saline
EBM	Endothelial Basal Medium
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EP	Prostaglandin E Receptor
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFR	Growth Factor Reduced

GPCR	G Protein-Coupled Receptor
HER2	Human Epidermal Growth Factor Receptor 2
hsa-miR-655	Homo Sapiens Micro Ribonucleic Acid 655
IDC	Invasive Ductal Carcinoma
IFN-gamma	Interferon-gamma
ILD	Invasive Lobular Carcinoma
iNOS	Inducible Nitric Oxide Synthase
LCIS	Lobular Carcinoma <i>In Situ</i>
MAb	Monoclonal Antibody
miRNA	Micro Ribonucleic Acid
MMPs	Matrix Metalloproteinases
M-PER	Mammalian Protein Extraction Reagent
mRNA	Messenger Ribonucleic Acid
NF-kB	Nuclear Factor-Kappa B
NS-398	N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide
NSAID	Non-Steroidal Anti-inflammatory Drug
ONO-AE8-208	4-(4cyano-2-(2-(4-fluoronaphthalen-1-yl)propionylamino)phenyl) butyric acid
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGE2	Prostaglandin E2
PGES	Prostaglandin E Synthase
PI3K	Phosphatidylinositol 3-Kinase
PLA2	Phospholipase A2
PLC	Phospholipase C
PR	Progesterone Receptor
Pro-uPA	Prourokinase Plasminogen Activator
RISC	RNA-Induced Silencing Complex

RNA	Ribonucleic Acid
RNU44	Small Nuclear RNA
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SFM	Serum Free Medium
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Tween-20
TIC	Tumor Initiating Cell
VEGF	Vascular Endothelial Growth Factor

CHAPTER ONE: INTRODUCTION

1.1 Breast Cancer: An Overview

Breast cancer is a malignant neoplasm that arises from the breast glandular tissue and often presents uniquely in each patient. Even though there have been advances in breast cancer diagnosis and treatment over the past few years, it is unfortunately the most common cancer among women in North America and there has been an insufficient change in mortality rate once the tumor has metastasized. Of those diagnosed, it is predicted that approximately one third of breast cancer patients will die of the disease given current treatment strategies (Canadian Cancer Statistics, 2011). Therefore, there is a pressing need to develop new treatment strategies and targeted therapies to manage this complex disease.

Breast cancer classification is based on the invasiveness, point of origin in the mammary glandular system, proliferative potential, and hormone receptor status of the neoplasm. Breast cancer may arise from either the ducts, referred to as ductal carcinoma (most common), or the lobules that produce milk, which is referred to as lobular carcinoma. Ductal carcinoma *in situ* (DCIS), or intraductal carcinoma, is breast cancer in the lining of the milk duct that has not yet invaded nearby tissues (Silverstein et al., 1996), but may progress to invasive cancer if left untreated. DCIS usually contain hormone-receptor positive cells that may respond to anti-hormone therapy and therefore have the best prognosis. Invasive ductal carcinoma (IDC) is the invasive variety of ductal carcinoma in which cancer cells have invaded through the basement membrane of the duct wall and into the fatty tissue of the breast, where they may metastasize via the bloodstream or lymphatic system to distant areas in the body (ACS, 2012). Lobular carcinoma *in situ* (LCIS) is a noninvasive form of lobular cancer. Unfortunately, patients

who are diagnosed with LCIS have an increased risk of invasive cancer in the same or both breasts (Simpson et al., 2005). Invasive lobular carcinoma (ILC) is the invasive form of lobular breast cancer and like IDC can metastasize.

Breast cancer is highly curable by lumpectomy or mastectomy and adjuvant therapy, provided that it has not metastasized. Current established treatment strategies to delay the progression of metastasized breast cancer include cytotoxic chemotherapy, radiation therapy and targeted therapies (Hobday and Perez, 2005). Similar to many other types of cancers, breast tumors tend to metastasize initially into the regional lymph nodes, a process that typically is followed by dissemination into distant organs. Metastasis is a multistep process that is initiated by primary tumor cells invading adjacent tissue and entering the vasculature (intravasation) (Fidler, 2003). From the systemic veins, cells will typically be arrested in the pulmonary circulation, to extravasate into the lungs. In the case of the lymphatic system, cells will travel to regional lymph nodes or to the venous system to extravasate into the lungs. In the case of the portal venous system draining the gastrointestinal tract, cells will arrest and extravasate into the liver. However, hemodynamics is not the only determinant of the metastatic site. For example, both breast and prostate cancer can metastasize to bones, best explained by the “seed and soil hypothesis,” initially proposed by Paget in 1889 and revived by Fidler (2003). This hypothesis suggests that tumor cells (“seeds”) interact with a specific organ microenvironment (“soil”), and metastasis occurs when the seed and soil are compatible. For example, chemokines made by the organ are believed to attract cancer cells with the appropriate chemokine receptors (Cheng and Hung, 2009). One well-studied chemokine is CXCL12 and its receptor CXCR4, which have been

shown to be involved in many cancers. CXCL12 is expressed in lung, liver, bone and lymph nodes where metastases are commonly located (Zlotnik, 2006).

Breast cancer classification can also be based on the expression of certain receptors, which influence the severity of the neoplastic disease. The receptors used to classify breast cancer include; estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2). The selective ER/PR+ tumors account for nearly 60-70% of diagnosed breast cancers, with the remaining 30-40% being split between HER2+ breast cancers and triple negative breast cancers (ER-, PR-, HER2-) (Slamon et al., 1989). Breast cancer classified as ER-positive is sensitive to estrogen and estrogen stimulation can lead to increased tumor growth. ER-positive cancer can therefore be treated by blocking this receptor and consequently the effect of this hormone. The selective ER antagonist tamoxifen and aromatase inhibitors are recommended as adjuvant endocrine therapy agents for the treatment of hormone receptor-positive early breast cancer (Sehdev et al., 2009). However, these agents are limited by their toxicity and moderate efficacy. HER2 positive patients have a more aggressive disease, a higher risk that the disease will recur and a higher propensity for metastasis to the brain (Serrano-Olvera et al., 2006). Trastuzumab is a recombinant humanized monoclonal antibody against HER2 protein that blocks the HER2-mediated activation of intracellular kinases and effectors (Valabrega et al., 2007). Although the combination of chemotherapy and trastuzumab prolongs survival in the adjuvant and metastatic settings, the majority of women with HER2+ metastasis disease will develop resistance to trastuzumab within one year of treatment or are not treatable with this agent because of cardiotoxicity. Triple negative breast cancer is considered the worst cancer

type because it tends not to respond to hormonal or HER2 based therapy. Although current treatment strategies are beneficial to some degree, future successful therapeutics must be directed toward multiple targets and individual patient subtypes.

Recently, gene expression profiling has been utilized to classify breast cancer for prognostic evaluation and therapeutic approaches. Breast cancer subtypes are associated with distinctly different gene expression patterns detectable by microarray analysis (Sorlie et al., 2006). Studies have shown that ER-positive and ER-negative breast cancers are distinct diseases at the transcriptomic level and additional subtypes might exist within these groups (Reis-Filho and Pusztai, 2011). Using DNA expression profiling, Sorlie et al. have identified signature genes characterizing two subtypes, *luminal A* and *basal-like*, suggesting that distinct molecular mechanisms might have been pre-programmed at an early stage in these particular subtypes (Sorlie et al., 2006). Gene expression profiling has created new laboratory methods, including a commercial tool called "Oncotype DX", which uses quantitative RT-PCR to measure the expression of 21 genes (16 cancer-related and 5 reference genes). This can be performed with RNA extracted from tissue samples (Paik et al., 2004). "Oncotype DX" provides genetic information that can be directly used to predict the response of therapy and likelihood of recurrence in breast cancer patients with ER-positive disease and therefore aid in their prognosis. These results support the concept that breast cancer subtypes represent biologically distinct diseases that require unique therapeutic approaches.

Breast cancer is a heterogeneous disease and each patient's tumor presents with unique characteristics. This fact has led to a movement towards personalized targeted therapy for the treatment of breast cancer. Targeted therapy utilizes biomarkers and

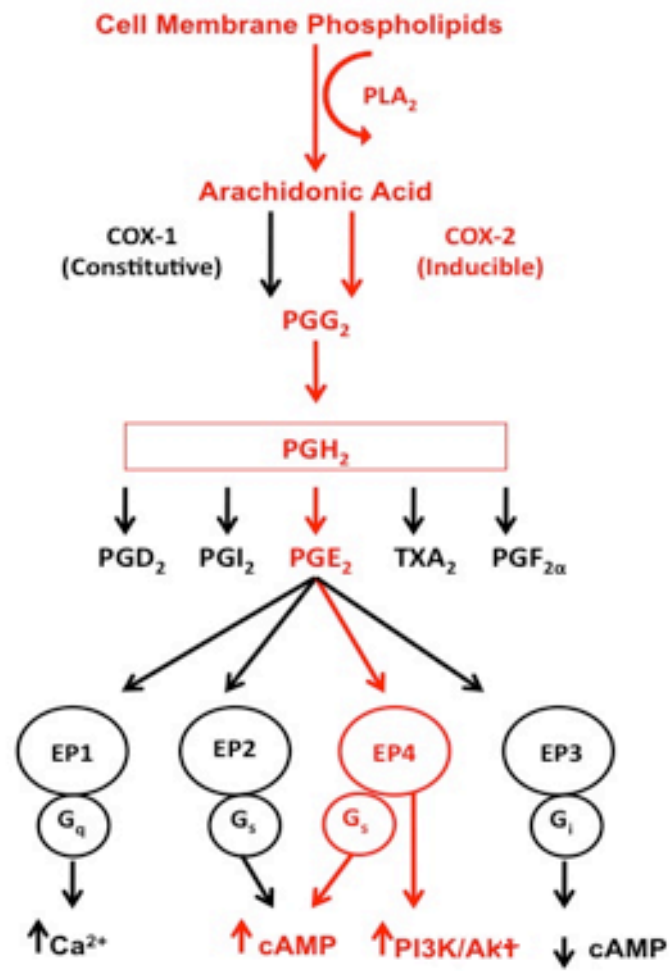
combinations of marker signatures to improve the diagnosis, prognostic classification and prediction of therapeutic benefit and toxicity for individual patients. Two novel therapeutic targets that hold great promise in advancing breast cancer treatment include tumor-initiating cells (TICs) or cancer stem cells (CSCs), and microRNAs (miRNAs). Tumor progression, metastasis, as well as recurrence after therapy-initiated remission are all believed to result from a minor tumor cell subpopulation known as the “Stem” or ‘tumor-initiating cells” (TICs). MicroRNAs (miRNAs) are small regulatory RNAs that are emerging as biomarkers for cancer and other diseases (Khoshnaw et al., 2009). The involvement of TICs and miRNAs in the development of novel targeted breast cancer therapies will be discussed in later chapters.

1.2 Prostanoids

1.2.1 Prostanoid Pathway

The prostaglandin E2 pathway and its mediators are currently under investigation for exploitation in breast cancer therapy (Figure 1). Prostaglandin E2 is a member of the prostanoid family. Prostanoids and leukotrienes are the two major divisions of the bioactive lipid compounds known as eicosanoids. Arachidonic acid (AA) is the precursor of the eicosanoids and is present in membrane bound glycerophospholipids. When cells receive an appropriate stimulus, either a secretory or cytoplasmic phospholipase A2 (PLA₂) is activated to cleave AA from the membrane and the free AA can serve as a substrate for the cyclooxygenase enzymes, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 and COX-2 isozymes catalyze the first two steps in the synthesis of prostanoids from AA (Li et al. 2007). The COX enzymes carry out a two-step reaction, where they first oxidize AA to produce prostaglandin G2 (PGG₂) and

Figure 1. Prostanoid Pathway. Following cellular activation, arachidonic acid (AA) is liberated from membrane phospholipids by phospholipase A2 (PLA₂). AA is then converted to the intermediate precursor prostaglandin G₂ (PGG₂) by the action of cyclooxygenase (COX) enzymes. COX-1 is constitutively active, while COX-2 is inducible following an inflammatory stimulus. COX enzymes contain both COX activity and the peroxidase activity, which converts PGG₂ to prostaglandin H₂ (PGH₂). PGH₂ is then converted by different prostanoid synthases to various prostanoids, including prostaglandin E₂ (PGE₂). PGE₂ elicits various signal transduction pathways by activation of one of the four EP receptor subtypes. All four EP receptors are G protein-coupled receptors. EP1 is coupled with phospholipase c inositol triphosphate signaling, leading to mobilization of intracellular calcium. EP2 and EP4 signaling both leads to activation of adenylate cyclase and increased cyclic AMP, via a G_s protein. The distinctive stimulation of the PI3K/Akt pathway makes EP4 unique from the EP2 receptor. EP3 is coupled to the inhibition of adenylate cyclase, via a G_i protein. (The pathway highlighted in red is the main focus of this project.) (Adapted from: Taketo, 1998, and Gualde & Harizi, 2004).



secondly carry out a hydroperoxidase reaction to convert PGG₂ to prostaglandin H₂ (PGH₂). PGH₂ is the common intermediate for the synthesis of the five primary series-2 prostanoids: prostaglandin D₂ (PGD₂), prostaglandin E₂ (PGE₂), prostaglandin F₂alpha (PGF_{2α}), prostacyclin (PGI₂) and thromboxane A (TXA₂) (Figure 1). The type of prostaglandin produced is dependent on the cell-type specific prostaglandin synthase present, and in the case of PGE₂ the appropriate synthase is prostaglandin E₂ synthase. PGE₂ plays physiological roles in inflammation, vasodilation, bone formation, hemostasis, wound healing, gastrointestinal epithelial integrity, and induction of labor (Li et al., 2007).

1.2.2 Cyclooxygenase (COX)-2 and Cancer Therapy

Three COX isozymes have been identified: constitutive COX-1, the inducible COX-2, and COX-3 (Xie et al., 1991). All mediate the conversion of AA to PGG₂ and then to PGH₂. COX enzymes are located on the luminal side of the endoplasmic reticulum membrane and on both inner and outer membranes of the nuclear envelope (Chandrasekharan and Simmons, 2004), and can free AA from phospholipids on the cytoplasmic side. COXs have short catalytic lifespans because they are autoinactivated. Unfortunately, not much is known about COX-3, other than that it is a splice variant of COX-1. COX-1 is constitutively expressed in virtually all tissues, including platelets, endothelial cells, cells of the gastrointestinal tract, and glomeruli among others (Xie et al., 1991).

COX-2 transcription is inducible and is increased in response to certain growth factors, inflammatory cytokines, tumor promoters or endotoxins (Needleman and Isakson,

1997). It is virtually undetectable in most tissues under basal conditions, but is inhibited at the level of transcription by glucocorticoids (Smith et al., 1994). COX-2 expression has also been reported in certain cells in the brain (Breder et al., 1995), lungs (Harris et al., 1994), kidney (Ermert et al., 1998) and reproductive organs (Lazarus et al., 2004). Since COX-2 is up-regulated in response to growth factors, tumor promoters and inflammatory associated cytokines (Herschman, 1996), it is significantly involved in tumorigenesis. Increased constitutive expression of COX-2 has been shown to be associated with many types of cancers, including breast, colorectal, lung, prostate, cervical, bladder, skin, head and neck, gastric and esophageal (Harris, 2003).

1.2.3 COX Inhibitors

Most cancers studied have increased levels of PGE₂ and other arachidonate products (Karmali, 1980, and Eberhart et al., 1994). Inhibiting various parts of this pathway has shown therapeutic potential for numerous types of cancer, especially colorectal cancer (Wang and Dubois, 2006). Analysis of COX expression in colorectal cancer revealed that COX-2 is up-regulated in 85% of colon carcinomas (Eberhart et al., 1994). Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin inhibit both COX-1 and COX-2 and it is widely accepted that aspirin has an antineoplastic effect on colorectal tumors. Several epidemiological studies (US Preventative Services Force, 2007), randomized controlled trials of colon polyp recurrence (Cole et al., 2009) and randomized trials in patients with hereditary colorectal cancer syndromes (Burn et al., 2011) have shown that NSAIDs reduce incidence of colorectal neoplasia. Recently, Rothwell et al. (2012) showed that aspirin has a chemo-preventative benefit for other

cancer types. They showed that a daily aspirin dose was associated with a 21% reduced risk of cancer death with benefit only apparent after 5 years. In six primary prevention trials, Rothwell and colleagues also showed that low-dose aspirin reduced the risk of cancer incidence by 12% and reduced the risk of cancer with distant metastasis (Rothwell et al., 2012). Since NSAIDs inhibit both COX-1 and COX-2 isoforms, they may cause serious side effects, due to the loss of the physiologically important roles of COX-1, such as inhibiting the production of PGs in the gastrointestinal tract leading to peptic ulcer disease (PUD)(Needleman and Isakson, 1997). This has led to the development of COX-2 specific inhibitor therapies for chronic inflammatory diseases and certain cancers. Celecoxib, a COX-2 specific inhibitor, is now approved adjuvant therapy in the treatment of colorectal cancer. Unfortunately, there are potentially serious side effects of COX-2 inhibitors such as an increased risk of atherothrombotic events, even with short-term use (Kearney et al., 2006). The PGE2 receptors (EP1, EP2, EP3 and EP4) may present as attractive alternatives because of their differential signaling abilities.

1.2.4 COX-2 and Breast Cancer

In breast cancer, elevated COX-2 mRNA-expression has been reported to vary between 50% (Yoshimura et al., 2003) and 100% (Kirkpatrick et al., 2002) in the literature, with most studies reporting elevated expression in all tumors. The proportion of immunohistochemically identified COX-2 positive tumors varies between 4% (Hwang et al., 1998) and 85% (Kelly et al., 2002). COX-2 expression in human breast cancer correlates with reduced survival and also poor prognostic markers, such as increased

tumor size, high tumor grade, negative hormone receptor status, HER2 overexpression (Ristimaki et al., 2002), lymphangiogenesis (Timoshenko et al., 2006) and lymph node metastases (Costa et al., 2002 & Zhang et al., 2008). All of these earlier studies reveal the importance of COX-2 overexpression in the promotion of tumor progression and malignant behavior by multiple mechanisms.

Previous work in our lab has shown that selective COX-2 inhibitors reduced tumor cell migration and invasiveness *in vitro* (Rozic et al., 2001). It was further shown that COX-2 mediated promotion of cancer cell migration was at least in part mediated by endogenous PGE₂, acting primarily via the EP₄ receptor (Timoshenko et al., 2003). The side effects of COX-2 therapy has led to the search for alternate targets in the prostanoid pathway, including the selective inhibition of individual downstream PGE₂ receptors, such as EP₄.

1.2.5 Prostaglandin E₂ (PGE₂) and Breast Cancer

PGE₂ is the prostanoid most implicated in tumor progression, and its involvement is through multiple mechanisms. For example, PGE₂ is involved in chronic inflammation (Schwartzburd, 2003) and has been shown to promote breast cancer progression by inactivation of host anti-tumor immune cells (Parhar & Lala, 1985 and 1986). PGE₂ has also been shown to promote tumor cell migration and invasiveness (Rozic et al., 2001 and Mayoral et al., 2005) and inhibit apoptosis (Munkarah et al., 2002 & Kawamori et al., 2003). Tumor derived PGE₂ resulting from COX-2 expression by murine and human breast cancer cells was also revealed to stimulate tumor-associated angiogenesis (Rozic et al., 2001) and production of lymphangiogenic factors such as

VEGF-C (Timoshenko et al., 2006). PGE2 can also induce actin polymerization and epithelial-mesenchymal transition (EMT), leading to increased metastasis of cancers through activation of the PI3K/Akt pathway (Sheen et al., 2006). Furthermore, COX-2 stimulation of VEGF-C production could also promote breast cancer cell migration by binding to a diverse family of VEGF-C receptors expressed by breast cancer cells (Timoshenko et al., 2007).

1.2.6 EP Receptors

The prostaglandin E (EP) receptor expression profile of a cell determines the effects that PGE2 will exert on it. In mammals, there are four isotypes of G-coupled receptors that can bind to PGE2, known as EP1, EP2, EP3 and EP4 (Figure 1). EP1 is coupled with phospholipase C/inositol signaling, leading to mobilization of intracellular calcium (Tabata et al., 2002). EP2 and EP4 signaling generates increased intracellular cyclic AMP (cAMP) levels (Sugimoto and Narumiya, 2007), while EP3 activation leads to a reduction in intracellular cAMP levels. However, EP2 and EP4 play distinct roles due to the fact that EP4 couples to phosphatidylinositol 3-kinase (PI3K/Akt) and EP2 does not (Sugimoto and Narumiya, 2007 & Sheng et al., 2001). Multiple EP receptors have been implicated in various aspects of breast cancer development and progression: EP1 in mammary carcinogenesis (Kawamori et al., 2001); EP2 in COX-2 induced mammary hyperplasia (Chang et al., 2005); and EP4 in promoting invasiveness in an inflammatory type breast cancer cell line (Robertson et al., 2010). Our laboratory has reported that PGE2 increases migration of breast cancer cells through binding to the EP4 receptor (Timoshenko et al., 2003). EP4 has likewise been shown to be responsible for

an up-regulation of iNOS gene expression under inducible conditions in murine breast cancer cells (Timoshenko et al., 2003). Up-regulation of iNOS expression leads to increased invasive capacity (Timoshenko et al., 2004). COX-2-mediated up-regulation of the lymphangiogenic factor, vascular endothelial growth factor-C (VEGF-C), in human breast cancer cell lines was shown to be at least in part dependent on EP1 and EP4 receptors (Timoshenko et al., 2006). EP4 antagonist ONO-AE3-208, but not EP1 antagonist, was highly and equally effective as the COX-2 inhibitor Celecoxib in inhibiting primary tumor growth, tumor-associated angiogenesis and lymphangiogenesis, and metastasis to the lymph nodes and the lungs in a murine breast cancer model (Xin et al, 2012). EP4 has also been shown to be up-regulated in macrophages in response to lipopolysaccharide (LPS) (Ikegami et al., 2001). The well-defined roles that the EP4 receptor plays in PGE2 mediated breast cancer progression makes targeting this particular receptor for the treatment of breast cancer very promising.

1.3 MicroRNAs (miRNAs)

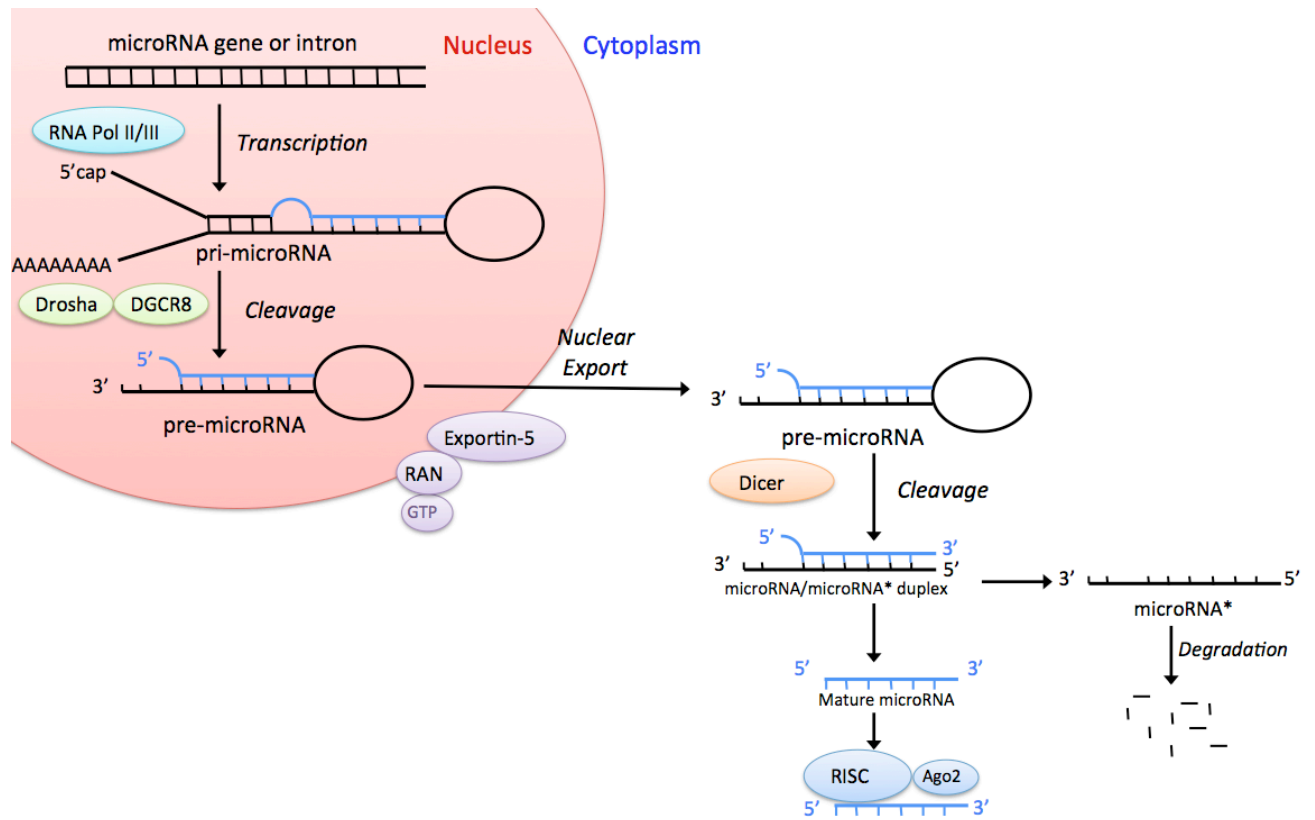
1.3.1 Background: Biogenesis of miRNAs

It is widely acknowledged that alterations in genes encoding proteins play a crucial role in cancer progression. Recent advancements in cancer research have unveiled that non-coding genes also significantly contribute to tumorigenesis (Calin and Croce 2006, & Esquela-Kerscher and Slack 2006), revealing that the genomic complexity of cancer cells is far greater than anticipated. Small non-coding miRNAs are now known to be involved in the initiation and progression of human cancer and this knowledge may have a revolutionary impact on cancer diagnosis, therapy and monitoring. MiRNAs are

small regulatory, evolutionally conserved RNAs of 19-24 nucleotides (nt) in length that lead to silencing of their cognate target genes by post-transcriptionally cleaving their target mRNA or inhibiting their translation (Bartel, 2004). Each miRNA may repress hundreds of mRNA transcripts, but do not exert the same degree of repression on each mRNA. Furthermore, a single mRNA sequence may present numerous miRNA-binding sites. It is estimated that miRNAs could target >30% of the human genome (Lewis et al., 2005) and subsequently directly or indirectly control the expression of thousands of proteins (Lim et al., 2005) and regulate a large portion of the genome (Friedman et al., 2009). Therefore, a disruption in the function of one miRNA could have exponential pathological consequences overall.

MiRNA biogenesis begins in the nucleus where the miRNA gene is transcribed by RNA polymerase II or III to produce primary miRNA (pri-miRNA) (Figure 2). The pri-miRNA is cleaved by the RNase III enzyme Drosha, coupled with its binding partner DGCR8. The resulting precursor hairpin (pre-miRNA), consisting of approximately 70 nt, is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, the RNase enzyme Dicer cleaves the pre-miRNA hairpin into an unstable 19-25 nt miRNA duplex structure. The passenger strand (microRNA*) is then degraded. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) into the RNA-induced silencing complex (RISC). The miRNA-RISC-Ago2 complex then binds to the 3'UTR region of the target mRNA and silences the target mRNA through cleavage or translational repression (Figure 2).

Figure 2. Pathway of MicroRNA (miRNA) biogenesis and action. In the nucleus, the miRNA gene is transcribed by RNA polymerase II or III to produce primary miRNA (pri-miRNA). The pri-miRNA is cleaved by the RNase III enzyme Drosha, coupled with its binding partner DGCR8. The resulting precursor hairpin (pre-miRNA), consisting of approximately 70 nt, is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, the RNase enzyme Dicer cleaves the pre-miRNA hairpin into an unstable 19-25 nt miRNA duplex structure. The passenger strand (microRNA*) is degraded. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) into the RNA-induced silencing complex (RISC). The miRNA-RISC-Ago2 complex then binds to the 3'UTR region of the target mRNA and silences the target mRNA through cleavage or translational repression. (Adapted from: Bartel, 2004 and Kim, 2005)



1.3.2 MiRNAs and Breast Cancer

MiRNAs control a wide array of physiological and pathological processes, including development, metabolism (Poy et al., 2004), differentiation (Chen et al., 2004), cellular proliferation, apoptosis (Bennecke et al., 2003), oncogenesis and metastasis (Filipowicz et al., 2008). This is achieved by modulating the expression of their target genes through imperfect base pairing with the target 3' UTR mRNA and cleaving mRNA molecules or inhibiting their translation (Brennecke et al., 2005). Unique miRNA signatures are found in different tissue types as well as in different tumor types. Calin et al. (2004) have shown that miRNA genes are frequently located at fragile sites or common breaking points in the genome and are susceptible to amplification, deletion, or translocation during the course of tumor development. This supports the idea that miRNAs are extensively involved in cancer. These fascinating findings reveal a promising role for miRNAs in serving as important predictors of disease risk and progression and thereby aiding in the translation of research from the bench to the bedside (Andorfer et al., 2011).

MiRNA expression signatures are being used advantageously to differentiate between tumor subtypes, tumor stage, receptor status, patient survival and the origin of metastatic tumors (Lu et al., 2005). MiRNAs that are abnormally expressed in cancer have great potential as biomarkers in the clinic due to their high resistance to degradation, extremely high stability in whole blood and serum and their ease of extraction and detection in patient samples (Andorfer et al., 2011). In particular, the stability of miRNAs in blood has evoked an emerging interest in utilizing miRNA profiling as a minimally invasive technique to aid in the diagnosis, prognosis and treatment of cancer.

Interestingly, it appears that circulating miRNAs elevated in the blood of cancer patients are derived from the primary tumor as removal of the primary tumor leads to the loss of the elevated levels of these miRNAs (Andorfer et al., 2011).

It has become apparent that alterations in miRNA expression contribute to breast cancer pathogenesis (Calin et al., 2004) and similar to genes, certain miRNAs can be classified either as oncogenic or tumor suppressive. This classification depends upon if the miRNA has the potential to cause cancer or protect cells from transformation into cancer respectively. Some miRNAs are also associated with the invasive and metastatic phenotype of breast cancer cell lines and have been identified in metastatic tumor tissues and lymph nodes (Dumont and Tisty, 2009).

Cumulating data have revealed that miRNAs exert their effects at multiple steps in the metastatic cascade by influencing cancer cell adherence, migration, invasion, motility, and angiogenesis (Dumont and Tisty, 2009). Recent expression profiling has identified breast cancer metastasis-associated miRNAs that are active players in breast cancer invasion and metastasis (Shi et al., 2010). MiRNA MiR-103/107 may directly bind the mRNA that codes Dicer therefore leading to the down-regulation of Dicer and causing extensive suppression of mature miRNAs (Martello et al., 2010). Overexpressing miR-103/107 *in vitro* enhanced cell invasion and migration and promoted metastasis *in vivo* (Martello et al., 2010). Another miRNA believed to play an important role in breast cancer metastasis is miR-31 (Valastyan et al., 2009). Valastyan et al. (2009) overexpressed miR-31 in aggressive SUM-159 human breast cancer cells and reduced invasion by 20-fold and motility by 10-fold *in vivo*. The molecular mechanisms in which miRNAs become deregulated and affect the process of breast cancer metastasis is newly

emerging and holds promising insights into breast cancer pathogenesis.

MiR-155 is another well known miRNA overexpressed in many cancers (Jiang, et al., 2010). MiR-155 overexpression in breast cancer has been found to be correlated with poor prognosis. Jiang et al. (2010) identified ‘suppressor of cytokine signaling 1’ (SOCS1) as a novel target of miR-155 in breast cancer cells. SOCS1 is a tumor suppressor and when miR-155 is overexpressed in breast cancer cells, proliferation, colony formation and xenograft tumor growth is increased via the repression of SOCS1.

One of the best-characterized oncogenic miRNAs is miR-21 (Frankel et al., 2008, Iorio et al., 2005, Si et al., 2007, & Zhu et al., 2007). Inhibition of miR-21 was shown to increase apoptosis, inhibit breast cancer cell growth and survival *in vitro* and *in vivo* (Si et al., 2007). Frankel et al. (2008) and Zhu et al. (2007, 2008) have identified tropomyosin 1 (TPM1), maspin (SERPINB5) and the tumor suppressor gene programmed cell death-4 (PDCD4) as direct targets of miR-21 in breast cancer. These tumor suppressor genes, when down-regulated by miR-21 lead to increased proliferation, growth, migration and invasion. Huang et al. reported that HER2 initiates signaling that leads to miR-21 up-regulation, resulting in down-regulation of PDCD4 and therefore an increased ability of breast tumor cells to invade (Huang et al., 2009).

Members of the let-7 family of miRNAs are known to act as tumor suppressors (Kumar et al., 2008). Let-7 has been found to be frequently down-regulated in human cancer, leading to an up-regulation of the proto-oncogene RAS (Johnson et al., 2005). Let-7 also seems to be involved in the modulation of the cancer stem cell (CSC) phenotype. It has been reported that let-7 miRNAs are under-expressed in CSCs and TICs (Droge and Davey, 2008).

MiRNA profiles are just as heterogeneous as breast cancer tumors themselves. MiRNA profiles have been correlated with pathological variables such as HER2+, ER+, or PR+ status, tumor stage, vascular invasion or proliferation indexes (Andorfer et al., 2011). A recent analysis of 453 known miRNAs in 29 early-stage breast cancer tumors identified predicative signatures corresponding to ER+ (miR-342, miR-299, miR-217, miR-190, miR-135b and miR-218), PR+ (miR-520, miR-377, miR-527-518a, and miR-520f-520c) and HER2+ (miR-520d, miR-181c, miR-302c, miR-376b, and miR-30e) status (Lowery et al., 2009). These subtype specific miRNA signatures will be of significant value to complement current methods of classification (Andorfer et al., 2011).

The development of tailored breast cancer profiles that define a potential relation among circulating miRNAs, disease status, tumor subtype, response to therapy and risk of metastasis is a promising avenue that will help treat individual breast cancer patients more effectively. Although the progress made thus far is truly noteworthy, there is still much that needs to be investigated concerning the aberrant expression of miRNAs in breast cancer and the mechanisms by which they regulate breast cancer progression.

1.4 Cancer Stem Cells

1.4.1. The Cancer Stem Cells (CSC) Hypothesis

Cancer stem cells (CSCs) or “stem-like” cells are a subset of cancer cells that are distinct from the other cells that form the bulk of a tumor. They possess stem cell properties, such as their abilities to self-perpetuate and to produce progenitor cells that undergo multi-lineage differentiation (Wicha et al., 2006). Putative CSCs are sometimes referred to as “tumor-initiating cells” (TICs). The idea that cancer might arise from a rare population of cells with stem cell properties was proposed many years ago (Wicha et al., 2006). CSCs are capable of repopulating the tumor at all stages if not fully eliminated (Wicha et al., 2006). It is believed that conventional chemotherapy kills the bulk of the tumor containing differentiated or differentiating cells, yet CSCs are resistant to such treatment and therefore can lead to recurrence of tumors (Al-Hajj, 2007 & Al-Hajj et al., 2003). Therefore, CSCs have been hypothesized to be the source of cancer. The mechanisms leading to the metastatic dissemination of tumor cells is also thought to be due to this subpopulation of CSCs (Sleeman and Cremers, 2007). In order to develop new successful treatments for cancer it is important to elucidate the characteristics of CSCs and develop therapeutics that can target this tumorigenic CSC population.

Multiple pathways and processes can give rise to CSCs. The molecular pathways that maintain the stem cell phenotype in stem cells are also active in numerous cancers. There are three hypotheses describing how CSCs may arise (Goldthwaite, 2011). First, CSCs may arise by mutation from normal stem cells. The similarity between CSCs and normal stem cells suggests that cancers may arise when some event produces a mutation

in a stem cell, causing it to become deregulated. Secondly, a progenitor cell may undergo two or more mutations that allow it to regain the properties of self-renewal and production of the heterogeneous lineages of cancer cells that comprise a tumor (Jamieson et al., 2004). Lastly, a fully differentiated cell may undergo several mutations that drive it back to a stem-like state. In all three circumstances, the resultant CSC has lost the ability to regulate its own cell division.

1.4.2 CSCs and Breast Cancer

Rare CSCs have been isolated from a number of human tumors, such as hematopoietic, brain, colon, pancreatic, prostate and breast cancers (Farnie and Clarke, 2007). In breast cancer, previous studies have shown that CSC subgroups can be propagated *in vitro* from both primary tumors and established cell lines by culturing the cells as tumor spheres in suspension. Mammary stem cells are able to detach from the extracellular matrix and grow into spheroids and this property has been utilized in a technique that has been successfully used to obtain highly enriched and functional mammary stem cells from breast cancer cell lines (Dontu et al., 2003). One tumor sphere clonally originates from a single breast CSC and usually contains ~300 cells at various levels of differentiation (Dontu et al., 2003). In anchorage-independent conditions breast CSCs also exhibit a CD44⁺CD24^{-/low} surface marker, also noted *in situ*, and can therefore be isolated by immunosorting based on the expression of these two markers (Dontu, 2008). The development of agents that specifically kill breast CSCs has promising efficacy in the treatment of breast cancer. Gupta et al. (2009) looked at the effect of salinomycin, an antibacterial therapeutic drug, on treating breast CSCs. They found that salinomycin reduces the proportion of CSCs in mice by >100-fold relative to paclitaxel,

a commonly used breast cancer chemotherapeutic drug. Thus, analysis of CSCs is a promising avenue to further understand mechanisms underlying tumor cell migration, invasion and metastasis. Concordantly, identifying agents with specific toxicity for CSCs holds great promise in improving breast cancer treatment (Gupta et al., 2009).

Identification of CSC-inducing or sustaining molecules may also unveil innovative therapeutic targets for eliminating CSCs (Tysen, 2010) and maintaining recurrence free survival.

1.4.3 CSCs and miRNAs

Investigating the important roles that miRNAs and CSCs play in various cancers is an innovative and popular area of research. MiRNAs may play a role in the initial stages of breast cancer pathogenesis by regulating the phenotype of CSCs. Yu et al. (2007) looked at miRNA expression in breast TIC's and found that miRNA let-7 was reduced in TICs and increased with cell differentiation. Lentivirus transfection of let-7 into breast cancer TICs reduced proliferation, tumor sphere formation and the proportion of undifferentiated cells. It seemed that let-7 silenced two oncogenes: *RAS* and *HMGA2*. *HMGA2* maintains pluripotency while *RAS* maintains self-renewal, so their overexpression in TICs gives them the stem-like phenotype of being able to self-renew and differentiate (Yu et al., 2007). Shimono et al. (2009) demonstrated that miR-200c repressed the expression of BMI1, a known regulator of stem cell self-renewal. Moreover, overexpression of miR-200c notably inhibited clonogenicity and tumor formation of breast CSCs *in vitro* and *in vivo* (Shimono et al., 2009). These results suggest that miRNAs may play an important role in CSC activity. Hence, further investigation of miRNA expression profiles in CSCs will greatly facilitate the

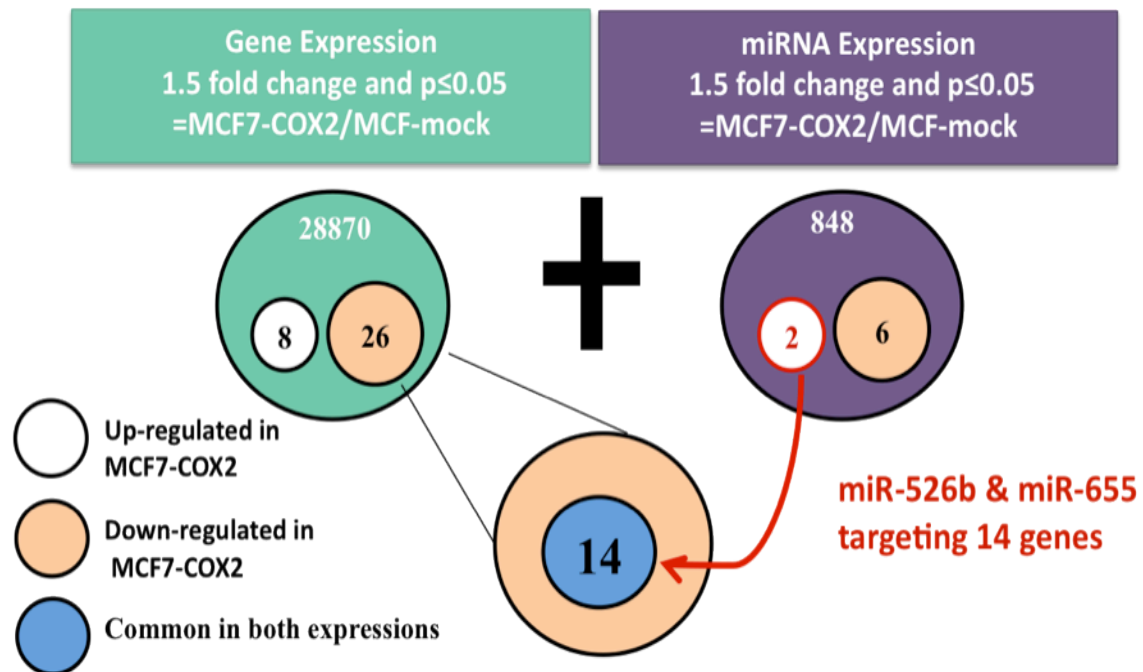
development of novel therapeutic methods targeting the root of tumorigenesis and recurrence.

1.5 Rationale

Preliminary data obtained by Dr. Mousumi Majumder in our laboratory by introducing the COX-2 gene into the non-metastatic MCF-7 (COX-2 negative, HER-2 negative) and into the weakly metastatic SKBR-3 (COX-2 negative, HER-2 overexpressing) human breast cancer cell lines, suggest that COX-2 expression induces and enhances sphere formation (Figure A2). After successful transfection of COX-2 cDNA in MCF-7 and SKBR-3 cells, stable integration of COX-2 was verified by DNA resequencing, quantitative-PCR (qPCR) and Western blotting. The resultant cell lines named MCF-7-COX-2 and SKBR-3-COX-2, when compared with mock (empty vector)-transfected cells, showed: accelerated growth, migration and invasiveness *in vitro* and EMT (down-regulation of E-cadherin and up-regulation of vimentin, quantitated with qPCR); markedly increased TIC content, as indicated by tumor sphere (spheroid) forming ability of single cells in ultra-low attachment plates for successive generations (Figure 3); increased vascular mimicry on matrigel; up-regulation of the PGE2 receptor EP4 and angiogenic and lymphangiogenic factors VEGF- A, C and D (as quantitated by Western blots and qPCR). Treatment of the stable transfected cell lines with selective COX-2 inhibitor or EP4 antagonist abrogated most of the aggressive properties including migration, invasiveness, ability for vascular mimicry as well as TIC-associated tumor sphere forming ability, indicating the need for COX-2 and EP4 activity for these functions.

Through combined gene expression and miRNA micro array analysis, Dr. Majumder has subsequently identified two miRNAs (miR-526b and miR-655) that are up-regulated by stable transfection of the COX-2 gene in the human breast cancer cell line MCF-7 (Figure 3), in association with sphere formation and acquisition of an aggressive phenotype commonly associated with enhanced malignancy. These two COX-2 up-regulated miRNAs were shown to down-regulate 14 target genes linked with tumor-suppressor functions. Since miR-526b and miR-655 appear to be up-regulated by COX-2 in an aggressive breast cancer cell line, they may be classified as oncogenic miRNAs that most likely target tumor-suppressor genes. We suggest that they may potentially contribute to the induction of the TIC phenotype in breast cancer.

Figure 3. Combined Gene Expression (Affymatrix Human Gene Array 1.0 ST) and miRNA (Affymatrix Genechip miRNAs Array) Micro Arrays. Differential gene (28870 genes covered) and microRNA (miRNA) arrays (848 human miRNA covered) were used to identify miRNAs and their target genes that were up- or down-regulated by introducing COX-2 into MCF-7 cells. We identified 26 genes that are down-regulated and 8 genes that are up-regulated in MCF7- COX-2 cells, with high statistical stringency. In the miRNA array only 2 miRNAs were up-regulated and 6 were down-regulated. In combined gene and miRNA data analysis we identified two miRNAs that are up-regulated (≥ 1.5 fold) in MCF7-COX-2 cells that down regulate 14 genes, (≤ 1.5 fold), including TP53, CDK6, INSIG2, and NDRG1 among others (Majumder M et al., 2012).



1.6 Hypothesis and Objectives

Based on the previously summarized results, the **hypothesis** of the present study is that the COX-2 up-regulated miRNA, miR-655, plays an important role in COX-2 and EP4 mediated induction of the TIC phenotype and the progression of human breast cancer. The overall objective will be to examine the regulatory role of COX-2 on the expression of miR-655 (Figure 4) and define the functional role that this miRNA has on the induction of TIC associated properties in human breast cancer cell lines.

The proposed hypothesis will be tested through the following **objectives**:

Use MCF-7, MCF-7-COX-2, SKBR-3 and SKBR-3-COX-2 cell lines:

1. a) To examine the role of COX-2 in breast cancer progression by performing functional assays, including transwell migration and invasion assays and the tumorsphere formation assay.
- b) To validate micro array data by quantifying expression of miR-655.
2. To determine if the expression of miR-655 is dependent on COX-2 or EP4 activity. This will be determined by blocking COX-2 (with NS398) or EP4 activity (with ONO-AE3-208) and then measuring changes in miRNA expression.
3. To determine the effect of expressing miR-655 (in non-expressing breast cancer cell lines) on COX-2 expression, breast cancer cell migration, invasion and proliferation.

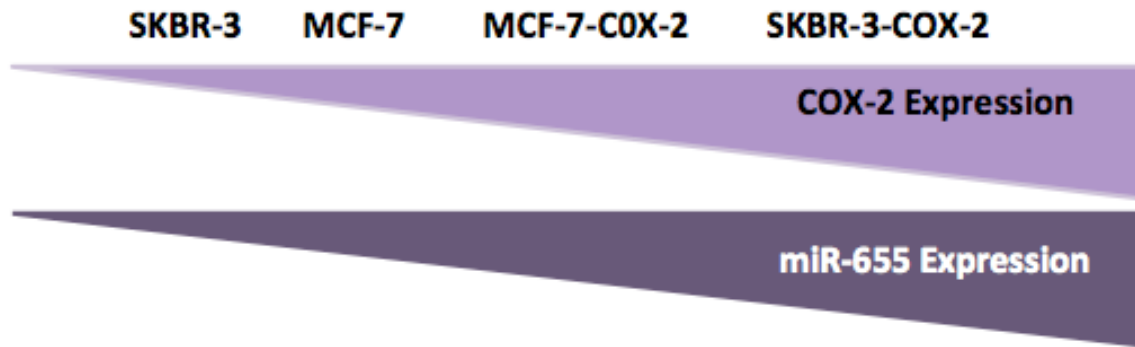


Figure 4. Proposed correlation of cyclooxygenase-2 (COX-2) expression and microRNA expression (miR-655) in the four COX-2 disparate cell lines.

CHAPTER TWO: EXPERIMENTAL PROCEDURES

2.1 Cell Lines and Culture

The MCF-7 human breast cancer cell line is low COX-2 expressing, estrogen receptor (ER) positive HER-2 negative and is non-metastatic in immunodeficient mice. MCF-7 cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD) and were grown as a monolayer in Minimum Essential Medium Eagle (MEME) (ATCC, Rockville, MD) supplemented with 8% fetal bovine serum (FBS), 100U/ml penicillin, and 100ug/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C. The weakly metastatic SKBR-3 human breast cancer cell line, also purchased from ATCC, does not express COX-2 and over-expresses HER-2. SKBR-3 cells were maintained as above, however they were grown in McCoy's 5A Modified Medium with L-glutamine.

In order to stably overexpress COX-2 in MCF-7 and SKBR-3 cells, Dr. Majumder in our lab transfected COX-2 cDNA into MCF-7 and SKBR-3 cell lines. Stable integration of COX-2 was verified by resequencing, quantitative real-time PCR and Western blots. The resultant cell lines named MCF-7-COX-2 and SKBR-3-COX-2, exhibited an increased aggressive phenotype as predicted with high COX-2 expression (see p.26 for details). MCF-7-COX-2 cells were maintained similar to MCF-7 cells and SKBR-3-COX-2 cells were maintained similar to SKBR-3 cells, however Geneticin® (Invitrogen, GIBCO, ON) was added to the media of COX-2 overexpressing cell lines at 500µg/ml to maintain selective pressure for the transfected cells.

2.2 Quantitative Real-time Polymerase Chain Reaction (PCR)

2.2.1 COX-2 mRNA Expression

For RNA extraction and complementary DNA (cDNA) synthesis, cells were grown to 80-90% confluence, trypsinized and collected as cell pellets. Total RNA was extracted using the miRNeasy Minikit (Qiagen, MD) following the manufacture's instructions. The total RNA was quantified with a spectrophotometer (NanoDrop 2000, Thermo Scientific, IL). Synthesis of cDNA from the RNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) with 2µg of RNA per 20µl volume reaction, using a thermo cycler (C2000™, Bio Rad). Quantitative real-time PCR was performed in single micro capillary tubes on a Rotor Gene 3000 (Corbett Research, SF) with TaqMan® Universal PCR Master Mix (Applied Biosystems, CA) for both the housekeeping gene (TaqMan® Gene Expression Assay GAPDH probe, for monolayer cells, or TaqMan® Gene Expression Assay β-Actin probe, for tumor spheres), and the target gene expression (TaqMan® Gene Expression Assay COX-2 probe). Each quantitative real-time PCR reaction was prepared to a volume of 20µl, including 1 µl of the appropriate TaqMan probe, and 2µl of the cDNA. The quantitative real-time PCR profile was 95°C for 15 seconds of denaturation and 58°C for 1 minute of anneal-extension for 40 cycles.

2.2.2 MiR-655 Expression

Quantitative real-time PCR for miR-655 (hsa-miR-655) expression was performed following the previously described protocol (2.2.1) with the following modifications. Total miRNA was extracted from the cells using the miRNeasy Minikit

and the RNeasy MiniElute Cleanup kit (Qiagen, MD) following the manufacturer's instructions. Complementary DNA was synthesized from the extracted miRNA using specific stem-loop primers for the miRNA under investigation (TaqMan® MicroRNA Assays, miR-655 primer) and the endogenous housekeeping miRNA (TaqMan® MicroRNA Assays, RNU 44 primer) and the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, CA) following the manufacturer's protocol. Quantitative real-time PCR was performed in single micro capillary tubes on a Rotor Gene 3000 (Corbett Research, SF) with TaqMan® Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, CA) for both the housekeeping miRNA (TaqMan® MicroRNA Assays, RNU 44 probe) and the target miRNA (TaqMan® MicroRNA Assays, miR-655 probe) as recommended by the manufacturer.

2.2.3 Roles of COX-2 and EP4 Activity on the Expression of miR-655

MCF-7-COX-2 and SKBR-3-COX-2 cells were plated at 1 million cells/well in 6-well plates and incubated overnight in complete MEME and McCoy's 5A respectively. The next day, cells were incubated in serum-free medium for 2 hours and then treatments of 20 μ M selective COX-2 inhibitor NS-398, 2 μ M EP4 antagonist ONO-AE3-208, or control (0.02% DMSO) were added. After 24 hours of treatment, cDNA was synthesized from miRNA extracted from the cells. Quantitative real-time PCR for miR-655 expression was performed following the previously described protocol (2.2.2).

2.2.4 Quantitative Real-time PCR for mRNA or miRNA Expression

Quantitative real-time PCR reactions were performed in triplicate. The delta-delta Ct method was employed for quantification of the fold change in mRNA or miRNA

expression for the samples under investigation. Each sample was repeated in triplicate in each run and the mean Ct (mean cycle threshold) value was calculated for each sample. The Ct value of the endogenous housekeeping gene (either GAPDH/ β -actin for mRNA quantification, or RNU44 for miRNA quantification) was subtracted from the mean Ct value of the sample under investigation to determine the resulting delta Ct value (Δ Ct). The Δ Ct value of the reference sample was then subtracted from the Δ Ct of the sample under investigation to produce the delta-delta Ct value ($\Delta\Delta$ Ct). The fold difference was finally determined ($2^{-\Delta\Delta\text{Ct}}$).

2.3 Western Blot Analysis: COX-2 Protein Expression

Cells were grown to 80-90% confluence in 75cm² flasks (BD falcon, CA). For analysis of total cell lysates, cells were rinsed in ice cold PBS and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, IL) supplemented with HALT protease inhibitor cocktail (Thermo Scientific, IL). After five minutes of shaking on ice, cells were scraped from tissue culture dishes and transferred to 1.5 ml Eppendorf tubes. For tumorspheres (described below in 2.6), cells were pelleted in 1.5 ml Eppendorf tubes, rinsed with ice cold PBS and re-suspended in the M-PER/HALT solution and left to shake on ice for five minutes. Lysates were sonicated (eight pulses at level four) and then centrifuged at 13 000 RCF for 20 minutes at 4°C, to remove cell debris. The supernatants were collected and protein concentration was quantified in triplicate using the Pierce® BCA Protein Assay Kit (Thermo Scientific, IL) following the manufacturer's protocol. Fifteen micrograms of total protein from the cell lysate was run per well on a 1.5 mm 10% SDS-PAGE gel (polyacrylamide gel electrophoresis) at

90V (voltage) for one and a half hours. The proteins were then transferred to an Immobilon-FL PVDF membrane at 5V for 3 hours (semi-dry transfer). After the transfer the membrane was blocked for one hour in a blocking buffer consisting of TBST (20mM tris-base, 0.14M NaCl, 0.01% Tween, pH 7.4) and 2% albumin from bovine serum (BSA) (Sigma-Aldrich®, MO). The membrane was then incubated in a mixture of primary antibodies; for cells grow as a monolayer: mouse monoclonal GAPDH (1:5000) (Millipore, MA) and goat polyclonal COX-2 (1:1000) (Abcam, MA), and for cells grown as tumorspheres: mouse monoclonal β -actin (1: 5000) (Santa Cruz Biotechnology, CA) and goat polyclonal COX-2 (1:1000) (Abcam, MA), diluted in a solution of TBST (pH 7.4) with 2% BSA overnight at 4°C. After being washed in TBST (pH 7.4) three times 15 minutes each, the membrane was probed with a mixture of a donkey anti-goat (1:5000) and donkey anti-mouse (1:10000) IRDye polyclonal secondary antibodies (LI-COR, NE) diluted in a solution of TBST (pH 7.4) with 2% BSA for one hour in the dark. Finally, the membrane was washed three times 15 minutes each in TBST (pH 7.4) before scanning on an Odyssey infrared imaging system (LI-COR, NE).

2.4 Transwell Assays

2.4.1 Cellular Migration

Cellular migration (chemokinesis) was measured using 24-well cell culture chambers (BD Biosciences, CA) fitted with multiporous (8 μ m pore size) polycarbonate membranes (BD Biosciences, CA) (Timoshenko et al., 2006). The uncoated upper chamber (insert) with membrane was filled with 300 μ l of serum-starved cells in suspension (2×10^5 cells/ml) in appropriate media, while the lower chamber (wells)

contained 700 μ l of the same cell free media with no FBS or 2% FBS as indicated. Plates were placed in a humidified CO₂ incubator for 24 hours at 37°C. It has been observed in our lab that the peak point of migration occurs at 24 hours (Timoshenko et al., 2003). The inserts were then removed and the upper surface of the membranes were gently wiped with cotton swabs to remove the non-migratory cells. The membranes were then fixed with methanol and stained with eosin and thiasine and then mounted onto slides. The number of cells appearing on the undersurface of the polycarbonate membranes were counted visually using a light microscope (LEICA DFC 295). For each sample, the cells on the entire membrane were counted and a mean value for each sample was calculated.

2.4.2 Cellular Invasion

Cellular invasion is similar to cellular migration; however, it requires a cell to migrate through an extracellular matrix (ECM) or basement membrane (BME) barrier by enzymatic degradation. Therefore, the multiporous (8 μ m pore size) polycarbonate membranes (BD Biosciences, CA) were coated with Matrigel (BD Biosciences, CA), a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells that resembles the complex extracellular environment found in many tissues (Zhou et al., 2010). Growth Factor Reduced (GFR) Matrigel (BD Biosciences, CA) was thawed overnight at 4°C, diluted with cold media (1:100) and placed on the transwell membranes to solidify. The Matrigel coated upper chamber membranes were then filled with 300 μ l of cell suspension (2×10^5 cells/ml) in appropriate media, while the lower chamber (wells) were filled with 700 μ l of cell free media with either no FBS or 2% FBS

as indicated. The cells were allowed to invade for 48 hours in a humidified CO₂ incubator at 37°C. It has been observed in our lab that the peak point of invasion occurs at 48 hours (Majumder et al., 2012). The membranes were then fixed, stained and quantified as previously described (2.4.2).

2.5 Nucleotransfection with miR-655 Overexpression Plasmid

The MicroRNA Expression Plasmids (purchased from OriGene, MD) were synthesized by cloning the amplified precursor microRNA into OriGene's pCMV6-Mir vector (Appendix figure 1). The expression of the miRNA is driven by the CMV promoter and with the human growth factor 1 poly(A) tailing signal (OriGene, MD). Confirmation of transfection and miR-655 over expression was done microscopically by validating expression of the GFP reporter and by performing quantitative real-time PCR (2.2.2).

MCF-7 and SKBR-3 cells were grown to 80-90% confluence in 75cm² flasks (BD Falcon, CA) and then gently harvested from the substrate. The harvested cells, at a concentration of 2 x10⁶ cells/ml, were distributed into certified cuvettes and transfected with 2ug of either the pCMV-MIR mock vector (control empty vector) or the pCMV-MIR miR-655 Expression Plasmid (Origene, MD) using the Amaxa Cell Line Nucleofactor Kit V (Lonza, MO) and the E-009 or P-020 program for MCF-7 cells or SKBR-3 cells respectively according to the manufacturer's protocol. After nucleofection, cells were placed in an antibiotic free medium and incubated at 37°C, 5% CO₂ for 48 hours. MCF-7 and SKBR-3 cell lines transiently transfected with the pCMV-MIR mock (empty) vector are referred to as MCF-7-mock and SKBR-3 mock respectively. Likewise,

MCF-7 and SKBR-3 cell lines transiently transfected with the pCMV-MIR miR-655 Expression Plasmid are referred to as MCF-7-655 and SKBR-3-655 respectively.

2.6 Tumorsphere Formation Assay

Sphere formation is an *in vitro* assay, which analyzes the ability of cells to self-renew and form tumorspheres, which is indicative of the cancer stem cell (CSC) phenotype. Primary tumorspheres were generated from cultured cells by seeding on 6-well ultra-low attachment plates (Corning, MA) at 2×10^4 cells/ml in HuMEC media (GIBCO, ON) supplemented with epidermal growth factor (EGF), basic fibroblast growth factor basic (bFGF) and B27 (Invitrogen, ON). Tumorspheres were grown from MCF-7, MCF-7-COX-2, SKBR-3, and SKBR-3-COX-2 human breast cancer cell lines for 10 -14 days, or until tumorspheres reached a size of at least 60 μ m in diameter. The tumorspheres were then harvested and used for either quantitative real time PCR to quantify COX-2 mRNA expression (2.2.1) and miR-655 expression (2.2.2), or for Western blot analysis for COX-2 protein expression (2.3).

2.7 Cell Proliferation ELISA, BrdU Assay

To examine whether miR-655 stimulates MCF-7 and SKBR-3 cellular proliferation, transiently transfected cells (2.5) were used in a BrdU assay 48 hours post-transfection. MCF-7, MCF-7-mock, MCF-7-655, SKBR-3, SKBR-3-mock, and SKBR-3-655 cells were harvested and seeded onto 96-well tissue-culture microplates (BD Biosciences, CA) at 1×10^4 cells/ml and incubated at 37°C, 5% CO₂ for 24 hours. After incubation, the quantification of cell proliferation was performed by the measurement of

BrdU incorporation in newly synthesized cellular DNA using the Cell Proliferation ELISA, BrdU (colorimetric) Kit purchased from Roche, following the manufacture's protocol. Proliferating cells were labeled by the addition of BrdU labeling solution for six hours. During this labeling period, BrdU was incorporated into the DNA of dividing cells. After removing the labeling medium, the cells were fixed, and the DNA was denatured in one step by adding FixDenat. After removing FixDenat, the anti-BrdU-POD antibody was added, which becomes bound to the BrdU incorporated into the newly synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction. The reaction product was measured with an ELISA plate reader (Infinite M200, TECAN) at a wavelength of 370 nm (reference wavelength 492 nm).

2.8 Statistical Analysis

Statistical calculations were performed using GraphPad Prism Software Version 5 (GraphPad Software, La Jolla, CA). Quantitative real-time PCR results for COX-2 mRNA and miR-655 expression in MCF-7, MCF-7-COX-2, SKBR-3, and SKBR-3-COX-2 cells grown as a monolayer or tumorsphere, were tested by Student's t-test. All other data were analyzed with a one-way ANOVA followed by a post-hoc TUKEY's test. A p-value of less than 0.05 was considered statistically significant as indicated in each figure.

CHAPTER THREE: RESULTS

3.1 Introduction of COX-2 Increases the Migration and Invasion of MCF-7 and SKBR-3 Human Breast Cancer Cell Lines

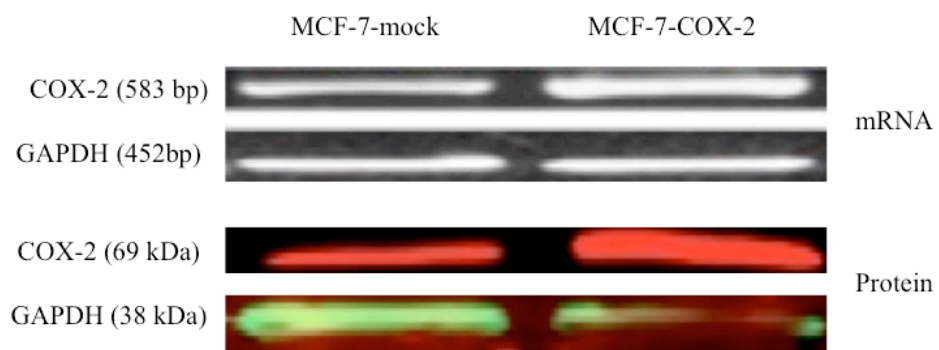
3.1.1 Verification of COX-2 Expression

Our laboratory has shown that overexpression of COX-2 in human, as well as murine breast cancer cells, promotes tumor progression and metastasis by multiple mechanisms. These include host immune cell inactivation (Lala et al., 1986), as well as stimulation of cancer cell migration, invasion, tumor-associated angiogenesis (Rozic et al., 2001) and lymphangiogenesis (Timoshenko et al., 2006), which support blood-borne and lymph-borne metastasis. Recently, by stable transfection of COX-2 cDNA into COX-2 negative non-metastatic MCF-7 and COX-2 negative HER-2 positive weakly metastatic SKBR-3 human breast cancer cell lines, it was further shown that COX-2 induces all the phenotypic properties of aggressive breast cancer, including induction of the CSC phenotype (Majumder et al., 2012). These cell lines were respectively named MCF-7-COX-2 and SKBR-3-COX-2. Correspondingly, empty vector transfected cells were named MCF-7-mock and SKBR-3-mock.

Overexpression of COX-2 in breast cancer cell lines was tested with RT-PCR and quantitative real-time PCR to determine COX-2 mRNA expression and Western blot to analyze COX-2 protein expression (Figure 5 & Figure 6). Lysates and RNA from cultured MCF-7-mock, MCF-7-COX-2 (Figure 5 (A)), SKBR-3-mock, and SKBR-3-COX-2 (Figure 6 (A)) cells were assayed by RT-PCR and Western blot respectively. The levels of GAPDH mRNA and protein were measured and used as the internal normalization control. Real-time PCR analysis determined that COX-2 mRNA

Figure 5. Expression of COX-2 mRNA and protein in MCF-7-mock and MCF-7-COX-2 human breast cancer cells as detected by RT-PCR, Western blot, and quantitative real-time PCR. (A) Lysates and RNA from cultured MCF-7-mock and MCF-7-COX-2 cells were assayed by RT-PCR and Western blot respectively. GAPDH (housekeeping gene) was used as an internal control. (B) COX-2 mRNA expression by quantitative real-time PCR (with Taqman probes) is presented as relative fold change ($2^{-\Delta\Delta CT}$ method). Relative fold changes were normalized to internal control GAPDH. MCF-7 results were plotted as a value of one. MCF-7-COX-2 was observed to express COX-2 mRNA at ~300 fold higher level, as compared to MCF-7-mock cells. The data are represented as mean +/- standard deviation (SD) for three independent experiments. (*) indicates significant difference ($p < 0.05$).

A



B

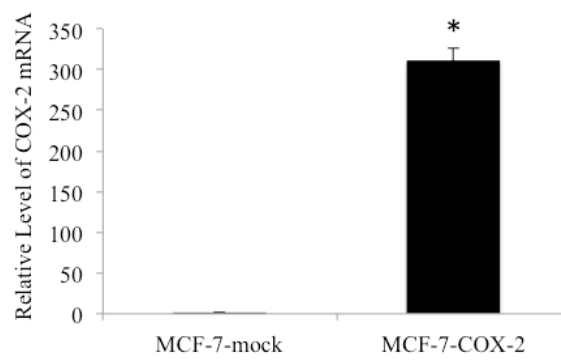
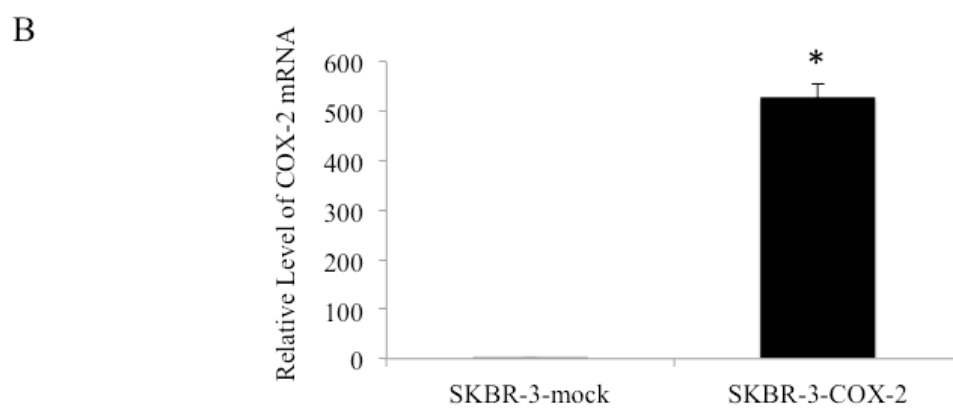
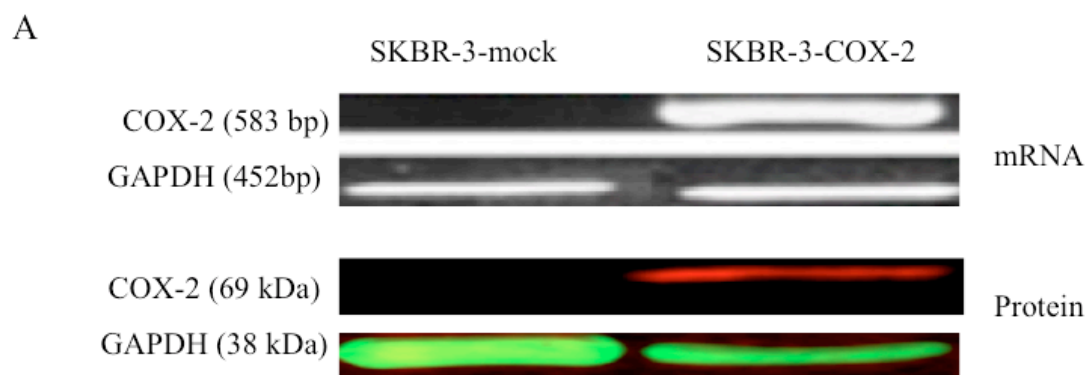


Figure 6. Expression of COX-2 mRNA and protein in SKBR-3-mock and SKBR-3-COX-2 human breast cancer cells as detected by RT-PCR, Western blot, and quantitative real-time PCR. (A) Lysates and RNA from cultured SKBR-3-mock and SKBR-3-COX-2 cells were assayed by RT-PCR and Western blot respectively. GAPDH (housekeeping gene) was used as an internal control. (B) COX-2 mRNA expression by quantitative real-time PCR (with Taqman probes) is presented as relative fold change ($2^{-\Delta\Delta CT}$ method). Relative fold changes were normalized to internal control GAPDH. SKBR-3 results were plotted as a value of one. SKBR-3-COX-2 was observed to express COX-2 mRNA at ~500 fold higher level as compared to SKBR-3-mock cells. The data are represented as mean +/- standard deviation (SD) for three independent experiments. (*) indicates significant difference ($p < 0.05$).



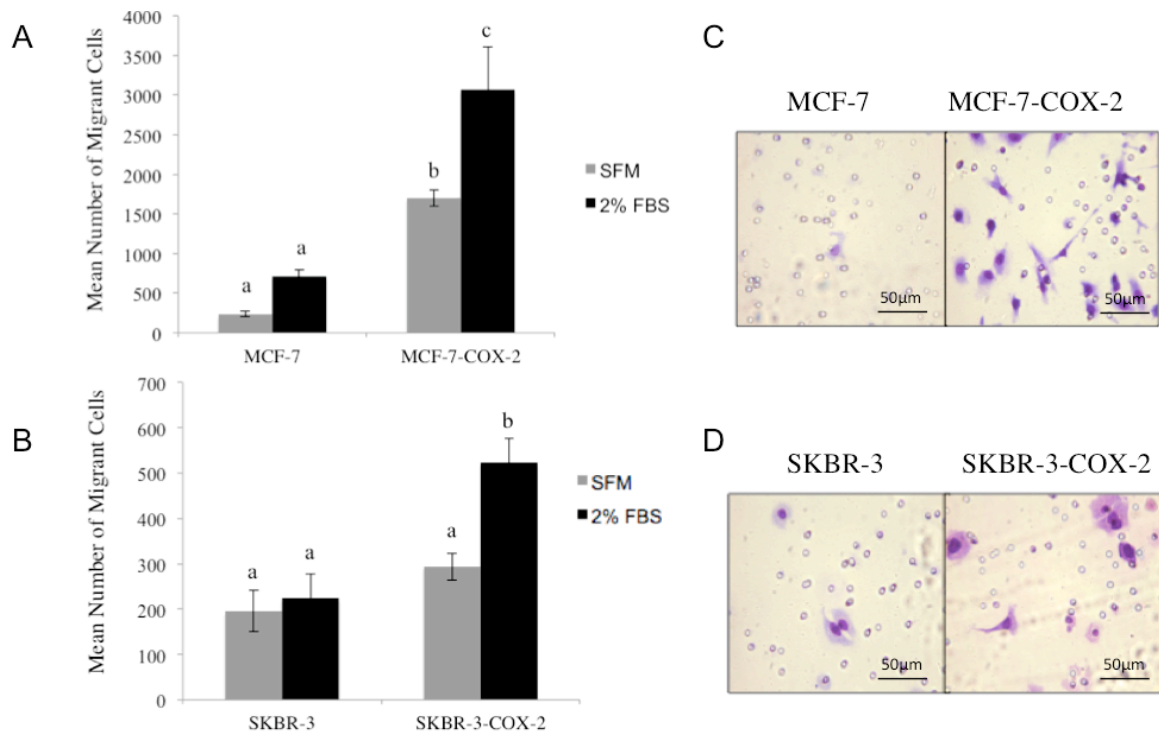
expression levels in the cells were ~300 fold higher compared to MCF-7-mock cells (Figure 5 (B)). There was ~500 fold change in SKBR-3-COX-2 cells compared to SKBR-3-mock cells (Figure 6 (B)).

3.1.2 Effects of COX-2 Overexpression on Cellular Migration and Invasion

Functional assays were performed for migration (across microporous membranes inserted in transwells) and invasion (using transwells containing matrigel-coated microporous membranes) using MCF-7, MCF-7-COX-2, SKBR-3 and SKBR-3-COX-2 cell lines. Previous studies in our laboratory have shown that the mock transfected and parental cell lines show no difference in COX-2 expression and therefore the parental cell lines were used throughout these experiments as the negative control (Majumder et al., 2012). For migration (Figure 7), cells were separately plated on the top chambers of transwell migration inserts and allowed to migrate for 24 hours. The bottom chambers contained serum free medium (SFM) or medium with 2% FBS as indicated. Three independent experiments with each cell line were carried out in triplicate. The cells on each membrane were counted manually. Overexpressing COX-2 in MCF-7 (Figure 7 (A)) breast cancer cell lines significantly increased the ability of these cells to migrate, supporting the idea that COX-2 stimulates migration of human breast cancer cells. In the case of the SKBR-3 cells, the differences become significant when migration was done in 2% FBS (Figure 7 (B)). Representative images (Figure 7 (C) and (D)) are shown.

Cellular invasion is defined by the ability of a cell to migrate through the ECM or basement membrane barrier by enzymatic degradation. Therefore, for the invasion assay (Figure 8), cell lines were plated on the top chamber of transwell inserts coated with

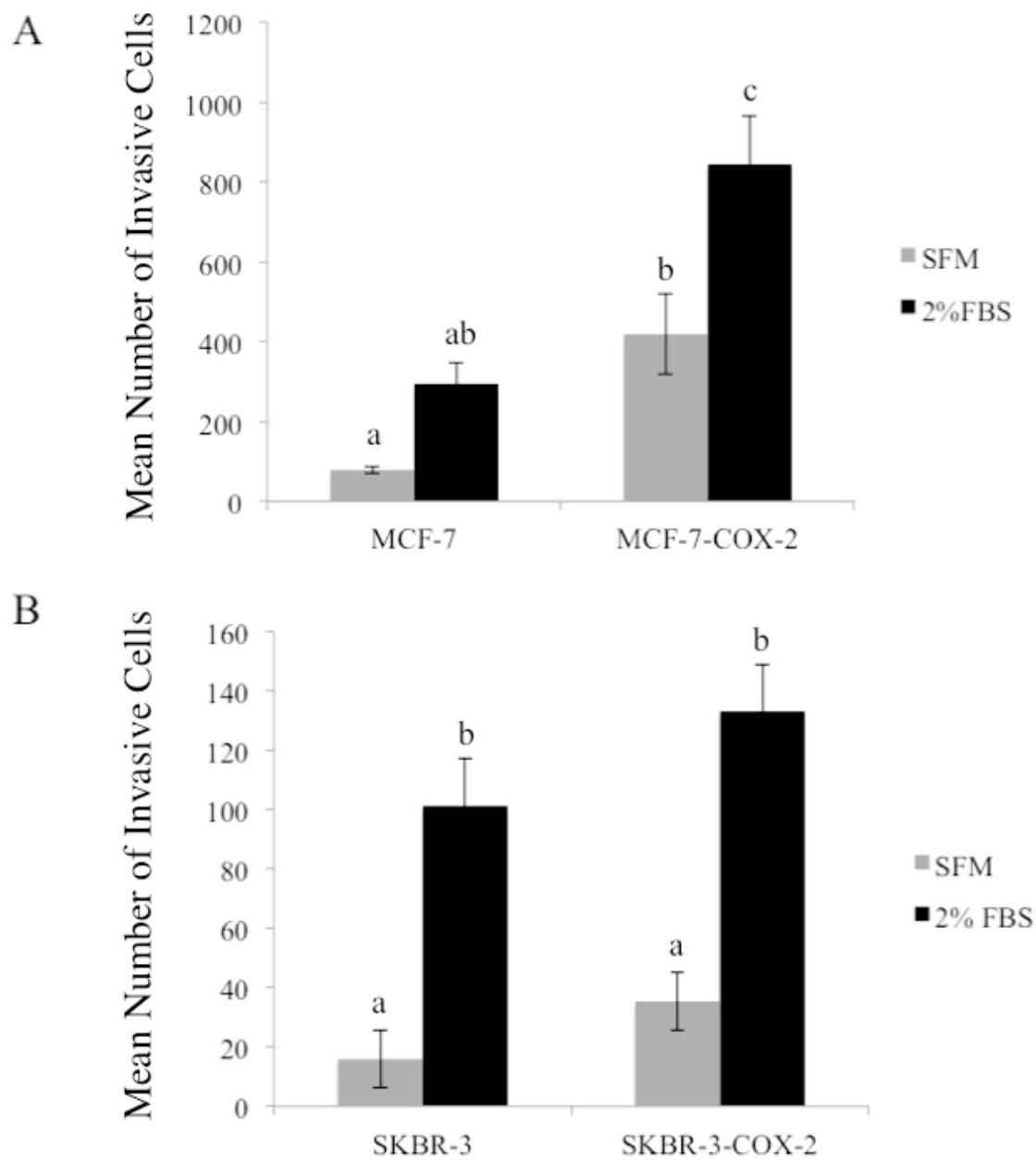
Figure 7. Introduction of COX-2 increases migration of MCF-7 and SKBR-3 human breast cancer cells. MCF-7 & MCF-7-COX-2 (A & C), and SKBR-3 & SKBR-3-COX-2 (B & D) cell lines were plated on the top chambers of transwell migration inserts and allowed to migrate for 24 hours. The bottom chambers contained serum free medium (SFM) or medium with 2% FBS as indicated. Three independent experiments with each cell line were carried out in triplicate. The cells on each membrane were counted manually. Overexpressing COX-2 in MCF-7 (A) and SKBR-3 (B) breast cancer cell lines increased the ability of these cells to migrate, supporting the idea that COX-2 stimulates migration of human breast cancer cells. The data are represented as mean +/- standard deviation (SD) for three independent experiments. Different superscripts indicate statistically significant differences, while shared superscripts indicate no significant difference ($p < 0.01$). (C & D) Images of the migration transwell membranes for each cell line with 2% FBS (40X), showing the morphology of the migratory cells.



Matrigel, a solubilized basement membrane preparation, and allowed to invade for 48 hours. The bottom chambers contained SFM or medium with 2% FBS as indicated. Three independent experiments with each cell line were carried out in triplicate. The cells on each membrane were counted manually (Figure 8). In particular, MCF-7-COX-2 cells showed a significant increase in ability to invade in both the SFM and 2% FBS treated groups, supporting the idea that COX-2 stimulates cellular invasion in human breast cancer.

Based on these results, it can be concluded that COX-2 plays an important role in promoting the aggressive breast cancer phenotype, indicated by the increased ability of the COX-2 overexpressing cells to migrate and invade.

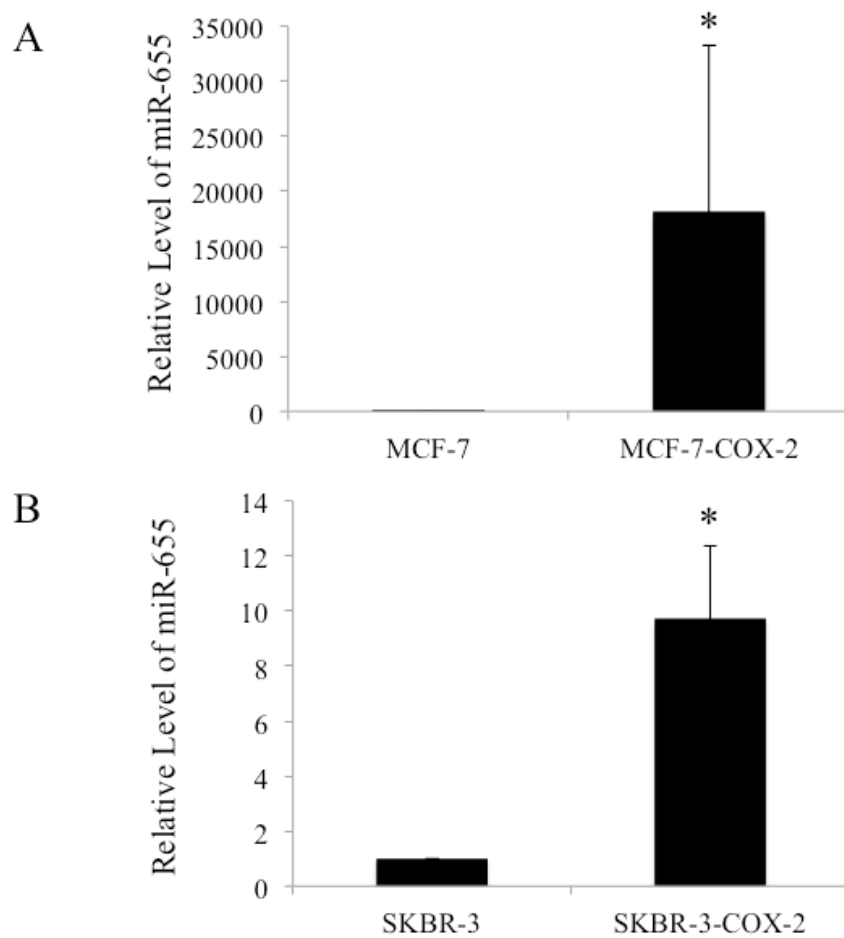
Figure 8. Introduction of COX-2 increases invasion of MCF-7 and SKBR-3 human breast cancer cells. MCF-7 & MCF-7-COX-2 (A), and SKBR-3 & SKBR-3-COX-2 (B) cell lines were plated on the top chamber of transwell inserts coated with Matrigel and allowed to invade for 48 hours. The bottom chambers contained serum free medium (SFM) or medium with 2% FBS as indicated. Three independent experiments with each cell line were carried out in triplicate. The cells on each membrane were counted manually. Overexpressing COX-2 in MCF-7 (A) breast cancer cell lines increased the ability of these cells to invade, supporting the idea that COX-2 stimulates cellular invasion in human breast cancer. A trend was seen in the SKBR-3 cell lines. The data are represented as mean +/- standard deviation (SD) of three independent experiments. Different superscripts indicate statistically significant differences, while shared superscripts indicate no significant difference ($p < 0.01$).



3.2 MiR-655 Expression in MCF-7, MCF-7-COX-2, SKBR-3 and SKBR-3-COX-2 Human Breast Cancer Cell Lines

Through combined gene expression and miRNA microarray analysis our laboratory has recently identified two miRNAs, miR-526b and miR-655, that are up-regulated in MCF-7-COX-2 cells and associated with a down-regulation of 14 target genes linked with tumor-suppressor functions. This thesis focused on the specific role of miR-655 in relation to COX-2 expression and human breast cancer progression. The data for miR-655 expression was validated in the MCF-7, MCF-7-COX-2, SKBR-3 and SKBR-3-COX-2 cell lines by quantification of miR-655 expression with Applied Biosystems Taqman miRNA expression assay (Figure 9). Overexpressing COX-2 in MCF-7 (Figure 9 (A)) and SKBR-3 (Figure 9 (B)) cell lines significantly increased the expression of miR-655. MCF-7-COX-2 showed ~18,000 fold increase in miR-655 expression relative to the MCF-7 parental cell lines. SKBR-3-COX-2 showed ~ 10 fold increase in miR-655 expression relative to the SKBR-3 parental cell line. To conclude, it appears that miR-655 expression is increased in response to COX-2 up-regulation.

Figure 9. Expression levels of miR-655 in MCF-7, MCF-7-COX-2, SKBR-3, and SKBR-3-COX-2 human breast cancer cell lines. Analysis of miR-655 expression by quantitative real-time PCR (with Taqman probes). Results presented in relative fold change ($2^{-\Delta\Delta CT}$ method). Relative fold changes were normalized to internal control RNU44. Overexpressing COX-2 in MCF-7 (A) and SKBR-3 (B) cell lines significantly increased the expression of miR-655. The data are represented as mean +/- standard deviation (SD) for three independent experiments. (*) indicates significant difference ($p < 0.05$) relative to parental cell line. Parental cell lines were given a value of 1.



3.3 Treatment with COX-2 Inhibitor and EP4 Antagonist Decreases the Expression of miR-655 in COX-2 Overexpressing Cell lines

Previous work in our lab has shown that selective COX-2 inhibitors reduced tumor cell migration and invasion *in vitro* (Rozic et al., 2001). It was further shown that COX-2 mediated promotion of migration was at least in part mediated by endogenous PGE₂, acting primarily via the EP4 receptor (Timoshenko et al., 2003). Looking at the effects of blocking COX-2 was performed in order to test the casual relationship between COX-2 activity and miR-655 expression in the COX-2 expressing breast cancer cell lines. Blocking EP4 activity was performed to determine if miR-655 up-regulation is dependent on this particular receptor. The working hypothesis tested was that blocking COX-2 or EP4 activity in the high COX-2 expressing cell lines would reduce the level of miR-655 expression.

To test this hypothesis, MCF-7-COX-2 and SKBR-3-COX-2 cells were plated at 1 million cells/well in 6-well plates and incubated overnight in complete DMEM or McCoy's respectively. The next day, cells were incubated in serum-free medium for 2 hours, and then treatments of 20 μ M selective COX-2 inhibitor NS-398, 2 μ M EP4 antagonist ONO-AE3-208, or control (0.02% DMSO) were added. After 24 hours of treatment, cDNA was synthesized using specific primers from miRNA extracted from the cells. Quantitative real-time PCR was performed to analyze the expression levels of miR-655.

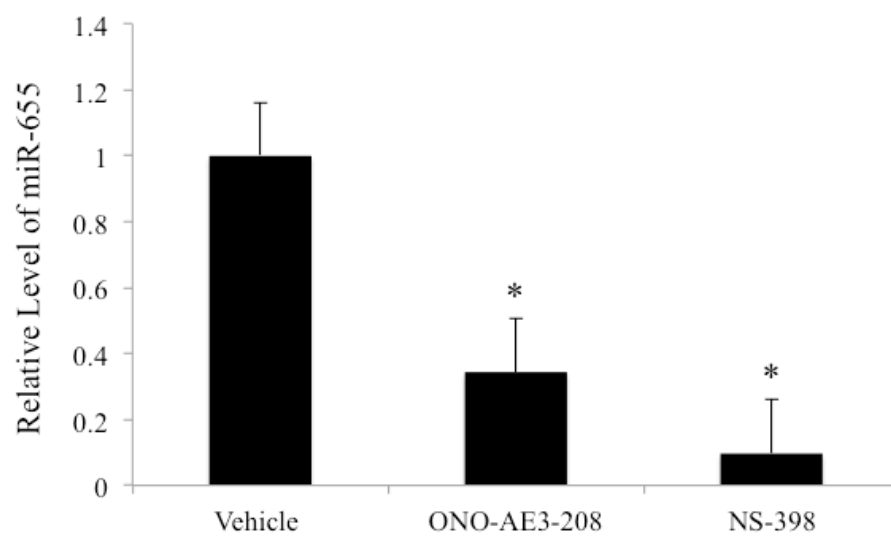
In both MCF-7-COX-2 and SKBR-3-COX-2 human breast cancer cell lines, treatment with the COX-2 inhibitor NS-398 and the EP4 antagonist ONO-AE3-208

significantly decreased the expression of miR-655 (Figure 10). This indicates that the expression of miR-655 in human breast cancer cell lines is dependent on both COX-2 and EP4 activity.

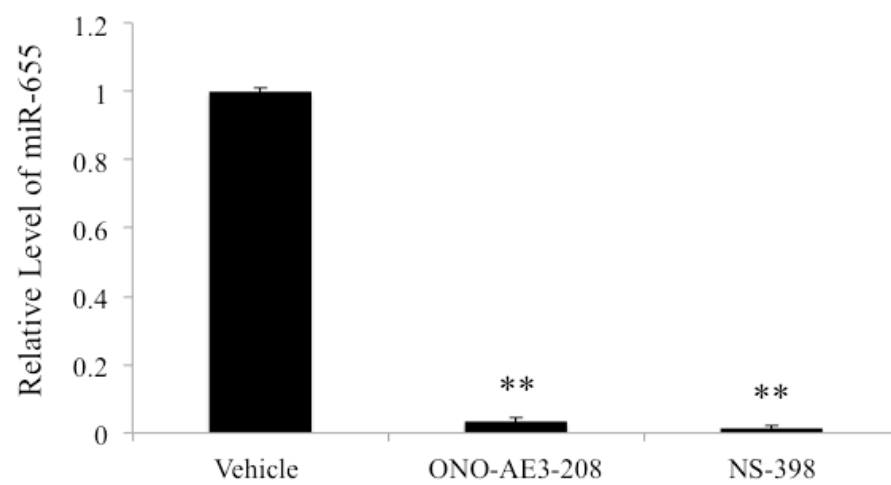
Figure 10. MiR-655 expression in response to COX-2 inhibitor and EP4 antagonist in MCF-7-COX-2 (A) and SKBR-3-COX-2 (B) human breast cancer cell lines.

MCF-7-COX-2 (A) and SKBR-3-COX-2 (B) cells were treated with either 20 μ M selective COX-2 inhibitor (NS-398), 2 μ M EP4 antagonist (ONO-AE3-208), or vehicle (0.02% DMSO) for 24 hours. After treatment, cDNA was synthesized from miRNA extracted from the cells using specific Taqman primers. Analysis of miR-655 expression was performed by quantitative real-time PCR (with Taqman probes). Results presented in relative fold change ($2^{-\Delta\Delta CT}$ method). Relative fold changes were normalized to internal control RNU44. In both MCF-7-COX-2 (A) and SKBR-3-COX-2 (B) human breast cancer cell lines, treatment with the COX-2 inhibitor NS-398 and the EP4 antagonist ONO-AE3-208 significantly decreased the expression of miR-655. This indicates that the expression of miR-655 in human breast cancer cell lines is dependent on both COX-2 and EP4 activity. The data are represented as mean +/- standard deviation (SD) for three independent experiments. (*) indicates significant difference ($p < 0.05$) and (**) indicates significant difference ($p < 0.001$) relative to the cell line treated with vehicle (0.02% DMSO).

A



B



3.4 COX-2 and miR-655 Expression in Tumorspheres in Comparison to Cells Grown as a Monolayer

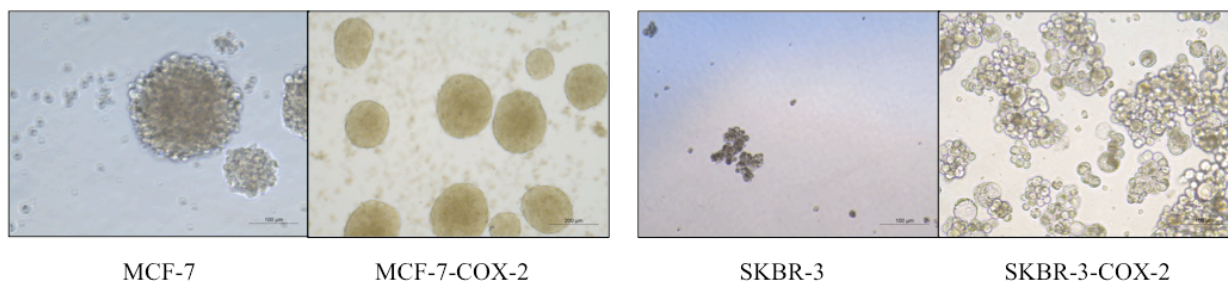
3.4.1. Cells Derived from Tumorspheres Have a Higher COX-2 Expression in Comparison to Cells Grown as a Monolayer

The tumorsphere formation assay is an *in vitro* correlate of the CSC phenotype by analyzing the ability of cancer stem-like cells to self-renew. MCF-7, MCF-7-COX-2, SKBR-3, and SKBR-3-COX-2 cells were grown in 6-well low attachment plates and allowed to form tumorsphere (as detailed in the methodology 2.6) for 7-14 days or until tumorspheres reached a size of at least 60 μ m in diameter (Figure 11). The tumorspheres in each well were pooled and harvested for either quantitative real-time PCR (Figure 11 (B)) or Western blot analysis (Figure 11 (C)). Expression levels of β -actin were used as an internal control. MCF-7 and MCF-7-COX-2 cells formed more spherical tumorspheres in comparison to SKBR-3 and SKBR-3-COX-2 cells, which formed irregular spheres (Figure 11 (A)). COX-2 mRNA expression was significantly increased in the cells grown as tumorspheres in comparison to the same cells grown as a monolayer (Figure 11 (B)). MCF-7 tumorspheres exhibited a 14 fold increase in COX-2 mRNA expression, while MCF-7-COX-2 tumorspheres exhibited a 6 fold increase in COX-2 mRNA expression relative to cells grown as a monolayer (Figure 11 (B)). SKBR-3 tumorspheres exhibited a 6 fold increase, while SKBR-3-COX-2 exhibited a 5 fold increase in COX-2 mRNA expression relative to cells grown as a monolayer (Figure 11 (B)). Analysis of COX-2 protein expression by Western blot revealed that COX-2 protein is also up-regulated in the cell lines grown as tumorspheres in comparison to cells grown as a monolayer (Figure 11 (C)).

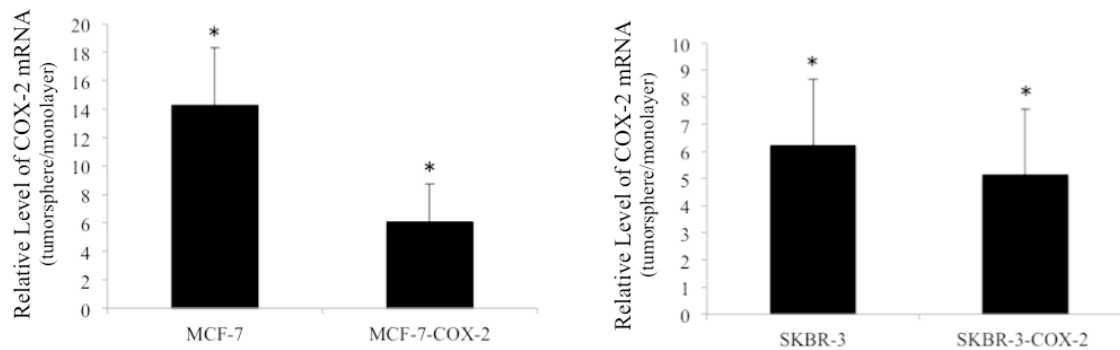
To conclude, COX-2 expression is significantly increased at the mRNA and protein levels in cell lines grown as tumorspheres relative to the same cell lines grown in monolayer. COX-2 therefore appears to play an important role in promoting and maintaining the CSC phenotype *in vitro*.

Figure 11. Cells derived from tumorspheres have a higher COX-2 expression in comparison to cells grown as a monolayer. Cells were grown in 6-well low attachment plates at 2×10^4 cells/ml and allowed to form tumorspheres (*in vitro* correlate of CSC growth) for 7-14 days or until tumorspheres reached a size of at least 60 μ m in diameter. The tumorspheres in each well were pooled and harvested for either quantitative real-time PCR (B) or Western blot analysis (C). (A) Images of MCF-7, SKBR-3 and SKBR-3-COX-2 tumorspheres at 10 days and MCF-COX-2 tumorspheres at 7 days. MCF-7 and MCF-7-COX-2 cells formed more spherical tumorspheres in comparison to SKBR-3 and SKBR-3-COX-2 cells, which formed irregular spheres. (B) Analysis of COX-2 mRNA expression by quantitative real-time PCR (with Taqman probes). Results presented in relative fold change ($2^{-\Delta\Delta CT}$ method). Relative fold changes were normalized to internal control β -actin. COX-2 mRNA expression was significantly increased in the cells grown as tumorspheres in comparison to the same cells grown as a monolayer. The data are represented as mean \pm standard deviation (SD) for three independent experiments. (*) indicates significant difference ($p < 0.05$) relative to the same cell line grown as a monolayer. (C) Analysis of COX-2 protein expression by Western blot. β -actin was used as a loading control. COX-2 protein is observed to be up-regulated in MCF-7 and MCF-7-COX-2 cell lines grown as tumorspheres (Tumor) in comparison to cells grown as a monolayer (Mono).

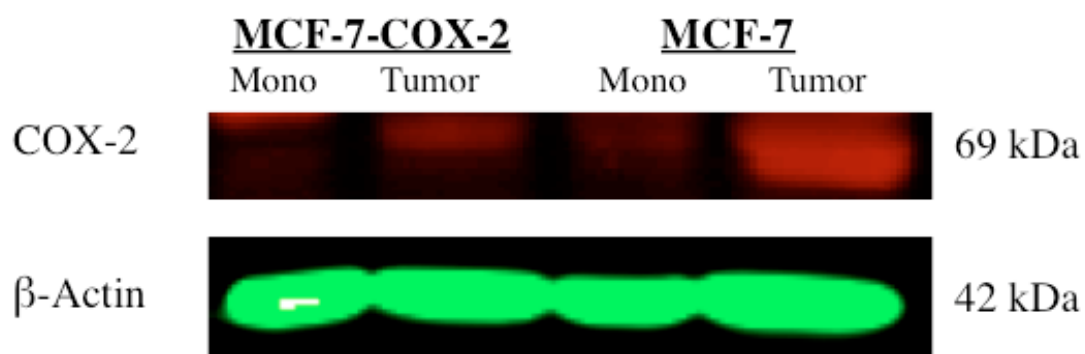
A



B



C



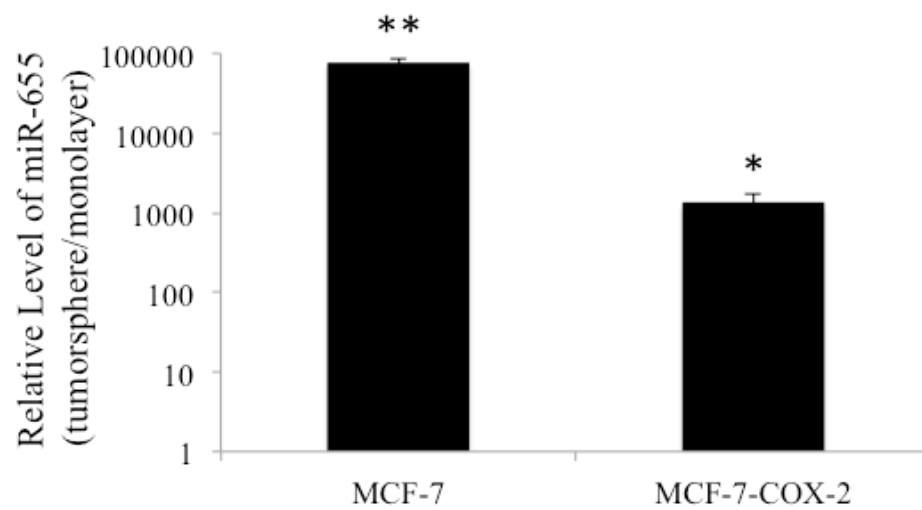
3.4.2 Cells Derived from Tumorspheres have a Higher miR-655 Expression in Comparison to Cells Grown as a Monolayer

To determine the expression of miR-655 in cells grown as tumorspheres, MCF-7, MCF-7-COX-2, SKBR-3, and SKBR-3-COX-2 cells were grown in 6-well low attachment plates and allowed to form tumorspheres for 7-14 days or until tumorspheres reached a size of at least 60 μ m in diameter (Figure 12). The tumorspheres in each well were pooled and harvested for quantitative real-time PCR for miR-655 quantification. MiR-655 expression was significantly increased in the cells grown as tumorspheres in comparison to the same cells grown as a monolayer (Figure 12). MCF-7 tumorspheres exhibited ~ 100,000 fold increase in miR-655 expression, while MCF-7-COX-2 tumorspheres exhibited ~1000 fold increase in miR-655 expression relative to cells grown as a monolayer (Figure 12 (A)). SKBR-3 tumorspheres exhibited a 5-fold increase in miR-655 expression relative to cells grown as a monolayer (Figure 12 (B)).

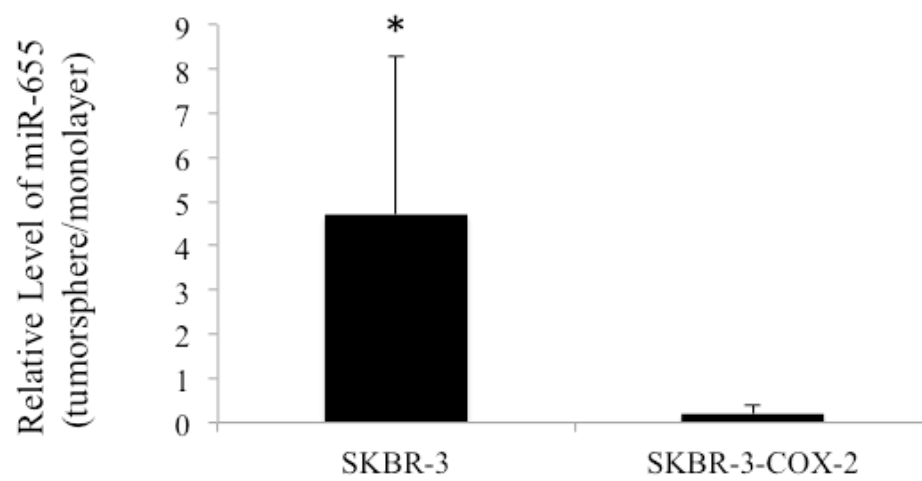
To conclude, miR-655 expression is significantly increased in MCF-7, MCF-7-COX-2, and SKBR-3 cell lines grown as tumorspheres relative to the same cell lines grown in monolayer. MiR-655 therefore appears to play an important role in promoting and tumorsphere formation *in vitro*.

Figure 12. Tumorspheres grown from MCF-7, MCF-7-COX-2 and SKBR-3 cells exhibit overexpression of miR-655. Cells were grown in 6-well low attachment plates at 2×10^4 cells/ml and allowed to form tumorspheres for 7-14 days or until tumorspheres reached a size of at least $60 \mu\text{m}$ in diameter. The tumorspheres in each well were pooled and harvested for quantitative real-time PCR analysis of miR-655 expression (with Taqman probes). Results presented in relative fold change ($2^{-\Delta\Delta\text{CT}}$ method). Relative fold change was normalized to internal control RNU44. MCF-7 and MCF-7-COX-2 cells (A) and SKBR-3 (B) cells grown as tumorspheres showed a significantly higher miR-655 expression compared to the same cells grown as a monolayer. The data are represented as mean \pm standard deviation (SD) for three independent experiments. (*) indicates significant difference ($p < 0.05$) and (**) indicates significant difference ($p < 0.001$) relative to cells grown as a monolayer.

A



B



3.5 Introduction of miR-655 Increases the Migration and Invasion of MCF-7 and SKBR-3 Breast Cancer Cell Lines

3.5.1 Introduction of miR-655 into MCF-7 and SKBR-3 Human Breast Cancer Cells

Our lab has recently shown that knocking down miR-655 expression with morpholino oligos significantly reduces the function that COX-2 plays in breast cancer progression (Majumder et al., 2012). When the expression of miR-655 is knocked down, MCF-7-COX-2 and SKBR-3-COX-2 cell lines show a significant decrease in ability to invade, migrate, and proliferate. Since our laboratory has and is currently looking at the role of knocking down miR-655, this study looked at the consequences of miR-655 overexpression in the human breast cancer cell lines MCF-7 and SKBR-3.

MCF-7 and SKBR-3 breast cancer cells, which express very little miR-655, were transfected via nucleofection with either the pCMV-MIR mock vector (control empty vector) or the miR-655 Expression Plasmid.

To determine if the transfection was successful, miRNA was isolated from the cells and analysis of miR-655 expression was performed by quantitative real-time PCR (with Taqman probes). Relative fold changes were normalized to internal control RNU44. MCF-7 cells transiently transfected with the miR-655 expression plasmid exhibited a 1000–10,000 fold increase in miR-655 expression in comparison to the parental cell line, and a 100-1000 fold increase in comparison to the mock transfected cell line (Figure 13 (A)). SKBR-3 cells transiently transfected with the miR-655 expression plasmid exhibited a 10,000-100,000 fold increase in miR-655 expression in comparison to the

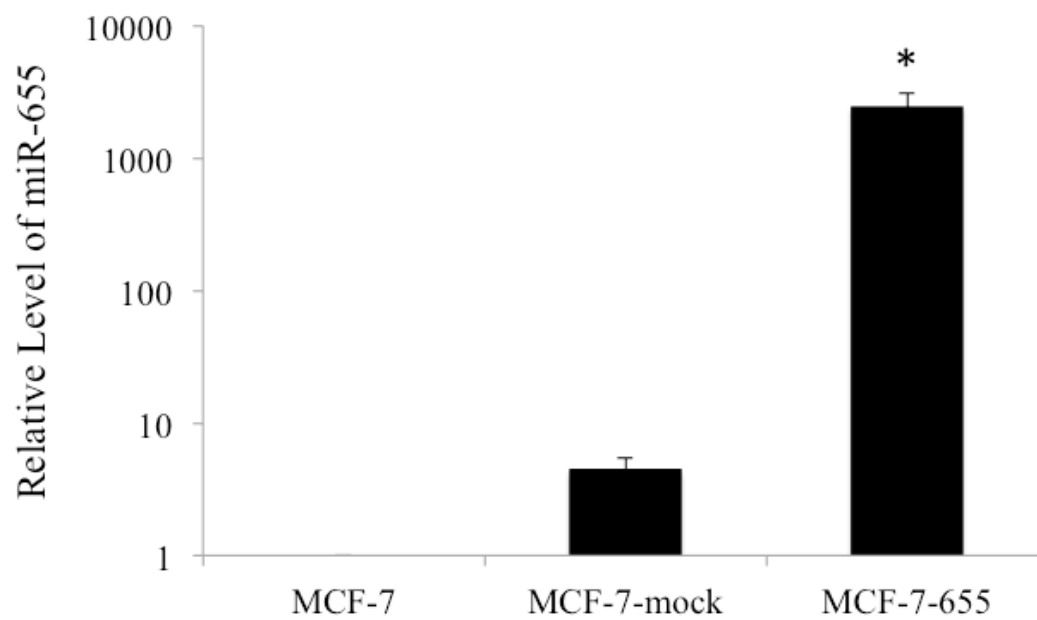
parental cell line, and a 1000-10,000 fold increase in comparison to the mock transfected cell line (Figure 13 (B)). Transfection with the miR-655 expression plasmid was therefore successful.

3.5.2 MiR-655 Expression Increases the Ability of MCF-7 and SKBR-3 Cells to Migrate and Invade

To determine the effect that miR-655 overexpression plays in cellular migration and invasion, cell lines were separately plated on the top chamber of transwell migration inserts 72 hours post transfection and allowed to migrate for 24 hours (Figure 14 (A)). Cell lines were also plated on the top chamber of transwell inserts coated with Matrigel 72 hours post transfection and allowed to invade for 48 hours (Figure 14 (B)). The bottom chambers contained 2% FBS. The cells on each membrane were counted manually. The cells that were transfected with the miR-655 Expression Plasmid showed a significant increase in ability to migrate and invade. Even though SKBR-3-655 did show an increase in invasion, it was not statistically significant. Based on these results, it can be suggested that miR-655 expression plays a role in making breast cancer cells more aggressive in their ability to migrate and invade.

Figure 13. MCF-7 cells (A) and SKBR-3 cells (B) transfected with miR-655 expression plasmid significantly overexpress miR-655. Cells were transiently transfected using either a mock (empty) or a miR-655 expression plasmid (supplied from Origene) by nucleofection. Seventy-two hours post-transfection miRNA was isolated from the cells and analysis of miR-655 expression was performed by quantitative real-time PCR (with Taqman probes). Results presented in relative fold change ($2^{-\Delta\Delta CT}$ method). Relative fold changes were normalized to internal control RNU44. (A) MCF-7 cells transiently transfected with the miR-655 expression plasmid exhibited a 1000–10,000 fold increase in miR-655 expression in comparison to the parental cell line. (B) SKBR-3 cells transiently transfected with the miR-655 expression plasmid exhibited a 10,000-100,000-fold increase in miR-655 expression in comparison to the parental cell line. The data are represented as mean +/- standard deviation (SD) for three independent experiments. (*) indicates significant difference ($p < 0.001$). The values for the parental cell lines were taken as one.

A



B

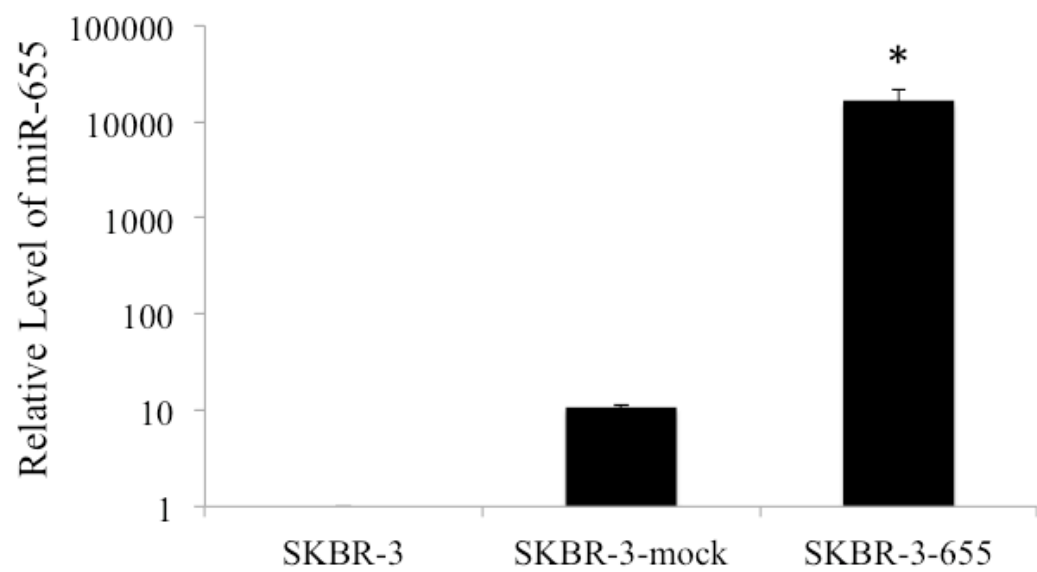
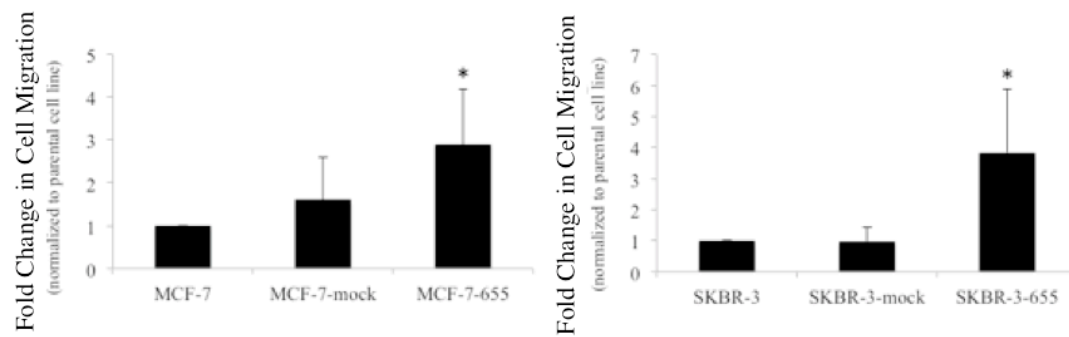
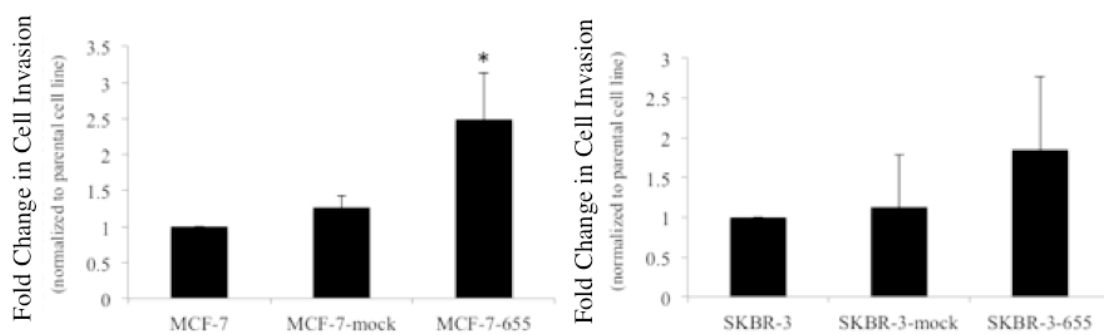


Figure 14. Introduction of miR-655 increases migration and invasion of MCF-7 and SKBR-3 cells. (A) Cell lines were separately plated on the top chamber of transwell migration inserts 72 hours post transfection and allowed to migrate for 24 hours. (B) Cell lines were separately plated on the top chamber of transwell inserts coated with Matrigel 72 hours post transfection and allowed to invade for 48 hours. The bottom chambers contained 2% FBS. The cells on each membrane were counted manually. (*) Indicates significant difference ($p < 0.05$). The data represent triplicates \pm standard deviation (SD) for three independent experiments normalized to the parental cell line. The values for the parental cell lines was taken as 1.

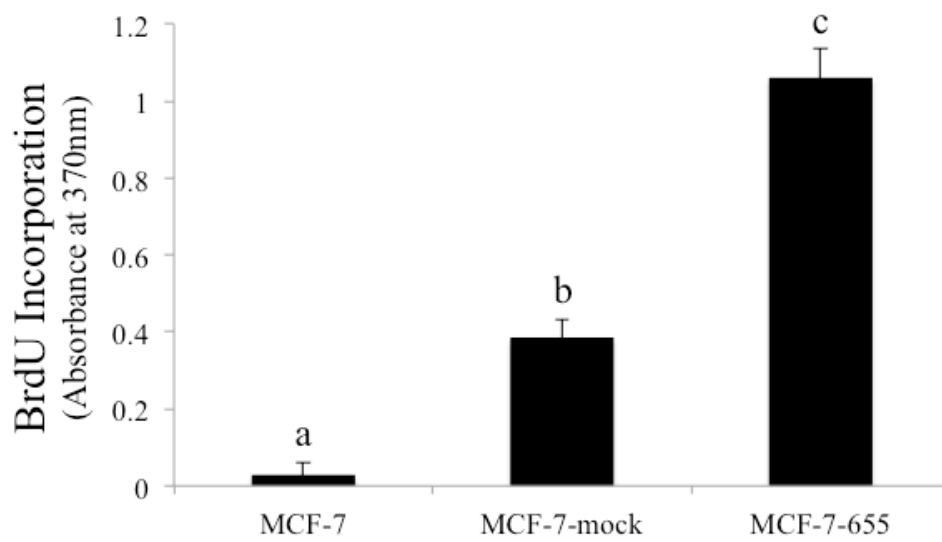
A**B**

3.6 Introduction of miR-655 Increases the Proliferation of MCF-7 Human Breast Cancer Cells

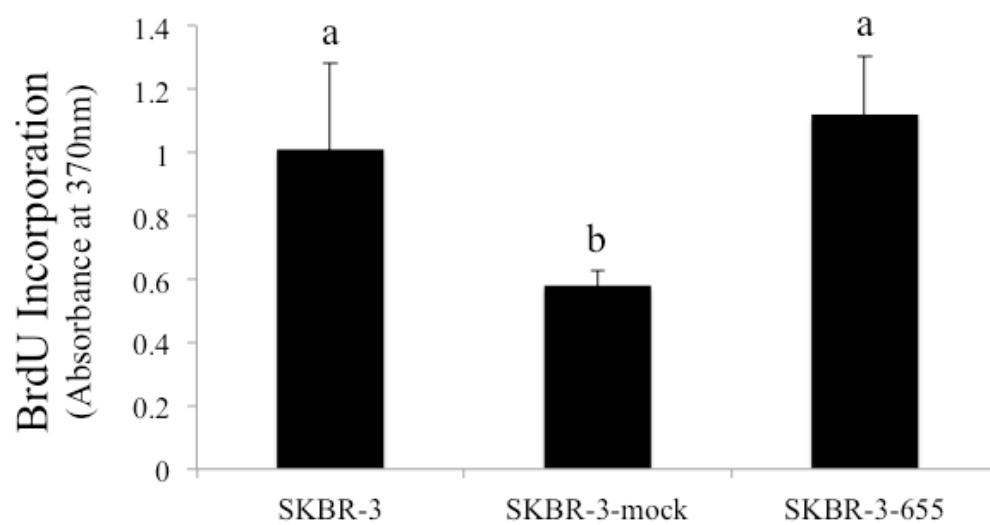
BrdU colorimetric assay was performed to determine the effect of miR-655 overexpression on MCF-7 and SKBR-3 proliferation. BrdU incorporation is presented as an absorbance value at 370nm (Figure 15) and the experiment was carried out in triplicate. MCF-7 cells transiently transfected with miR-655 (MCF-7-655) exhibited a significant increase in cellular proliferation in comparison to the parental MCF-7 cell line (Figure 15 (A)). SKBR-3 cells transiently transfected with miR-655 (SKBR-3-655) showed no difference in cellular proliferation in comparison to the parental SKBR-3 cell line (Figure 15 (B)).

Figure 15. Introduction of miR-655 increases the proliferation of MCF-7 human breast cancer cells. Seventy-two hours post transfection with either the mock (empty) or the miR-655 expression plasmid and cell proliferation ELISA was performed using transiently transfected MCF-7 cells (A) and SKBR-3 cells (B). BrdU incorporation is presented as an absorbance value at 370nm. (A) MCF-7 cells transiently transfected with miR-655 (MCF-7-655) exhibited a significant increase in cellular proliferation in comparison to the parental MCF-7 cell line. (B) SKBR-3 cells transiently transfected with miR-655 (SKBR-3-655) showed no difference in cellular proliferation in comparison to the parental SKBR-3 cell line. However, the proliferative ability was significantly higher than the mock-transfected cells. The data are represented as mean +/- standard deviation (SD) for three independent experiments. Different superscripts indicate statistically significant differences, while shared superscripts indicate no significant difference ($p < 0.05$).

A



B



3.7 Introduction of miR-655 Increases the Expression of COX-2 in MCF-7 and SKBR-3 Human Breast Cancer Cells

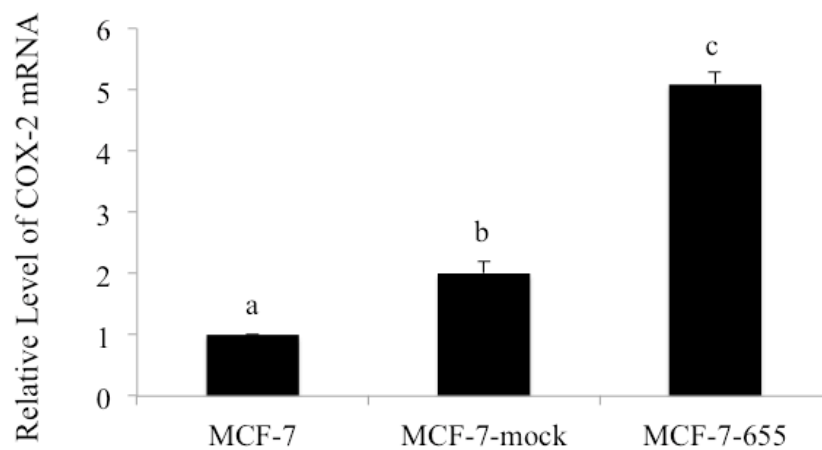
To determine the effect that miR-655 has on COX-2 expression, RNA and protein were isolated from transfected cells for analysis of mRNA expression by quantitative real-time PCR and protein expression by Western blot analysis. COX-2 mRNA expression by quantitative real-time PCR (with Taqman probes) was normalized to the internal control GAPDH and was carried out in triplicate (Figure 16 (A)). MCF-7-655 significantly overexpresses COX-2 mRNA relative to the parental and mock cell lines (Figure 16 (A)). MCF-7-655 expressed COX-2 mRNA at a 4 fold increase in comparison to the MCF-7 parental cell line, and a 3 fold increase in comparison to the mock transfected cell line (MCF-7-mock). Even though the data for SKBR-3 are the result of a single experiment, SKBR-3-655 appeared to express COX-2 mRNA at ~1800 increase level in comparison to the mock and parental cell lines (Figure 16 (B)).

Protein was isolated from MCF-7, MCF-7-mock and MCF-7-655 for COX-2 protein expression by Western blot. MCF-7-COX-2 protein was used as a positive control and GAPDH was used as a loading control. MCF-7 transfected with the miR-655 expression plasmid appeared to overexpress COX-2 at the protein level as well, however this needs to be repeated and quantified with densitometry.

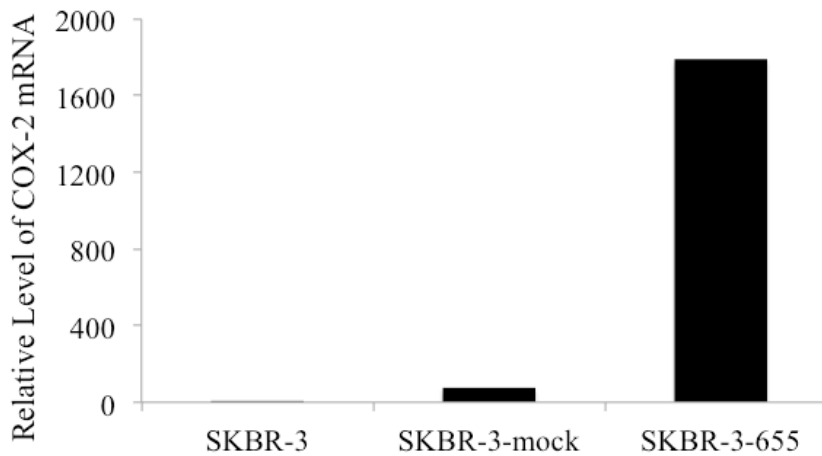
Based on these results it can be concluded that miR-655 plays a role in increasing COX-2 expression in MCF-7 human breast cancer cells.

Figure 16. Introduction of miR-655 in MCF-7 and SKBR-3 human breast cancer cell lines increases COX-2 expression. Cells were transiently transfected using either a mock (empty) or a miR-655 expression plasmid (supplied from Origene) by nucleofection. Seventy-two hours post-transfection RNA was isolated from the cells for analysis of COX-2 mRNA expression by quantitative real-time PCR (with Taqman probes) (A & B). Results presented in relative fold change ($2^{-\Delta\Delta CT}$ method). Relative fold changes were normalized to internal control GAPDH. (A) The data are represented as mean +/- standard deviation (SD) for three independent experiments. Different superscripts indicate statistically significant differences, while shared superscripts indicate no significant difference ($p < 0.001$). MCF-7-655 significantly overexpressed COX-2 mRNA relative to the parental and mock cell lines. (B) The data for SKBR-3 are the result of a single experiment (n=1), revealing that SKBR-3-655 cells show an up-regulation of COX-2 mRNA expression. (C) Protein was isolated from MCF-7, MCF-7-mock and MCF-7-655 for COX-2 protein expression by Western blot. MCF-7-COX-2 protein was used as a positive control and GAPDH (housekeeping gene) was used as a loading control (n=1).

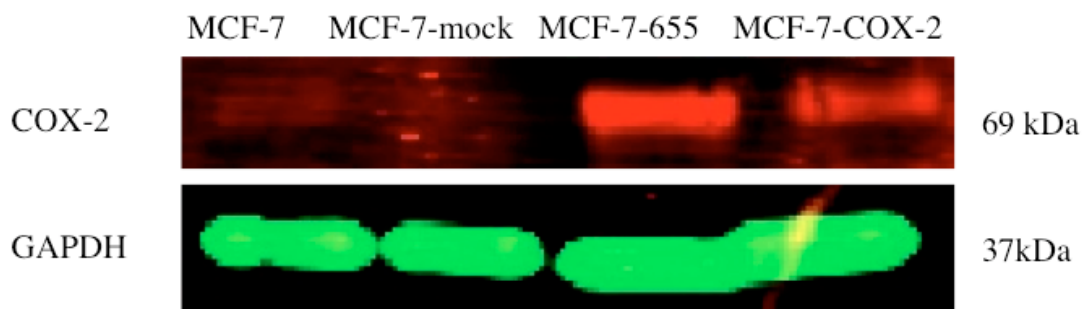
A



B



C



CHAPTER FOUR: SUMMARY AND DISCUSSION

4.1 Summary

Based on the results presented in Chapter 3, the following remarks can be made:

- The migratory and invasive capacities of the COX-2 disparate cell lines went hand in hand with COX-2 expression.
- Quantification of miR-655 expression and COX-2 mRNA and COX-2 protein revealed that COX-2 expression is associated with an up regulation of miR-655.
- Expression of miR-655 was markedly inhibited by treating MCF-7-COX-2 and SKBR-3-COX-2 cells with a COX-2 inhibitor (NS398) or an EP4 antagonist (ONO-AE3-208), indicating that the expression depended on both COX-2 and EP4 activity.
- MCF-7, MCF-7-COX-2, SKBR-3 and SKBR-3-COX-2 cell lines grown as tumorspheres significantly overexpressed COX-2 in comparison to the same cell lines grown as a monolayer.
- MCF-7, MCF-7-COX-2, and SKBR-3 cell lines grown as tumorspheres significantly overexpressed miR-655 in comparison to the same cell lines grown as a monolayer.
- MCF-7 and SKBR-3 cell lines transfected with a miR-655 Expression Plasmid showed a significant increase in miR-655 expression. The transfected cell lines also showed an increase in migratory and invasive capacity. MCF-7-655 also showed an increase in proliferation in comparison to both the parental and mock transfected cell lines.

Original contribution: The COX-2 regulated miRNA, miR-655, is implicated in the stimulation of breast cancer cell migration, invasion and tumorsphere formation.

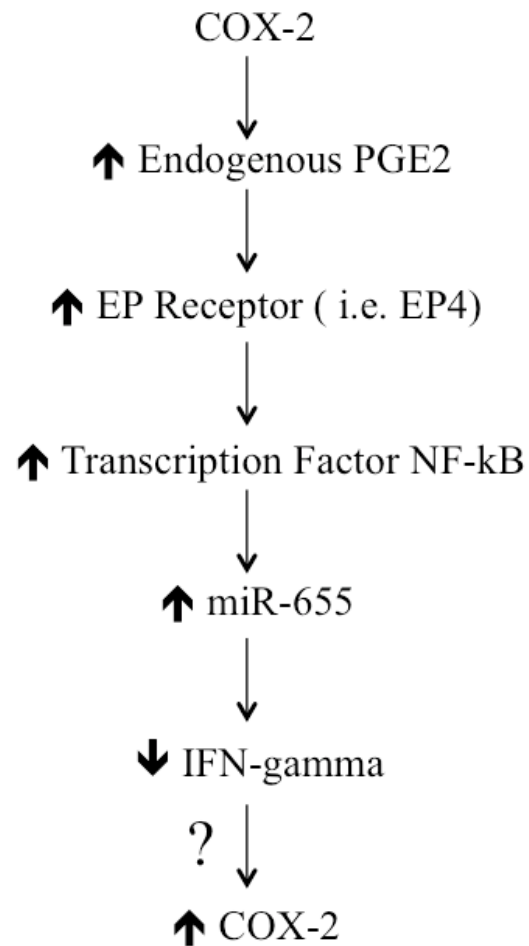


Figure 17. Speculated Mechanism of COX-2 and miR-655 signaling in human breast cancer cell lines. COX-2 overexpression leads to the production of endogenous PGE2, which binds to the EP receptors to elicit its response. PGE2 induced activation of the EP receptors may result in the recruitment of the NF-kB transcription factor binding to the miR-655 gene (Shin et al., 2000 and Bhattacharjee et al., 2009). MiR-655 may then target IFN-gamma, which may lead to a further up-regulation of COX-2 expression (Nares, 2011).

4.2 Overview

Present studies on miR-655 and reports on other miRNAs in the literature have revealed that certain miRNAs play important roles in breast cancer cell proliferation, invasion and migration (Martello et al., 2010 and Shi et al., 2010). These miRNAs may serve as potential biomarkers for breast cancer. Once validated, miRNAs could have significant clinical impact by allowing clinicians to monitor disease progression by measuring circulating miRNAs that are stable in the blood (Andorfer et al., 2011). MiRNA signatures may be used as novel prognostic indicators that will help in the development of improved personalized therapy for breast cancer.

The distinction between miRNAs and other RNA interfering molecules (i.e. siRNA/shRNA) is that they are natural small RNAs synthesized in the body and are present from early development to any disease stage (Bartel, 2004). These miRNAs can target hundreds of mRNAs due to their imperfect complementarity to their target mRNA. A single miRNA therefore can affect multiple targets, regulating several genes on a similar pathway and a whole network of interacting molecules (Bartel 2004 and 2009). Therefore, it is a challenge to determine the mechanisms of how COX-2 regulates miR-655, and vice versa, in breast cancer cells.

4.3 The Role of COX-2 in Breast Cancer Progression

In the present study, stable transfection of COX-2 into two poorly migratory and invasive cell lines, MCF-7 and SKBR-3 stimulated their migratory and invasive capacity both under serum free and FBS stimulated conditions. These findings are in support of earlier data from this and other laboratories.

Our laboratory has shown that COX-2 expression by breast cancer cells in both murine and human breast cancer models promotes tumor progression by multiple mechanisms. These include the inactivation of host anti-tumor immune cells by tumor-derived PGE₂ (Parhar and Lala, 1985, 1986), enhanced cell migration and invasiveness (Rozic et al., 2001, Timoshenko et al., 2003), enhanced tumor-associated angiogenesis (Rozic et al., 2001), an up regulation of VEGF-C (Timoshenko et al., 2006) and thereby lymphangiogenesis and lymphatic metastasis (Bhattacharyya et al., 2010) as well as VEGF-C mediated stimulation of cancer cell migration (Timoshenko et al., 2001). Furthermore, it was shown that COX-2 induced migration as well as VEGF-C up-regulation (Timoshenko et al., 2003) was mediated at least in part by endogenous PGE₂ acting primarily via the EP4 receptor (Timoshenko et al., 2006).

Recently, in a high COX-2 expressing murine breast cancer cell line C3L5, selected from lung metastasis in a spontaneous mammary tumor, COX-2 was shown to up-regulate the lymphangiogenic factors VEGF-C and VEGF-D *in vitro* and *in vivo* primarily via EP4 receptor activation. In this tumor model, therapy with the COX-2 inhibitor Celecoxib, as well as an EP4 antagonist ONO-AE3-208, abrogated growth of the primary tumors, tumor-associated angiogenesis and lymphangiogenesis, as well as metastasis to the lymph nodes and the lungs (Xin et al., 2012).

In both MCF-7-COX-2 and SKBR-3-COX-2 breast cancer cell lines, earlier data by Majumder et al. (AACR 2012, abstract) revealed that the enhanced migratory and invasive capacity of these cells was dependent on COX-2 and EP4 activity. Both the COX-2 inhibitor NS-398 and EP4 antagonist ONO-AE3-208 inhibited their migratory and invasive capacity.

Several laboratories have examined mechanisms underlying the roles of COX-2 in promoting breast cancer cell invasion. For example, Singh et al. (2005) transfected COX-2 cDNA into the highly invasive estrogen-independent human breast cancer cell line MDA-MB-231 to show that the migratory and invasive capacity of the cells were stimulated further, in association with an increased expression of prourokinase plasminogen activator (pro-uPA). UPA can activate plasmin, which in turn can activate matrix metalloproteinases (MMPs) capable of degrading basement membrane components. Singh et al. (2005) illustrated a mechanism of COX-2 promotion of cellular invasion by showing that the level of pro-uPA was significantly higher (approximately 5-fold) in COX-2 transfected MDA-MB-231 cells compared to untransfected MDA-MB-231 cells via Western blotting. Additionally, they showed that increased COX-2 activity correlates with uPA in an *in vivo* mouse model of breast cancer metastasis to bone.

As previously mentioned, in order for cancer cells to migrate and invade and therefore metastasize, the cells must digest the basement membrane components (e.g., laminin, collagen IV, entactin and heparin sulfate proteoglycan) and the ECM, typically requiring the secretion and activation of MMPs. In particular, the gelatinases (MMP-2 and MMP-9) have been associated with high potential for metastasis in several human cancers including breast (Barsky et al., 1983 and Pacheco et al., 1998). Takahashi et al. (1999) have shown that Hs578T breast cancer cells transfected with COX-2 resulted in the activation of MMP-2. Sivula et al. (2005) also found increased COX-2 expression in breast cancer specimens in association with increased levels of MMP-2. Larkins et al. (2006) have likewise shown that inhibition of COX-2 using NS-398, decreases breast cancer cell motility and invasion due to the decrease in MMP expression. These studies

together reveal that the role of COX-2 in modulating the expression of MMP-9 and MMP-2 may be an important component of the molecular mechanisms by which COX-2 promotes cellular invasion and migration.

4.4 The Role of COX-2 in the Induction of miR-655 Expression

Our discovery of the induction of miR-655 by COX-2 and its role in promoting breast cancer aggressiveness presents a novel mechanism in breast cancer progression. A possible mechanism for the induction of miR-655 by COX-2 is that the gene encoding miR-655 may be the target of certain transcription factors that are activated in response to COX-2 in aggressive breast cancer cells. Whether downstream effectors in the prostaglandin signaling pathway are binding to and activating the promoter of miR-655, remains to be examined. It is also possible that downstream effectors in the prostaglandin signaling pathway bind to processing complexes within the miRNA biogenesis pathway, such as Drosha/DGCR8 or Dicer, leading to increased miR-655 expression.

In a study similar to ours, Shin et al. (2010) identified two miRNAs, miR-16 and miR-21, that were up-regulated in nicotine induced gastric cancer. They showed that the up-regulation of miR-16 and miR-21 were dependent on the transcription factor nuclear factor-kappa B (NF-kB), which induces COX-2 expression. Using bioinformatics, they determined that the miR-16 and miR-21 genes are in fact the direct targets of NF-kB. Furthermore, they demonstrated that antagonists of the EP2 and EP4 receptors attenuated nicotine activation of NF-kB. Additionally, it has been shown that COX-2 mediated up-regulation of VEGF-C, due to endogenous PGE₂ induced activation of the EP receptors, results from the recruitment of the NF-kB transcription factor binding to the VEGF-C

promoter site (Bhattacharjee et al., 2009). It is possible that miR-655 up-regulation by COX-2 results from NF-kB binding to the corresponding gene of miR-655 (Figure 19). To test this hypothesis, we would have to look at the transcription factor-binding sites in the miR-655 gene to determine if the NF-kB-binding site is present.

4.5 The Role of COX-2 and miR-655 Expression in Inducing the Tumorsphere Formation

This study has shown that COX-2 expression and miR-655 expression are both implicated in tumorsphere formation. Since COX-2 and miR-655 were significantly overexpressed in cells grown as tumorspheres compared to cells grown as a monolayer, it is likely that both play a role in maintaining the CSC *in vitro*. The role of COX-2 in the maintenance of the stem-like state has also been reported for in mesenchymal stem cells involved in osteogenesis (Yoon et al., 2010). Similarly Liou et al. (2007) state that COX-2 derived PGE2 protects embryonic stem cells from apoptosis.

COX-2 overexpression associated with tumorsphere formation can be explained by two possibilities: (1) Sphere-initiating cells can survive under stress and hypoxia, both of which can up-regulate COX-2 and COX-2 activity in turn can promote their expansion and survival. Colony formation in ultra-low attachment plates maybe subjected to stress and hypoxia. In support of this hypothesis it has been shown that direct transcriptional up-regulation of COX-2 by the hypoxia inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhanced HIF-1 transcriptional activity during hypoxia (Kaidi et al., 2006). (2) The CSC, even if rare in a cancer cell line, is high COX-2 expressing to start with and the COX-2 expressing CSC then expands as a colony. Singh et al. (2010)

speculated that the rare cells highly expressing COX-2 from MCF-7 tumorspheres induce the CSC state. This hypothesis could only be tested if the rare high COX-2 expressing cancer cells could be separated out by cytofluorometry and subsequently have their CSC activity measured. While such fluorometry is yet to be developed, the design could be similar to the measurement of Aldefluor activity used as a CSC marker to separate out ALDH-1 positive cells.

Since there is a significant up-regulation of miR-655 in tumorspheres with a concomitant up-regulation of COX-2 expression, this miR-655 up-regulation may be the consequence of COX-2 expression. On the other hand, the reverse cannot be ruled out since MCF-7-655 cells also showed increased COX-2 expression.

Interestingly, MCF-7 and MCF-7-COX-2 cells formed more spherical tumorspheres in comparison to SKBR-3 and SKBR-3-COX-2 cells, which formed irregular spheres. One main difference between the MCF-7 and SKBR-3 cell lines is that SKBR-3 overexpresses HER-2. However, breast cancer cell lines BT474 and MDA361 also have amplified HER-2 expression and they still form morphologically spherical spheres similar to MCF-7 cells (Wang et al., 2011). Therefore, HER-2 may not be the reason why SKBR-3 cells formed irregular spheres. Other laboratories have reported the formation of spherical SKBR-3 tumorspheres (Fengyan et al., 2007), however, in our hands we were unable to get ideal spheres. Due to the heterogeneity of cell lines, it is possible that the progeny of the stem-like cells may also be heterogeneous in their growth rate. This may explain the irregular formation of tumorspheres *in vitro* in cell lines such as SKBR-3 and SKBR-3-COX-2. In conclusion, the morphology of the

spheres formed may be context dependent or a result of the heterogeneity of a breast cancer cell line.

We have established that COX-2 plays a role in inducing miR-655 expression and tumorsphere formation, but the exact mechanisms remain to be investigated. Wang et al. (2011) showed that transforming growth factor- β (TGF- β) regulates the sphere-initiating stem cell-like feature in breast cancer cells through miR-181. Similarly, it is possible that COX-2 regulates the sphere formation via miR-655. To test this hypothesis, several approaches should be utilized. First, we would have to use a cell line with stable miR-655 expression in the absence of COX-2 expression and grow tumorspheres in order to determine if miR-655 overexpression alone could induce spheres in non-COX-2 expressing cells. Secondly, we would have to treat COX-2 negative cells, such as MCF-7, with exogenous PGE2 and EP4 agonist and observe if these breast cancer cells could form spheres more effectively and if miR-655 is simultaneously up-regulated. Finally, it would be important to look at the downstream targets of miR-655 to see if any of the genes that are inhibited by miR-655 play a role in influencing the sphere forming ability of breast cancer cells.

One important target of miR-655, as well as the second COX-2 up-regulated miRNA miR-526b, is the CPEB-2 tumor suppressor gene. This gene is a senescence-associated molecule. CPEB-2 null mice do not survive and the embryonic fibroblasts in these mice fail to mature (Groisman et al., 2006). The role of the CPEB-2 gene could be tested by down-regulating it in MCF-7 cells or up-regulating it in MCF-7-COX-2 cells and then looking at the subsequent efficiency of tumorsphere formation.

One miRNA known to play a role in maintaining the stem cell phenotype in embryonic cells is miR-302 (Lin et al., 2008). MiR-302 is expressed most abundantly in slow-growing human embryonic stem cells, and quickly decreases after cell differentiation and proliferation. Lin et al. (2008) transfected miR-302 into several human cancer cell lines. The miR-302-transfected cells not only expressed many key stem cell markers, such as Oct3/4, Sox2, and Nanog, but also had a highly demethylated genome similar to a reprogrammed zygotic genome. These miR-302-transfected cells also maintained pluripotency since they could differentiate into distinct tissue cell types, such as neuron, chondrocyte, fibroblast, and spermatogonia like primordial cells (Lin et al., 2008). In our study it would be very interesting to examine the expression of these stem cell markers in MCF-7-655 and SKBR-3-655 cells.

4.6 The Effect of COX-2 Inhibition and EP4 Antagonism on miR-655 Expression

In both MCF-7-COX-2 and SKBR-3-COX-2 human breast cancer cell lines, treatment with the COX-2 inhibitor (NS-398) and the EP4 antagonist (ONO-AE3-208) significantly decreased the expression of miR-655. This indicates that the expression of miR-655 in human breast cancer cell lines is dependent on both COX-2 and EP4 activity.

Both EP2 and EP4 are linked with a Gs protein and thus activation of these receptors leads to an increase in intracellular cAMP followed by activation of protein kinase A (PKA). In addition, EP4 activation can also stimulate the PI3K/Akt pathway (Sugimoto et al., 2007) to promote PGE2 dependent cell survival. In order to determine the role of the EP4 receptor in miR-655 expression we would have to examine whether the up-regulation of miR-655 depends on the stimulation of the PKA or PI3K/Akt pathways. It is possible that components of one or both of these pathways play a role in

up-regulating miR-655 expression.

There is compelling epidemiological evidence that intake of NSAIDs, including COX-2 inhibitors, leads to significant risk reductions for the development of cancers in various organs including the breast (Harris et al., 2003 and 2009). Since COX-2 inhibitors cause cardiovascular side effects (Fitzgerald, 2004) alternative and safer therapies need to be tested in preclinical models.

Recent studies have demonstrated that the EP4 antagonist ONO-AE3-208, but not EP1 antagonist ONO-8713, was highly and equally effective as a COX-2 inhibitor (Celecoxib) in inhibiting primary tumor growth and tumor-associated lymphangiogenesis and lymphatic metastasis *in vivo*. Furthermore, ONO-AE3-208 produced no drug-related toxicities (Xin et al., 2012).

Since EP4 has been shown to promote breast cancer progression and it may have a role in inducing miR-655 and thereby the CSC phenotype, we suggest that EP4 antagonism may prove to be a safe therapy in breast cancer patients in preventing and reducing metastasis in combination with other agents.

4.7 The Effect of miR-655 Expression on COX-2 Expression

We have shown that miR-655 up-regulates COX-2 expression in MCF-7 breast cancer cells, however the underlying mechanisms remain unknown.

Literature reporting on the role of miR-655 is very limited. One study suggests that miR-655, amongst other miRNAs, regulate amyloid precursor protein expression *in vitro*, therefore having a potential role in Alzheimer's disease progression (Delay et al., 2011). One interesting study by Nares (2011) used microarrays to identify differentially-

expressed miRNA profiles of periodontal diseased tissues and found that miR-655 targets the 3' UTR of interferon-gamma (IFN-gamma), an inflammatory cytokine present in the tumor microenvironment. Several studies report an anti-inflammatory role of IFN-gamma as it has been shown to suppress COX-2 transcription in tissues such as the placenta (Hanna et al., 2004) and in vascular lesions (Deng et al., 2005). Therefore, miR-655 could be inhibiting IFN-gamma leading to an up-regulation of COX-2 expression (Figure 19). However, IFN-gamma has also been shown to up-regulate a variety of pro-inflammatory mediators such as interleukin (IL)-12, tumor necrosis factor- α (TNF α) and inducible nitric oxide synthase (iNOS) (reviewed by Muhl and Pfeilshofer, 2003). Therefore the role of this cytokine in inflammation is ambiguous.

Another mechanism by which miR-655 could up-regulate COX-2 expression is via the down-regulation of DNA methyltransferase (DNMT). DNMTs epigenetically silence gene expression by methylation. Fang et al. (2012) showed that miRNAs of the miR29 family induce COX-2 expression in lung epithelial cells by regulating DNMTs, and this is worth investigating in our future studies.

4.8 The Role of miR-655 in the Promotion of Migration, Invasion and Proliferation of MCF-7 and SKBR-3 Breast Cancer Cells

Our laboratory has recently shown that knocking down miR-655 expression with morpholino oligos significantly reduced COX-2 mediated function in breast cancer progression (Majumder et al., 2012). When the expression of miR-655 is knocked down, MCF-7-COX-2 cells show a significant decrease in their ability to invade, migrate, and proliferate and SKBR-3 showed noted variability. Similarly, as presented here, when

miR-655 is overexpressed, MCF-7 and SKBR-3 cell lines showed an increase in ability to migrate and invade. The introduction of miR-655 also increased the ability of MCF-7 cells, but not SKBR-3 cells, to proliferate. There are several explanations for this difference. The increase in proliferation of MCF-7-655 cells may be context dependent or cell line specific and needs to be further investigated in other breast cancer cell lines. Another possibility as to why there was no difference seen in SKBR-3-655 proliferation is that the incubation period with BrdU needs to be longer than six hours, since in culture SKBR-3 is a relatively slower growing breast cancer cell line.

If miR-655 increases the expression of COX-2 as discussed earlier then the increase in migration, invasion and proliferation seen in the transfected cell lines is likely the result of COX-2 up-regulation. As discussed previously, it is well documented that COX-2 increases migration, invasion and proliferation in human breast cancer cells (Timoshenko et al. 2003, Rozic et al. 2001, Sobolewski et al. 2010, and Singh et al. 2005).

4.9 Clinical Implications and Significance

MiRNAs can be easily extracted from nearly every cell and tissue type due to their high resistance to degradation and their small size. Circulating miRNAs can easily be measured in whole blood or serum (Andorfer et al., 2011). Identification of miRNAs up-regulated in human breast cancer that are associated with the CSC phenotype and COX-2 overexpression are promising for use as prognostic markers for screening, monitoring, and therapeutic responses in the clinic.

COX-2 inhibitors and EP4 antagonists have been successfully utilized to abrogate tumor growth, tumor-associated angiogenesis, lymphatic and lung metastasis in a COX-2 overexpressing murine breast cancer model (Xin et al., 2012). Thus, EP4 may prove to be a safe target in the clinic for preventing and mitigating lymphatic metastasis of breast cancer in combination with other agents.

4.10 Possible Limitations

One important limitation of the present study design is the use of a limited number of cell lines. Although similar results were seen in both the MCF-7 and SKBR-3 breast cancer cell lines, results varied somewhat, especially with respect to proliferation. In order to establish a universal role of miR-655 in COX-2 mediated breast cancer progression it is important to examine its expression and function in other breast cancer cell lines and *in vivo*.

Another limitation of this study is that the CSC phenotype was only analyzed using a single assay, the tumorsphere formation assay. In order to strengthen our present results it would be helpful to revalidate the CSC phenotype of our cells by testing for other possible stem cell markers (e.g., Oct-4, Nanog, Sox-2 and CD44⁺/CD24⁻).

4.11 Future Directions

4.11.1 Mechanism in COX-2 and miR-655 signaling?

The present study was unable to discern the mechanisms responsible for COX-2 up-regulation of miR-655 expression and vice versa in MCF-7 and SKBR-3 human breast cancer cells. Our results show that COX-2 up-regulation leads to miR-655

expression and likewise miR-655 up-regulation leads to COX-2 overexpression. Future studies are needed to examine this relationship, including functional testing of the predicted miR-655 target genes (i.e. CPEB2, TP53 etc.). Treating breast cancer cells with exogenous PGE2 or EP4 agonist (PGE-alcohol) and looking for a possible up-regulation in miR-655 and increased spheroid formation would also help to elucidate the mechanism of action of COX-2 and miR-655 expression.

4.11.2 Future *in vivo* experiments

Additional work will be performed *in vivo* to strengthen the acquired results and thereby the level of confidence in the hypothesis being tested. In complementary studies, Dr. Mousumi Majumder will assess the effects of miR-655 knockdown in MCF-7-COX-2 cells and overexpression of miR-655 in MCF-7 cells on the lung-metastatic ability in NOD/SCID/GUSB null mice (in collaboration with Dr. David Hess, Roberts Institute). Our laboratory also plans to analyze the expression of miR-655 in human breast cancer tissues and blood samples.

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APPENDIX

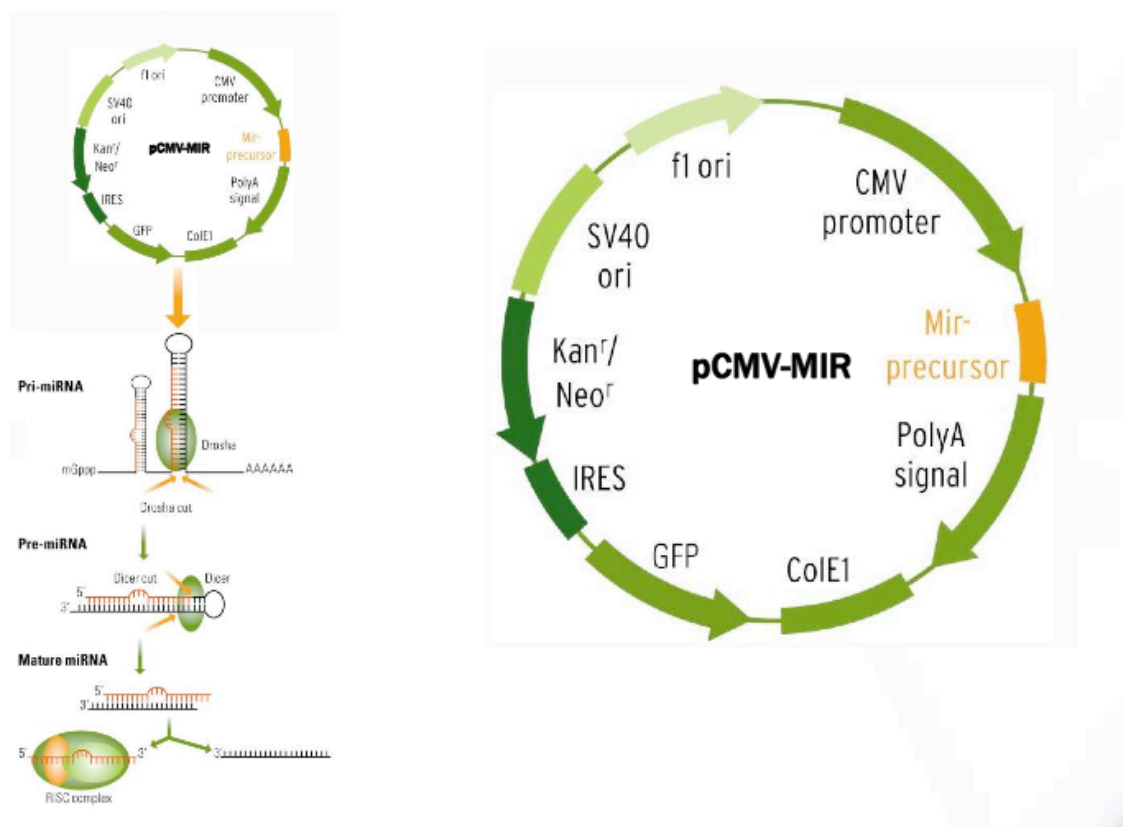


Figure A1. MicroRNA Overexpression Plasmid. A vector map of the MicroRNA pCMV6-MiR Expression Plasmid (OriGene). The expression of miRNA is driven by the CMV promoter and with human growth factor 1 poly(A) tailing signal.

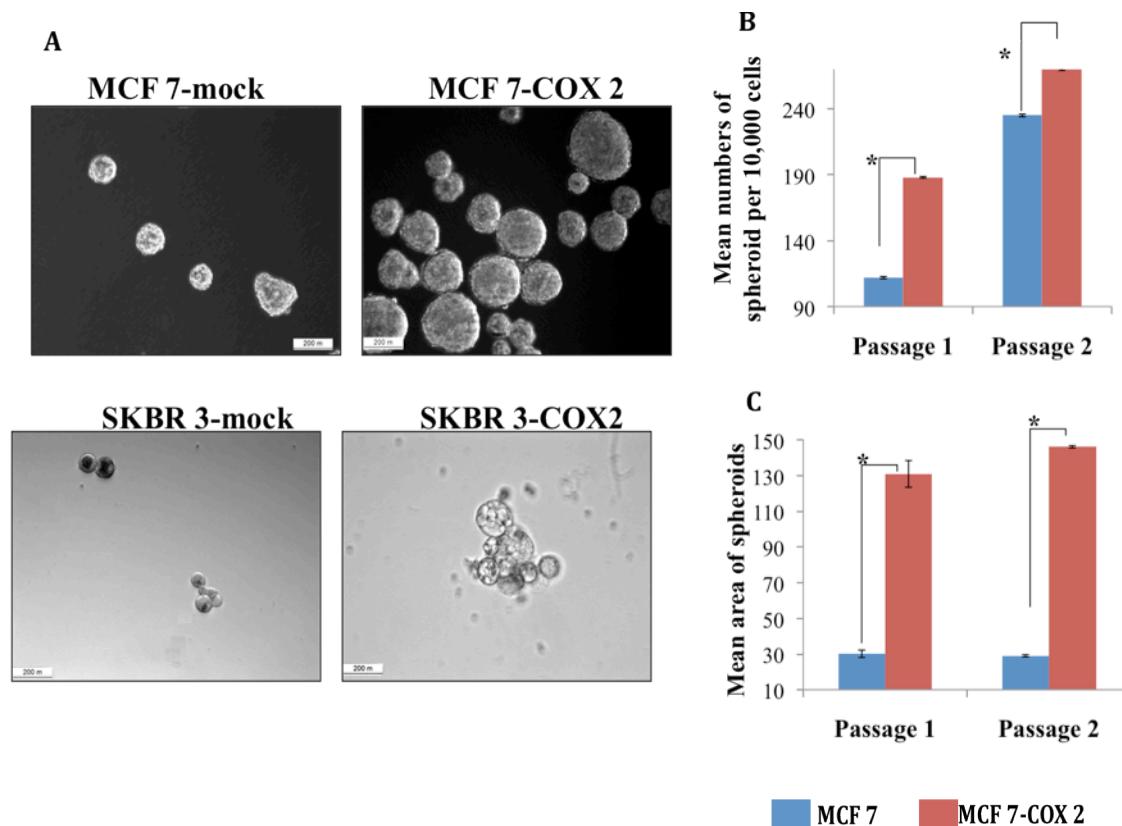


Figure A2. COX-2 overexpression increases the incidence and size of spheroids.

Passage zero (A) shows that incidence and size of spheroids in COX-2-expressing MCF-7 and SKBR-3 cells. (B) and (C) Serial passage of spheroid-derived cells from MCF-7-COX-2 shows increased spheroid-forming capacity both in number (B) and sizes (C). *P*-values for each passage are <0.001. (Majumder M et al., 2012.)

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