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The Role of miR-526b in COX-2 Mediated Human Breast Cancer Progression and Induction of Stem-Like Phenotype Via EP4 Receptor Signaling

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

THE ROLE OF MIR-526b IN COX-2 MEDIATED HUMAN BREAST CANCER PROGRESSION AND INDUCTION OF STEM-LIKE PHENOTYPE VIA EP4 RECEPTOR SIGNALING

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ABSTRACT

Our laboratory previously established that aberrant expression of cyclo-oxygenase (COX)-2 promotes breast cancer progression and metastasis via multiple mechanisms, including stem-like cell (SLC) induction, owing to activation of the prostaglandin E2 receptor EP4. COX-2 expression was linked to up-regulation of miRNA-526b. We hypothesized that miR-526b is regulated by EP4 activity, and that miR-526b supports breast cancer progression and induction of SLCs. Using stably miR-526b transfected MCF-7 and SKBR-3 cells in functional assays, including tumorsphere formation *in vitro* and lung colony formation *in vivo*, we observed enhanced migration, invasion, proliferation, tumorsphere formation, and *in vivo* tumorigenecity compared to controls. EP4 receptor activation and inhibition resulted in respective increases or decreases in miR-526b expression in PKA and PI3K-AKT dependent manners. We conclude that miR-526b promotes breast cancer progression and SLC induction, is up-regulated by EP4, and holds promise as a biomarker for monitoring and personalizing breast cancer treatment.

KEYWORDS: Cyclooxygenase-2 (COX-2), EP4 receptor, prostaglandin E2 (PGE2), microRNA, miR-526b, stem-like cells (SLCs), breast cancer.

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TABLE OF CONTENTS

Abstract ..... **ii**

Acknowledgements ..... **iii**

Table of Contents ..... **iv**

List of Figures ..... **ix**

List of Abbreviations ..... **xi**

CHAPTER ONE: INTRODUCTION **1**

1.1 Overview of Breast Cancer 2

1.2 Prostanoids 6

1.2.1 Cyclooxygenases (COX) and Prostanoid Production 6

1.2.2 COX-2, PGE2, and Breast Cancer 10

1.2.3 COX Inhibitors and Cancer 11

1.3 EP Receptors 12

1.3.1 EP Receptor Physiology 12

1.3.2 EP4 Receptor and Breast Cancer 13

1.3.3 EP4 Signaling Pathways and Cancer 15

1.4 Cancer Stem Cells (CSCs) 19

1.4.1 Cancer Stem Cell Hypothesis 19

1.4.2 CSCs in Breast Cancer 23

1.5 MicroRNAS 25

1.5.1 Biogenesis and Activity of miRNAS 25

1.5.2 miRNAs and Breast Cancer 29

1.5.3. miRNAs and CSCs 33

1.6 Rationale 34

1.7 Hypothesis and Objectives 38

CHAPTER 2: EXPERIMENTAL PROCEDURES 39

2.1 Cell Lines and Culture 40

2.2 Nucleotransfection with miR-526b Over-Expression Plasmid 41

2.3 Quantitative Real-Time RT- Polymerase Chain Reaction (qPCR) 42

2.3.1 MiR-526b Expression 42

2.3.2 Comparing Effects of COX-2 and EP4 Receptor Activity on miR-526b Expression 43

2.3.3 Effects of EP 1-4 or EP4 Activation on miR-526b Expression 43

2.3.4 Effects of PI3K Inhibition on miR-526b Expression 43

2.3.5 Effect of PKA Inhibition on miR-526b Expression 44

2.3.6 Qualitative Analysis of PKA Stimulation and Inhibition 44

2.3.7 Quantitative Real Time RT- PCR for miRNA Expression 45

2.4 Western Blot Analysis: pAKT Protein Expression 46

2.5 Transwell Assays 47

2.5.1 Cellular Migration 47

2.5.2 Cellular Invasion 48

2.6 Cell Proliferation ELISA, BrdU Assay 49

2.7 Tumorsphere Formation Assay 50

2.7.1 Establishing Tumorspheres in Culture 50

2.7.2 Tumorsphere Forming Efficiency 51

2.8 *In Vivo* Lung Colony Formation 51

2.8.1 Model Organism 51

2.8.2 Lung Colony Formation 51

2.8.3 Cell Proliferation *In Vivo* 52

2.9 Statistical Analysis 53

CHAPTER 3: RESULTS 54

3.1 EP4 Receptor Activity Regulates miR-526b Expression in MCF-7 and MCF-7-COX-2 Human Breast Cancer Cells 55

3.1.1 Treatment with COX-2 Inhibitor or EP4 Receptor Antagonist Decreases miR-526b Expression in MCF-7-COX-2 Cells 55

3.1.2 Treatment with PGE2 and PGE1OH Increases miR-526b Expression in MCF-7 Monolayer and Tumorsphere Culture Conditions 57

3.1.3 Treatment with PGE2, PGE1OH, or L-902-688 Stimulates pAKT Protein Levels in MCF-7 Cells 59

3.1.4 Treatment with PGE2 and PGE1OH Stimulates SLC Phenotype in MCF-7 Cells 61

3.2 miR-526b Expression in MCF7-COX-2 Cells is Dependent on PI3K/AKT Signaling Pathway 63

3.2.1 Treatment with PI3K Inhibitors Decreases miR-526b Expression and pAKT in MCF-7-COX-2 Cells 63

3.2.2 Treatment with PI3K Inhibitors Reduces SLC Phenotype in

MCF-7-COX-2 Cells 65

3.3 cAMP Signaling Regulates miR-526b Expression in MCF-7 and MCF-7-COX-2 Cells 67

3.3.1 Treatment with EP4 Agonist PGE1OH Increases miR-526b Expression in MCF-7 Cells 67

3.3.2 Treatment with PKA Inhibitor Decreases miR-526b Expression in MCF-7-COX-2 Cells 69

3.4. Validation of Stable miR-526b Over-Expression in MCF-7 and SKBR-3 Cells Transfected with Over-Expression Plasmid 71

3.5 MCF-7 and SKBR-3 Human Breast Cancer Cells Over-Expressing miR-526b Display Enhanced Lung Colony Forming Ability in NOD/SCID/GusB-Null Female Mice 73

3.5.1 MCF-7-526b and SKBR-3-526b Cells Display Enhanced Ability to Form Lung Colonies *In Vivo* Compared to Mock Controls 73

3.5.2. MCF-7-526b and SKBR-3-526b Lung Sections Display Increased Cellular Proliferation *In Vivo* Compared to Mock Controls 76

3.6 Over-Expression of miR-526b Increases Cellular Migration and Invasion in MCF-7 and SKBR-3 Cells 79

3.7 Over-Expression of miR-526b Increases Cellular Proliferation in MCF-7 and SKBR-3 Cells 82

3.8 Over-Expression of miR-526b Stimulates SLC Phenotype in MCF-7 and SKBR-3 Cells 84

3.8.1 MCF-7-526b and SKBR-3-526b Cells Form Spheroids of Greater

Number and Size Compared to Parental Controls 84

3.8.2 MCF7-526b Cells Form Spheroids at a Higher Efficiency Compared to Parental Control 87

CHAPTER 4: DISCUSSION AND CONCLUSIONS 89

4.1 Summary of Novel Findings and Conclusions 90

4.2 Contribution of Novel Findings to Current Field of Research 92

4.3 Potential Limitations of Study 101

4.4. Future Directions 102

4.5 Conclusion 103

REFERENCES 104

APPENDIX 116

CURRICULUM VITAE 120

LIST OF FIGURES

Figure 1. Prostanoid Pathway and EP Receptors 8

Figure 2. Cancer Stem Cells as Therapeutic Targets 21

Figure 3. MicroRNA (miR) Biogenesis and Activity 27

Figure 4. Comparative Analysis of Combined Gene and miRNA Expression in MCF-7 and MCF-7-COX-2 Human Breast Cancer Cells 36

Figure 5. MiR-526b Expression Decreases with COX-2 Inhibitor and EP4 Antagonist Treatment in MCF-7-COX-2 56

Figure 6. MiR-526b expression increases with PGE2 and PGE1OH treatment in MCF-7 58

Figure 7. pAKT Protein Expression Increases in Response to Treatment with PGE2, L-902688, or PGE1OH in MCF-7 60

Figure 8. PGE2 and PGE1OH stimulate SLC phenotype in MCF-7 62

Figure 9. PI3K Inhibition Reduces miR-526b Expression and Reduces pAKT Levels in MCF-7-COX-2 64

Figure 10. PI3K Inhibition Reduces SLC Phenotype in MCF-7-COX-2 66

Figure 11. PKA Activity Increases in Response to Treatment with EP4 Agonist PGE1OH in MCF-7 68

Figure 12. PKA Inhibition Reduces miR-526b Expression in MCF-7-COX-2 70

Figure 13. Validation of Stable miR-526b Over-Expression in

MCF-7 and SKBR-3 Cells Transfected with Over-Expression Plasmid 72

Figure 14. MCF-7-526b and SKBR-3-526b Cells Display Enhanced Ability to Form Lung Colonies *In Vivo* Compared to Mock Controls 74

Figure 15. MCF-7-526b and SKBR-3-526b Lung Sections Display Increased Cellular Proliferation *In Vivo* Compared to Mock Controls 77

Figure 16. MiR-526b Increases Cellular Migration in MCF-7 and SKBR-3 80

Figure 17. MiR-526b Increases Cellular Invasion in MCF-7 and SKBR-3 81

Figure 18. MiR-526b Increases Cellular Proliferation in MCF-7 and SKBR-3 83

Figure 19. MiR-526b Promotes the Induction of SLC phenotype in MCF-7 85

Figure 20. MiR-526b Promotes the Induction of SLC Phenotype in SKBR-3 86

Figure 21. MiR-526b Promotes Enhanced Spheroid Formation Efficiency in MCF-7 88

Figure 22. Possible Mechanism of COX-2 and miR-526b Signaling in Human Breast Cancer Cell Lines 99

Figure A1. MicroRNA over-expression plasmid structure 117

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AKT	Protein Kinase B
ANOVA	Analysis of Variance
BAD	Bcl-2-Associated Death promoter
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
COX	Cyclooxygenase
CSC	Cancer Stem Cell
CREB	cAMP Response Element-Binding
CPEB	Cytoplasmic Polyadenlyation Element Binding
DCIS	Ductal Carcinoma *In Situ*
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
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
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
hsa-miR-526b	*Homo Sapiens* Micro Ribonucleic Acid 526b
IDC	Invasive Ductal Carcinoma
ILD	Invasive Lobular Carcinoma
iNOS	Inducible Nitric Oxide Synthase
LCIS	Lobular Carcinoma *In Situ*
MAb	Monoclonal Antibody
miRNA	Micro Ribonucleic Acid
M-PER	Mammalian Protein Extraction Reagent
mTOR	Mammalian Target of Rapamycin
mRNA	Messenger Ribonucleic Acid
NF- κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NS-398	N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide
NSAID	Non-Steroidal Anti-inflammatory Drug
ONO-AE8-208	4-(4cyano-2-(2-(4-fuoronaphthalen-1-yl)propionylamino)phenyl) butyric acid
PCR	Polymerase Chain Reaction

PG	Prostaglandin
PGE2	Prostaglandin E2
PGES	Prostaglandin E Synthase
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PLA2	Phospholipase A2
PLC	Phospholipase C
PR	Progesterone Receptor
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNU44/48	Small Nuclear RNA
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SLC	Stem-Like Cell
SDS-PAGE	Sodium Dodoecyl Sulfate-Polyacrylamide Gel Electrophoresis
SFM	Serum Free Medium
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Tween-20
TGF-ß	Transforming Growth Factor Beta
VEGF-C, D	Vascular Endothelial Growth Factor

CHAPTER 1: INTRODUCTION

1.1 Overview of Breast Cancer

Breast cancer is a disease that arises from the epithelial lining in the lobular or ductal tissue of the breast, resulting in the formation of a malignant tumor mass within the tissue. A tumor can be described as a mass of cells within a tissue that have lost the ability to regulate the rate at which cells renew and differentiate, leading to aberrant growth. Often, they exhibit a rapid proliferation rate. Cancer cells also have the ability to invade surrounding tissues and leave their primary site of origin, leading to metastasis and the formation of a secondary tumor. In Canada, breast cancer is the most frequently diagnosed cancer in women, with 1 in 9 women expected to develop the disease within their lifetime (Canadian Cancer Society, 2014). In Canadian women, breast cancer continues to have a major impact on mortality rates, currently established to have the 2nd highest number of cancer-related deaths, behind lung cancer, in 2014 (Canadian Cancer Society, 2014). While advances in screening procedures and earlier diagnosis have been promising, the treatment and survival rates of breast cancer still have much room for improvement. In particular, the treatment of breast cancers that have already metastasized to other locations in the body remains especially challenging. Therefore, new therapeutic strategies are greatly needed to improve breast cancer patient outcomes.

Breast cancer is generally characterized by a few key characteristics: origin of disease within the mammary tissue, relative level of invasiveness, hormone receptor status, and most recently by the expression of specific genetic markers. An account of these distinct features is important in patient diagnosis, and has crucial implications for the course of treatment prescribed. Breast cancer almost always develops within the glandular epithelial lining of two main structures of the breast, the ducts or the lobules of

the mammary glands, and is hence termed adenocarcinoma. Most commonly, breast cancer arises in the ducts of the mammary gland, and is called ductal carcinoma. Alternatively, the cancer can arise in the lobules of the mammary glands that are required for milk production, and is then referred to as lobular carcinoma. If the malignancy arising in the tissue of origin has not yet invaded other tissues, it is referred to as either ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS). However, if the cancer cells begin to invade nearby tissues, the treatment inherently becomes more difficult because cancer cells occupy multiple locations simultaneously. At this stage, the name of the cancer changes to either invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) (American Cancer Society, 2014).

At the pre-invasive stage of breast cancer, the identified malignant tissue can be removed via a lumpectomy and the patient may be treated with other traditional therapies such as chemotherapy or radiation. However, invasive carcinomas present a greater challenge due to the complex nature of the metastasis. Metastasis in carcinomas begins with malignant cells losing their polarity, and acquiring the ability to move out of their normal confines by a variety of changes including epithelial-mesenchymal transition (EMT) (Bartuma *et al.,* 2014). The next step in the metastatic cascade is for the malignant cell to move into a newly established blood or lymphatic vessel and enter the bloodstream in a process called intravasation. The ability of cancer cells to invade blood vessels has been linked to a variety of tumor-stroma cell interactions, including those with pericytes and endothelial cells, which normally maintain vascular integrity. Intravasation is facilitated by formation of new blood and lymphatic vessels at the primary tumor site (angiogenesis and lymphangiogenesis), which have weaker endothelial barriers

(Valastyan and Weinberg, 2011). Once inside the blood or lymphatic system, the cancer cells can and travel to a secondary location within the body. At the secondary site, the cancer cell may extravasate from the vessel into the surrounding tissue and may begin to establish a secondary tumor. The ability of the cancer cell to grow and proliferate at a secondary location is thought to depend on a variety of factors, including cellular and molecular components of the microenvironment (Fidler *et al.*, 2003). The most common sites of metastasis in breast cancer tend to be the auxillary lymph nodes, lungs, liver, and bone marrow. A comprehensive study by Müller *et al.* (2001) demonstrated that specific ligands for the chemokine receptors CCR7 and CXCR4, which are highly expressed by human breast cancer cells, malignant breast tissue and metastases, display peak levels of expression in all of these organs. Therefore, the microenvironment of the secondary tumor site likely plays crucial roles in mediating establishment of a new tumor outside of the primary site.

Traditional classification of breast cancer includes the expression of two hormone receptors, estrogen (ER) and progesterone (PR), as well as human epidermal growth factor 2 (HER2) receptors. The pattern of expression of these three receptors via immuno-histological analysis has been used to formulate treatment plans and predict disease outcomes for many years. Approximately 75% of all diagnosed breast cancers are found to be ER+, and estrogen-blocking (e.g. tamoxifen) therapies initially show promising results. Unfortunately, most patients experience resistance to these drugs over time, as the ER status is thought to change via several epigenetic mechanisms (Sommer and Fuqua, 2001). An estimated 65% of ER+ tumors are also PR+, and like ER status, PR status can change over time. The HER2 receptor status of a tumor is also very important

when considering a course of treatment. It is estimated that this receptor is over-expressed in 20-25% of all breast cancer patients, and like ER+ tumors, HER2 blocking agents have shown promising results in the initial stages of treatment (Slamon *et al.*, 1989). One such agent is a monoclonal antibody for HER2, called trastuzumab (Herceptin), which is currently considered the gold standard for treating HER2+ patients (Arteaga *et al.,* 2012). However, limitations exist in targeting this receptor, as many patients develop resistance over time and the risk of cardiac toxicity remains high in patients with other risk factors. Even given the challenges associated with changes in receptor status over the course of treatment, hormone and HER2 receptor positive cancers tend to have better outcomes than receptor negative cancers (Foulkes *et al.,* 2010). Cancers of this "triple negative" class account for approximately 15% of diagnoses, and they tend to be much more difficult to treat due to the fact that they do not express hormone receptors that can be targeted pharmacologically.

With technological advancements in recent years surrounding the human genome, large-scale gene expression profiles can now be used to categorize breast cancer sub-types in comparison to normal breast tissue, and in comparison to each other. For instance, Sørlie *et al.* (2006) used three different DNA expression platforms (DNA, cDNA, whole genome) to identify distinct molecular profiles for the basal and luminal A sub-types of breast cancer. These sub-types display unique physical and pathological features, in addition to distinct gene expression profiles. Luminal A cancers are characterized by a high expression of estrogen receptors, in addition to expression of several genes normally expressed in luminal epithelial cells that line the ducts of the mammary gland, and clinically have better prognostic outcomes (Sørlie *et al.,* 2003).

Conversely, basal-like tumors do not express estrogen receptors, tend to express genes normally found in the basal epithelial tissue (such as laminin), and tend to be associated with poorer patient outcomes (Sørlie *et al.,* 2003). In addition, gene expression profiles of tumors are also capable of revealing information about specific genetic mutations that can predict breast cancer outcomes, such as the expression of the *BRCA1* gene in basal-like tumors (Lakhani *et al.,* 2005; Perou 2011).

Given the complex and inherently heterogeneous nature of breast cancer, establishing different sets of reliable genetic markers that can accurately distinguish between sub-types of the disease will be extremely useful in creating more effective and personalized treatment regimes. In addition to gene expression profiles, large-scale miRNA microarray profiling may also be a useful tool in discovering miRNA biomarkers of cancer sub-types. A study conducted by Bockmeyer *et al.* (2011) demonstrated that miRNA expression profiles in normal basal and luminal breast epithelial tissue display distinct differences, and that these differences are also observed in basal and luminal A breast cancer sub-types. Therefore, a combination of several gene expression and miRNA expression profiles could be of great use in the early diagnosis of patients via a simple blood test, and could also assist clinicians in formulating personalized treatment plans that will better target the unique aspects of each patient's disease.

1.2 Prostanoids

1.2.1 Cyclooxygenases (COX) and Prostanoid Production

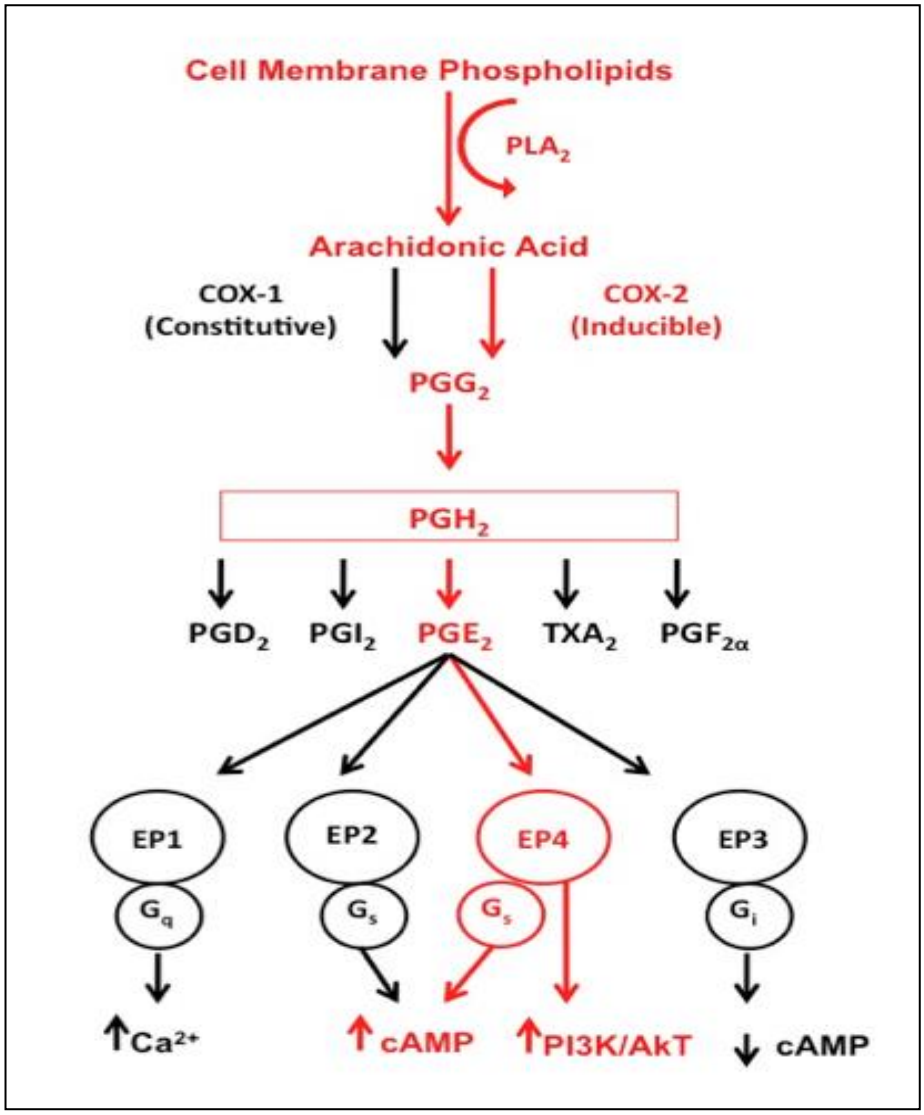
The two well-characterized COX iso-enzymes, COX-1 and COX-2, play distinct roles in normal physiological and pathological processes. COX-1 is constitutively expressed in almost all tissues of the body, while COX-2 is inducible and characterized

as an inflammation-associated enzyme (Marnett *et al.*, 1999) expressed in response to mitogens, hormones, cytokines, and growth factors (Costa *et al.*, 2002). Both COX enzymes tend to be located on the luminal side of endoplasmic reticulum and the nuclear envelope (Chandrasekharan and Simmons, 2004), and display cyclooxidase and peroxidase activity.

Prostanoids are a class of signaling molecules, known as eicosanoids, which are generated through the oxidation of fatty acids found within the lipid bilayer. The main prostanoid categories include prostaglandins (PG), prostacyclins (PGI), and thromboxanes (TXA). In recent years, studies investigating the inflammatory PGE2 have come to the forefront in the field of cancer research. PGE2 production involves a number of complex steps involving several enzymes, the most important being the cyclooxygenase (COX) isoenzymes COX-1 and COX-2 **(Figure 1)**. Given an appropriate stimulus, phospholipase A2 (PLA_2) becomes activated and cleaves the prostanoid pre-cursor arachidonic acid (AA) from the lipid bilayer. A two-step rate-limiting reaction then occurs via the activity of COX-1 and COX-2, where AA undergoes a primary oxidation reaction to produce prostaglandin G2 (PGG2), followed by a hydroperoxidase reaction to produce prostaglandin H2 (PGH2). PGH2 can then be converted into 5 key prostanoid products, depending on the physiological requirements of the tissue and the availability of prostaglandin, prostacyclin, and thromboxane synthase enzymes. These prostanoid products are: PGE2, PGI2, PGD2, $PGF2_a$, and TXA2. PGE2 has been implicated in several aspects of cancer, including cell proliferation, angiogenesis, apoptosis, and stability of the immune system (Nakanishi and Rosenburg, 2012).

Figure 1. Prostanoid Pathway and EP Receptors

COX-1 and COX-2 are key enzymes in the synthesis of prostanoids, including prostaglandin E2 (PGE2). Under appropriate conditions, cell membrane phospholipids are converted to arachidonic acid (AA) by the enzyme Phospholipase A2, and AA serves as the substrate for COX-1 and COX-2. COX enzyme activity controls the rate-limiting steps of prostaglandin production. These enzymes convert AA to the prostaglandin precursors PGG2 (via COX activity) and PGH2 (via peroxidase activity). PGH2 is then converted to various prostanoids (including PGE2) via several prostanoid synthases. PGE2 can bind to four G-protein-coupled prostaglandin E2 (EP) receptors to elicit various physiological outcomes. PGE2 binding the EP1 receptor leads to G_q protein activation of phospholipase C inositol triphosphate signaling, and an increase in intracellular calcium levels. PGE2 binding to EP3 elicits an inhibitory cell response, via coupling with a G_i protein to inhibit adenylate cyclase activity. PGE2 binds the EP2 and EP4 receptors to elicit a stimulatory cell response via coupling with a G_s protein. EP2 receptor stimulation leads to activation of the cyclic adenosine monophosphate (cAMP) signaling pathway, while EP4 receptor stimulation leads to activation of both the canonical cAMP and non-canonical phosphatidylinositol-3-kinase (PI3K/AKT) signaling pathways. Activation of the PI3K-AKT pathway is unique to the EP4 receptor, and not shared by EP2. This pathway is highlighted in red and is the focus of this project. (Adapted from: Taketo 1998; Gualde & Harizi, 2004).


Cell Membrane Phospholipids
PLA2
Arachidonic Acid
COX-1
(Constitutive)
COX-2
(Inducible)
PGG2
PGH2
PGD2
PGI2
PGE2
TXA2
PGF2α
EP1
EP2
EP4
EP3
Gq
Gs
Gs
Gi
↑Ca2+
↑cAMP
↑PI3K/AkT
↓cAMP

1.2.2 COX-2, PGE2, and Breast Cancer

Aberrant COX-2 expression has been implicated in a wide range of epithelial cancers, including breast (Hwang *et al.,* 1998; Parrett *et al.,* 1997), lung (Hida *et al.,* 2008), colon (Eberhart *et al.,* 1994), head and neck (Chan *et al.,* 1999), and pancreas (Tucker *et al.,* 1999). In breast cancer, COX-2 up-regulation has been observed in about 40% of primary breast cancer cases (Denkert *et al.,* 2003) and has been linked to cancer progression and metastasis (Costa *et al.,* 2002). In addition, COX-2 expression has been shown to be inversely correlated with disease-free survival (Denkert *et al.,* 2003; Ristimäki *et al.,* 2002). In both pre-invasive (Ristimäki *et al.,* 2002; Perrone *et al.,* 2006) and invasive (Bhattacharjee *et al.,* 2010) stages of breast cancer, COX-2 over-expression has been observed, suggesting the promoting role of COX-2 in establishment and progression of the disease. COX-2 expression occuring both at the pre-invasive (Ristimäki *et al.,* 2002; Perrone *et al.,* 2006) and invasive (Bhattacharjee *et al.,* 2010) stages implicate COX-2 in perpetuation of the disease.

Previous work in our lab has shown that tumor-derived PGE2 arising from COX-2 expression by tumor or host cells promotes breast cancer progression through several mechanisms, including inactivation of host anti-tumor immune cells (Lala *et al.,* 1997), stimulation of tumor cell migration, invasiveness, angiogenesis (Rozic *et al.,* 2001; Timoshenko *et al.,* 2004 & 2006) and lymphangiogenesis promoting lymphatic metastasis (Timoshenko *et al.,* 2006). COX-2 up-regulated vascular endothelial growth factor (VEGF)-C also promoted tumor cell motility by binding to a diverse group of VEGF-C receptors expressed by breast cancer cells (Timoshenko *et al.,* 2007). Using naturally low COX-2-expressing or COX-2-deficient human breast cancer cell lines

(MCF7 and SKBR3, respectively) our lab has also shown that over-expression of COX-2, by stable transfection with a COX-2 expression plasmid, results in many changes including increased migration, invasion, proliferation, and self-renewal ability (Majumder *et al.,* 2012). COX-2 over-expressing breast cancer cell lines also displayed an increased expression and secretion of angiogenic (VEGF-A) and lymphangiogenic factors (VEGF -C, -D) (Timoshenko *et al.,* 2006 & 2007). In addition, our lab has also observed an association between COX-2 over-expression and up-regulation of the prostaglandin E2 (PGE2) cell surface receptor EP4 in human breast cancer cell lines and in tumors obtained from xenotransplants in immune-compromised mice (Majumder *et al.,* 2014).

1.2.3 COX Inhibitors and Cancer

Non-specific COX inhibitors, such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have been used for many years to reduce inflammation, and several studies have noted that their use correlates with a reduction in cancer incidence. A comprehensive epidemiological study by Harris *et al*. (2005) demonstrated that daily NSAID intake resulted in a significant reduction in the risk of developing colon (63%), lung (36%), prostate, (39%) and breast (39%) cancer after a period of 5 years. Another study by Rothwell *et al.* (2012) reported that preventative low-dose aspirin intake was associated with a significant reduction in cancer incidence and cancer mortality, and that such benefits were observed after a period of 3-5 years. However, NSAIDs have limitations due to potential severe side effects that often present with chronic use. Since COX-1 and COX-2 enzymes have different physiological roles in the body, non-specific blocking of both enzymes has been shown to lead to an increase risk of gastric ulcers due

to loss of COX-1 activity that supports normal gastric mucosa (Peterson and Cryer, 1999). Such risks lead to the production of COX-2 specific inhibitors, such as Celecoxib, for the treatment of inflammatory conditions. Several studies have investigated the potential of using COX-2 inhibitors in the prevention and treatment of cancers. Reddy *et al.* (2000) showed that treating later stage colon cancer patients with Celecoxib lead to a reduction in disease progression, and Celecoxib therapy in colon cancer has also been shown to induce cell apoptosis and induce cell cycle arrest (Gröesch *et al.*, 2005). Nevertheless, COX-2 inhibitors have been reported to increase the risk of adverse cardiovascular events, due to a potential imbalance in production of COX-2-derived PGI2 (vasodilation) and COX-1 derived aggregating agent TXA2 (platelet aggregation) (Funk and FitzGerald, 2007). Therefore, new therapeutics that target alternative aspects of the PGE2 pathway, without altering COX-1 and COX-2 enzyme production, are greatly needed. One strategy that has arisen in recent years is to target downstream effectors of the prostanoid pathway, such as the prostaglandin E (EP) receptors, to which PGE2 binds to exert its diverse effects in the body.

1.3 EP Receptors

1.3.1 EP Receptor Physiology

PGE2 exerts a wide range of physiological effects in the body by binding to one of four EP receptors: EP1, EP2, EP3, and EP4 **(Figure 1)**. These receptors are transmembrane G-protein coupled receptors, and their activation leads to distinct cell signaling pathways through G-protein coupled activity. They exhibit differences in signal transduction, localization in tissues, and regulation of expression (Sugimoto and

Narumiya, 2007). Upon binding PGE2, the EP1 receptor couples to an unidentified G-protein (G_q), and intracellular free calcium (Ca^{2+}) levels increase. The EP3 receptor couples to an inhibitory G (G_i) protein, to inhibit activity of adenylate cyclase and to decrease intracellular cyclic-AMP (cAMP) levels; while the EP2 and EP4 receptors both signal mainly through coupling with a stimulatory G (G_s) protein, to increase cAMP levels (Sugimoto and Narumiya, 2007). While the EP2 and EP4 receptors are both capable of signaling via the cAMP pathway, EP4 also signals through the phosphatidylinositol 3-kinase (PI3K) pathway. In support, a study by Fujino *et al.* (2002) established that while PGE2 treated EP2 and EP4 receptors were both able to stimulate T-cell factor signaling, this was achieved through cAMP signaling in EP2 receptors, and by PI3K signaling in EP4 receptors. This alternative signaling mechanism, unique to the EP4 receptor, makes it an attractive strategy for specific pharmacologic targeting which could have implications in cancer therapy (Fugino and Regan 2003).

1.3.2 EP4 Receptor and Breast Cancer

Several of the EP receptors have been implicated in various aspects of establishment and progression of cancers. For example, the EP1 receptor has been implicated in up-regulation of vascular endothelial growth factors (VEGFs) in human colorectal cancer cells (Fukada *et al.,* 2003), leading to increases in angiogenesis and lymphangiogenesis. In breast cancer, the EP receptors have demonstrated diverse roles in various steps of carcinogenesis: EP1 receptor antagonism has been shown to significantly reduce tumorigenesis in mouse xenotransplants (Kawamori *et al.,* 2001); EP2 receptor activation has been linked to COX-2-induced mammary gland hyperplasia (Chang *et al.,*

2005) and lymphangiogenesis (Pan *et al.,* 2008). Recently, the EP4 receptor has emerged as a leader providing the most promising advancements in understanding EP involvement in human breast cancer, and in providing avenues for targeted therapeutics. EP4 activity has been associated with several important aspects of breast cancer, including: inactivation of host natural killer T-cells (Ma *et al.,* 2013); cellular invasion in inflammatory breast cancer (Robertson *et al.,* 2010); enhancement of breast cancer metastasis (Xin *et al.,* 2012; Ma *et al.,* 2013; Kundu *et al.,* 2014); and induction of stem-like cell phenotype *in vitro* (Kundu *et al.,* 2014) and *in vivo* (Majumder *et al.,* 2014). Most recently, our laboratory has demonstrated a strong link between COX-2 over-expression in human breast cancer cell lines, and up-regulation of EP4 receptors in these cell lines and also in their respective murine xenotransplants (Majumder *et al.,* 2014).

Previous work in our lab by Timoshenko *et al.* (2003) has helped establish a key link between COX-2 induced PGE2 overproduction and enhanced cellular migration in human and murine breast cancer cell lines. This study also demonstrated that this migration-promoting role of PGE2 was primarily mediated by EP4 receptor activity upon binding PGE2. Additionally, Timoshenko *et al.* (2004) observed that up-regulation of EP4 activity in the murine breast cancer cell line C3L5 resulted in up-regulation of the inducible nitric oxide synthase (iNOS) enzyme, which promoted increased cellular invasiveness via nitric oxide activity. In several human breast cancer cell lines and in primary breast tumors, up-regulation of VEGF-C via endogenous COX-2-induced PGE2 has also been shown to be regulated in part by the EP4 receptor, implicating its activity in lymphangiogenesis and lymphatic metastasis (Timoshenko *et al.,* 2006).

Most recently, our lab has validated the EP4 receptor as an important therapeutic

target in murine breast cancer. Using a spontaneously lung- and lymph node-metastasizing murine breast cancer model, Xin *et al.* (2012) discovered that chronic oral administration of a COX-1/COX-2 inhibitor (Indomethacin), a COX-2 specific inhibitor (Celecoxib), or specific EP4 antagonist (ONO-AE3-208), could significantly reduce tumor growth, angiogenesis, lymphangiogenesis, and metastasis to the lymph nodes and lungs. Interestingly, these therapeutic effects were not seen with a specific EP1 antagonist.

Due to its demonstrated involvement in various aspects of disease progression, these results suggest that the EP4 receptor is a promising therapeutic target in the treatment of human breast cancer. Understanding the unique signaling mechanisms through which the EP4 receptor acts, namely PI3K signaling, could lead to important advances in translational applications from bench to bedside.

1.3.3 EP4 Signaling Pathways and Cancer

cAMP was the first molecule to be identified in eukaryotes as a second messenger in cell signaling, and activation of several receptors, including G_s-protein coupled and growth factor receptors, can result in elevated levels of cAMP (Fimia and Sassone-Corsi, 2001). In the case of G_s-protein coupled cAMP-dependent signaling, binding a specific stimulatory ligand leads to activation of the adenylyl cyclase enzyme, which catalyzes the conversion of ATP to cAMP. Protein kinase A (PKA) is an established target of cAMP, and binding of cAMP to PKA leads to its activation. PKA is an important regulator of several downstream transcription factors, such as cAMP response element-binding protein (CREB) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-

κB), which alter the transcription profile of a cell and ultimately lead to changes in gene expression (Fimia and Sassone-Corsi, 2001).

cAMP signaling plays important roles in normal cell growth and proliferation. However, dysregulation of various components of this pathway, including PKA activity, has been implicated carcinogenesis (Cho-Chung *et al.,* 2006). Miller (2006) discovered that the over-expression of regulatory subunits of the PKA enzyme is associated with high proliferation in normal and malignant breast tissues, poor prognosis in established breast cancer, and correlates with tamoxifen-resistance. In human breast cancer, ER status has been correlated with cAMP activity and proliferation in MCF-7 cells (Zivadinovic *et al.,* 2004). Phosphorylation of a specific region of ER-α by PKA has also been shown to induce resistance to tamoxifen (Michalides *et al.,* 2004), a commonly prescribed ER antagonist in the treatment of ER+ breast cancer. The same group also established that in clinical breast cancer samples, reduction of an endogenously expressed PKA down-regulating molecule, PKA-RIα, was also associated with tamoxifen resistance prior to treatment (Michalides *et al.,* 2004).

Specific ligand binding to certain receptor tyrosine kinases or G-protein coupled receptors within the cell membrane leads to a series of phosphorylation reactions, and ultimately the activation of PI3K (Cantley *et al.,* 2002). The PI3K family of proteins encompasses several lipid and serine/threonine kinases that act on their targets via a phosphorylation reaction. Following activation of PI3K via conversion of phosphatidylinositol-4,5-diphosphate (PIP_2) to phosphatidylinositol-3,4-triphosphate (PIP_3), several downstream targets can be activated. One crucial intracellular protein that is activated by PI3K is AKT, also known as protein kinase B (PKB). AKT is found in

three isoforms (AKT1, AKT2, AKT3), and activation of a particular isoform upon phosphorylation by PI3K has been implicated in various physiological and pathological outcomes, including cancer. For instance, AKT2 has effects on cellular motility and invasiveness, while AKT3 has been linked to hormone independence in cancers (Hennessy *et al.*, 2005). Of great relevance to cancer, activation of AKTs results in the induction of pathways involved in cell growth and survival (mammalian target of rapamycin, mTOR), as well as inhibition several pro-apoptotic factors (BAD, Forkhead, pro-caspase-9), and up-regulation of transcription factors for certain anti-apoptotic genes (CREB) (Hennessy *et al.,* 2005). Dysregulated mTOR signaling is of particular importance in cancer, since activation of mTOR leads to phosphorylation events downstream of PI3K and AKT that ultimately enhance gene expression of many proteins regulating the cell cycle (Engelman, 2009).

Mutations in the various molecules exerting regulatory effects on PI3K signaling are often observed in cancer, and have been studied intensely in recent years as potential therapeutic targets. In human breast cancer, the most frequently mutated element of the PI3K signaling pathway is the *PIK3CA* gene, encoding the alpha catalytic subunit of the PI3K protein. Somatic mutations in *PIK3CA* have been observed at frequencies as high as 27% in primary human breast cancer tumors (Woo Lee *et al.*, 2004), and these mutations appear most frequently in hormone-receptor positive breast cancers (Hennessy *et al.*, 2005). The PTEN protein is another regulatory molecule, acting to convert PIP_3 back to PIP_2, and by so doing suppressing PI3K signaling. A study by Depowski *et al.* (2001) demonstrated that *PTEN* loss is significantly correlated with disease related death, lymph node metastasis, and loss of estrogen receptor staining in invasive breast cancer tumor

samples. Since *PTEN* and *PIK3CA* mutations occur most frequently in human breast cancer, their roles in tumor progression and in resistance to treatment are being studied actively with promising results. A comprehensive study by Berns *et al.* (2007) used a wide-scale RNA interference scan to identify genes involved in trastuzumab (Herceptin) resistance, and established the *PTEN* genetic mutation as a the primary target. Additionally, this group also showed that combined mutation in *PTEN* and *PIK3CA* was associated with enhanced resistance to therapy *in vitro*, and was a predictor of poor prognosis in breast cancer patients treated with trastuzumab (Berns *et al.*, 2007).

Regulatory molecules of the PI3K pathway have been emerging as potential therapeutic targets in recent years. Four main classes of inhibitors targeting components of the PI3K-AKT pathway are at various stages of pre-clinical and clinical testing: PI3K-specific inhibitors, AKT-specific inhibitors, dual PI3K-mTOR inhibitors, and mTOR catalytic site inhibitors. Several factors contribute to the efficacy of each inhibitor, such as which iso-form of the molecule being inhibited is most effective in a particular cancer. For instance, AKT1 and AKT2 inhibitors can be used to target a specific phenotype of cancer, namely cellular invasiveness and EMT for AKT1, and cell growth and survival for AKT2 (Engelman, 2009). The ability to target specific aspects of cancer could allow for enhanced personalization of treatment, and could mitigate potential side effects. It has also been suggested that blocking one key signaling pathway could lead to a feedback up-regulation of other pathways, potentially leading to therapy resistance. For example, treatment with mTOR inhibitors, such as rapamycin, has been shown to induce feedback activation of PI3K in certain cancers (O'Reilly *et al.*, 2006). Therefore, combination treatments inhibiting multiple key molecules within the PI3K-AKT pathway might prove

to be effective tools in treating patients with cancer, and preventing therapy resistance and disease recurrence.

1.4 Cancer Stem Cells (CSCs)

1.4.1 Cancer Stem Cell Hypothesis

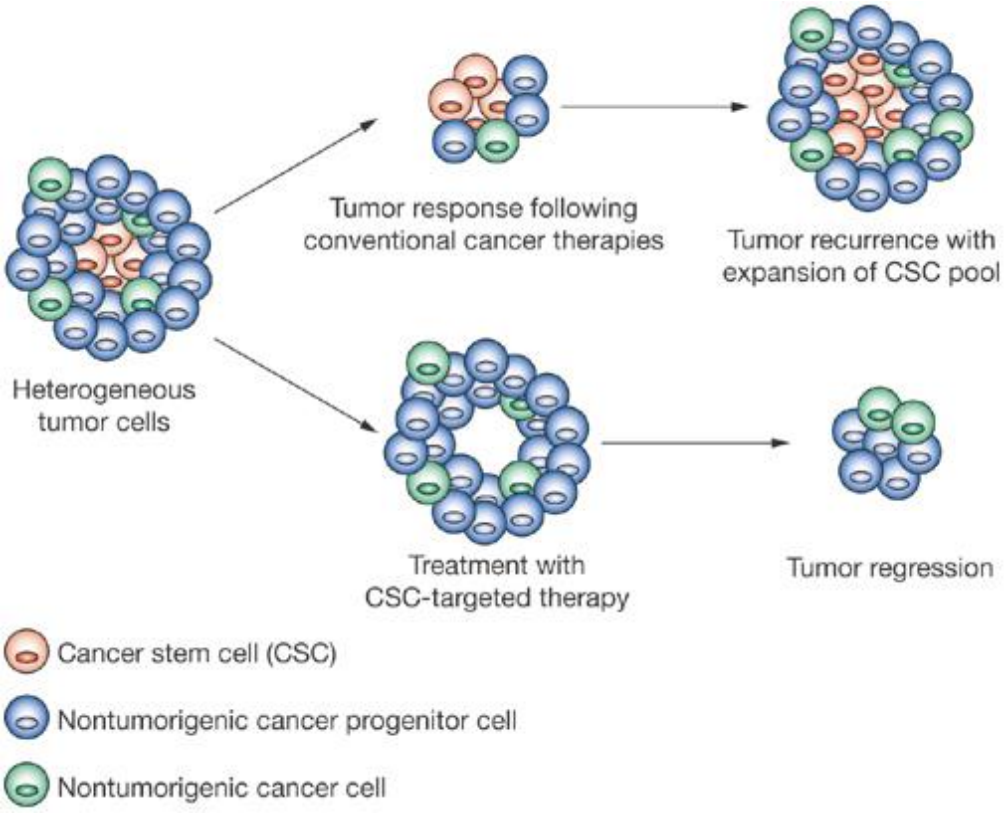
Cancer stem cells (CSCs) comprise a small subset of cells within the tumor, and are thought to be responsible for tumor induction and recurrence **(Figure 2)**. This population of cells is thought to be distinct from other malignant cells within the tumor in that they possess the ability to self-renew, as well as to produce progenitor cells that are capable of taking several differentiation paths; ultimately contributing to the heterogeneous nature of solid tumors (Wicha *et al.,* 2006). While traditional chemotherapeutics have become quite adept at eliminating non-stem proliferative malignant cells, the inherent differences between these cells and CSCs may prevent eradication of this small but potent subset from the tumor. As such, CSCs left behind by non-targeted treatments may give rise to the genesis of a new tumor and cancer recurrence in the patient (Wicha *et al.,* 2006). In addition, failure to eliminate CSCs is also thought to play a role in the progression of cancer to a metastatic disease (Sleeman and Cremers, 2007). Therefore, newer CSC-linked markers and new therapeutic agents that can successfully target and eliminate the CSC population of a tumor should provide lasting solutions in combating cancer.

Stem cells are commonly found in most tissues in the body, including the breast, and are necessary for cell renewal, growth and development. However, the exact mechanisms by which cancer stem cells arise within a tumor are still uncertain. Currently, there are three main hypotheses that postulate the genesis of CSCs (Reya *et al.,* 2001). The first hypothesis suggests that CSCs arise from stem cells normally found

in the tissue of origin, but that acquire a specific mutation that transforms the cell into a CSC. Support for this idea has been shown in acute myeloid leukemia, where specific induced chromosomal abnormalities for genes encoding haematopoetic stem cells have been linked to expression of CSC markers (Reya *et al.*, 2001). The second hypothesis for the creation of CSCs proposes that normal progenitor cells within the tissue acquire mutations that return it to a permanent stem-like state. Support for this idea has also been shown in myeloid leukemia cells, where specific mutations targeting only the progenitor cell population can result in a stem-like phenotype in the resulting population (Reya *et al.*, 2001). Finally, the last hypothesis for the generation of CSCs is that a differentiated cell in the tissue acquires random mutations that result in a CSC genetic pattern (Wicha *et al.*, 2006). In all cases, the result is the production of a CSC that has lost its own ability to differentiate, exhibits clonal self-renewal, and proliferates in an uncontrolled manner to promote tumorigenicity in its tissue of origin.

Figure 2. Cancer Stem Cells as Therapeutic Targets

The cancer stem cell (CSC) hypothesis suggests that a small sub-population cells within a tumor drive tumorigenicity and disease recurrence. These CSCs may arise from mutated stem or progenitor cells, and differ from other cancer cells in their enhanced ability to proliferate and self-renew. It is also thought that the CSC population is able to give rise to a variety of differentiated malignant cells within a tumor, therefore contributing to tumor heterogeneity. Traditional therapies such as chemotherapy and radiation may initially reduce tumor burden due to their efficacy in targeting differentiated cancer cells within a tumor. However, the CSC hypothesis suggests that failure to eradicate the CSC population of a tumor is a likely cause of disease recurrence following conventional therapies. Therefore, new therapeutics that effectively target the CSC population of a tumor could completely eliminate cancer at its CSC source, and could have great potential as adjuvants to traditional therapies that eradicate differentiated cancer cells in the tumor (adapted from Das *et al*. 2008).


Tumor response following
conventional cancer therapies
Tumor recurrence with
expansion of CSC pool
Heterogeneous
tumor cells
Treatment with
CSC-targeted therapy
Tumor regression
Cancer stem cell (CSC)
Nontumorigenic cancer progenitor cell
Nontumorigenic cancer cell

1.4.2 CSCs in Breast Cancer

There are currently three distinct markers that are most frequently used to describe the CSC population in human breast cancer. High expression of the cell surface marker CD44 and low expression of the cell surface marker CD24 ($CD44^{high}CD24^{low}$) currently describe the CSC population in breast tumors (Al-Hajj *et al.*, 2002). Additionally, high expression of the Aldehyde dehydrogenase 1 (ALDH1) enzyme, in addition to expression of the above cell surface markers, is also a distinctive marker of CSCs in breast cancer (Croker *et al.*, 2009). Therefore, the CSC population in human breast cancer can be sorted from the bulk tumor cells on the basis of expression of these markers, and can be achieved through fluorescence-activated cell sorting (FACS) techniques (Croker *et al.*, 2009).

In addition to these CSC cell surface and enzyme markers, an *in vitro* functional assay can be used to assess CSC phenotype in a cell population. This assay uses anchorage-independent growth on ultra-low attachment plates for singly plated cells, and assesses the ability of cells to self-renew to form clonal spheroids in culture (Dontu *et al.*, 2003). In human breast cancer, it was observed that such spheroids contain a significantly greater number of CSCs when they were dissociated and sorted for traditional breast cancer CSC markers (Dontu *et al.*, 2003). Additionally, spheroids were enriched with progenitor cells capable of differentiating into multiple lineages, and serial passages of CSCs derived from spheroids had an enhanced capability for self-renewal (Dontu *et al.*, 2003).

Breast CSCs have indeed been implicated in disease progression in primary tumors, and validated in *in vivo* studies. In a study conducted by Pece *et al.* (2010), it was

observed that in primary breast tumors, poorly differentiated cancers displayed a significantly greater CSC content when compared to well-differentiated cancers. Moreover, when cells from poorly differentiated and well-differentiated tumors were xenotransplanted into immune-compromised mice, the poorly differentiated tumors formed also displayed a significantly higher content of CSCs.

With the CSC population becoming better characterized at both molecular and functional levels, the investigation of targeted therapeutics has become a popular area of research. Recently, Bhutia *et al.* (2013) found that expression of the melanoma differentiation-associated gene-7/interleukin-24 (MDA-7/IL-24) protein has the ability to reduce proliferation, induce apoptosis, and initiate endoplasmic reticulum stress in breast CSCs without affecting normal breast stem cells. Perhaps most promising, this group also discovered that MDA-7/IL-24 also significantly reduced the self-renewal capabilities of breast CSCs, by suppressing the Wnt/ß-catenin signaling pathway within these cells. The Wnt pathway has been shown to play an important role in maintaining the self-renewal properties of breast CSCs (Han and Crowe, 2009). Widespread screening for small molecular agents that can selectively inhibit breast CSCs have identified certain compounds that show promise in reducing CSC growth *in vitro* and *in vivo* (Gupta *et al.,* 2009 and Germain *et al.,* 2012). Understanding the key molecular and functional characteristics of breast CSCs opens new avenues for developing targeted treatments that kill cancer at its source. Therefore, combining targeted CSC therapeutics with traditional chemotherapy and radiation may become the most effective way to improve patient outcomes and disease-free survival in the treatment of breast cancer.

1.5 MicroRNAs

1.5.1 Biogenesis and Activity of miRNAs

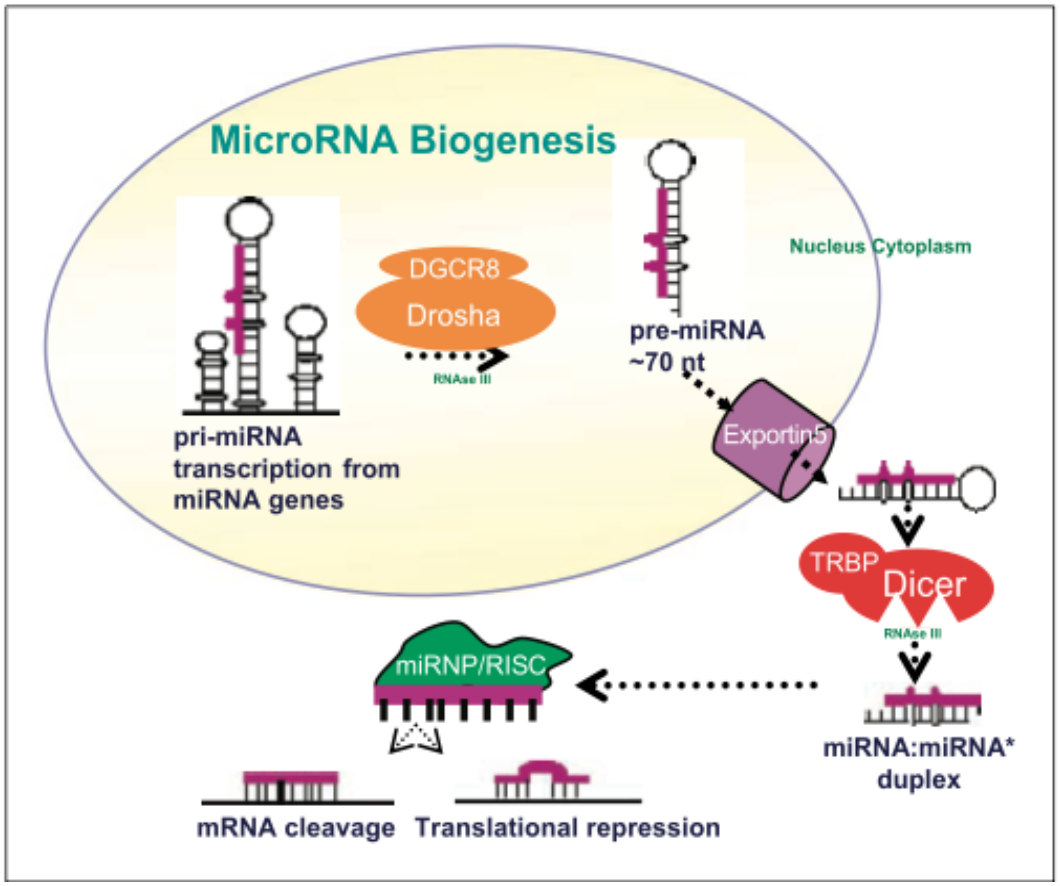
MicroRNAs (miRNAs, miRs) are short (19-24 base pairs), single-stranded, non-coding regulatory RNA molecules that help to control gene expression post-transcriptionally in several eukaryotic species, including humans (Bartel, 2004). Although they are small, miRNAs play a significant role in regulating the translation of their target mRNAs, and their effects can be far-reaching as a single miRNA may regulate hundreds or thousands of genes (Dumont and Tlsty, 2009). In addition, miRNAs are often implicated in cancers due to the fact that miRNA genes are frequently located in fragile sites of the chromosome, and are therefore at increased susceptibility for mutation or damage (Calin *et al.,* 2004). Since miRNAs alter gene expression at the post-transcriptional level, increases or decreases in expression of specific miRNAs may contribute to the establishment and progression of the cancer.

miRNA biogenesis begins with transcription of the specific gene encoding a specific miRNA by RNA Poly II or III in the nucleus, creating a primary miRNA (pri-miRNAs) transcript with a distinct hairpin-loop structure **(Figure 3)**. The pri-miRNA is then cleaved by the RNAse endonuclease Drosha to create the precursor miRNA (pre-miRNA) transcript. With the help of Ran-GTP and Exportin5 in the nucleus, the pre-miRNA is exported out of the nucleus and into the cytoplasm, where the RNase enzyme Dicer cleaves the hairpin-loop structure and to create a linear, double-stranded miRNA:miRNA* duplex. A helicase enzyme then binds to separate the double-stranded miRNA complex, leading to synthesis of the mature miRNA and degradation of the miRNA* passenger strand. To carry out its regulatory role, the mature miRNA and the

protein Argonaute 2 (Ago2) associate with the RNA-Induced Silencing Complex (RISC), and can then bind to the 3'UTR of its target mRNA to down-regulate gene expression. If the miRNA base pairs with its target with complete complementarity, the mRNA transcript will be degraded directly. However, if the miRNA binds its target with incomplete complementarity, translation will be blocked but the transcript will remain intact (Hutvágner and Zamore, 2002). Both scenarios result in down-regulation of the target mRNA, and therefore a reduction in gene expression.

Figure 3. MicroRNA (miR) Biogenesis and Activity

miRNA biogenesis begins in the nucleus where miRNA genes are transcribed to yield the corresponding primary miRNA (pri-miRNA) transcripts. The RNase III enzyme Drosha couples with DGCR8 to cleave the pri-miRNA transcripts into f70 nucleotide precursors (pre-miRNA), containing an imperfect stem-loop structure. Pre-miRNAs are then exported out of the nucleus via the Ran-GTP /Exportin 5 complex. In the cytoplasm, the enzyme Dicer and its partner, the transactivaor RNA-binding protein (TRBP), cleave the hairpin precursors from the double-stranded pre-miRNA. Following cleavage of the pre-miRNA, the small double stranded duplex (miRNA: miRNA*) contains both the mature miRNA strand and its passenger strand. The single-stranded mature miRNA and Argonaute 2 (Ago2) are then incorporated into the RNA-induced silencing complex (RISC), and target complementary mRNA sequences, either repressing translation of the mRNA target (incomplete complementarity), or degrading the transcript directly (complete complementarity) (adapted from Lowery *et al.*, 2009).


MicroRNA Biogenesis
Nucleus Cytoplasm
DGCR8
Drosha
RNAse III
pri-miRNA
transcription from
miRNA genes
pre-miRNA
~70 nt
Exportin5
TRBP
Dicer
RNAse III
miRNP/RISC
miRNA:miRNA*
duplex
mRNA cleavage
Translational repression

1.5.2 miRNAs and Breast Cancer

miRNAs are important regulators of gene expression, and play important roles in the normal physiological processes within the body. MiRNAs have been shown to be key players in circulatory (Li *et al.*, 2010; Yang *et al.*, 2005), musculoskeletal (Crist *et al.*, 2009), and neurological development (Lagos-Quintana *et al.*, 2002), as well as metabolism (Morita *et al.*, 2009). In addition to regulation of normal development, miRNA expression profiles are emerging as valuable tools that can be used to diagnose a wide range of pathological conditions and diseases. For example, altered miRNA expression profiles have been established in several neurodegenerative diseases such as Alzheimer's (Hebert *et al.*, 2008) and Parkinson's disease (Xu *et al.*, 2007).

In recent years, miRNA expression profiling has come to the forefront in the area of cancer research since samples from patients and healthy individuals can be easily compared using tissue or blood. Differential miRNA expression profiles have been found to be reliable for classifying human cancers, including both the developmental lineage and differentiation of solid tumors (Lu *et al.*, 2005). A study by Arroyo *et al.* (2011) suggested that the stability of miRNAs in human plasma is largely due to association with the Argonaute 2 protein complex, preventing RNase degradation in the body. Thus, since miRNAs are stable in the blood and can be easily extracted from tissues, they show excellent promise as biomarkers of cancer (Arroyo *et al.*, 2011). It has been suggested that in patients with breast cancer, miRNA expression profiles may have the ability to accurately distinguish between basal and luminal tumor sub-type, ER and HER2 status, and to even predict tumor response to traditional chemotherapeutics (Andorfer *et al.*, 2011). For instance, miR-155 has been used accurately distinguish ER(-) and ER(+)

tumors in the clinic , and miR-7 expression has been associated with pathological grade and cell cycle deregulation (Van ’T Veer *et al.,* 2002).

Gene expression profiles in human breast cancer have been well studied, leading to the discovery of a set of tumor-promoting and tumor-suppressive genes that can be used to predict a person’s susceptibility to breast cancer. One prominent example is mutation occurring in the *BRCA1* tumor suppressor gene, leading to significantly increased risk of breast cancer (Welcsh and King, 2001). Similar to such genes, expression of specific oncogenic miRNAs or repression of tumor-suppressive miRNAs could contribute to progression of the disease (Calin *et al.,* 2004). For example, a study comparing tumor and normal breast tissues identified miR-125b (down-regulated), miR-145 (down-regulated), miR-21 (up-regulated), and miR-155 (up-regulated) as the most commonly deregulated miRNAs in human breast cancer (Iorio *et al.*, 2005).

It has been established that altered miRNA expression can have significant effects on several steps of the metastatic cascade, including cell motility, invasiveness, and adherence, as well as resistance to apoptosis (Dumont and Tlsty, 2009). Indeed, large scale miRNA microarray profiles have provided impressive lists of miRNAs that are dysregulated in human breast cancer, and that likely play active roles in the invasive and metastatic processes leading to disease progression (Shi *et al.,* 2010). A family of miR-103/107 oncogenic miRNAs has recently been shown to play a key role in repressing the expression of Dicer, an enzyme that is required for mature miRNA biogenesis and thus affects global miRNA expression profiles (Martello *et al.,* 2010). Over-expression of miR-103/107 in human breast cancer cell lines enhanced cellular migration and invasion, and repression of miR-103/107 lead to restoration of mature miRNA levels and reduced

metastatic colonization in an *in vivo* study (Inui *et al.,* 2010).

Oncogenic miRNAs have important impacts not only on metastasis, but also on cancer cell survival and avoidance of programmed cell death. miR-21 has been studied extensively in a number of cancers, and is currently the only oncogenic miRNA to be over-expressed in 9 types of solid tumors, including breast cancer (Lu *et al.,* 2008). miR-21 has also been considered an anti-apoptotic agent, and miR-21 knock-down resulted in an increased caspase activation and cellular apoptosis (Chan *et al.,* 2005). The anti-apoptotic role of miR-21 in human breast cancer is thought to be a repression in translation of its target gene, programmed cell death 4 (*PDCD4*), as over-expression of miR-21 in MCF-7 cells lead to significantly reduced PDCD4 protein levels (Lu *et al.,* 2008).

miR-31 was recently shown to be an important metastasis suppressor, as its expression was inversely correlated with metastasis-free survival in breast cancer patients (Valastyan *et al.,* 2009). Additionally, re-expression of miR-31 in human breast cancer cells lead to reduced migration, invasion, and resistance to apoptosis *in vitro*, and also suppressed metastatic ability *in vivo* in an orthotopic mouse model (Valastyan *et al.,* 2009). This study provides important evidence for the impact of a single miRNA in breast cancer progression, and demonstrates the potential of targeted miRNA therapeutics in reducing metastasis.

Of the tumor suppressor miRNAs characterized to date, members of the let-7 family are often the most significantly and consistently reduced miRNAs in human cancers (Shi *et al.,* 2010). In the human breast cancer cell line SKBR-3, over-expression of let-7 resulted in a reduction of cellular proliferation and spheroid formation *in vitro*, as

well as a reduction in undifferentiated cells (Shi *et al.,* 2010). The key genes targeted by the let-7 miRNAs are the established *proto*-oncogenes High mobility group AT-hook 2 (*HMGA2*) and *Ras*, both shown to play a role in maintaining the self-renewal properties displayed by cancer cells (Dangi-Garimella *et al.,* 2009 and Johnson *et al.,* 2005). Further investigation revealed that let-7-induced *Ras* silencing targeted the self-renewal ability of the cancer stem cell population; whereas let-7-induced *HMGA2* silencing enhanced cellular differentiation (Yu *et al.,* 2007). This suggests that let-7 may play a key role in regulating the cancer stem cell sub-population phenotype in human breast cancer.

A key benefit of establishing miRNA expression profiles for human breast cancer is the great potential they hold for a translational approach to cancer diagnosis and therapy. Since cancer is inherently a heterogeneous disease both between and within patients, it will be beneficial if plasma and tissue samples could be screened and have their miRNA expression profiles compared to established baselines for their specific type of disease. For example, miRNA expression profiles have already begun to be established to accurately predict HER2, ER, and PR receptor status in human breast cancer (Lowery *et al.,* 2009). In addition to implementing miRNA profiles into the diagnostic aspect of breast cancer, such profiles may also prove to be extremely useful in monitoring disease progression, predicting responses to traditional chemotherapies, and ultimately creating a more personalized approach to administering treatment. While great advances have been made in the last decade in the area of miRNA expression profiling, the number of miRNAs implicated in the various aspects of the disease continues to grow and additional research is needed to continue to establish miRNA gene targets, and mechanisms of action on these targets before therapeutic interventions can have an impact on this

disease.

1.5.3. miRNAs and CSCs

Similar to mRNA profiling in gene expression, dysregulated miRNA expression may also help to distinguish CSC and non-CSC populations in human breast cancer. For instance, Shimono *et al.* (2009) discovered that miR-200c expression is down regulated in human breast CSC cells, and showed that over-expression of miR-200c could inhibit tumor formation from breast CSCs *in vitro* and *in vivo*. Moreover, miR-200c has been shown to play an important role in suppressing EMT phenotype in human breast cancer (Burk *et al.,* 2008). The let-7 family of miRNAs has also been found to be an important regulator of stemness and down regulation of let-7 miRNAs has been established in CSC populations sorted from breast cancer cell lines and from primary breast tumors (Yu *et al.*, 2007). Furthermore, Yu *et al.* (2007) observed that over-expression of let-7 miRNAs resulted in a reduction of CSC population size, proliferation, tumorsphere formation *in vitro*, as well as a reduction in tumor formation and metastatic ability *in vivo*. These results demonstrate the important roles that miRNAs have in maintaining breast CSCs, and suggest that with further research, miRNAs could have great diagnostic and therapeutic potential in human breast cancer.

1.6 Rationale

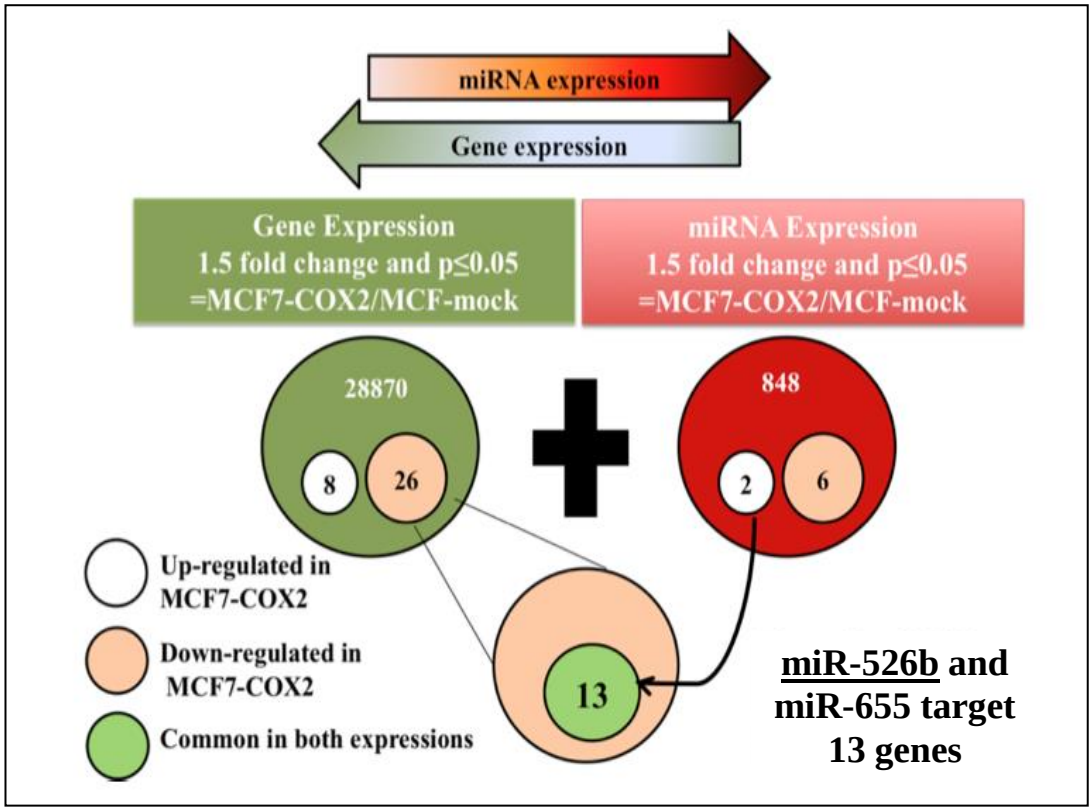
Previous work in our lab by Majumder *et al.* (2012) established that stable transfection and over-expression of the COX-2 gene in MCF-7 (ER+, HER2-, COX-2-) and SKBR-3 (ER-, HER2+, COX-2-) human breast cancer cell lines is associated with enhanced aggressiveness of the cells compared to empty vector controls. This was achieved via multiple mechanisms, including: increased growth rate, cellular migration, invasiveness, and proliferation; increased abilities to promote lymphangiogenesis (via enhanced VEGF-C, D expression) and angiogenesis (via enhanced VEGF-A expression); EMT (reduction of E-cadherin and increase in vimentin, twist, N-cadherin protein levels); and the induction of stem-like cell (SLC) phenotype (enhanced spheroid formation on ultra-low attachment plates). Additionally, MCF-7 and SKBR-3 cells over-expressing COX-2 also showed an up-regulation of the PGE2 receptor EP4, and treatment with a COX-2 inhibitor or EP4 antagonist mitigated the above aggressive phenotypes including the SLC phenotype of spheroid formation in both MCF-7-COX-2 and SKBR-3-COX-2 cell lines. In xenograft models *in vivo*, EP4 antagonist treated or EP4 silenced MCF-7-COX-2 cells exhibited also reduced tumorigenicity. Majumder *et al.* (2012) also performed a combined gene expression and miRNA microarray and observed that MCF-7-COX-2 displayed an up-regulation of two miRNAs, miR-655 and miR-526b, targeting a total of 13 COX-2 down-regulated genes with known tumor-suppressor functions **(Figure 4)**.

Functional analysis of miR-655 by Dunn *et al.* (2012) demonstrated that over-expression of this miRNA in MCF-7 and SKBR-3 human breast cancer cell lines lead to increased migration, invasion, proliferation, and induction of SLC phenotype. Moreover, expression of both miR-655 and miR-526b correlated with COX-2 expression in a series

of COX-2 disparate human breast cancer cell lines (Dunn *et al.*, 2012). These results suggested that miR-655 is a pro-oncogenic miRNA induced by COX-2 expression in human breast cancer cells, suggesting that both COX-2 and miR-655 expression play an interconnected role in human breast cancer aggressiveness and progression.

Figure 4. Comparative Analysis of Combined Gene and miRNA Expression in MCF-7 and MCF-7-COX-2 Human Breast Cancer Cells

Following the introduction of the COX-2 gene into MCF-7 human breast cancer cells, a differential gene (Affymatrix Human Gene Array 1.0 ST; 28 870 genes) and micro-RNA (miRNA) microarray (Affymatrix Genechip miRNAs Array; 848 human miRNA) were used to compare miRNA and gene expression between MCF-7 and MCF-7-COX-2 human breast cancer cells. Majumder *et al.* (2012) identified 26 down-regulated genes, and 8 up-regulated genes in MCF7-COX-2 cells. In the miRNA microarray, only 2 miRNAs were up- regulated and 6 were down-regulated in MCF-7-COX-2 cells. Using a combined gene and miRNA data analysis, it was observed that the two up-regulated miRNAs (≥1.5 fold) in MCF7-COX-2 cells are targeting a total 13 genes, with documented tumor suppressive activity, that are down- regulated in the same cells (≤-1.5 fold) (Adapted from Majumder *et al.*, 2012).


miRNA expression
Gene expression
Gene Expression
1.5 fold change and p≤0.05
=MCF7-COX2/MCF-mock
miRNA Expression
1.5 fold change and p≤0.05
=MCF7-COX2/MCF-mock
28870
8
26
848
2
6
13
Up-regulated in MCF7-COX2
Down-regulated in MCF7-COX2
Common in both expressions
miR-526b and miR-655 target 13 genes

1.7 Hypothesis and Objectives

Given the results previously obtained in our laboratory as summarized above, the hypothesis of the present study is that COX-2 induced miR-526b expression is associated with an aggressive phenotype in human breast cancer cells, and that miR-526b expression is regulated in part by EP4 receptor signaling. The overall objective of this study is to determine the functional roles of miR-526b *in vitro* and *in vivo*, including induction of SLC phenotype *in vitro*, and to investigate a potential regulatory role of EP4 receptor activity on miR-526b expression.

This hypothesis will be tested using via the following project objectives:

Using the following human breast cancer cell lines: MCF-7, MCF-7-Mock, MCF-7-526b, MCF-7-COX-2 and SKBR-3, SKBR-3-Mock, SKBR-3-526b:

1. To investigate the functional roles of miR-526b in breast cancer progression:
   (a) *In vitro* via transwell migration, invasion, proliferation, and SLC phenotype (spheroid formation) assays.
   (b) *In vivo* using a xenograft in an immune-compromised mouse model followed by assessment of lung colony formation.

2. To investigate the regulatory role of the EP4 receptor in miR-526b expression, using EP4 receptor agonists to stimulate and EP4 antagonists to block EP4 receptor activity followed by quantitative evaluation of miR-526b expression *in vitro*.

3. To investigate the role of the canonical cAMP-PKA and non-canonical PI3K-AKT signaling pathways of the EP4 receptor in regulating miR-526b expression *in vitro*.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 Cell Lines and Culture

The MCF-7 human breast cancer cell line expresses low levels of COX-2, is estrogen receptor (ER) positive and 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 Eagle's Minimum Essential Medium (EMEM) (ATCC, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 0.4 µl/mL human insulin (company), 100 U/mL penicillin, and 100 ug/mL streptomycin in a humidified incubator with 5% CO_2 at 37°C. The weakly metastatic SKBR-3 human breast cancer cell line, also purchased from ATCC, does not express COX-2, is ER negative and HER-2 positive. SKBR-3 cells were maintained as above, however they were grown in McCoy's 5A Modified Medium with L-glutamine (Invitrogen, GIBCO, ON) not supplemented with insulin.

In order to stably over-express COX-2 in MCF-7 cells, our lab's senior post-doctoral fellow, Dr. Majumder, had previously transfected COX-2 cDNA or an empty-vector control into the MCF-7 cell line. Stable over-expression of COX-2 was verified by resequencing, quantitative real-time PCR, and Western blot analysis. The resultant cell lines were named MCF-7-COX-2 and MCF-7-Mock. MCF-7-COX-2 cells displayed an enhanced aggressive phenotype as would be predicted with high COX-2 expression (Majumder *et al.*, 2012) MCF-7-COX-2 and MCF-7-Mock cells were maintained in identical culture conditions as MCF-7 cells, however Geneticin® (Invitrogen, GIBCO, ON) was added to the media of COX-2 over-expressing and Mock cell lines at 500 µg/ml to maintain selective pressure for the transfected cell population.

2.2 Nucelotransfection with miR-526b Over-Expression Plasmid

MCF-7 and SKBR-3 cells were grown to 80% confluence in 75 cm^2 flasks (BD Falcon, CA). Cells were enzymatically dissociated using 0.25% Trypsin and the pellet collected via centrifugation. Cells were then re-suspended in appropriate media, counted, and transferred to into certified cuvettes at a concentration of $2x10^6$ cells/mL. Cells were then transfected with 2 μg of either pCMV-MIR Mock vector (control) or pCMV-MIR miR-526b 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. Following transfection, MCF-7 and SKBR-3 cells were incubated in appropriate media without antibiotic treatment for 48 hours at 37°C and 5% CO_2. After 48 hours, the cells were treated with the antibiotic-resistance selection agent Geneticin® at 500 μg/mL. MCF-7 and SKBR-3 cell lines stably transfected with the pCMV-MIR mock (empty) vector are referred to as MCF-7-Mock and SKBR-3-Mock respectively. Similarly, MCF-7 and SKBR-3 cell lines stably transfected with the pCMV-MIR miR-526b Expression Plasmid are referred to as MCF-7-526b and SKBR-3-526b respectively. Once cells had reached 80% confluence, total miRNA was extracted, and miR-526b expression levels were quantified using real-time RT-PCR and the ΔCt method for fold-change in expression (Schmittgen and Livak, 2008).

The miR-526b over-expression plasmid was synthesized by cloning the amplified precursor microRNA into OriGene's pCMV6-Mir vector. The Cytomegalovirus promoter (CMV) is used in this plasmid to drive expression of miR-526b (OriGene, MD). The promoter also drives expression of a Neomycin resistance gene, which is used as a

selection agent for cell populations over-expressing miR-526b, as well as a green fluorescent protein (GFP) reporter gene, which can be used to assess the success of the transfection reaction.

2.3 Quantitative Real-Time RT-Polymerase Chain Reaction (qPCR)

2.3.1 MiR-526b Expression

MCF-7-526b, MCF-7-Mock, SKBR-3-526b and SKBR-3-Mock human breast cancer cell lines were grown under the conditions described above until they reached 80% confluence. Cells were then trypsinized using 0.25% Trypsin and pelleted down using centrifugation. Total miRNA was extracted using the miRNeasy Minikit and the RNeasy MiniElute Cleanup kit (Qiagen, MD) as per the manufacturer's instructions. The total miRNA was then quantified with a spectrophotometer (NanoDrop 2000, Thermo Scientific, IL). Complementary DNA (cDNA) was synthesized from the extracted miRNA using specific stem-loop primers for the miRNA under investigation (TaqMan® MicroRNA Assays, miR-526b primer) and the endogenous housekeeping miRNAs (TaqMan®MicroRNA Assays, RNU 44 and RNU 48 primer) and the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, CA) following the manufacturer's protocol and 2 μg of miRNA per 24 μl volume reaction. cDNA synthesis was carried out using a thermo cycler (C2000TM, Bio Rad).

Quantitative real-time PCR for miR-526b (hsa-miR-526b) 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 miRNAs (TaqMan® MicroRNA Assays, RNU 44 and RNU 48 probe) and the target miRNA (TaqMan®MicroRNA Assays, miR-526b probe), as

suggested by the manufacturer. Each quantitative real-time PCR reaction was prepared to a volume of 20 µl, including 1 µl of the appropriate TaqMan probe, and 2 µl of the cDNA. The quantitative real-time PCR profile was 95°C for 10 seconds of denaturation followed by 50°C for 2 minutes of anneal-extension for 40 cycles.

2.3.2 Comparing Effects of COX-2 and EP4 Receptor Activity on miR-526b Expression

MCF-7-COX-2 cells were grown to 80% confluence in a 6-well plate. Cells were then serum starved overnight, washed with DPBS, and then incubated for 24h with either the COX-2 specific inhibitor NS-398 (10 µM, Biomol, PA), the EP4 receptor antagonist ONO-AE3-208 (10 µM, ONO Pharmaceutical, Japan), or a control (0.003% DMSO). Total miRNA was extracted and cDNA was synthesized for each condition, followed by qPCR to assess miR-526b expression as described previously (2.2.1).

2.3.3 Effects of EP 1-4 or EP4 Activation on miR-526b Expression

MCF-7 cells were grown to 80% confluence in a 6-well plate. Cells were then serum starved overnight, washed with DPBS, and then incubated for 24h with either PGE2 (10 µM, Cayman Chemical), the natural ligand for all EP receptors, the EP4 specific agonist PGE1OH (10 µM, Cayman Chemical) or a control solvent (0.003% DMSO). Total miRNA and was extracted and cDNA was synthesized for each condition, followed by qPCR to assess miR-526b expression as described previously (2.2.1).

2.3.4 Effects of PI3K Inhibition on miR-526b Expression

MCF-7-COX-2 cells were grown to 80% confluence in a 6-well plate. Cells were then serum starved overnight, washed with DPBS, and then incubated for 24h with one of two PI3K inhibitors, Wortmannin (WT) (10 µM, Sigma-Aldrich, MO, an irreversible

inhibitor) or LY-204002 (LY) (10 μM, Sigma-Aldrich, MO, a reversible inhibitor), or a control solvent (0.003% DMSO). Total miRNA was extracted and cDNA was synthesized for each condition, followed by qPCR to assess miR-526b expression as described previously (2.2.1).

2.3.5 Effect of PKA Inhibition on miR-526b Expression

MCF-7-COX-2 cells were grown to 80% confluence in a 6-well plate, and then serum starved overnight. Cells were then washed with DPBS, and incubated with the specific PKA inhibitor H89 (30 μM, Sigma-Aldrich, MO) or a control solvent (0.003% DMSO). Total miRNA was extracted and cDNA was synthesized for each condition, followed by qPCR to assess miR-526b expression as described previously (2.2.1).

2.3.6 Qualitative Analysis of PKA Stimulation and Inhibition

To validate the effects of the PKA stimulating and inhibiting agents described above (2.3.5), a qualitative fluorescent PKA assay was used (PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay, Promega). MCF-7 and MCF-7-COX-2 cells were treated with EP4 stimulating (PGE2, PGE1OH) and a PKA inhibiting agent (H89), respectively, as previously described (2.3.5). This assay gives a qualitative representation of PKA activity within a sample using a fluorescent substrate. When the substrate is phosphorylated by PKA, its charge changes from positive to negative. As such, samples can be run on agarose gel with a voltage of 100 V and can be separated based on their charge, with phosphorylated substrate migrating towards the positive electrode (Promega, Canada). Cells were lysed with a solution of 25 mM TRIS-HCl, EDTA, EGTA, HALT protease inhibitor cocktail, and BME. Cells were gently scraped on ice, physically disrupted by pipetting, and then pelleted down using centrifugation at 13 000 RCF and

4°C for 5 minutes. The supernatant was collected and used for the PKA assay. For each reaction, 10 µl of supernatant for each treatment condition under investigation was mixed with PKA reaction buffer, PKA activator solution, ddH_2O, and a fluorescent PepTag® A1 Peptide, to a final volume of 25 µl in an 1.5mL Eppendorf tube, as per the manufacturer's protocol. The positive control, provided by the manufacturer, was comprised of the catalytic subunit of cAMP-dependent PKA, diluted in a small volume of cold DPBS. Reactions were activated for 1 minute by submerging the tubes in a warm water bath, and then covered with aluminum foil and allowed to incubate for 30 minutes at room temperature. Following incubation, the samples were heated to 95°C to stop the reaction and were briefly centrifuged at room temperature and 2000 RCF for 3 minutes. After completing the reactions, 1 µl of 0.8% glycerol in ddH_2O was added to each sample and samples were run on a 0.8% agarose gel at 100 V in a buffer of 25 mM TRIS-HCl for 40 minutes. Once separation of the phosphorylated and non-phosphorylated substrate bands had been achieved, the bands were imaged using a fluorescence-imaging machine (Gel Doc, Bio Rad).

2.3.7 Quantitative Real-Time RT-PCR for miRNA Expression

All quantitative real-time RT-PCR reactions were performed in triplicate, and the delta-delta Ct method was used to quantify the fold change in miRNA expression level between cell lines and treatments. For each experiment, each sample was replicated in triplicate in each run, and the mean Ct value (mean cycle threshold) value was calculated for each condition. The Ct value of the endogenous housekeeping gene (RNU 44 or RNU 48 for miRNA quantification) was subtracted from the mean Ct value of the sample under examination to obtain the delta Ct value (ΔCt). The ΔCt value of the control sample was

then subtracted from the ΔCt of the sample under review to obtain the delta-delta Ct value (ΔΔCt). The fold change in expression was then calculated using the formula: Fold change = $2^{-\Delta\Delta Ct}$.

2.4 Western Blot Analysis: pAKT Protein Expression

MCF-7 and MCF-7-COX-2 cells were grown to 70% confluence and then serum-starved overnight. Cells were then washed with DPBS, and incubated at a concentration of 10 μM with PGE2 (MCF-7), PGE1OH (MCF-7), L-902-688 (highest specificity for EP4; MCF-7), LY (MCF-7-COX-2), WT (MCF-7-COX-2), or a control solvent (0.003% DMSO) for 24h in serum-free media. After the incubation period, cells were washed with cold DPBS, and lysed with 250-300 μl of mammalian protein extraction reagent (M-PER) (Thermo Scientific, IL) supplemented with HALT protease inhibitor cocktail (Thermo Scientific, IL). After gently scraping the cells on ice, the cell lysate was collected in a 1.5mL Eppendorf tube, sonicated (8 pulses on level 4), and centrifuged at 13 000 RCF and 4°C for 20 minutes. Following centrifugation, the supernatant was collected and protein concentration was quantified in triplicate using the Pierce® BCA Protein Assay Kit (Thermo Scientific, IL) and a photometric plate reader (Infinite M200, TECAN), as per the manufacturer's protocol. Twenty 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 90 V (voltage) for approximately one hour. Following electrophoresis, the proteins were transferred to an Immobilon-FL PVDF membrane (Bio Rad) at 100 V for 1.5 hours (wet transfer). The membrane was then blocked for one hour in a blocking buffer solution composed of 4% albumin from bovine serum (BSA) (Sigma-Aldrich, MO) in 1x TBST (20 mM tris-base, 0.14 M NaCl, 0.01% Tween-20, pH 7.4). Next, the

membrane was incubated overnight in a primary antibody solution: mouse monoclonal AKT (1:1000) (Cell Signaling, MA) and rabbit monoclonal pAKT Ser473 (1:500) (Cell Signaling, MA) diluted in a solution of TBST (pH 7.4) with 4% BSA overnight at 4°C. The next morning, the membrane was washed three times (15 minutes per wash) with 1x TBST (pH 7.4), and then probed with a solution of secondary antibodies: goat anti-mouse (1:5000) and donkey anti-rabbit (1:1000) IRDye polyclonal secondary antibodies (LI-COR, NE) diluted in a solution of TBST (pH 7.4) with 4% BSA for one hour in the dark. Finally, the membrane was washed an additional three times (15 minutes per wash) with 1 x TBST (pH 7.4, pH 8.0 for final wash), and then scanned using an Odyssey infrared imaging system (LI-COR, NE).

2.5 Transwell Assays

2.5.1 Cellular Migration

MCF-7, MCF-7-Mock, MCF-7-526b, and SKBR-3, SKBR-3-Mock, SKBR-3-526b were assessed for their ability to migrate in response to serum-enriched media over 24 hours. Cells grown to 70% confluence were serum starved overnight, trypsinized, and re-suspended at a concentration of $2x10^5$ cells/mL. Next, 300 µl of this cell solution was added to the upper chamber of a multi-porous polycarbonate (8 µm pore size) insert (BD Falcon, CA), and placed in a 24-well plate (BD Falcon, CA). The lower chamber contained 700 µl of either serum free media or 2% FBS supplemented media. Plates were incubated for 24h at 37°C and 5% CO_2, as it had been previously established in out lab that the peak of migration occurs after this period of time (Timoshenko *et al.,* 2006). Following incubation, transwell inserts were removed from the plate, and the membranes gently wiped with a cotton swab in order to remove non-migratory cells. Cells were

subsequently fixed with pure methanol, stained with eosin and thiazine, respectively, and washed with ddH_2O. Membranes were then carefully excised using a scalpel blade, and fixed on microscope slides with mounting media. The number of migrating cells on the underside of the membrane was then captured using a light microscope imaging system (LEICA DFC 295), and the entire surface was used to calculate an average number of migrating cells for each condition using the ImageJ program.

2.5.2 Cellular Invasion

MCF-7, MCF-7-Mock, MCF-7-526b, and SKBR-3, SKBR-3-Mock, SKBR-3-526b were assessed for their ability to invade through an artificial basement membrane in response to serum-enriched media over 48 hours. The same protocol used for cellular migration (2.5.1) was carried out for cellular invasion, with the following modifications: microporous membrane inserts (8 µm) were coated with a thin film of growth factor reduced Matrigel (BD Biosciences, CA) diluted in ddH_2O (1:100 dilution). Matrigel is a protein rich gelatinous secretion from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells that mimics the complex extracellular environment encountered by a cancer cell invading surrounding tissues (Zhou *et al.,* 2010). The Matrigel solution was plated in the top chamber and allowed to solidify over night under the cell culture hood, prior to adding any cells. The following morning, 300 µl of cell solution ($2x10^5$ cells/mL) was added to the top chamber of the insert containing the Matrigel, with 700 µl of supplemented media (SFM or 2%FBS) added underneath the insert. Plates were incubated for 48h at 37°C and 5%CO_2, as it had been previously established in out lab that the peak of invasion occurs around this period of time (Majumder *et al.,* 2012). Following incubation, membranes were gently wiped with a cotton swab, fixed, stained,

counted, and analyzed as previously described for cellular migration (2.5.1).

2.6 Cell Proliferation ELISA, BrdU Assay

The relative level of cellular proliferation was assessed and compared in MCF-7, MCF-7-Mock, MCF-7-526b, and SKBR-3, SKBR-3-Mock, SKBR-3-526b breast cancer cell lines using a Bromodeoxyuridine (BrdU) assay followed by an Enzyme-Linked Immunosorbent Assay (ELISA) (Cell Proliferation ELISA, BrdU (colormetric) Kit, Roche). BrdU is a synthetic analog of the nitrogenous base thymidine, and is therefore capable of base pairing with adenine during cell division and being incorporated into newly synthesized DNA. Therefore, quantifying the amount of BrdU incorporated into the cells acts as a proxy for the amount of cellular proliferation occurring. Next, 100 µl of cell solution was plated (1-2x 10^3cells/mL) in a 96-well plate and incubated overnight to allow cells to attach. The next day 10 µl of BrdU (10µM) labeling solution was added to the cells, and the plate was re-incubated for 6 hours at 37°C and 5% CO_2. Next the culture medium containing BrdU was removed with a vacuum pipette, and washed with buffer as per the manufacturer's instructions. The adherent cells were then fixed with 200 µl of FixDenat solution provided by the manufacturer, and incubated for 30 minutes at room temperature. The FixDenat solution was then removed by flicking, and 100 µl of anti-BrdU-POD working solution was added to each well and incubated for 30 minutes at room temperature, allowing it to bind to the exposed BrdU within the DNA. Following incubation, the antibody conjugate was removed by flicking, and the wells were washed three times with 200 µl of DPBS. Following this step, 100 µl of substrate solution was added to each well, and allowed to incubate at room temperature for approximately 30 minutes, until colour development was sufficient for photometric analysis. Finally, the

BrdU reaction was quantified using an ELISA plate reader at a wavelength of 370 nm, with 492 nm as a reference.

2.7 Tumorsphere Formation Assay

2.7.1 Establishing Tumorspheres in Culture

In order to assess the ability of cells to form spheroids *in vitro*, a tumorsphere formation assay was conducted. This assay is an *in vitro* surrogate of the stem-like cell (SLC) phenotype, and examines the ability of single cells to form clones in an anchoarage-independent manner (Dontu *et al*, 2003). Cells grown to 70% confluence were trypsinized, counted, and re-seeded at a concentration of $1x10^4$ cells/mL in 6-well ultra-low attachment plates (Corning, MA). In order to plate cells singly in the well, the cell suspension was first passed through a 8 µm filter (Falcon, BD) and a syringe fitted with a 27-gauge needle. All tumourspheres were grown in HuMEC (GIBCO, ON) media supplemented with epidermal growth factor (EGF; 20 ng/mL), fibroblast growth factor (FGF; 20 ng/mL), and B27 (20 ng/mL) (Invitrogen, ON), and allowed to grow for 7-10 days, or until the majority of spheroids reached a diameter of 60 µm (Dontu *et al.*, 2003). Tumorspheres were harvested and their miRNA was extracted, quantified, and used for real-time qPCR to assess miR-526b expression for (1) MCF-7 and SKBR-3 transfected cell lines, (2) DMSO, PGE2, PGE1OH treated MCF-7 cells (2.3.3), and (3) DMSO, LY, WT treated MCF-7-COX-2 cells (2.3.4). In addition, tumorspheres were imaged using a light microscope for the above conditions, and the number and size of spheroids for each condition was calculated using the ImageJ program.

2.7.2 Tumorsphere Forming Efficiency

To assess the efficiency of MCF-7-526b cells to form spheroids in comparison to MCF-7 cells, the protocol for culturing tumorspheres was carried out as described previously (2.7) with the following modifications. Cells were counted, and re-suspended in tumorsphere culture media at a concentration of $1x10^4$ cells/mL and plated in 6-well ultra low attachment plates so that the total number of cells plated in each well was 96 000. After 4 days, the number of spheroids was counted in each well, and the percentage of cells forming a spheroid was calculated for the MCF-7 and MCF-7-526b cell lines.

2.8 *In Vivo* Lung Colony Formation

2.8.1 Model Organism

Seven-week old female NOD/SCID/*GusB*-null mice were used as an *in vivo* model to assess the ability of stably transfected miR-526b over-expressing human breast cancer cell lines to establish colonies in the lung. These animals are non-obese diabetic, with a severe combined immune deficiency that leads to a deficiency in T and B-lymphocytes (Hofling *et al.,* 2003). In addition, these animals also lack the beta-glucuronidase (*GusB*) enzyme, which is important for the metabolism of complex carbohydrates. The absence of *GusB* in host cells allows donor cell colonization at the single cell level (Hofling *et al.,* 2003*).* However our lab observed that with increasing tumor age, some tumor cells lose *GusB* staining. For this reason we adopted HLA antibody as a tool for identification of colonizing tumor cells (below).

2.8.2 Lung Colony Formation

Animals were given a tail vein injection of MCF-Mock, MCF-7-526b, SKBR-3, or SKBR-3-526b at an inoculum dose of $5x10^5$ cells. Animals were maintained on a

normal diet, and were sacrificed after 4-weeks to assess micro-metastases to the lung. Lungs were harvested after inflation with media and frozen, and 3 semi-serial sections in the mid-coronal plane (to expose maximal lung surface area) were obtained for each animal. The serial sections were then stained for Human Leukocyte Antigen (HLA) and nuclei stained with DAPI using an immunoflourescence protocol. Primary mouse anti-human HLA antibody (1:100 dilution, Sigma-Aldrich, MO), followed by secondary horse anti-mouse antibody (1:1000 dilution, Vector Scientific), and 4', 6-diamidino-2-phenylindole (DAPI) stain were applied to the sections. Entire serial sections were imaged using a fluorescence microscope and the number of HLA stained lung colonies formed (8 or more confined cells) was calculated for each condition. Negative controls were provided by an equivalent concentration of mouse Ig isotype replacing the primary antibody to exclude nonspecific staining.

2.8.3 Cell Proliferation *In Vivo*

To assess the number of proliferating cells in the lung sections described above (2.8.2), a 5-ethynyl-2'-deoxyuridine (EdU) proliferation kit was purchased from Life Technologies Inc. (Click-iT® EdU Alexa Fluor® 488 Imaging Kit). Similar to the BrdU assay described previously for *in vitro* cellular proliferation (2.6), EdU is also a synthetic analog of uridine that is incorporated into newly synthesized DNA and can be used as a marker for proliferation. However, it can be used to assess *in vivo* cell proliferation, since DNA does not need to be denatured in order for fluorescence to occur (Life Technologies, CA). Twenty-four hours prior to sacrifice, animals were injected with EdU solution, as per the manufacturer's protocol. Animals were sacrificed and lung tissue was frozen. Serially sections were obtained, followed by a 30 min EdU reaction as described

by the manufacturer's protocol. The sections were also stained with Hoechst, as suggested by the manufacturer. Entire serial sections were imaged using a fluorescence microscope, and the number of proliferating cells in each section was calculated for each condition. Since attempts to obtain double labeling for HLA and EdU were unsuccessful, we used adjacent sections for these procedures.

2.9 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism Software Version 5.01 (GraphPad Software, La Jolla, CA). Real-time qPCR data comparing miR-526b expression in non-transfected and transfected MCF-7 and SKBR-3 cell lines was calculated using a Student's T-test, and miR-526b expression levels comparing DMSO, PGE2, PGE1OH, LY, WT, and H89 treated monolayer and tumorsphere conditions were calculated using One-Way ANOVA, followed by Tukey's *post-hoc* test. A result was considered statistically significant with a p-value of less than 0.05. The respective p-values were designated as < 0.05, 0.01, or 0.001, as indicated in each figure.

CHAPTER 3: RESULTS

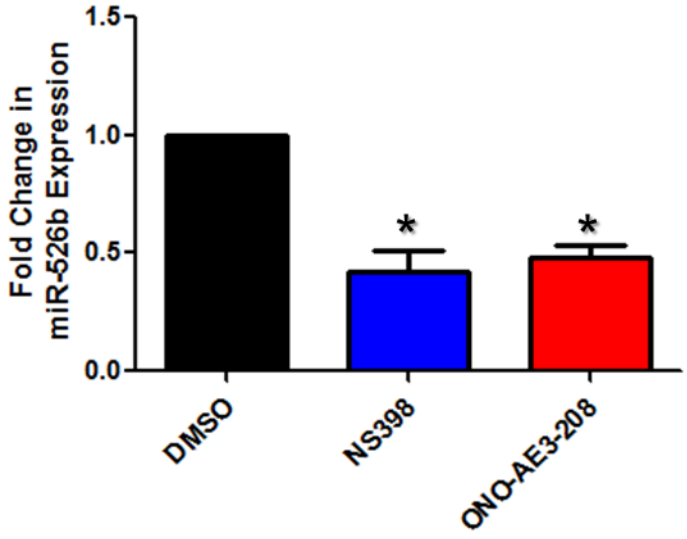
3.1 EP4 Receptor Activity Regulates miR-526b Expression in MCF-7 and MCF-7 COX-2 Human Breast Cancer Cells

3.1.1 Treatment with COX-2 Inhibitor or EP4 Receptor Antagonist Decreases miR-526b Expression in MCF-7-COX-2 Cells

A previous study in our lab demonstrated that targeting the EP4 receptor with a selective antagonist (ONO-AE3-208) treatment *in vivo* reduced the growth of primary tumors and spontaneous metastases in mice injected orthotopically with a syngeneic COX-2 expressing C3L5 cell line (Xin *et al.*, 2012). Furthermore, treating with an EP4 antagonist or EP4 knockdown of MCF-7-COX-2 cells markedly reduced their lung colony forming ability *in vivo* (Majumder *et al.*, 2014). To examine the potential of the EP4 receptor to regulate COX-2 induced miR-526b expression *in vitro*, MCF-7-COX-2 cells were treated with an EP4 antagonist (ONO-AE3-208, 10 μM), COX-2 inhibitor (NS-398, 10 μM), or vehicle (0.003% DMSO) for 24 h. It was observed that MCF-7-COX-2 cells treated with COX-2 inhibitor and EP4 antagonist showed a significant decrease in miR-526b expression levels, compared to vehicle treated cells **(Figure 5)**. These results suggest that miR-526b expression in MCF-7-COX-2 cells may be reliant on both COX-2 expression and EP4 activity.

Figure 5. MiR-526b expression in response to treatment with COX-2 inhibitor and EP4 antagonist in MCF-7-COX-2 human breast cancer cells.

MCF-7-COX-2 cells were treated with 10 μM of a COX-2 specific inhibitor (NS-398), an EP4 receptor antagonist (ONO-AE3-208), or vehicle treatment (0.003% DMSO) for 24h. Total miRNA was extracted, cDNA was synthesized, and real-time RT-PCR was performed (ΔCt method). Treatment with either the COX-2 inhibitor or EP4 antagonist resulted in a significant decrease in miR-526b expression, compared to the vehicle treated cells. The data are represented as fold change ± standard error mean (SEM). (*) Indicates a significant difference ($p<0.001$) relative to the cell line treated with vehicle (n=3, One-Way ANOVA).

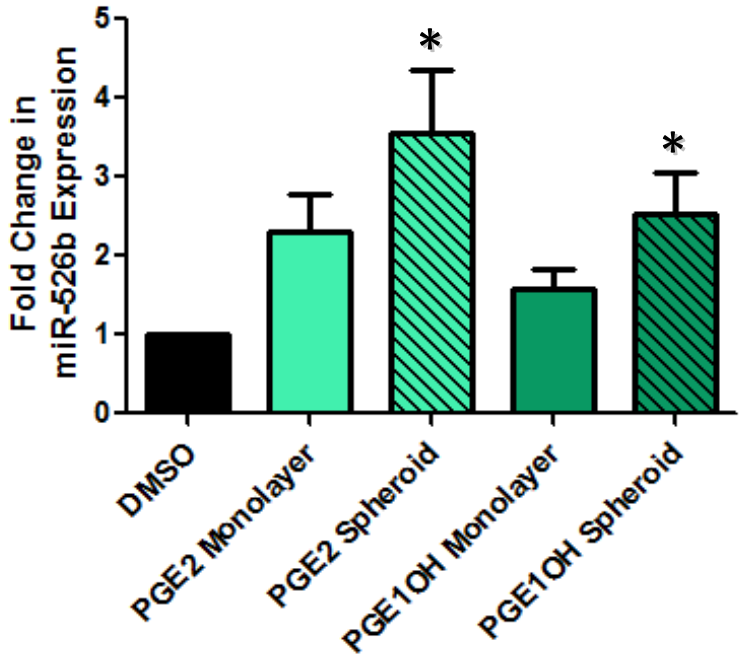


3.1.2 Treatment with PGE2 and PGE1OH Increases miR-526b Expression in MCF-7 Monolayer and Tumorsphere Culture Conditions

PGE2 is the major prostanoid product of COX-2 enzyme activity, and can bind to and activate all EP receptors including cAMP-stimulatory EP receptors, EP2 and EP4. Conversely, PGE1OH binds EP4 (both cAMP and PI3K-AKT signaling) but not the EP2. MCF-7 human breast cancer cells grown in monolayer and tumorsphere culture were treated with PGE2 (10 μM), PGE1OH (10 μM), or vehicle (0.003% DMSO) for a period of 9 days. MCF-7 cells cultured in monolayer and tumorsphere conditions and treated with either PGE2 or PGE1OH displayed increases in miR-526b expression, and these increases were significant for both treatments under tumorsphere conditions **(Figure 6)**. These results suggest that miR-526b expression in human breast cancer cells is positively regulated in part by EP4 receptor activity. Since PGE2 also stimulates EP2 receptor activity, the increases in miR-526b expression observed were greater with PGE2 compared to PGE1OH treatment.

Figure 6. MiR-526b expression in response to treatment with PGE2 and PGE1OH alcohol in MCF-7 human breast cancer cells.

MCF-7 cells were cultured under monolayer and tumorsphere conditions, and treated with PGE2, PGE1OH, or vehicle (0.003% DMSO) for a period of 9 days. In both monolayer and tumorsphere culture conditions, treatment with either PGE2 or PGE1OH resulted in increases in miR-526b expression compared to the vehicle treated cells, with significant increases observed fro PGE2 and PGE1OH treated tumorspheres. The data are represented as fold change ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.05$) relative to the cell line treated with vehicle (n=3, One-Way ANOVA).

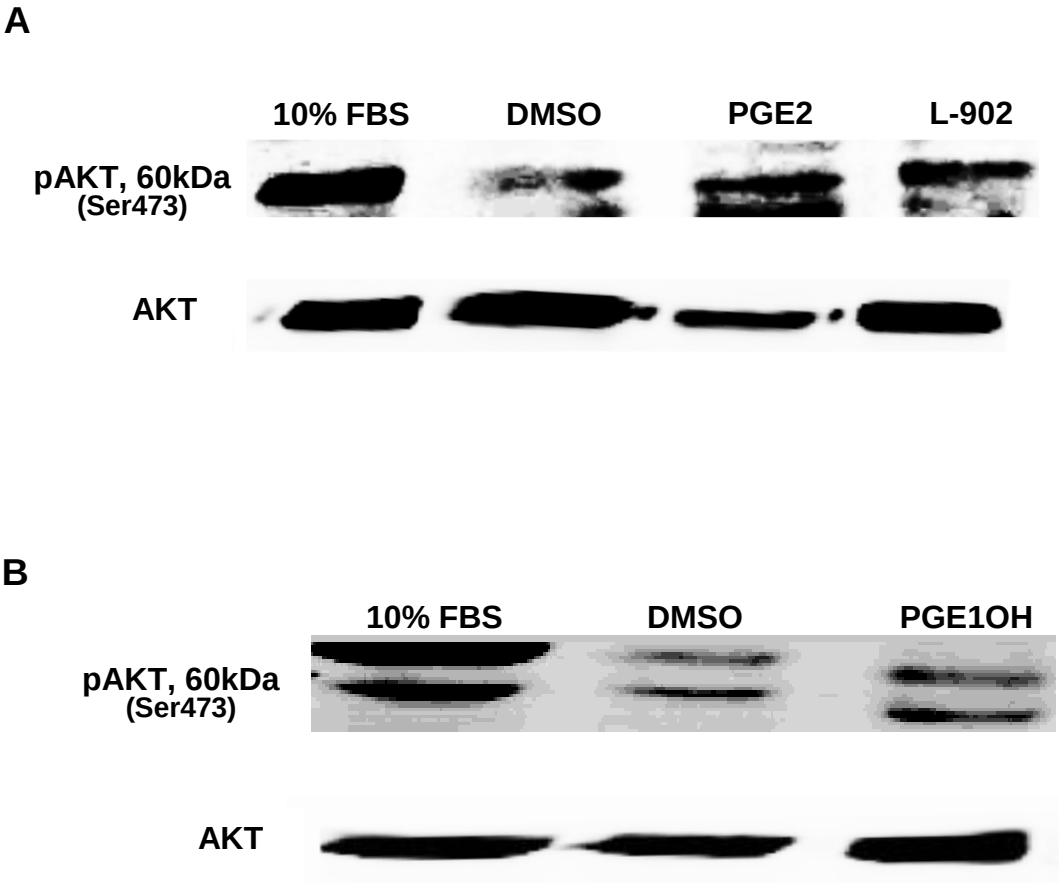


3.1.3 Treatment with PGE2, PGE1OH, or L-902688 Increases pAKT Stimulation in MCF-7 Cells

Activation of the EP4 receptor initiates PI3K-AKT signaling, in addition to the canonical cAMP pathway. In order to validate that activation of the EP4 receptor did indeed result in activation of the PI3K pathway, Western blot analyses were performed. MCF-7 cells were treated for 24h with each treatment, and 30 µg of protein for each sample was run on an SDS-PAGE for 1.5 h. The membrane was blocked for 1h and incubated over night in a mixture of primary antibodies: pAKT (monoclonal mouse, 1:500, Ser473), and AKT (monoclonal rabbit, 1:1000). It was observed that PGE2 or EP4 agonists L-902-866 **(Figure 7A)** or PGE1OH **(Figure 7B)** stimulated pAKT in MCF-7 cells, compared to DMSO treated cells. This result provides validation that stimulation of the EP4 receptor activates the PI3K-AKT signaling pathway in MCF-7 human breast cancer cells.

Figure 7. pAKT protein expression in response to treatment with PGE2, L-902688, or PGE1OH in MCF-7 human breast cancer cells.

MCF-7 cells were cultured in monolayer in 100 cm^2 Petri dishes, and serum starved overnight. Cells were then treated with PGE2 (100µM), L-902 (100 µM), PGE1OH (100 µM), or vehicle (0.003% DMSO) for 24 h and total protein was extracted. 30 µg of protein for each treatment was run on an SDS-PAGE at 100 V for 1.5 hours, and Western blot analyses were performed probing for pAKT and AKT as an internal control. For PGE2, L-902-688 (7A) and PGE1OH (7B) treated cells, pAKT protein expression was stimulated compared DMSO treated controls.

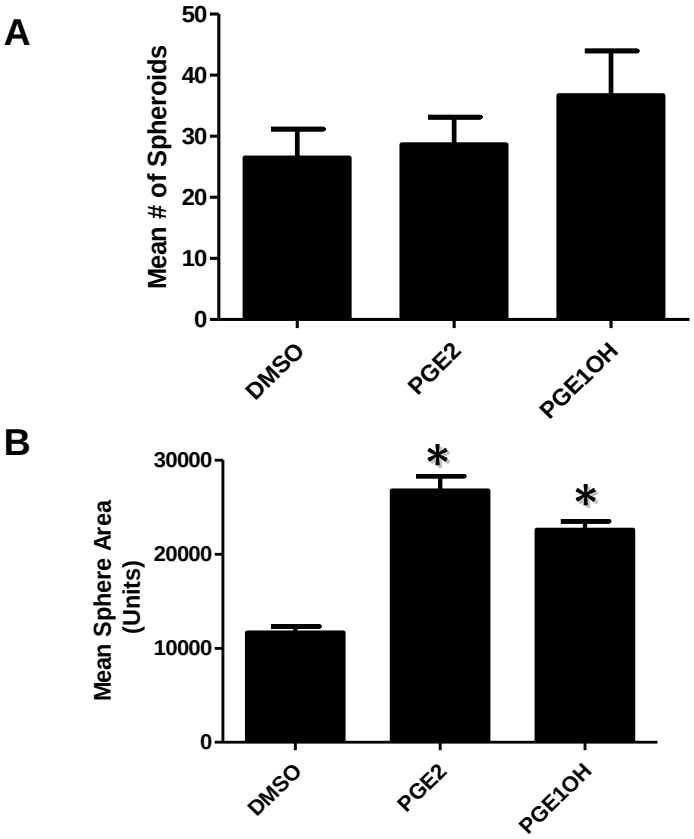


3.1.4 Treatment with PGE2 and PGE1OH Stimulates SLC Phenotype in MCF-7 Cells

The ability to form spheroids on ultra-low attachments plates can be used as an *in vitro* surrogate for functional SLC phenotype in human breast cancer cells. Cells cultured in such tumorsphere conducive conditions grow in an anchorage-independent manner, and spheroids are formed via clonal regeneration. MCF-7 cells were plated at single cell suspensions on ultra-low attachment plates and treated with PGE2, PGE1OH, or vehicle control (0.003% DMSO) for 9 days. Following treatment, each well of the plate was imaged using a bright-field microscope, and representative images were obtained for each treatment. The number and size of spheroids for each treatment was analyzed using ImageJ software. Compared to vehicle-treated cells, MCF-7 tumorspheres treated with PGE2 or PGE1OH displayed notable increases in spheroid number **(Figure 8A),** and significant increases in spheroid size **(Figure 8B)**. These results suggest that activation of both EP2 and EP4 can have stimulatory effects on SLC phenotype in human breast cancer cells.

Figure 8. Stimulation of SLC phenotype in response to PGE2 and PGE1OH in MCF-7 human breast cancer cells.

MCF-7 cells were cultured under tumorsphere conditions in 6-well ultra-low attachment plates and treated with PGE2, PGE1OH, or vehicle (0.003%) DMSO for a total period of 9 days. Using ImageJ software, the number of spheroids in each treatment condition was calculated. Additionally, the area of each spheroid was obtained using ImageJ software, and the mean spheroid area for each treatment condition was calculated. PGE2 and PGE1OH treated cells displayed a notable increase in spheroid number **(A)**, and a significant increase in spheroid size **(B)** compared to vehicle-treated controls. The data are represented as mean ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.0001$) relative to the cell line treated with vehicle (n=3, One-Way ANOVA).



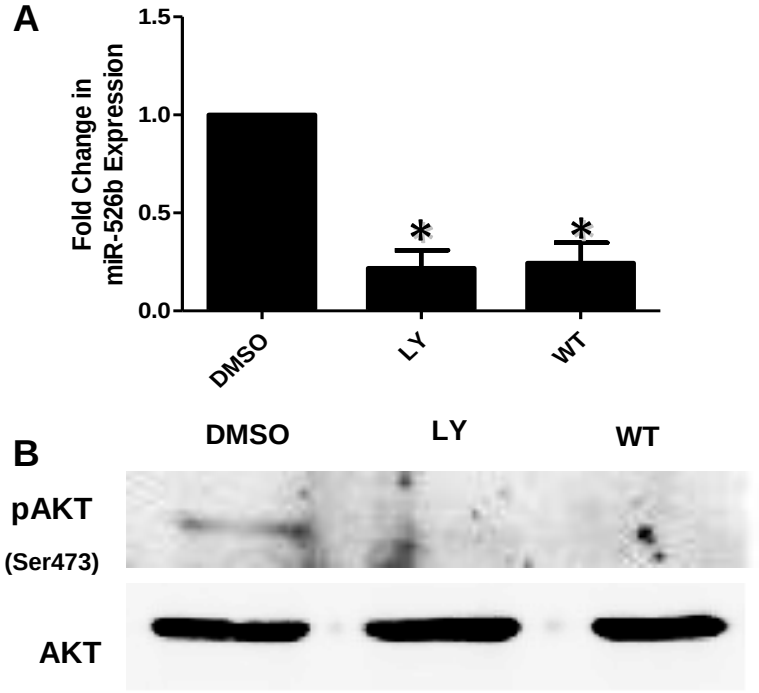
3.2 miR-526b Expression in MCF7-COX-2 Cells is Dependent on PI3K/AKT Signaling Pathway

3.2.1 Treatment with PI3K Inhibitors Decreases miR-526b Expression and pAKT Stimulation in MCF-7-COX-2 Cells

Stimulation of the EP4 receptor results in activation of the PI3K-AKT signaling pathway, in addition to the canonical cAMP pathway, also shared by EP2 receptor stimulation. To investigate the role of PI3K-AKT activity in regulating miR-526b expression, MCF-7-COX-2 cells were grown as a monolayer and treated with either an irreversible PI3K inhibitors Wortmannin (WT), a reversible PI3K inhibitor LY-204002 (LY), or vehicle (0.003% DMSO) for 24 h. It was observed that MCF-7-COX-2 cells treated with both PI3K inhibitors showed a significant decrease in miR-526b expression levels **(Figure 9A)**, as well as a decrease on pAKT protein levels relative to total AKT **(Figure 9B)** compared to vehicle treated controls. This result suggests that miR-526b expression in MCF-7-COX-2 cells is associated with PI3K-AKT signaling activity, due in part to EP4 receptor activity.

Figure 9. MiR-526b expression and pAKT protein levels in response to treatment with PI3K inhibitors in MCF-COX-2 human breast cancer cells.

Cells were serum-starved overnight and treated for 24h with one of two PI3K inhibiting agents, LY-204002 (LY), Wortmannin (WT), or a vehicle (0.003% DMSO). Total miRNA was extracted from cells and real-time RT-PCR was performed. It was observed that cells treated with either LY or WT displayed a significant decrease in miR-526b expression levels (A). Total protein was extracted from treated cells, and pAKT (Ser473) protein levels between samples were assessed using Western blot analysis. It was observed that cells treated with either PI3K inhibitor displayed a reduced level of pAKT, compared to DMSO treated cells (B). The qPCR data are represented as fold change ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.0001$) relative to the cell line treated with vehicle (n=3, One-Way ANOVA).

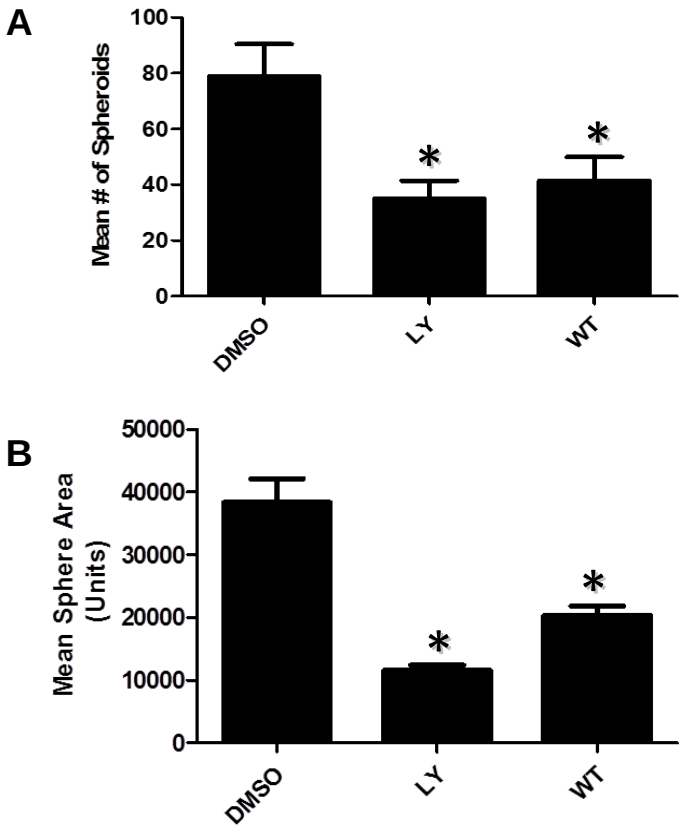


3.2.2 Treatment with PI3K Inhibitors Reduces SLC Phenotype in MCF-7-COX-2 Cells

To investigate the importance of PI3K-AKT signaling in promoting SLC phenotype in MCF-7-COX-2 cells, a tumorsphere formation assay was conducted in the presence of two PI3K inhibitors. Cells were plated on ultra-low attachment 6-well plates and were treated with PI3K inhibitors LY-204002 (LY) or Wortmannin (WT), or a vehicle control (0.003% DMSO). It was observed that MCF-7-COX-2 cells treated with LY or WT displayed a significant decrease in spheroid number **(Figure 10A)** and spheroid size **(Figure 10B)**. These results suggest that PI3K-AKT signaling is involved in promoting SLC phenotype in human breast cancer cells.

Figure 10. Reduction of SLC phenotype in response to LYand WT in MCF-7-COX-2 human breast cancer cells.

MCF-7-COX-2 cells were grown on ultra-low attachment 6-well plates and treated with the PI3K inhibitors LY, WT, or a vehicle control (0.003% DMSO). Using ImageJ software, the number of spheroids in each treatment condition was calculated. Additionally, the area of each spheroid was obtained using ImageJ software, and the mean spheroid area for each treatment condition was calculated. LY and WT treated cells displayed a significantly fewer number of spheroids (A) with a significantly decreased size (B) compared to vehicle-treated controls. The data are represented as mean ± SEM for three independent experiments. (*) Indicates a significant difference (A: $p<0.005$, B: $p<0.0001$) relative to the cell line treated with vehicle (n=3, One-Way ANOVA).



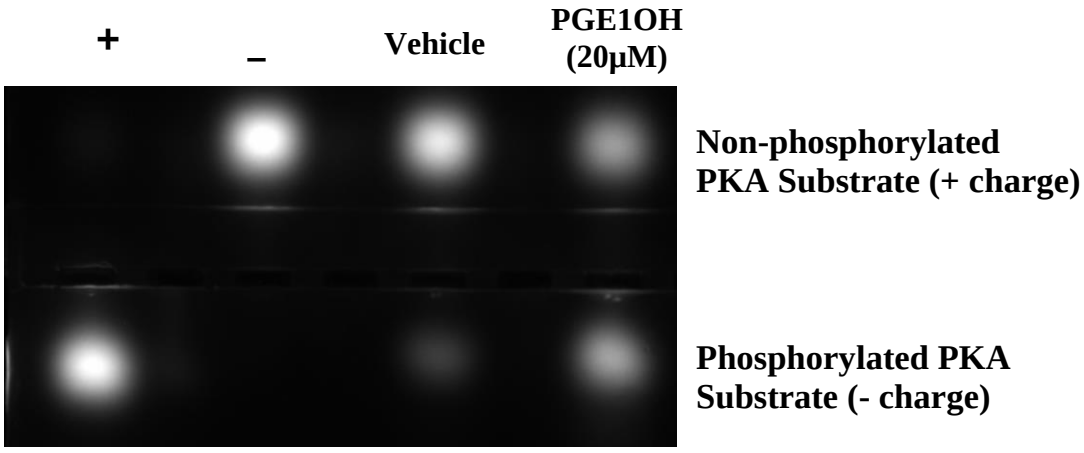
3.3 cAMP Signaling Regulates miR-526b Expression in MCF-7 and MCF-7-COX-2 Cells

3.3.1 Treatment with EP4 Agonist PGE1OH Increases miR-526b Expression in MCF-7 Cells

EP2 and EP4 receptor stimulation activates the canonical cAMP-PKA pathway, and can be stimulated using an EP4 agonist. As previously demonstrated (3.1.4), treatment with the EP4 agonist PGE1OH resulted in a significant increase in miR-526b expression. To validate that up-regulation of miR-526b was occurring due in part to cAMP activity, a qualitative PKA assay was performed. MCF-7 cells grown in monolayer were treated for 24h with PGE1OH (20μM) or vehicle (0.003%) DMSO and total protein was extracted. A non-radioactive cAMP-dependent PepTag assay was carried out using the extracted protein lysates in order to validate PKA activity qualitatively in the treated cells. The cAMP-dependent PKA assay results demonstrated that cells treated with PGE1OH displayed an increase in overall PKA activity, compared to DMSO treated controls **(Figure 11)**. These results indicate the potential importance of EP4-induced cAMP signaling in miR-526b over-expression in MCF-7 human breast cancer cells.

Figure 11. PKA activity in response to treatment with EP4 agonist PGE1OH in MCF-7 human breast cancer cells.

MCF-7 cells were cultured in monolayer, serum-starved overnight, and treated for 24h with the EP4 agonist PGE1OH (20 μM) or vehicle (0.003% DMSO). Total protein was extracted from cells and a cAMP-dependent PKA assay was carried out. Cells treated with PGE1OH displayed a visible increase in PKA activation, as demonstrated by the brighter fluorescent PKA substrate bar, compared to DMSO treated controls. The (+) sign indicates a positive control sample, containing cAMP-Dependent Protein Kinase, Catalytic Subunit in addition to the components in each sample condition. The (-) sign indicates a negative control sample, containing no sample, but all other components present in the other reactions.

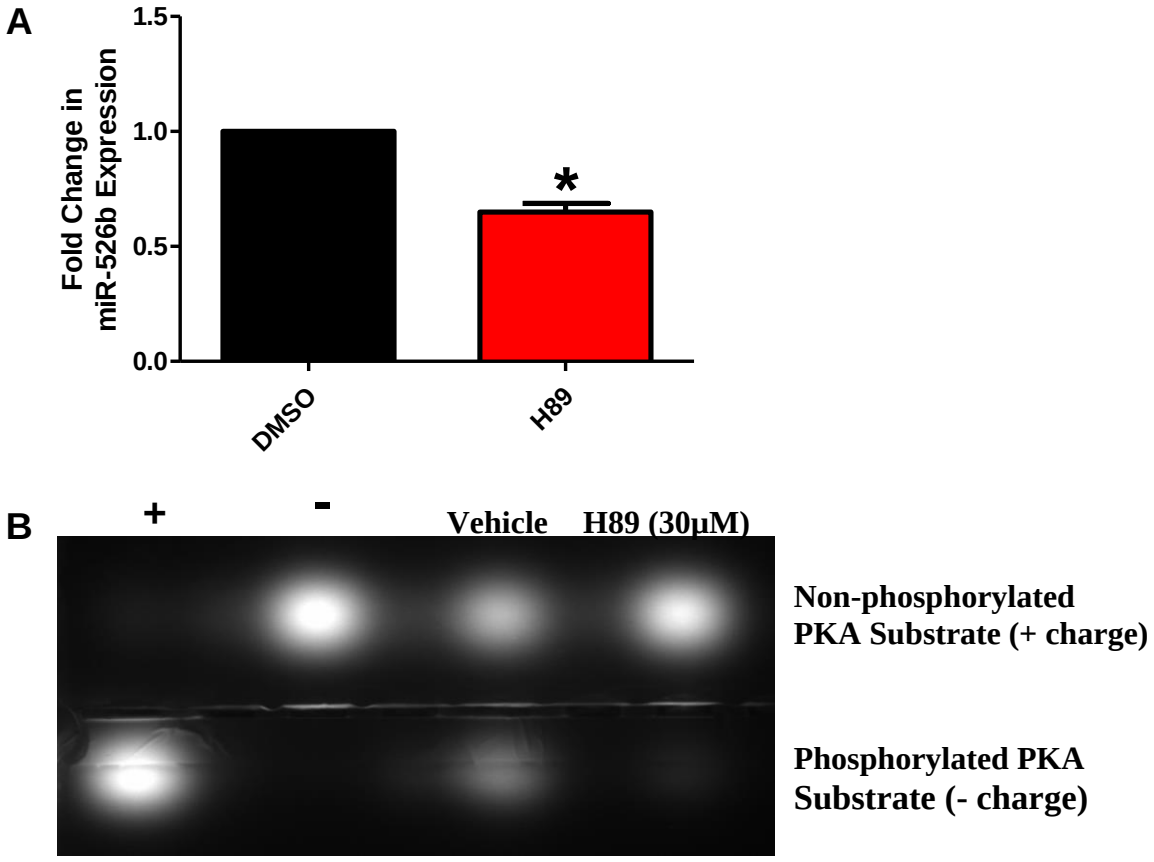


3.3.2 Treatment with PKA Inhibitor Decreases miR-526b Expression in MCF-7-COX-2 Cells

To investigate the effects of inhibiting cAMP signaling on miR-526b expression, MCF-7-COX-2 cells were treated with the PKA specific inhibitor H89 (30 μM). Cells were grown in monolayer and treated with H89 for a period of 24 h. Total miRNA and total protein was extracted from the cells, cDNA was synthesized, and real-time RT-PCR was carried out. A non-radioactive cAMP-dependent PepTag assay was also carried out using the extracted protein lysates to validate PKA activity in treated cells. Analysis of qPCR revealed that H89 treated cells had a significantly lower expression of miR-526b, compared to DMSO treated controls **(Figure 12A)**. Additionally, the cAMP-dependent PKA assay results demonstrated that cells treated with H89 displayed a decrease in overall PKA activity, compared to DMSO treated controls **(Figure 12B)**. Together, these results validate the importance of EP2/EP4-mediated PKA activity and cAMP signaling in miR-526b expression in MCF-7-COX-2 human breast cancer cells.

Figure 12. MiR-526b expression and PKA activity in response to treatment with a PKA inhibitor in MCF-7-COX-2 human breast cancer cells.

MCF-7-COX-2 cells were grown in monolayer and treated with the PKA specific inhibitor H89 (30 µM) or vehicle (0.003% DMSO) for 24h. Total miRNA was extracted from cells followed real-time RT-PCR was carried out. It was observed that cells treated with H89 displayed a significant decrease in miR-526b expression levels (A). Cells treated with H89 displayed a visible decrease in PKA activation, as demonstrated by the dimmer fluorescent PKA substrate bar, compared to DMSO treated controls (B). The qPCR data are represented as fold change ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.005$) relative to the cell line treated with vehicle (n=3, Student's T-test).

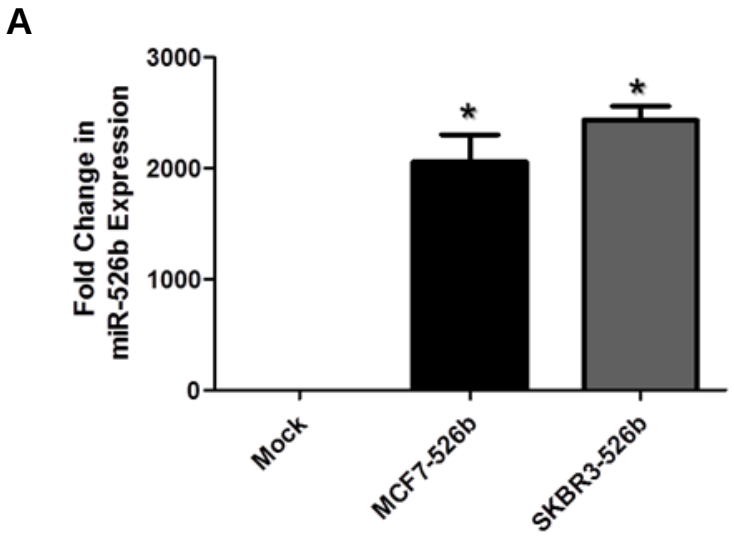


3.4. Validation of Stable miR-526b Over-Expression in MCF-7 and SKBR-3 Cells

In order to investigate the functional roles of miR-526b *in vitro* and *in vivo*, stable over-expression of miR-526b in two weakly metastatic, low COX-2 and low miR-526b expressing human breast cancer cell lines was achieved. MCF-7 and SKBR-3 breast cancer cells lines were stably transfected using a miR-526b over-expression plasmid, and treated with the anti-biotic selection agent Geneticin® as per the manufacturer's protocol. Over-expression of miR-526b was validated using real-time RT-PCR (ΔCt method) to compare expression between miR-526b over-expressing cell lines and their appropriate empty vector controls. MiR-526b was significantly over-expressed in miR-526b transfected MCF-7 and SKBR-3 cell lines, compared to empty vector controls **(Figure 13)**. The miR-526b over-expressing cell lines were named MCF-7-526b and SKBR-3-526b, with their empty vector controls being named MCF-7-Mock and SKBR-3-Mock, respectively.

Figure 13. MCF-7 and SKBR-3 cells stably transfected with miR-526b over-expression plasmid significantly express miR-526b.

MCF-7 and SKBR-3 cell lines were stably transfected with either a miR-526b over-expression plasmid or empty vector (Mock) control plasmid using electroporation. Cells expressing either plasmid were selected for using a Neomycin resistance gene. MiR-526b expression levels were validated in both cell lines using real-time RT-PCR (ΔCt method). MCF-7 and SKBR-3 cells stably transfected with the miR-526b expression plasmid displayed approximate 2000-fold, and 2400-fold increases, respectively, in miR-526b expression compared to empty vector (Mock) control cells. The qPCR data are represented as fold change ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.0001$) relative to the Mock cell line (n=3, Student's T-test).

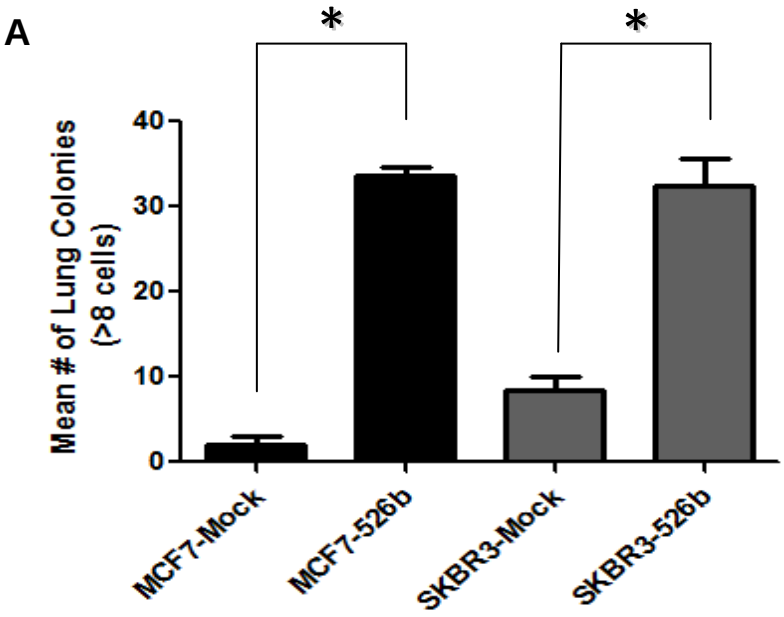


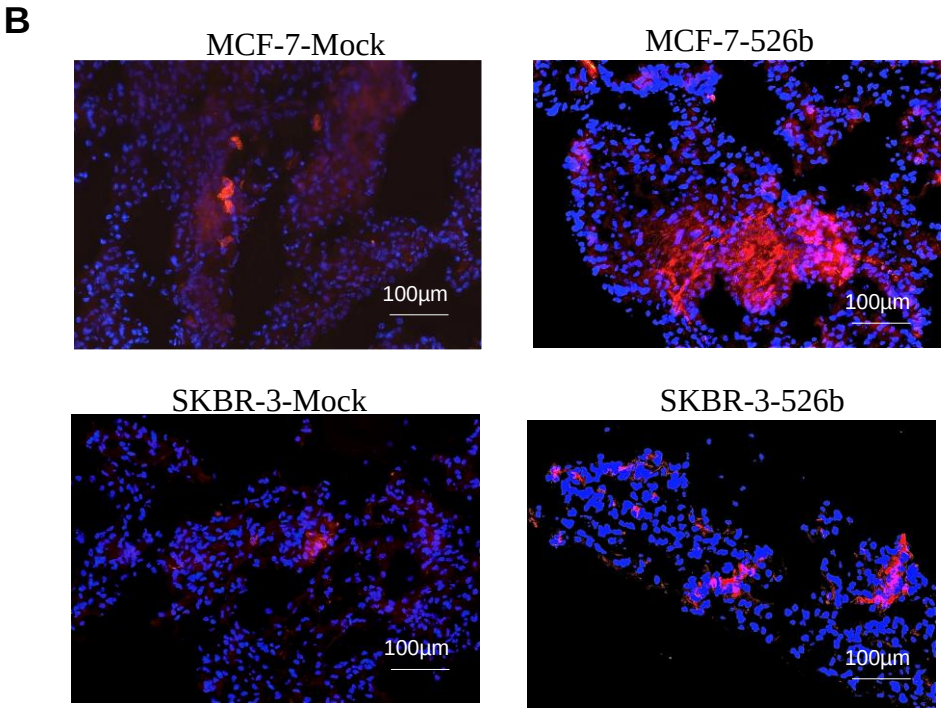
3.5 MCF-7 and SKBR-3 Human Breast Cancer Cells Over-Expressing miR-526b Display Enhanced Lung Colony Forming Ability in *NOD/SCID/GusB*-Null Female Mice

3.5.1 MCF-7-526b and SKBR-3-526b Cells Display Enhanced Ability to Form Lung Colonies *In Vivo* Compared to Mock Controls

In order to investigate tumorigenic functions of miR-526b *in vivo*, miR-526b over-expressing cell lines and their respective Mock control cell lines were injected into the tail-vein of 7-week old *NOD/SCID/GusB* null female mice (inoculum dose of $5x10^5$ cells). Animals were sacrificed after 4 weeks, and an intra-peritoneal EdU injection was given to each 24h prior to sacrifice in order to identify proliferating cells. Following sacrifice, lungs, liver, spleen, kidney, and brain were collected from each animal. Lungs were frozen on dry ice in mounting media, serially sectioned, mounted on slides, and stained with anti-Human Leukocyte Antigen (HLA) anti-body and the nuclear marker DAPI. The number of HLA-positive colonies (>8 cells) was counted for each of the three serial sections in each animal using the ImageJ program. It was observed that MCF-7 and SKBR-3 breast cancer cells over-expressing miR-526b established a significantly greater number of lung colonies, compared to empty vector controls **(Figure 14)**. This result supports the hypothesis that over-expression of miR-526b in human breast cancer cells supports tumorigenicity in this *in vivo* model.

Figure 14. Lung colony formation in *NOD/SCID/GusB* null mice following tail vein injection with MCF-7-526b, SKBR-3-526b, and empty vector controls after 4-weeks. MCF-7-526b, SKBR-3-526b, and respective Mock control cells were injected into the tail vein of 7-week old female mice. After 4-weeks, animals were sacrificed and lung were collected, frozen, serially sectioned (3 sections per animal, 150 pictures per section), and stained with anti-HLA anti-body (red) and the nuclear marker DAPI (blue). Fluorescent images were obtained for each serial section and the number of colonies (>8 cells) was counted using ImageJ. (A) Quantification of lung colony formation in the lung of mice injected with MCF-7-526b, SKBR-3-526b, or appropriate Mock control cell lines. The data are represented as mean ± SEM for all three sections in all animals (n=2 Mock, n=3 miR-526b). (*) Indicates a significant difference ($p<0.001$) comparing miR-526b over-expressing cell line and its appropriate empty vector control (Student's T-test). (B) Representative images of MCF-7-526b, SKBR-3-526b, and Mock control serial lung sections stained with anti-HLA antibody (red) and DAPI (blue). Images are shown at a magnification of 20x.


A
*
*
Mean # of Lung Colonies (>8 cells)
40
30
20
10
0
MCF7-Mock
MCF7-526b
SKBR3-Mock
SKBR3-526b

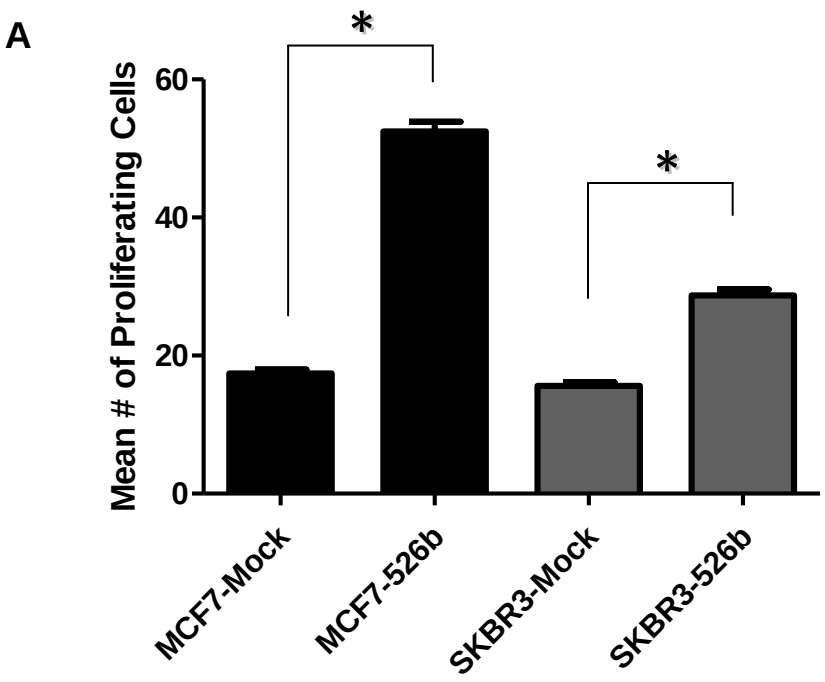

B
MCF-7-Mock
MCF-7-526b
100µm
100µm
SKBR-3-Mock
SKBR-3-526b
100µm
100µm

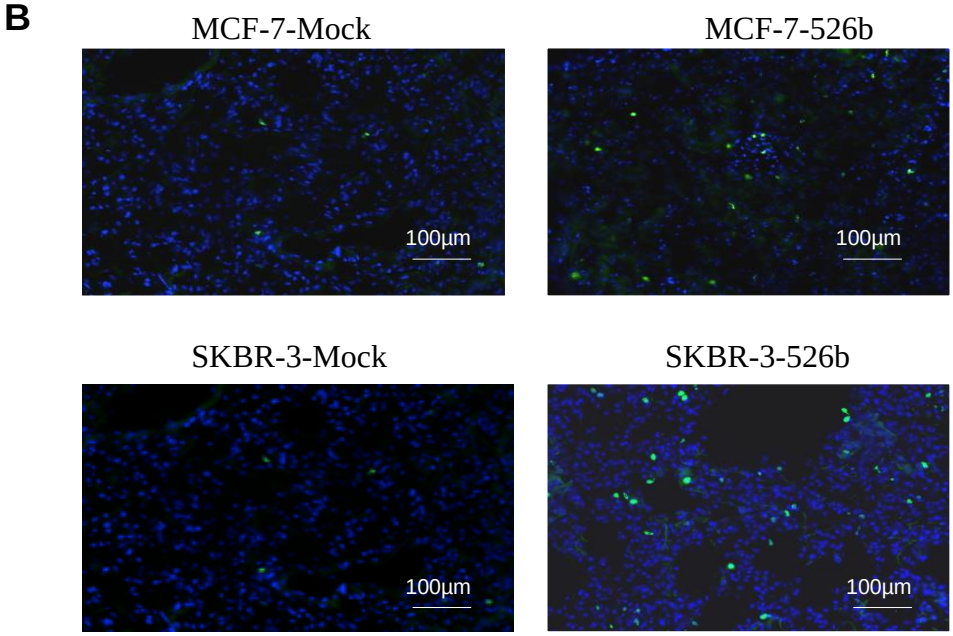
3.5.2. MCF-7-526b and SKBR-3-526b Lung Sections Display Increased Cellular Proliferation *In Vivo* Compared to Mock Controls

In order to determine if the lung colonies established in the *in vivo* mouse model previously discussed (3.5.1) also contained a greater number of proliferating cells in the lungs, an EdU-Alexa Flour488® proliferation kit (Life Technologies) was used. Briefly, animals were injected intra-peritoneally with an EdU reaction, as per the manufacturer's protocol. MCF-7 and SKBR-3 breast cancer cells over-expressing miR-526b displayed significantly greater number of proliferating cells, compared to empty vector controls **(Figure 15)**. This result suggests that miR-526b is involved in the establishment of proliferating lung colonies *in vivo* for MCF-7 and SKBR-3 human breast cancer cell lines.

Figure 15. Lung colony proliferation in *NOD/SCID/GusB*-Null mice following tail vein injection with MCF-7-526b, SKBR-3-526b, and empty vector controls after 4-weeks.

MCF-7-526b, SKBR-3-526b, and respective Mock control cells were injected into the tail vein of 7-week old female mice. After 4-weeks and 24h prior to sacrifice, animals were injected with a fluorescent-tagged EdU reaction. Lungs were collected, frozen, serially sectioned (3 sections per animal, 150 pictures per section), and stained with anti-EdU anti-body (green) and the nuclear marker Hoechst (blue). (A) Quantification of proliferating cells in the lung of mice injected with MCF-7-526b, SKBR-3-526b, or appropriate Mock control cell lines. The data are represented as mean ± SEM for all three sections in all animals (n=2 Mock, n=3 miR-526b). (*) Indicates a significant difference ($p<0.0001$) comparing miR-526b over-expressing cell line and its appropriate empty vector control (Student's T-test). (B) Representative images of MCF-7-526b, SKBR-3-526b, and Mock control serial lung sections stained with anti-EdU antibody (green) and Hoechst (blue). Images are shown at a magnification of 20x.


A
*
*
Mean # of Proliferating Cells
60
40
20
0
MCF7-Mock
MCF7-526b
SKBR3-Mock
SKBR3-526b


B
MCF-7-Mock
MCF-7-526b
100µm
100µm
SKBR-3-Mock
SKBR-3-526b
100µm
100µm

3.6 Over-Expression of miR-526b Increases Cellular Migration and Invasion in MCF-7 and SKBR-3 Cells

In order to investigate the effects on migratory ability in over-expressing miR-536b in MCF-7 and SKBR-3 human breast cancer cell lines, functional transwell migration and transwell invasion assays were performed. MCF-7 and SKBR-3 cell lines over-expressing miR-526b demonstrated a significantly enhanced ability to migrate through the membrane, compared to parental controls **(Figure 16A)**. This result supports the notion that over-expression of miR-526b stimulates migration in human breast cancer cell lines. These quantification data were also supported by qualitative images, demonstrating the morphology and relative number of migrating cells **(Figure 16B)**.

The ability of miR-526b over-expressing cells to enzymatically break down and invade through a basement membrane mimetic was assessed using a transwell invasion assay. MCF-7 and SKBR-3 cell lines over-expressing miR-526b demonstrated a significantly enhanced ability to invade through the matrigel and membrane, compared to parental controls **(Figure 17A)**. This result supports the notion that over-expression of miR-526b stimulates invasion in human breast cancer cell lines.

Together these results support the hypothesis that miR-526b promotes the induction of an aggressive phenotype in MCF-7 and SKBR-3 human breast cancer cell lines, as evidenced by the increased ability of miR-526b over-expressing cells to migrate and invade.

Figure 16. Over-Expression of miR-526b Increases Cellular Migration in MCF-7 and SKBR-3 Human Breast Cancer Cell Lines.

MCF-7, MCF-7-Mock, MCF-7-526b (A & C) and SKBR-3, SKBR-3-Mock, SKBR-3-526b (B & D) cells were separately plated in the top well of a transwell chamber. Over-expressing miR-526b in MCF-7 (A) and SKBR-3 (B) human breast cancer cells significantly increased the ability of cells to migrate through a microporous membrane. The data are presented as a mean of triplicates ± SEM. (*) Indicates a significant difference ($p<0.0001$) comparing miR-526b over-expressing cell line and its appropriate empty vector control (n=3, One-Way ANOVA). Representative images of the transwell membranes showing the number and morphology of migratory cells for MCF-7 (C) and SKBR-3 (D) cell lines. Magnification in 20x.

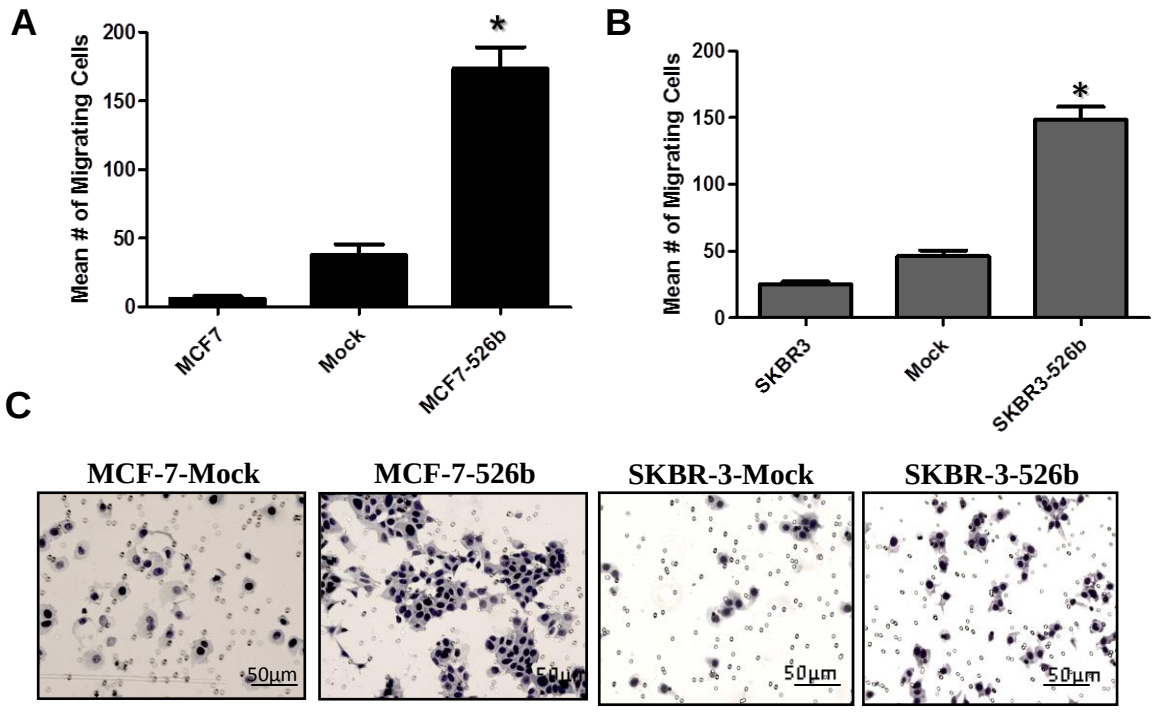
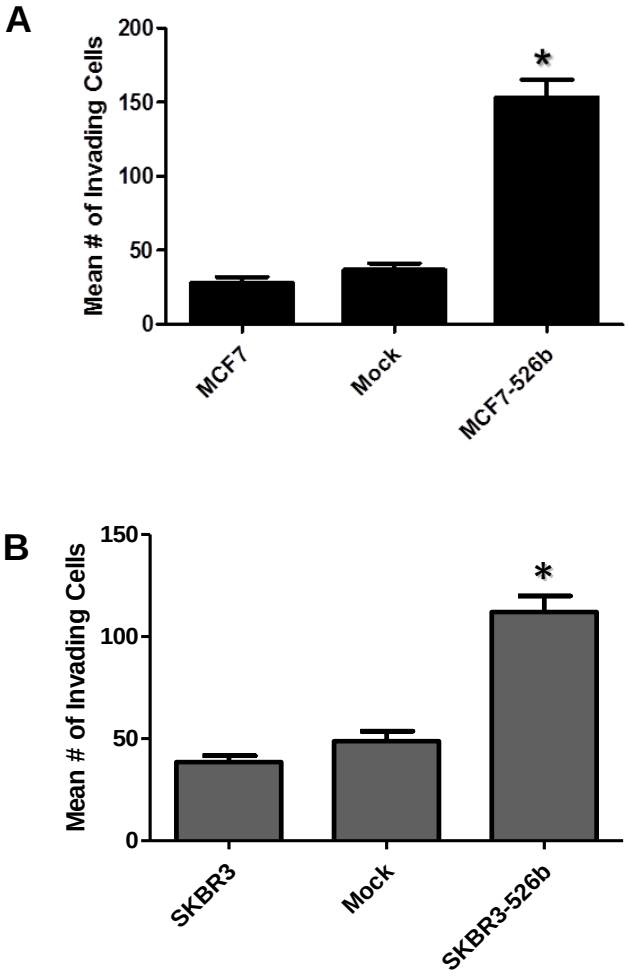


Figure 17. Over-Expression of miR-526b Increases Cellular Invasion in MCF-7 and SKBR-3 Human Breast Cancer Cell Lines.

MCF-7, MCF-7-Mock, MCF-7-526b (A) and SKBR-3, SKBR-3-Mock, SKBR-3-526b (B) cells were separately plated in the top well of a transwell chamber coated with a thin layer of GFR Matrigel. Over-expressing miR-526b in MCF-7 (A) and SKBR-3 (B) human breast cancer cells significantly increased the ability of cells to invade through a basement membrane mimetic. The data are presented as a mean of triplicates ± SEM. (*) Indicates a significant difference ($p<0.0001$) comparing miR-526b over-expressing cell line and its appropriate empty vector control (n=3, One-Way ANOVA).

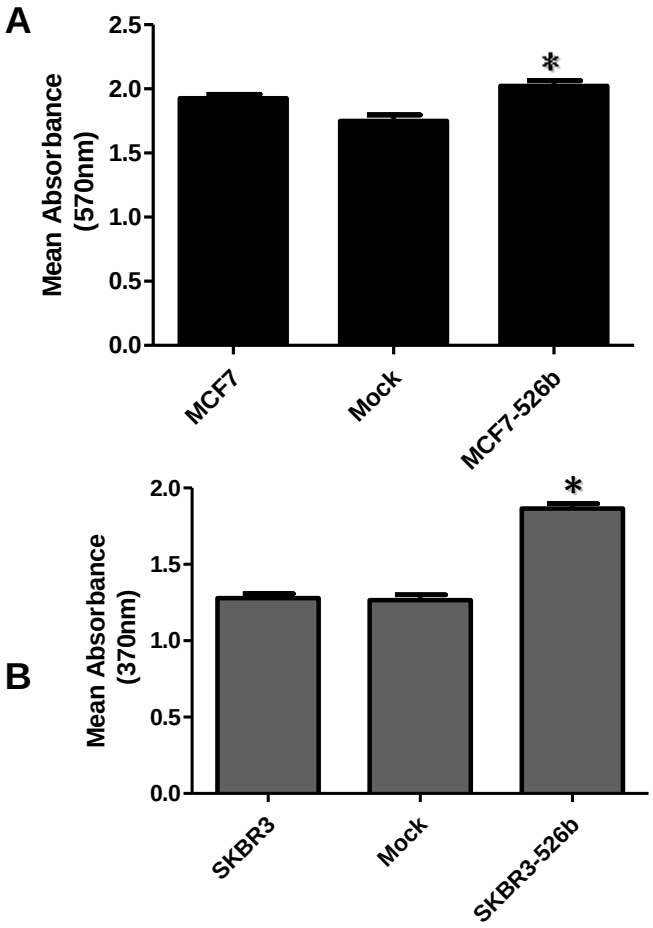


3.7 Over-Expression of miR-526b Increases Cellular Proliferation in MCF-7 and SKBR-3 Cells

To assess the effect of miR-526b over-expression on cellular proliferation in MCF-7 and SKBR-3 human breast cancer cells, a BrdU colormetric assay was performed. MCF-7-526b **(Figure 18A)** and SKBR-3-526b **(Figure 18B)** cells displayed a significant increase in cellular proliferation compared to their respective Mock cells. This result suggests that over-expression of miR-526b supports cellular proliferation in MCF-7 and SKBR-3 cell lines.

Figure 18. Introduction of miR-526b increases cellular proliferation in MCF-7 and SKBR-3 human breast cancer cell lines.

MCF-7, MCF-7-Mock, MCF-7-526b and SKBR-3, SKBR-3-Mock, SKBR-3-526b were assessed for their ability to proliferate using a BrdU ELISA assay. BrdU incorporation is presented as a mean absorbance value at 370 nm for each cell line under investigation, following a 6 h incubation period. (A) MCF-7-526b and (B) SKBR-3-526b cells displayed significant increases in cellular proliferation, compared to their respective parental control cell lines. The data are presented as mean ± SEM. (*) Indicates a significant difference ($p<0.0001$) comparing miR-526b over-expressing cell line and its appropriate parental control (n=96, One-Way ANOVA).



3.8 Over-Expression of miR-526b Stimulates SLC Phenotype in MCF-7 and SKBR-3 Cells

3.8.1 MCF-7-526b and SKBR-3-526b Cells Form Spheroids of Greater Number and Size Compared to Parental Controls

In order to investigate the role of miR-526b in promoting SLC phenotype in MCF-7 and SKBR-3 human breast cancer cell lines, miR-526b over-expressing cells and their respective Mock and parental controls were plated as single cells in 6-well ultra-low attachment plates. MCF-7 **(Figure 19A)** and SKBR-3 **(Figure 20A)** cell lines over-expressing miR-526b displayed a significant increase in the number of spheroids formed, compared to parental controls. Additionally, miR-526b over-expressing MCF-7 **(Figure 19B & C)** and SKBR-3 **(Figure 20B & C)** cell lines also produced spheroids of a significantly greater average area, compared to their respective parental controls. These results suggest that over-expression of miR-526b is associated with the induction of SLC phenotype in MCF-7 and SKBR-3 human breast cancer cell lines.

Figure 19. Over-Expression of miR-526b Promotes the Induction of SLC Phenotype in the MCF-7 Human Breast Cancer Cell Line.

MCF-7, MCF-7-Mock, and MCF-7-526b cells were cultured as tumorspheres, and the number and size of spheroids formed for each cell line was counted manually using a light microscope and the ImageJ program. MCF-7-526b cells formed a significantly greater number of spheroids, compared to the parental control cell line MCF-7 (A). Additionally, MCF-7-526b spheroids displayed a significantly greater average size when compared to MCF-7 spheroids (B). The data are presented as a mean of triplicates ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.001$) comparing miR-526b over-expressing cell line and its parental control cell line (n=3, One-Way ANOVA). Representative images comparing MCF-7-526b and MCF-7 spheroids support the quantitative data and demonstrate the change in size of spheroids with over-expression of miR-526b (C).

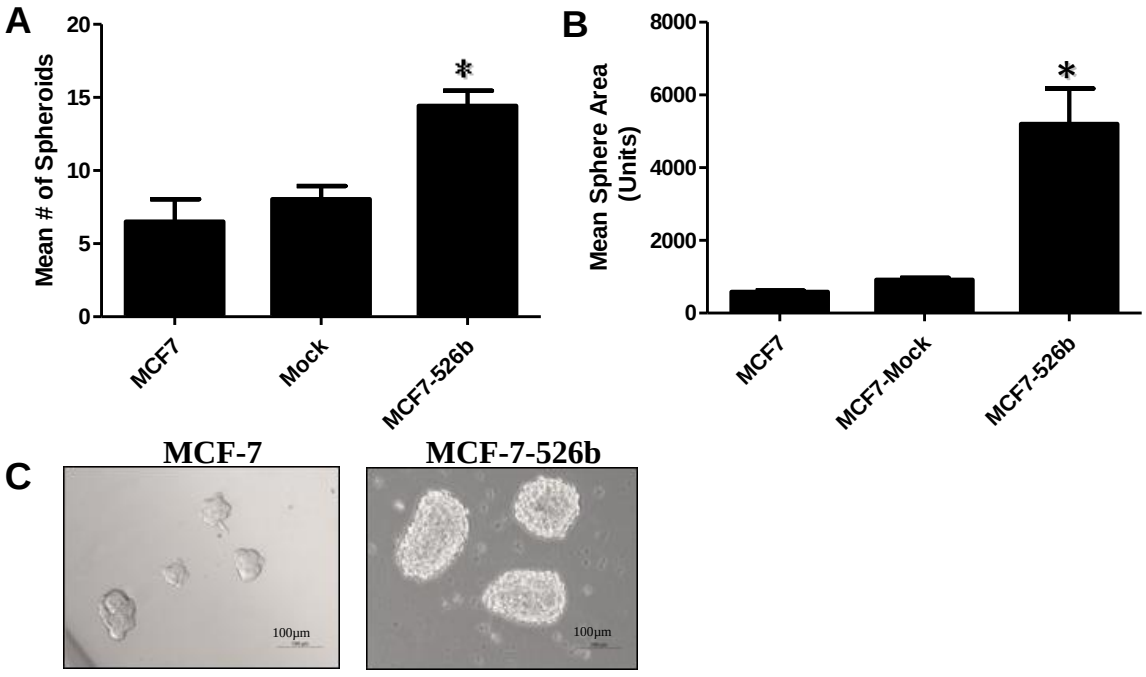
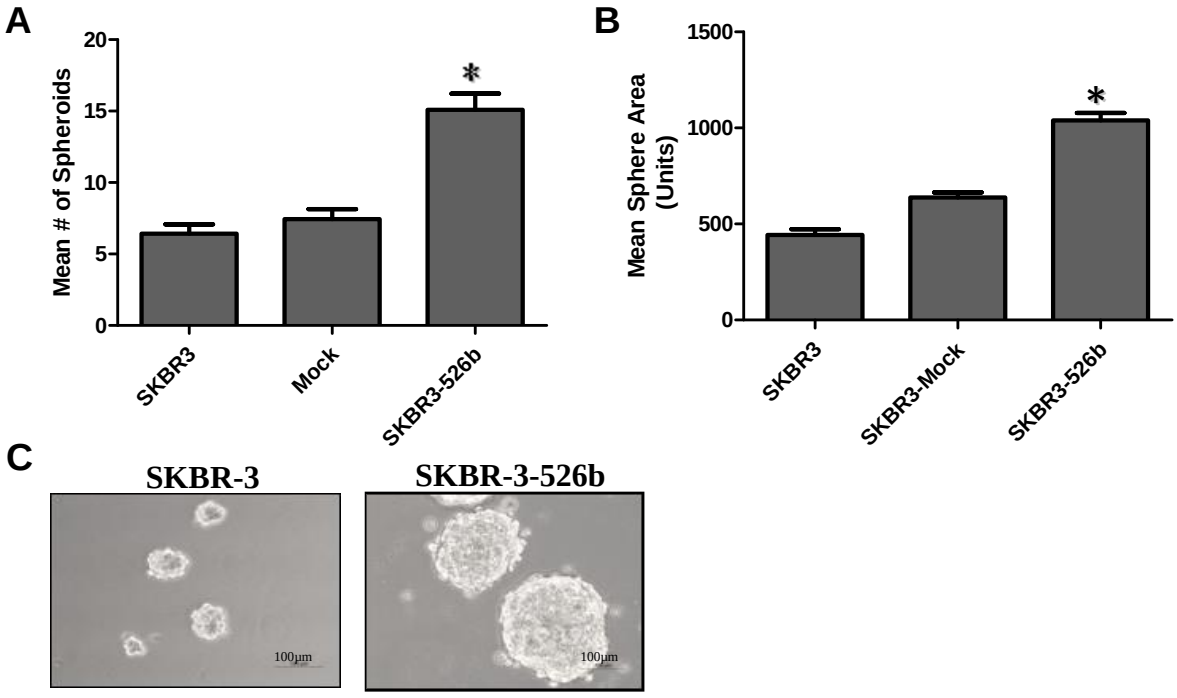


Figure 20. OverExpression of miR-526b Promotes the Induction of SLC Phenotype in the SKBR-3 Human Breast Cancer Cell Line.

SKBR-3, SKBR-3-Mock, and SKBR-3-526b cells were cultured as tumorspheres, and the number and size of spheroids formed for each cell line was counted manually using a light microscope and the ImageJ program. SKBR-3-526b cells formed a significantly greater number of spheroids, compared to the parental control cell line SKBR-3 (A). Additionally, SKBR-3-526b spheroids displayed a significantly greater average size when compared to SKBR-3 spheroids (B). The data are presented as a mean of triplicates ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.0001$) comparing miR-526b over-expressing cell line and its parental control cell line (n=3, One-Way ANOVA). Representative images comparing SKBR-3-526b and SKBR-3 spheroids support the quantitative data and demonstrate the change in size of spheroids with over-expression of miR-526b (C).

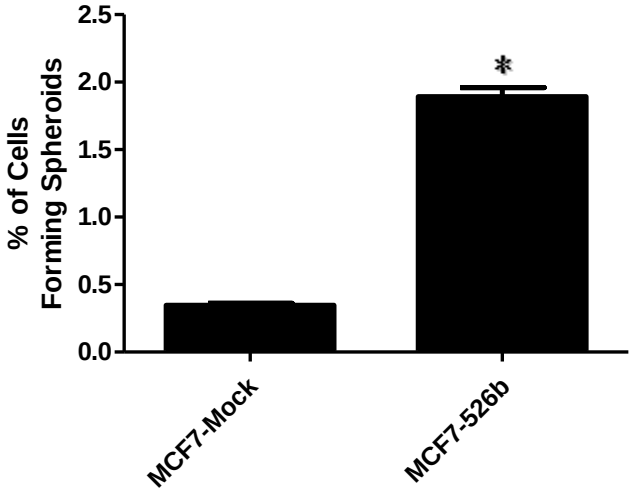


3.8.2 MCF7-526b Cells Form Spheroids at a Higher Efficiency Compared to Parental Control

In addition to assessing the ability of the MCF-7 cell lines to form spheroids (3.8.1), the efficiency at which these cells formed spheroids was also investigated. MCF-7 and MCF-7-526b cells were separately plated at a dilution of $1x10^4$ cells/mL and 96 000 cells per well. By using a shorter period of time for growth, the ability of cells to initiate spheroid formation could be evaluated. The number of spheroids formed for each cell line was converted into a percentage of the total number of cells plated at day one. It was observed that MCF-7-526b cells displayed a significant increase in spheroid forming efficiency after 4 days of incubation, compared to MCF-7 control cells **(Figure 21)**. This result supports the idea that over-expression of miR-526b induces SLC phenotype in MCF-7 human breast cancer cells, increasing the cells' efficiency to form spheroids.

Figure 21. Over-Expression of miR-526b Promotes Enhanced Efficiency of Spheroid Formation in the MCF-7 Human Breast Cancer Cell Line After 4 Days of Incubation.

MCF-7 and MCF-7-526b cells were separately plated in ultra-low attachment plates with 96 000 cells per well. Cells formed spheroids over 4 days, after which time the number of spheroids in each well was counted manually using a light microscope and the ImageJ program. The number of cells forming spheroids in each well was calculated as a percentage of the total number of cells plated on day one. MCF-7-526b cells displayed a significant increase in spheroid formation efficiency after 4 days of incubation, compared to parental control cells. The data are presented as a mean of triplicates ± SEM for three independent experiments. (*) Indicates a significant difference ($p<0.0001$) comparing miR-526b over-expressing cell line and its parental control cell line (n=3, One-Way ANOVA).



CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1 Summary of Novel Findings and Conclusions

Objective 1: To investigate the regulatory role of the EP4 receptor in miR-526b expression in vitro.

Stimulation of the EP4 receptor with exogenous PGE2 or the EP4 receptor agonist PGE1OH resulted in a significant increase in miR-526b in MCF-7 cells. Additionally, stimulation of EP4 with PGE2, or the EP4 agonists PGE1OH and L-902-688 stimulated pAKT protein levels. Conversely, inhibition of the EP4 receptor with an EP4 antagonist (ONO-AE3-208) or COX-2 inhibitor (NS-398) resulted in a significant reduction in miR-526b in MCF-7-COX-2 cells. These data indicate that COX-2 and the EP4 receptor play a positive regulatory role in miR-526b expression in human breast cancer cells. Thus COX-2 mediated miR-526b upregulation in breast cancer cells is partially due to EP4 activation by endogenous PGE2.

Objective 2: To investigate the role of the canonical cAMP-PKA and non-canonical PI3K-AKT signaling pathways of the EP4 receptor in mediating miR-526b up-regulation.

Stimulation of the cAMP-PKA (shared by EP2 and EP4) and the PI3K-AKT (unique to EP4) pathways with the EP4 agonist PGE1OH resulted in a significant increase in miR-526b expression, stimulated pAKT protein levels, and an increase in SLC phenotype in MCF-7 cells. Inhibition of PKA with H89 and PI3K with LY-204002 and Wortmannin resulted in significant decreases in miR-526b expression in MCF-7-COX-2 cells. These data indicate that both cAMP signaling (shared by EP2/EP4) and PI3K-AKT signaling (unique to EP4) pathways are involved in regulating miR-526b expression and possibly

SLC induction. If miR-526b is shown to be an SLC-linked marker, EP4 may present as an attractive therapeutic target in human breast cancer.

Objective 3: To investigate the functional roles of miR-526b in human breast cancer progression:

(a) In vivo: lung colony formation by cells xenografted by the intravenous route

Delivery of MCF-7 and SKBR-3 cells over-expressing miR-526b via intravenous tail vein injection in NOD/SCID/*GusB*-Null mice resulted in a significant increase in lung colony formation and proliferation, compared to empty vector controls. Therefore, our data suggest that miR-526b promotes tumorigenesis *in vivo*.

(b) In vitro: migration, invasion, proliferation, and stem-like cell (SLC) phenotype (tumorsphere) assays.

Over-expression of miR-526b in MCF-7 and SKBR-3 cell lines resulted in a significant increase in cellular proliferation, migration, and invasion; the latter two characteristics play an important role in cancer progression and metastasis. In addition, miR-526b over-expression in both cells lines was associated with significant increases in tumorsphere formation ability, an *in vitro* surrogate of SLC phenotype. MCF-7 and SKBR-3 cells over-expressing miR-526b formed tumorspheres of significantly greater number and size, and efficiency per number of cells plated. We conclude that miR-526b is a pro-oncogenic microRNA that promotes several aggressive phenotypes, including SLC induction, of COX-2 over-expressing human breast cancer. Thus, we support its investigation as a potential biomarker in breast cancer patient serum and tissue samples.

4.2 Contributions to Current Field of Research

The stimulatory role of COX-2 in human breast cancer is well established; its expression has been linked to disease progression, metastasis, and a reduction in overall survival rates (Costa *et al.*, 2002; Denkert *et al.,* 2003; Ristimäki *et al.*, 2002). Previous work in our laboratory and others has demonstrated that EP4 is up-regulated in COX-2 over-expressing cancer cells (Majumder *et al.,* 2012, Chell *et al.,* 2006). EP4 receptor antagonists have recently demonstrated great therapeutic promise in murine breast cancer models, reducing tumorigenesis, tumor-associated angiogenesis and lymphangiogenesis, spontaneous metastases to lungs and lymph nodes (Xin *et al.,* 2012), and maintaining normal host natural killer (NK) cell activity (Ma *et al.,* 2013). Furthermore, the EP4 receptor has recently been identified as a potential therapeutic target in breast cancer stem-like cells (SLCs) (Majumder *et al.,* 2012 and 2014; Kundu *et al.,* 2014). Additionally, our lab had previously shown that miR-526b is one of two miRs up-regulated in human breast cancer cells over-expressing both COX-2 and EP4 receptors (Majumder *et al.,* 2012). Therefore, establishing a link between the COX-2-induced oncogenic miR-526b and EP4 receptor activity may identify miR-526b as a potential biomarker of human breast cancer. As a first step, the purpose of this study was to examine the role of the prostaglandin E2 receptor EP4 and its signaling pathways, cAMP and PI3K-AKT, in regulating the expression of COX-2-induced miR-526b, and to investigate the role of this miRNA in breast cancer progression.

4.2.1 *Role of miR-526b in Migration, Invasion, Proliferation, and SLC Induction and Tumorigenicity*

In combination with traditional gene expression profiling, dysregulated miRNA expression patterns are emerging as biomarkers in human breast cancer diagnosis and prognosis. Previous research using the non-metastatic SUM149 breast cancer cell line revealed that over-expression of miR-10b resulted in significant increases in cellular migration and invasion, as well tumorigenicity *in vivo* (Ma *et al., 2007*). Additionally, miR-10b expression levels in primary breast carcinomas were found to correlate with clinical disease progression (Ma *et al.,* 2007). Similarly, our laboratory has begun to investigate whether up-regulation of the COX-2-induced miR-526b could also contribute to breast cancer progression. Combined gene expression and microRNA microarrays completed in our lab established that miR-526b is one of only two up-regulated miRs in MCF-7 cells over-expressing COX-2. Since expression of COX-2 has been associated with poor outcomes in breast cancer patients, we wanted to examine the functional roles of miR-526b in promoting aggressive phenotypes. We observed an increase in cellular migration, invasion, and proliferation in MCF-7 and SKBR-3 cells made to over-express miR-526b, supporting our hypothesis that this is a cancer-promoting (pro-oncogenic) miRNA. Our study is one of several to date supporting the role of miRNA dysregulation in breast cancer progression. The functional activity of miR-526b strongly mimics data on another well-established pro-oncogenic miRNA, miR-106b, in breast cancer. Pan *et al.* (2009) reported that over-expression of miR-106b using a lentiviral system in MDA-MB-231 cells (high COX-2) resulted in a significant increase in cellular migration and invasion. Moreover, miR-106b was found to play a role in mediating in TGF-β induced

epithelial to mesenchymal transition (EMT). Clinically, miR-106b was also elevated in non-metastatic breast cancer patients compared to metastatic patients, suggesting its potential as a biomarker of early stage breast cancer. Similarly, preliminary evidence in our lab revealed that miR-526b levels are also elevated in a sample of human breast tumors compared to healthy tissue. A larger sample size is currently being recruited in order to further support our preliminary data. We propose that miR-526b may have promise as a biomarker of COX-2 positive breast cancers.

Recently, certain miRNAs have been shown to negatively correlate with established CSC markers (CD44+/CD24-, $ALDH^{High}$), suggesting their use as clinical markers. Yu *et al.* (2007) reported that in primary breast tumors, down-regulation of the *let*-7 family of miRNAs could accurately distinguish CSC populations from non-CSC populations. To further support these findings, they over-expressed *let*-7 miRNAs in human breast cancer cell lines and observed a reduction in CSC population size after sorting cells using traditional CSC markers, as well as a reduced ability to form spheroids. Another study by Shimono *et al.* (2009) found that under-expression of a single miRNA, miR-200c, could accurately distinguish the CSC population in primary breast tumor samples. Moreover, human breast cancer cells made to over-express miR-200c could inhibit tumor formation from CSCs both *in vitro* and *in vivo.* Here we tested if miR-526b could promote SLC phenotype *in vitro*, and therefore have potential as a biomarker for CSC phenotype. Using an *in vitro* tumorsphere formation assay, we observed that miR-526b over-expressing MCF-7 and SKBR-3 cells had markedly increased ability to form spheroids that were much larger than parental controls. Both our study and Shimono *et al.*'s study demonstrate that dysregulation of a single miRNA can

have functional importance in promoting breast cancer progression, and can also be used as a biomarker to identify CSC and SLC populations in human breast cancer.

4.2.2 *Role of miR-526b in Promoting In Vivo Tumorigenicity*

miRNA de-regulation in primary tumors of the breast may suggest an important implication of such miRs in the later stages of metastatic disease, including tumor establishment at a secondary site. Intravenous delivery of cancer cell is a well-established method of studying the later stages of metastatic cascade such, as extravasation and colonization in secondary sites. Valastyan *et al.* (2009) showed that over-expression of a single miRNA, miR-31, in highly metastatic MDA-MB-231 cells greatly reduced tumor formation in the lungs. We used a similar intravenous strategy to deliver MCF-7 and SKBR-3 cells over-expressing miR-526b to *NOD/SCID/GusB*-Null mice and observed that over-expression of miR-526b resulted in increased ability to form proliferating colonies in the lung. In combination with the supportive *in vitro* data, we conclude that miR-526b is pro-oncogenic in breast cancer cells, and it is possible that this happens in the context of COX-2 expression. Interestingly, when the other COX-2 up-regulated miRNA observed in our lab, miR-655, was introduced into MCF-7 cells or SKBR-3 cells, both cell lines exhibited a marked up-regulation of COX-2 (Majumder *et al.*, 2012). We are currently testing whether similar up-regulation occurs in miR-526 over-expressing cells. If so, this may reveal a positive feedback loop for SLC sustenance.

4.2.3 *Role of EP4 Receptor in miR-526b Expression*

Up-regulation of EP4 activity has been implicated in human breast cancer progression, owing to the activation of its two signaling pathways, cAMP and PI3K-AKT. Ma *et al*. 2013 observed an up-regulation of EP4 receptors in highly metastatic

66.1 and 410.4 cells derived from a spontaneously occurring murine mammary adenocarcinoma, and subsequent treatment with EP4 antagonists reduced *in vivo* tumor formation owing to a decrease in cAMP activation. Previous studies in our lab showed comparable results, including up-regulation of EP4 receptors in COX-2 over-expressing MCF-7 cells (Majumder *et al.,* 2012). Additionally, our lab previously demonstrated that oral administration of EP4 receptor antagonists could reduce tumor growth and metastasis to lymph nodes and lungs in syngeneic C3L5 tumor-bearing mice with similar efficacy to traditional COX-2 inhibitors. The residual tumors also exhibited reduced AKT phosphorylation, indicating EP4 inactivation (Xin *et al.,* 2012). Hence, in the present study we explored the possible regulatory roles of EP4 receptor activation, via both cAMP and PI3K-AKT signaling, on the COX-2-induced miR-526b expression and induction of the SLC phenotype *in vitro*. Our data revealed that MCF-7 cells treated with a selective EP4 receptor agonist resulted in up-regulation of miR-526b expression, while MCF-7-COX-2 cells treated with an EP4 antagonist, PKA inhibitor, or PI3K inhibitors resulted in down-regulation of this miRNA. Thus, for the first time we have shown that EP4 receptor activity, via both cAMP and PI3K-AKT signaling, plays a regulatory role in mediating the expression of the COX-2 induced miR-526b. However, the mechanism by which EP4 and its downstream signaling pathways upregulate miR-526b expression remains to be investigated. Since we observed in this study that EP4 agonists did not upregulate miR-526b expression to levels as high as the endogenous ligand PGE2, it is likely that miR-526b expression is regulated through a combination of EP2 and EP4 receptor activity.

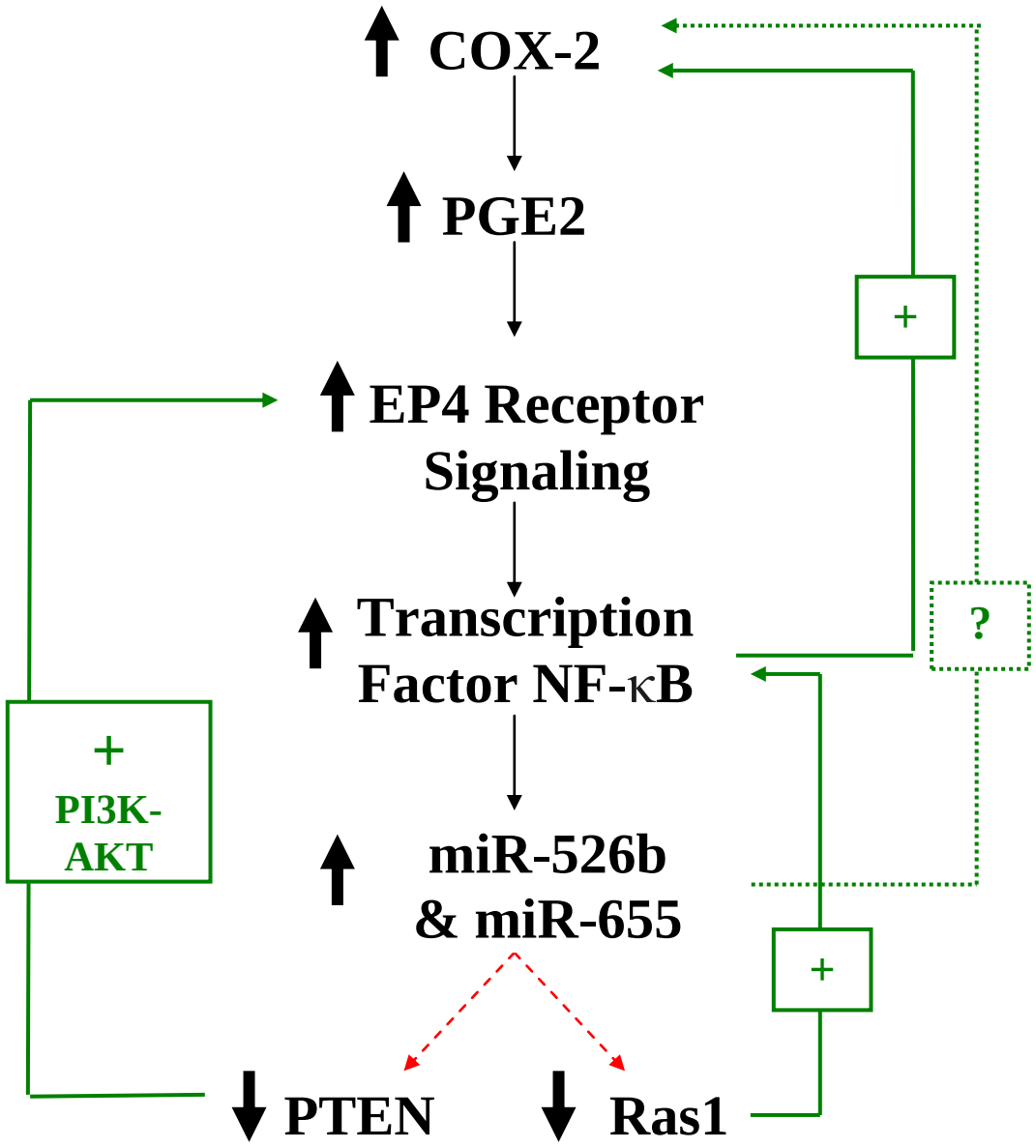
One factor that may be involved in miR-526b expression in response to EP4 receptor activation is activation of the nuclear factor-kappa B (NF-κB) transcription factor. NF-κB plays an important role in up-regulation of COX-2 in a variety of human cancers (Dolcet *et al.*, 2005), and has demonstrated regulatory activity on miRNA gene expression. Similar to our study, Shin *et al.* (2010) observed up-regulation of two miRNAs (miR-16 and miR-21) in gastric cancer. Further investigation revealed expression of both miR genes was regulated by NF-κB activity, and that this activity could be controlled by EP4 receptor antagonism. Therefore, it is possible that in our study, COX-2 and EP4 may be regulating miR-526b expression via NF-κB activity. Genome data mining performed *in silico* in our lab (miRanda-mirSVR software) revealed that miR-526b targets the NF-κB negative regulator, Ras-like 1 (*Ras1*), a gene that down-regulates NF-κB. A down-regulation of *Ras1* could thus lead to up-regulation of NF-κB, and subsequent up-regulation of COX-2 and EP4. Additionally, miR-526b and miR-655 both target *PTEN*, suggesting a possible mechanism of *PTEN* down-regulation leading to up-regulation of PI3K-AKT signaling via the EP4 receptor **(Figure 22)**.

In a similar study to ours, Rao *et al.* (2012) showed that the miR-17~19 cluster of miRNAs is over-expressed in mantle cell lymphoma, and that these miRNAs exert their action by directly targeting the protein phosphatase PHLPP2, an important negative regulator of the PI3K/AKT pathway. Interestingly, the miR-17~19 cluster were also directly targeting *PTEN*, one of the most commonly mutated tumor suppressor elements of the PI3K-AKT pathway in human breast cancer (Rao *et al.*, 2012). Interestingly, a study by Chandramouli *et al.* (2012) reported that miR-101 post-transcriptionally regulates the expression of the EP4 receptor in colon cancer, and it is hypothesized that

feedback mechanisms occur to balance levels of EP4 and miRNA. Therefore in our present study, it is also possible that miR-526b up-regulation may be affecting EP4 receptor expression positively, and a positive feedback loop may be implicated in maintenance of miR-526b up-regulation and subsequent continued activation of PI3K-AKT signaling.

Figure 22. Possible mechanism of COX-2 and miR-526b signaling in human breast cancer cell lines.

COX-2 up-regulation in breast cancer results in up-regulated prostanoid production, including PGE2. Increased PGE2 levels lead to increased binding of this ligand to EP receptors, including EP4. Activation of EP4 and its signaling pathways, cAMP and PI3K-AKT, could result in recruitment of the transcription factor NF-κB, leading to increased expression of miR-526b and COX-2. Through a series of unknown mechanisms, it is possible that increased levels of miR-526b could then inhibit mRNA targets involved in repression of COX-2 via NF-κB activity (E.g. *Ras1*), in addition to mRNAs repressing EP4 signaling pathways (E.g. *PTEN* in PI3K-AKT signaling). Together, these interactions could result in a positive feedback system regulating COX-2, miR-526b, and miR-655 expression. (Adapted from Dunn *et al.*, 2012; Kaltschmidt *et al*., 2002).


COX-2
PGE2
EP4 Receptor Signaling
Transcription Factor NF-κB
miR-526b & miR-655
PTEN
Ras1
+
PI3K-AKT
+
+
?

4.2.4 *Role of EP4-mediated PI3K-AKT Signaling in Stem-Like Cell Phenotype*

Recently, treating human breast cancer cells with EP4 receptor antagonists *in vitro* has been shown to reduce breast CSCs (Kundu *et al.,* 2014). Furthermore, treating COX-2 expressing C3L5 mammary tumor cells with an EP4 antagonist *in vitro* revealed profound SLC-reductive effects (Majumder *et al*., 2014). Similarly, we observed a reduction of spheroid formation with EP4 antagonism in MCF-7-COX-2 and SKBR3-COX-2 cells. Furthermore, we show here for the first time that selectively blocking the PI3K-AKT pathway, unique to the EP4 receptor, could reduce SLC phenotype *in vitro.* In support of this observation, our lab has also shown that therapy with an EP4 antagonist reduced the incidence of SLC-associated markers, as well as decreased pAKT levels in residual tumors in a COX-2 expressing C3L5 tumor model (Majumder *et al.*, 2014) Thus, EP4 antagonists may hold promise in SLC reductive therapeutics in human breast cancer, in which miR-526b may serve as an SLC-linked marker.

4.3 Potential Limitations of Study

Considering the *in vitro* experiments completed in this study, one limitation is that only two cell lines, MCF-7 and SKBR-3, were used for all experiments. Both cells lines are considered weakly metastatic, and so it would be important to examine miR-526b expression levels via real-time RT-PCR in a variety of human breast cancer cell lines with intrinsically different levels of COX-2 and metastatic ability. For instance, it would be important to investigate the presence of high miR-526b levels in high COX-2-expressing breast cancer cells, such as MDA-MB-231.

Another limitation to consider is that only one *in vitro* assay was used to assess induction of the SLC phenotype in human breast cancer cell lines. Therefore, we make

the assumption that only the cells forming tumorspheres possess stem-like capabilities, but cannot definitively identify cancer stem cells *in vitro*. In order to comprehensively assess induction of the SLC phenotype, it would be necessary to use the identified human breast cancer stem cell markers in our *in vitro* tumorspheres. For example, we could fix and co-stain tumorspheres for the established breast cancer stem cell markers CD44+/CD24-, and $ALDH^{High}$ expression, along with embryonic stem cell markers such as Sox-2, Oct-4, and Nanog in order to validate that cells forming spheroids are indeed cancer stem cells.

The *in vivo* xenograft mouse model used in this study was *NOD/SCID/GusB*-Null, and cells were delivered via an intravenous tail vein injection. However, this type of injection only allows us to assess the ability of cells to form colonies in the lungs, and does not fully assess their metastatic ability or stem-like functions. In order to assess the ability of cells to intravasate into surrounding blood vessels and travel to the lung, we could use an orthotopic injection of cells into the mammary fat pad of the mouse and then assess lung colony formation, as well as metastases to other organs. Additionally, serial transfer of limiting cell numbers in orthotopic xenografts could be used to evaluate SLC activity.

4.4 Future Directions

Our lab is the first to discover up-regulation of miR-526b (and miR-655) in COX-2 over-expressing human breast cancer cells. However, understanding the target genes of miR-526b and miR-655, and their implications in cancer progression, could provide opportunities to create more comprehensive and targeted treatment strategies. For instance, these two miRNAs are targeting a total of 13 genes, including one common

gene target, cytoplasmic polyadenylation binding element (CPEB)-2. Very little is known about this particular gene product. Interestingly, down-regulation of another CPEB family member, the CPEB-4 protein, via miR-550 up-regulation has been observed in hepatocellular carcinoma (Tian *et al.*, 2012). Therefore, further investigation is needed to establish the role of miR-526b on its predicted gene targets, and potential involvement of these genes in breast cancer progression.

4.5 Conclusion

Our results demonstrate for the first time that miR-526b contributes to breast cancer progression, including stimulation of SLC phenotype, and that miR-526b expression is up-regulated in part by the EP4 receptor. *In vitro* experiments showing that miR-526b expression enhances cellular migration, invasion, and proliferation are supported by *in vivo* experiments revealing enhanced tumorigenicity in miR-526b over-expressing breast cancer cells. Additionally, our results support the role of EP4 and its pathways, cAMP and PI3K-AKT, in regulating miR-526b expression and induction of SLC phenotype in human breast cancer cell lines. Thus, we show for the first time that miR-526b may have potential as a biomarker of human breast cancer progression, and support continued pre-clinical investigation of EP4 as a therapeutic target for SLCs in human breast cancer.

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APPENDIX

Figure A1. MicroRNA Overexpression Plasmid. A vector map of the MicroRNA pCMV6-MiR Expression Plasmid (OriGene). Expression of miR-526b, Neomycin resistance, and a GFP marker is driven by the CMV promoter and with human growth factor 1 poly(A) tailing signal.

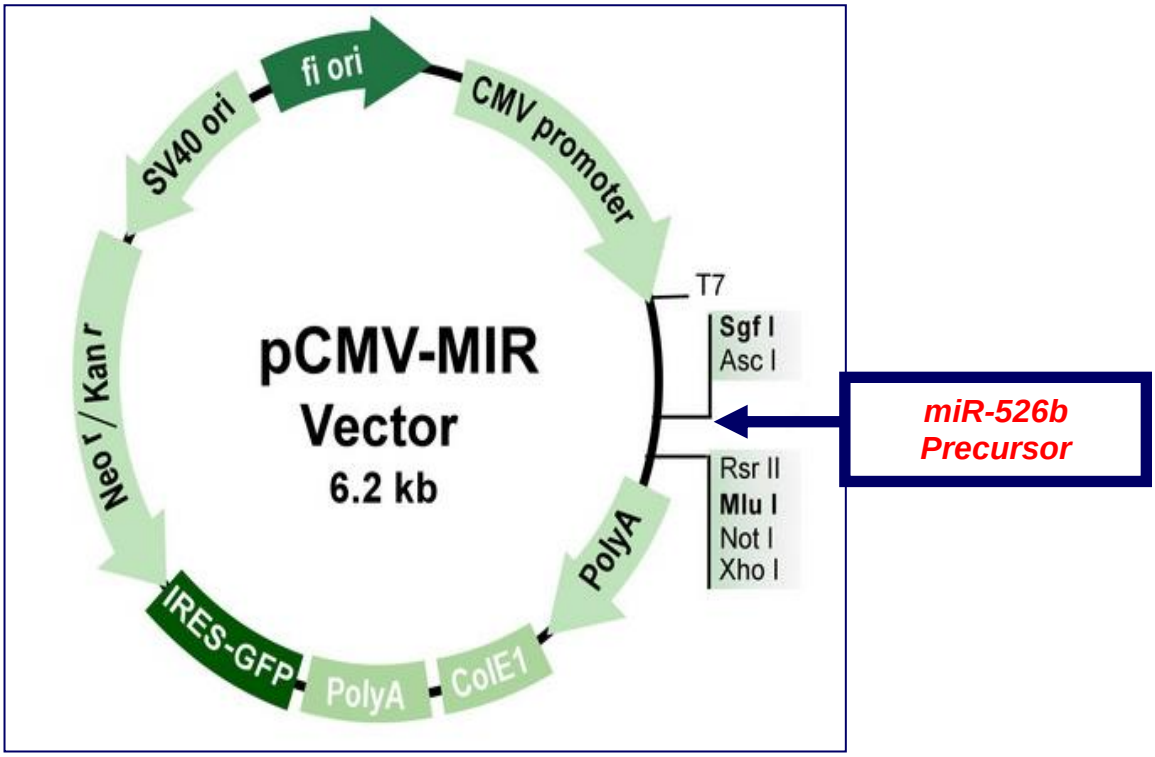

Table 1. Summary of Drugs Used

Drug	Target	Concentration	Reference
NS398	COX-2	10 µM	Majumder *et al*, 2012
ONO-AE3-208	EP4R	10 µM	Xin *et al*, 2012
PGE2	EPR1-4	10 µM	Ma *et al*, 2013
PGE1OH	EP3R, EP4R	10 µM, 20 µM	Ma *et al*, 2013
L-902-688	EP4R	10 µM	Young *et al*, 2006
LY-204002	PI3K	10 µM	Majumder *et al*, 2012, 2014
Wortmannin	PI3K	10 µM	Majumder *et al*, 2012, 2014
H89	PKA	30 µM	Ombra *et al*, *2013*

Approval- Tumor Protocol-2012-2016

AUP Number: 2007-057-04
P1 Name: Lala, Peeyush K
AUP Title: The Role Of Cyclo-oxygenase 2 In Breast Cancer Progression

Approval Date: 04/20/2012

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "The Role Of Cyclo-oxygenase **2 In Breast Cancer Progression**
"has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2007-057-04::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

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on behalf of the Animal Use Subcommittee
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