COX-2 Elevates Oncogenic miR-526b in Breast Cancer by EP4 Activation

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Running Title: miR-526b promotes human breast cancer progression

Key words: miR-526b, Breast Cancer, Cyclo-oxygenase-2, EP4, PI3K-AKT, and Stem-Like Cell.

Grant Support

This work was supported by a grant of the Ontario Institute for Cancer Research (OICR) to PKL, a Canadian Breast Cancer Foundation (CBCF) – Ontario Chapter Graduate Fellowship to EL; and a Translational Breast Cancer Research Unit Postdoctoral Fellowship to MM, funded by the Breast Cancer Society of Canada; MM and EL are honorary fellows of the Canadian Institutes of Health Research Strategic Training Program in Cancer Research and Technology Transfer (CIHR-CaRTT).

Disclosure/Duality of Interest

All authors declared no potential conflicts of interest.
Abstract

MicroRNAs (miRs) are small regulatory molecules emerging as potential biomarkers in cancer. Previously, it was shown that COX-2 expression promotes breast cancer progression via multiple mechanisms including induction of stem-like cells (SLC), owing to activation of the prostaglandin E2 receptor EP4 (PTGER4). COX-2 over-expression also up-regulated microRNA-526b (miR-526b), in association with aggressive phenotype. Here, the functional roles of miR-526b in breast cancer and the mechanistic role of EP4 signaling in miR-526b up-regulation were examined. A positive correlation was noted between miR-526b and COX-2 mRNA expression in COX-2 disparate breast cancer cell lines. Stable over-expression of miR-526b in poorly metastatic MCF7 and SKBR3 cell lines resulted in increased cellular migration, invasion, EMT phenotype and enhanced tumorsphere formation in vitro and lung colony formation in vivo in immunodeficient mice. Conversely, knockdown of miR-526b in aggressive MCF7-COX-2 and SKBR3-COX-2 cells reduced oncogenic functions and reversed the EMT phenotype, in vitro. Furthermore, it was determined that miR-526b expression is dependent on EP4 receptor activity and downstream PI3K/AKT and cyclic AMP (cAMP) signaling pathways. PI3K/AKT inhibitors blocked EP4 agonist-mediated miR-526b up-regulation and tumorsphere formation in MCF7 and SKBR3 cells. NF-κB inhibitor abrogates EP agonist stimulated miRNA expression in MCF7 and T47D cells indicating that NF-κB pathway is also involved in miR-526b regulation. Additionally inhibition of COX-2, EP4, PI3K and PKA in COX-2 overexpressing cells down-regulated miR-526b and its functions in vitro. Finally, miR-526b expression was significantly higher in cancerous than in non-cancerous breast tissues and associated with reduced patient survival. In conclusion, miR-526b promotes breast cancer progression, SLC-phenotype through EP4-mediated signaling, and correlates with breast cancer.
patient survival.

**Implications:** This study presents novel findings that miRNA 526b is a COX-2 up-regulated, oncogenic miRNA promoting stem-like cells, the expression of which follows EP4 receptor-mediated signaling and is a promising biomarker for monitoring and personalizing breast cancer therapy.

**Introduction**

Cyclo-oxygenase (COX)-2 is an inducible inflammatory enzyme found to be up-regulated in approximately 40% of primary breast cancer cases (1, 2) at both the pre-invasive (2, 3) and invasive (4) stages of the disease. Moreover, COX-2 expression has been linked to cancer progression and metastasis (5), and reduction of overall and disease-free survival (1, 2). COX-2 catalyzes the rate-limiting step of the prostanoid pathway, ultimately leading to the production of prostaglandin 2 (PGE2), the endogenous ligand of the transmembrane G-protein coupled prostaglandin E (EP) receptors, EP1-4 (6). Each EP has differential signaling abilities: EP1 is coupled with Gq, stimulating (Ca++ i; EP2 and EP4 are coupled with Gs, stimulating adenylate cyclase (AC)/PKA pathway, while most EP3 isoforms are coupled with Gi thus inhibiting AC (7). Unlike EP2, EP4 can additionally stimulate phosphatidylinositol 3-kinase (PI3K)/Akt mediated cell survival pathway as well as the pro-migratory ERK pathway (8). EP2 can only occasionally stimulate PI3K/Akt by transactivation of EGFR (9). This differential signaling mechanism, unique to the EP4 receptor (8, 10), protecting cells from apoptosis makes it an attractive target to replace COX-2 inhibitors. Indeed thrombo-embolic side effects of COX-2 inhibitors (11, 12), resulting from inhibition of cardio-protective prostanoids such as PGI2 (13) may possibly be avoided by targeting EP4. We and others have shown that EP4 activity
contributes to multiple mechanisms in breast cancer progression including: inactivation of host anti-tumor immune cells (14, 15); stimulation of tumor cell migration (16); invasiveness (16); angiogenesis (16, 17); and lymphangiogenesis due to EP4 mediated upregulation of lymphangiogenic factors VEGF-C (17, 18) or VEGF-D (19); induction of stem-like cell (SLC) phenotype in vitro (19, 20) and in vivo (19). The role of EP4 in tumor progression has also been reported in colonic tumors (21). While EP4 activity on breast cancer cells promoted SLC phenotype (19), VEGF-C and -D production by cancer cells as well as tumor-infiltrating macrophages in situ was also attributed to EP4, explaining strong anti-tumor, anti-metastatic and SLC-reductive effects of EP4 antagonists in a murine breast cancer model (19). Thus contribution of EP4 in both tumor and host cell mediated events make it a promising therapeutic target in breast cancer.

MicroRNAs (miRNAs; miRs) are short, non-coding regulatory RNA molecules that down-regulate gene expression at the post-transcriptional level, and are emerging as potential biomarkers of breast cancer. Many of them are implicated in carcinogenesis because miRNA genes are frequently located in fragile sites of the chromosome, having increased susceptibility to mutation or damage (22). Since miRNAs alter gene expression at the post-transcriptional level, increases or decreases in expression of a single miRNA or miRNA clusters may contribute to oncogenic behavior or signatures in cancer (23). Differential miRNA expression profiles were shown to be reliable for classifying both the developmental lineage and the differentiation stage of solid tumors (24). In breast cancer, miRNA expression profiles were reported to be distinct for basal and luminal sub-types, ER and HER2 status, and even predict tumor responses to traditional chemotherapies (24, 25). Altered miRNA expression can influence several steps of the metastatic cascade, including cell adhesion, motility, invasiveness, and resistance to apoptosis.
(26-28). Certain miRNAs were shown to play an important regulatory role on EMT phenotype in human breast (29, 30) and colon cancer (31). Given their stability in blood and their relative ease of extraction from tissues, miRNAs show excellent promise as biomarkers of cancer (32).

Stem-like cells (SLCs) comprise a small subset of cells within the tumor, believed to be responsible for tumor induction, perpetuation and recurrence after traditional therapies (33). This population is considered as distinct from other malignant cells within the tumor in having 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 (34). Certain miRNAs such as the Let7 family, and miR-200C were shown to be inversely associated with maintenance of SLCs in human breast cancer (35, 36), demonstrating their potential as biomarkers of the SLC population in this disease.

We identified miR-526b to be significantly up regulated in COX-2 high human breast cancer cell lines, both under natural and ectopically over-expressing conditions. The gene coding for miR-526b is located on chromosome 19. Although this miRNA is listed in breast cancer database, its role in human breast cancer has not previously been reported. The present study, employing in vitro and in vivo assays with miRNA-manipulated breast cancer cell lines examined the functional roles of miR-526b in breast cancer progression, including SLC stimulation and the role of EP4 and NF-κB signaling pathways in its regulation. We also measured its expression in human breast cancer tissues. We demonstrate for the first time that miR-526b is a COX-2-induced oncogenic miRNA linked with SLC-phenotype, up-regulated by EP4-mediated signaling pathways PI3K-AKT and PKA; that its expression is elevated in primary breast cancer tissues, high expression being associated with reduced survival.
Materials and Methods

Cell lines

All human breast cancer cell lines (MCF7, SKBR3, MDA-MB-231 and T47D) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). MCF10A (COX-2, ER, HER-2 negative) mammary epithelial cell line is a kind gift of Dr. Moshmi Bhattacharya, University of Western Ontario, purchased from ATCC and maintained in her lab. The MCF7 cell line expresses low levels of COX-2, is estrogen receptor (ER) positive and HER-2 negative, and is non-metastatic in immune-deficient mice. Both SKBR3 (COX-2 and ER negative, HER-2 positive and weakly metastatic) and MCF7 cells were transfected with 2 µg of either pCMV-IRES2-EGFP Mock vector (control) or pCMV-IRES2-EGFP-COX-2 expression plasmids (kind gift of Dr. Michael Archer, University of Toronto). Stable COX-2 over-expression was confirmed with qPCR, sequencing and Western blots. These cell lines were respectively named as MCF7-COX-2 and SKBR3-COX-2. MCF7 and MCF7-COX-2 cells were grown in Eagle’s Minimum Essential Medium (EMEM), supplemented, 0.4 µl/mL human insulin (ATCC). SKBR3 and SKBR3-COX-2 were grown in McCoy’s 5A Modified Medium with L-glutamine (GIBCO, ON). Other human breast cancer cell lines, MDA-MB-231 (high COX-2, ER and HER-2 negative) and T47D (low COX-2, ER positive and HER-2 negative) were grown in RPMI-1640 minimal essential medium (GIBCO, ON). All media were supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, ON) and maintained in a humidified incubator with 5% CO2 at 37°C. Additionally MCF7-COX-2 and SKBR3-COX-2 and their respective mock cell lines maintained with Geneticin® (GIBCO, ON) at 500 µg/mL.
**MiRNA micro array**

We conducted miRNA micro arrays (quadruplicate measurements) comparing miRNA expression changes in MCF7-COX-2 and Mock-transfected control cells, using Affymatrix Genechip miRNAs Array as per the manufacturer’s protocol. ANOVA with a nominal alpha value set to 0.05 was then used to identify significant changes, followed by Benjamini-Hochberg multiple testing correction in order to reduce the false positive rate. These results were then separated into significant increases or decreases, and used in a cross platform analysis.

**Drugs and reagents**

NS-398 (COX-2 inhibitor) was purchased from Cayman Chemical (Michigan, USA). ONO-AE3-208 (selective EP4 antagonist) was a gift of ONO Pharmaceuticals, Osaka, Japan. PGE2 (EP1-4 ligand) and PGE1OH (EP4 agonist) were purchased from Cayman Chemical. H89 (PKA inhibitor), Wortmannin (WM), an irreversible PI3K inhibitor, and LY-204002 (LY), a reversible PI3K inhibitor, were all purchased from Sigma-Aldrich, Canada. NF-κB inhibitor, Bay 11-7082 (Sigma-Aldrich, Cat # B5556) is a kind gift of Dr. Xiufen Zheng (Department of Pathology and Laboratory Medicine, University of Western Ontario). For all treatments *in vitro or in vivo*, respective vehicles served as the control.

**Real-time PCR**

Total RNAs were extracted using miRNeasy Mini Kit (Qiagen, ON) and reverse transcribed using the TaqMan microRNA and total RNA cDNA Reverse Transcription Kit (Applied Biosystems, USA). The TaqMan MiRNA Assay or Gene Expression Kit was used for quantitative PCR. C_t values were used for quantification of transcripts. MiRNA expression was
normalized to the values of RNU44 or RNU48, considered as internal control miRNAs. Total RNA expression for COX-2, EP4, E-Cadherin, Vimentin, SNAIL and TWIST were normalized to the values of GAPDH and RPL5 control genes expression.

**Western blot**

Cells were treated with M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) supplemented with HALT Protease Inhibitor Cocktail (Thermo Scientific) and Phosphatase Inhibitor Cocktail (Thermo Scientific) to extract protein. Twenty micrograms of total protein were electrophoresed per well on a SDS-polyacrylamide gel and transferred onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA, USA). Membranes were then incubated with the following primary antibodies: E-Cadherin (Cell Signaling, #3195S); Vimentin (Millipore, #MAB3400); TWIST (Santa Cruz Biotechnology Inc. Texas, USA, #sc15393), SNAIL (C15D3) from Cell Signaling, MA, USA (#3879) and β-actin (Santa Cruz, #sc47778) and probed with a mixture of IRDye polyclonal secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA). Images were read with an Odyssey infrared imaging system (LI-COR Biosciences).

**Stable miRNA knock-in**

Cells were transfected with 2 µg of either pCMV-MIR Mock vector (control) or pCMV-MIR miR-526b expression plasmid, also containing a Neomycin selection marker and a Green Fluorescent Protein (GFP) marker (OriGene, MD) using the Amaxa Cell Line Nucleofector Kit V (Lonza, MO) and the E-009 or P-020 program for MCF7 cells or SKBR3 cells respectively, according to the manufacturer’s protocol. Cells were treated with the antibiotic-resistance
selection agent Geneticin® at 500 μg/mL. MCF7 and SKBR3 cell lines stably transfected with the pCMV-MIR mock (empty) vector are referred to as MCF7-Mock and SKBR3-Mock respectively, and cell lines stably transfected with the pCMV-MIR miR-526b expression plasmid are referred to as MCF7-526b and SKBR3-526b.

**Transient miRNA knock-down**

We used morpholino oligonucleotides, which provide highly specific knockdown of miRNAs (37) for the target miR-526b. Morpholino oligos specific to miR-526b (MO$^{526b}$) purchased from Gene Tools LLC, at two different concentrations (10 μM and 20 μM), and respective control MO was used to transfect both MCF7-COX-2 and SKBR3-COX-2 cell lines as per the manufacturer’s protocol (Lonza, MO). Functional assays as described later (migration, invasion and spheroid formation) were performed within 48h of transient transfection using control and MO$^{526b}$ (20 μM). For spheroid formation, data were collected after seven days.

**Transwell migration and invasion assays**

Cells (2x10$^5$ cells/mL) in 300 μl of either EMEM or McCoy’s 5A media were 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). For invasion assays, cell inserts were coated with Matrigel (1:100 in basal media; BD Biosciences, CA). The lower chamber contained 700 μl of either serum free media or 2% FBS supplemented media as positive control. Plates were incubated for 24 h (migration assay) or 48 h (invasion assay) at 37°C and 5% CO$_2$, as previously established (16, 38). Cells were subsequently fixed with pure methanol, stained with eosin and thiazine, respectively, and washed with ddH$_2$O. The number of migrant/invaded 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 or invading cells for each condition using the ImageJ program.

**Tumorsphere formation *in vitro***

For testing the ability of single cells to form spheroids or tumorspheres (an *in vitro* surrogate of SLC), cells (1x10⁴ cells/mL) were passed through 8 µm filter (Falcon, BD) and a syringe fitted with a 27-gauge needle, and plated in 6-well ultra-low attachment plates (Corning, MA), as previously described (19, 39). All tumorspheres 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 (19, 39). Tumorspheres were harvested followed by miRNA extraction; quantification and real-time qPCR to assess miR-526b expression for treated cell lines. Tumorspheres were imaged using a light microscope and the number and perimeter of spheroids for each condition was calculated using the ImageJ program.

**Lung colony assay**

Seven-weeks-old female NOD/SCID/GUSB (Glucorunidase-beta)-null mice (Robarts Research Inst., London, ON) were maintained on standard mouse chow and tap water on a 12 h light/dark cycle, and treated in accordance with the guidelines set by the Canadian Council on Animal Care. Animals were given a tail vein injection of an inoculum dose of 5x10⁵ cells, and sacrificed after 4 weeks to assess micro-metastases to the lung. Lungs were harvested after inflation with PBS and flash frozen, with OCT compound. At least 3 semi-serial 10 µm thick sections in the
mid-coronal plane were obtained for each animal. 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 a 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 cells) was calculated for each condition. Negative controls were provided by an equivalent concentration of mouse Ig iso-type replacing the primary antibody to exclude nonspecific staining. Although NOD/SCID/GUSB hosts were chosen initially to identify tumor cells in the lungs by staining for the GUSB marker, we adopted HLA staining preferred to GUSB staining since our preliminary studies revealed that some human cancer cells lost GUSB staining as lung colonies grew bigger.

**Human tissue samples**

To examine the clinical relevance of miR-526b expression in breast cancer, we obtained frozen female human breast tumor (n=105) and control (n=20) tissues (adjacent non-tumor tissue from unrelated patients) from the Ontario Tumor Bank, maintained by the Institute for Cancer Research (OICR), following approval by the Ethic Review Board of the bank. Demographic, tobacco, alcohol habit and ER, PR and HER-2 status information of patient and control populations are summarized in Table 1. While a minority (25-27%) in both patient and control groups had occasional drinking habits, only a few (<3%) had regular alcohol-drinking habit. Less than 5% of cancer and control population had tobacco inhalation habit. Majority of the cancer patients (>80%, data not provided) had a history of some unspecified cancer in the family. Of the tumor tissues, 76% were ER positive, 62.9% PR positive, 20% HER2 positive, and 9.5% triple (ER/PR/HER2) negative.
Total RNA and miRNA were extracted using Qiagen RNA and miRNA extraction kits followed by cDNA synthesis and Taqman miR526b, COX-2 and EP4 gene expression analysis as described before. Negative ΔCt values are indicative of higher miRNA expression in tissue samples.

**Survival analyses**

Coded patient survival data was extracted from the TCGA clinical information file. Patient survival was calculated as time in months elapsed from date of diagnosis until date of last contact. Kaplan-Meier curves for overall survival associated with miR-526b expression were conducted in 639 primary breast carcinomas. A cut off p value (p <0.05) was determined using log rank test.

**Statistical analysis**

Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software, CA, USA). All parametric data were analyzed with one-way ANOVA followed by Tukey-Kramer or Dunnett post-hoc comparisons. Lung colony numbers were analyzed both by parametric and non-parametric (Wilcoxon rank sum test) methods, giving same results. Student’s t-test was used when comparing two datasets and Pearson’s coefficient was employed to assess statistical correlations. Statistically relevant differences between means were accepted at p < 0.05.
Results

Identification of up-regulated mir-526b in MCF7-COX-2 cells

Using miRNA micro array to compare MCF7-COX-2 and Mock-transfected cells, we identified numerous miRNAs whose expressions were up-or down regulated. However, this increased expression was statistically significant only for two miRNAs miR-526b and miR-655 (Supp. Table 1). In this study we investigated the role of miR-526b, up-regulated in concert with COX-2 expression, and whose role in human breast cancer has not yet been investigated.

Positive association of miR-526b with COX-2 expression in multiple COX-2 disparate human breast cancer cell lines

We tested multiple COX-2 disparate breast cancer cell lines varying in genetic background (40) to explore whether miR-526b expression levels were broadly correlated with COX-2 expression. Data presented in Supp. Figures 1A and 1B reveal that this was indeed the case, suggesting that, amongst many genes, COX-2 played a dominant role in miR-526b up-regulation. That COX-2 activity was instrumental in this up-regulation is shown later.

Validation of stable miR-526b over-expression in MCF7 and SKBR3 cells

Stable over-expression of miR-526b in MCF7 (non-metastatic, low COX-2), and SKBR3 (low metastatic, COX-2 negative), both low miR-526b expressing human breast cancer cell lines was achieved using nucelotransfection as per the manufacturer’s protocol. They were respectively named MCF7-526b and SKBR3-526b, with their empty vector controls named as MCF7-Mock and SKBR3-Mock, respectively. Over-expression of miR-526b was confirmed using real-time
RT-PCR, RNU44 and RNU48 miRNAs served as control miRNA. MiR-526b was significantly over-expressed in both cell lines, compared to empty vector controls (Supp. Figure 1C).

Validation of transient miR-526b knockdown in MCF7-COX-2 and SKBR3-COX-2 cells

Morpholino-mediated knock-down of miR-526b in high COX-2 expressing MCF7-COX-2 and SKBR3-COX-2 cell lines was confirmed using Taqman real-time RT-PCR, RNU44 and RNU48 miRNAs serving as control miRNAs. MiR-526b was significantly down regulated (more than 80%) in both cell lines at two different morpholino concentrations (Supp. Figure 1D), compared to mock transfected cells. The resulting cell lines (at 20 µM concentrations) were named MCF7-COX-2-526b KD, SKBR3-COX-2-526b KD, with their empty vector controls being named MCF7-COX-2-Mock and SKBR3-COX-2-Mock, respectively and used for functional assays.

MiR-526b promotes cellular migration, invasion, and EMT in MCF7 and SKBR3 cells

Migration/Invasion: To investigate an association between miR-526b expression and two key steps of metastasis, transwell migration and invasion assays were performed with MCF7-526b, SKBR3-526b, and their respective Mock cells. Over expression of miR-526b in both cell lines resulted in a significant increase in cellular migration (Figure 1A & C) and invasion (Figure 1B & D), images of migrant MCF7 and SKBR3 cells are presented in Supp. Fig 2. Conversely knocking-down miR-526b in both MCF7-COX-2 and SKBR3-COX-2 cells resulted in significantly reduced abilities to migrate (Figure 1E & G) and invade (Figure 1F & H).

EMT/MET: Cellular morphology changes after miR-526b over expression (Supp. Figure 3), combined with increased migration may suggest epithelial to mesenchymal (EMT) phenotype. Although miR-526b has no binding site on E-Cadherin, it binds to the 3’UTR of
CTNNB1 catenin (cadherin-associated protein), beta 1. The protein encoded by this gene is part of a complex of proteins that constitute adherens junctions in epithelial cells. Hence we decided to screen well-known epithelial marker E-Cadherin and mesenchymal markers Vimentin, TWIST and SNAIL in miRNA manipulated MCF7 cells. MiR-526b over expression in MCF7 cells induced EMT, evidenced by decrease in E-Cadherin and up regulation of Vimentin, TWIST and SNAIL (mRNA and protein; Figures 1I & J). Conversely, knockdown of miR-526b in MCF7-COX-2 cells resulted in a significant increase in the E-Cadherin and decrease in Vimentin, TWIST and SNAIL mRNA (Figure 1K), indicating mesenchymal to epithelial transition (MET) of the cells or a reversal of their EMT phenotype. Together, these results demonstrate an important role for miR-526b in promoting an aggressive phenotype in human breast cancer.

**Link between miR-526b and stem-like cell (SLC) phenotype**

The closest in vitro assay of SLC function is the ability of single cells to form spheroid-like structures (39). We allowed multiple human breast cancer cell lines to grow in regular monolayer culture condition and in ultra-low attachment plates to grow as tumorspheres followed by miRNA quantification. When grown as spheroids, miR-526b expression increased in all cell lines including COX-2 high and miRNA high cells, while this was more pronounced in COX-2 and miRNA low cell lines (Supp. Fig 1E). These results gave us the first hint of a positive association SLC phenotype in breast cancer cells with miR-526b.

**Introduction of miR-526b stimulates SLC phenotype in MCF7 and SKBR3 cells**
To test the role of miR-526b in SLC induction, MCF7-526b and SKBR3-526b human breast cancer cell lines, and their respective Mock and parental controls were plated in 6-well ultra-low attachment plates to perform tumorsphere assay. Compared to controls, both miR-526b over-expressing cell lines displayed a significant increase in the number of spheroids (Figure 2A & C), as well as spheroid sizes (given by average perimeter, Figure 2B & D). Conversely, transient knock-down of miR-526b in MCF7-COX-2 and SKBR3-COX-2 cells resulted in a significant reduction in the number of spheroids formed in both (Figure 2F & G); however, no significant difference was observed in the average size of spheroids compared to Mock-transfected cells (size data not presented). These results suggest that over-expression of miR-526b is associated with the stimulation of SLC phenotype in human breast cancer cell lines.

**EP receptor activation stimulates miR-526b expression**

PGE2 is the major prostanoid product of COX-2 enzyme activity, and is the endogenous ligand for all EP receptors including the cAMP-stimulatory EP2 and EP4 receptors (6). PGE1OH binds selectively to EP4 (both cAMP and PI3K-AKT signaling) but not EP2. We treated a panel of breast epithelial and cancer cell lines with both PGE2 and PGE1OH for 24 h and quantified miR-526b expression. While no change was noted with the MCF10A human mammary epithelial cells, low COX-2 expressing MCF7 and T47D human breast cancer cells showed highly significant increases in miRNA expression with both PGE2 and PGE1OH treatments (Figure 3A). High COX-2 expressing MDA-MB-231 cells showed no change in miRNA expression.

**EP activation stimulates SLC phenotype and miRNA expression in spheroids**
Based on the results above, MCF7 cells were plated at single cell suspensions on ultra-low attachment plates and treated with PGE2, PGE1OH, or vehicle control for 9 days. After treatment, each well of the plate was digitized, and representative images were obtained for each treatment (Figure 3B). Then miRNA was extracted. Compared to vehicle-treated cells, MCF7 cells treated with PGE2 or PGE1OH displayed notable increases in spheroid number (Figure 3C) and size (Figure 3D). Consistent with our murine data (16), these results show that EP4 activation contributes to SLC phenotype in human breast cancer cells. Furthermore, both PGE2 and PGE1OH treatments stimulated miR-526b expression in tumorsphere conditions in both low COX-2 expressing MCF7 cells and T47D cells (Figure 3E). These results, taken together reveal that both spheroid formation and miR-526b expression in human breast cancer cells is positively regulated, at least in part, by EP4 receptor activity.

**Treatment with COX-2 inhibitor or EP4 receptor antagonist (EP4A) decreases miR-526b expression in MCF7-COX-2**

Previous studies had demonstrated that inhibiting COX-2 or EP4 receptor activity reduced the growth of COX-2 expressing murine C3L5 primary breast carcinomas and their spontaneous metastases *in vivo* (17, 19). EP4A treatment or EP4 knockdown in the same breast cancer cells markedly reduced spheroid forming ability *in vitro* and incidence of SLC marker positive cells in tumor (19). To examine the potential role of the EP4 receptor in COX-2 induced miR-526b expression *in vitro*, MCF7-COX-2 cells were treated with an EP4A (ONO-AE3-208), COX-2 inhibitor (NS398), or vehicle for 24 h. Both treatments caused a significant decrease in miR-526b expression levels, compared to vehicle treated cells (Figure 3F). These results suggest that
miR-526b expression in COX-2 over-expressing cells is dependent on both COX-2 and EP4 activity.

**Treatment with PI3K inhibitors decreases miR-526b expression and SLC phenotype in MCF7-COX-2 cells**

To investigate the role of PI3K-AKT activity in regulating miR-526b expression and SLC phenotype, MCF7-COX-2 cells were grown as a monolayer and treated with either an irreversible PI3K inhibitors Wortmannin (WM), a reversible PI3K inhibitor LY-204002 (LY), or vehicle for 24 h. MCF7-COX-2 cells treated with either agent showed a significant decrease in miR-526b expression levels (Figure 4A) compared to vehicle treated controls. This result suggests that miR-526b expression in MCF7-COX-2 cells is reliant on PI3K-AKT signaling activity. A tumorsphere formation assay conducted in the presence of two PI3K inhibitors (LY or WM) with MCF7-COX-2 cells showed significant decreases in spheroid numbers (Figure 4B) and spheroid sizes (Figure 4C) compared to vehicle treated control cells. These results suggest that PI3K-AKT signaling is involved in promoting SLC phenotype by miR-526b in human breast cancer cells.

**Treatments with AKT inhibitors (LY and WM) significantly blocks PGE2 and PGE1OH induced miRNA expression at variable time points**

PGE2 can bind to all EP receptors and PGE1OH is a selective EP4 agonist. To test the distinctive role of EP4 in regulating miRNA stimulation, we examined whether blocking the PI3K-AKT pathway, stimulated by EP4, but not EP2 activation (8), could mitigate the stimulatory effects of PGE2 and PGE1OH on miR-526b expression. We first treated MCF7 and T47D cells with
PGE2 or PGE1OH, followed by one of two PI3K inhibitors for 12 and 24 h. Following miRNA extraction and qPCR analysis, we observed that treatment with both PI3K inhibitors significantly blocked miR-526b up-regulation in a time dependant manner in both cell lines (Figure 4E, 4F). These results reveal the role of PI3K/AKT signalling, presumably mediated by EP4 activation, in stimulating miR-526b expression in MCF7 human breast cancer cells.

Inhibition of cAMP signaling reduces miR-526b expression in MCF7 cells

To investigate the effects of inhibiting cAMP signaling (shared by EP2 and EP4), on miR-526b expression, MCF7-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. Taqman analysis showed that inhibiting PKA with H89 resulted in a significantly lower expression of miR-526b, compared to DMSO treated controls (Figure 4G). These results reveal the contribution of EP2/EP4-mediated PKA activity in miR-526b expression in MCF7-COX-2 human breast cancer cells.

Role of NF-κB in miR-526b Regulation

Interestingly, miR-526b knockdown in MCF-COX-2 and SKBR-COX2 cells also reduced COX-2 mRNA (Supp. Fig 4), indicating a positive feedback loop for COX-2/EP4/miR-526b mediated SLC perpetuation. Post-study 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 also targets PTEN, suggesting a possible mechanism of PTEN down-regulation leading to up-
regulation of PI3K-AKT signaling via the EP4 receptor (scheme presented in Figure 4H). To test the role of NF-κB in miR-526b regulation, we treated MCF7 and T47D cells with BAY-11-7082 (10 μM), a NF-κB inhibitor along with PGE2 and PGE1OH for 24 h. BAY-11-7082 significantly blocked PGE2 and PGE1OH stimulated miR-526b expression in both MCF7 and T47D cells (Figure 4I & 4J respectively), indicating the positive loop indeed exist.

**MiR-526b promotes lung colony formation in an experimental metastasis model**

In order to investigate the 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 5x10⁵ cells) and allowed to colonize for 4 weeks. Lung sections were stained with anti-Human Leukocyte Antigen (HLA) antibody and the nuclear marker DAPI. The number of HLA-positive colonies (8 or more cells) was counted for each of the three serial sections in each animal using the ImageJ program. Inocula of MCF7-526b and SKBR3-526b cells established a significantly greater number of lung colonies, compared to Mock controls (Figure 5). The increase in lung colonization (about nine fold relative to Mock controls) was higher than relative tumor-sphere forming efficiency (1.5-2 fold, Figs 2 A and C) in vitro. This result supports the hypothesis that over-expression of miR-526b in human breast cancer cells promoted tumorigenicity in an experimental in vivo model of metastasis.

**MiR-526b expression in human breast tissues and correlation with overall survival**

Primary data generated in our lab revealed significantly higher expression of miR-526b in breast cancer tissues compared to adjacent non-tumor tissues (Figure 6A). A weak but positive
correlation between miRNA-526b expression and COX-2 or EP4 expression in tumor tissues was noted (Figs 6B and 6C). The lack of a more robust correlation can be explained by the likelihood that levels of COX-2 or EP4 mRNA may not reflect COX-2 or EP4 activity earlier shown to upregulate this miRNA. On histo-pathological stratification of the tumors, we observed that 7.6% of grade 2 and 7.9% of grade 3 tumors expressed significant levels of miR-526b, while no expression was noted in well-differentiated Grade I tissues (Table 1). Thus this miRNA appears to be elevated in relatively advanced grade of the disease, suggesting its potential as a prognostic biomarker. In support, cancer genome atlas data mining revealed that high of miR-526b expression (80th percentile) in breast tumors is associated with decreased overall survival (Figure 6D).

**Discussion**

COX-2 is a major driver of human breast cancer progression (1-5), in which EP4 receptor activity plays a significant role (17, 19, 20). The role of COX-2 in regulating micro RNAs has never been tested. In the present study we show that miRNA-526b is an oncogenic, SLC-linked miRNA, upregulated by COX-2 and EP4 activity, detectable in higher-grade primary human breast cancer tissues. Moreover, high expression of miR-526b in breast cancer patients was negatively correlated with overall patient survival, indicating an association of miR-526b expression with disease progression. In aggressive breast cancer cell lines, treatment with EP4A and COX-2 inhibitor could reduce miR-526b expression. We suggest that miR-526b is an oncogenic miRNA and a potential biomarker of stem-like cell activity in human breast cancer, the expression of which can be blocked with EP4A.
MiRNA expression profiling has led to the identification of disease-associated miRNAs (41, 42). This presents as a newer tool for classifying human cancers, including both the developmental lineage and level of differentiation (22, 43). MiRNA profiling has been utilized in a large number of human breast cancer cell lines to identify subtypes and driver mutations (44). MiRNAs elevated in breast cancer tissues can also appear as blood bio-markers. For example, up-regulation of single miRNA miR-195 in human breast tumors was reliably reflected in the circulating plasma levels of these patients (45). Certain circulating miRNAs were reported to mark early stage breast cancer (46, 47). Future studies should reveal the potential of using miR-526b as a disease biomarker in patient plasma.

Micro-RNAs can be pro- or anti-oncogenic, as investigated in many tumor types (48). They can influence tumor progression, by affecting cellular migration, invasion, EMT and other events in the metastatic cascade (49, 50). In this study, by employing miRNA manipulated human breast cancer cell lines, we show that miR-526b promotes cancer cell migration, invasiveness, EMT and SLC phenotypes in vitro and lung colony forming ability in vivo. These attributes clearly establish miR-526b as an oncogenic and putatively metastasis-associated miRNA, detected in human COX-2 expressing breast cancer cell lines, and also in patient-derived breast cancer tissues of progressive stage. This miRNA adds to the list of other oncogenic miRNAs in breast cancer, such as miR-106b-25 cluster (49), miR 10b (50), miR-21 (51), and miR 9 (52), shown to be associated with one or more events in tumor progression and metastasis.

Expression levels of certain miRNAs have been shown to correlate inversely with established SLC markers (CD44+/CD24-, ALDHHigh) in human breast cancer. For example, a down-regulation of the let-7 family of miRNAs could distinguish SLC populations from non-
SLC populations within primary breast tumors (35). Forced over-expression of let-7 miRNAs in human breast cancer cell lines resulted in a reduction in SLC marker bearing cells as well as a spheroid forming ability (35). Of the many oncogenic micro-RNAs reported in breast cancer (44, 52), miR 9 has been indirectly linked with SLC phenotype (53). None of them have yet been directly linked with SLC functions. In the present study, we observed that ectopic over-expression of miR-526b in human breast cancer cell lines markedly increased their ability to form spheroids in vitro and lung colony forming ability in vivo. Pending further validation of the SLC promoting role of this miRNA in orthotopic transplants of miRNA over-expressing cells at limiting dilution, present study is the first to identify an oncogenic miRNA directly linked with SLC in human breast cancer.

Several studies including ours have reported the involvement of EP4 receptor on tumor cells (17, 19, 20) and host cells such as NK cells (20) and macrophages (19) in breast cancer progression. EP4 activation can result in stimulation of both cAMP and PI3K-AKT pathways (6, 8). In our murine C3L5 breast cancer model, therapy with EP4 antagonists inhibited tumor growth and metastasis to lymph nodes and the lungs, and residual tumors exhibited reduced AKT phosphorylation, indicating EP4 inactivation (17, 19). Furthermore, this therapy exhibited distinct SLC-reductive effects, as noted from the reduction of multiple SLC marker bearing cells in residual tumors (19). In the present study we explored the possible 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 MCF7 cells treated with a selective EP4 receptor agonist PGE1OH, resulted in up-regulation of miR-526b expression, while MCF7-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. Moreover, PI3K-AKT inhibitors could significantly block PGE2 and PGE1OH-induced miR-526b expression, indicating the importance of the COX-2, PGE2, EP4, and PI3K-AKT axis in regulating miR-526b expression in human breast cancer.

The mechanisms responsible for miR-526b stimulation of SLC remain to be investigated. This may involve one or more gene targets, many with putative tumor suppressor functions, listed in Supp. Table1. We already established the role of COX-2 in SLC induction in breast cancer (19) and based on our observation that miR-526b knock down markedly reduced COX-2 expression in breast cancer cell lines (Supp. Fig 4), we speculated that this phenomenon of COX-2 upregulation by miR526b creates a positive feedback loop, which maintains the SLC phenotype. NF-κB is known to play an important role in up-regulating COX-2 in a variety of human cancers (54). In this study we observed that miR-526b expression is regulated by NF-κB. The proposed scheme for COX-2/EP4/miR-526b mediated SLC perpetuation is presented in Fig 4H.

Taken together, results of this study provide support for classifying miR-526b as an oncogenic miRNA with a strong potential as a biomarker of human breast cancer, which could be applied to monitoring during SLC-targeted therapies. Multiple roles of EP4 receptor in tumor progression demonstrated in breast cancer models (15,17,19,20), including SLC induction (19) makes EP4 an attractive therapeutic target in preference to COX-2 inhibitors. This is because EP4 spares cardio-protective prostanoids such as PGI2 inhibited by COX-2 inhibitors (13). Presently demonstrated association of EP4 mediated signaling pathways, cAMP and PI3K-AKT, with miR-526b expression, and the oncogenic functions of this miRNA including SLC induction,
makes this miRNA an attractive biomarker for monitoring therapies with EP4 antagonists as adjuvant in selected breast cancer patients.

Acknowledgements

We thank Gillian Bell to perform all tail vain injections in mice and Krista Vincent to extract and analyze TCGA data. We are thankful to Asma Hasan to help generating data for migration, invasion and EMT experiments and Mehdi Amiri for helping with EMT data generation. We acknowledge the participation of Elena Tutunea-Fatan and Mauricio Rodriguez during miRNA and RNA extraction from human tissue. This study is supported by grants of the Ontario Institute of Cancer research and Canadian Breast Cancer Foundation, Ontario chapter, to PKL and a Canadian Breast Cancer Foundation (CBCF) – Ontario Chapter Graduate Fellowship to EL; and a Translational Breast Cancer Research Unit Postdoctoral Fellowship to MM, funded by the Breast Cancer Society of Canada; EL and MM are honorary fellows of the Canadian Institutes of Health Research Strategic Training Program in Cancer Research and Technology Transfer (CIHR-CaRTT).

Reference:

genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 2004; 101: 2999-3004.


Figure 1. Mir-526b Over-Expression Promotes Cellular Migration and Invasion and EMT, and Knock-Down of miR-526b Produces Opposite Effects. Transwell migration and invasion assays were performed as triplicate independent experiments. The data are presented as a mean of triplicates ± SEM. * Indicates p<0.05 compared to parental and mock transfected cell lines. Comparison of (A) migration and (B) invasion of parental MCF7, empty vector (Mock) transfected, and miR-526b over-expressing cells. Comparison of (C) migration and (D) invasion of parental SKBR3, Mock transfected, and miR-526b over-expressing cells. Comparison of (E) migration and (F) invasion of MCF7-COX-2-526b KD, compared to empty vector (Mock) transfected cells. Comparison of (G) migration and (H) invasion of SKBR3-COX-2-526b KD after transient miR-526b knock-down, compared to empty vector (Mock) transfected cells. Fold change in E-Cadherin, Vimentin, TWIST, SNAIL mRNA (I) and protein (J) in MCF7-526b cells indicating EMT. (K) MCF7-COX-2-526b KD cells showing MET with up regulation of E-Cadherin and down regulation of Vimentin, TWIST and SNAIL mRNA. In figure K, # indicates p<0.05 compared to MCF-7-COX-2.

Figure 2. Mir-526b Over-Expression Induces Stem-Like Cell Phenotype in MCF7 and SKBR3 and Knock-Down of miR-526b Reverses These Effects. The data are presented as a mean of triplicate independent experiments ± SEM. *Indicates p<0.05 compared to parental and mock cell lines. Scale bar is 50 µm. Comparison of spheroid (A) number and (B) size (perimeter) of parental MCF7; Mock transfected, and miR-526b over-expressing cells. Comparison of spheroid (C) number and (D) area of parental SKBR3, Mock transfected, and miR-526b over-expressing cells. (E) Representative images for MCF7 and SKBR3 cell lines demonstrating relative number and morphology of spheroids grown on ultra-low attachment plates. Comparison of spheroid formation with transient miR-526b knockdown in (F) MCF7-COX-2-526b KD and (G) SKBR3-COX-2-526b KD cells, compared to respective Mock controls.

Figure 3. EP4 Receptor Activation Promotes miR-526b Expression and Spheroid Formation. The data are presented as a mean of triplicate experiments ± SEM. * Indicates p<0.05 compared to Mock cell line. Scale bar is 100 µm. (A) Comparison of fold change in miR-526b expression levels, relative to controls (vehicle treatment) in a panel of breast cancer cell lines treated with a EP ligand (PGE2; 10 µM), EP4 receptor agonist (PGE1OH; 10 µM), or vehicle (DMSO). MCF7 cells were treated with PGE2, PGE1OH, or DMSO in spheroids culture condition and captured images presented in (B), spheroid number (C) and spheroids areas (perimeter) presented in (D). (E) Comparison of fold change in miR-526b expression levels for MCF7 and T47D cells treated as above and grown in spheroid culture conditions. Data presented as ratio of spheroid/monolayer. (F) Comparison of fold change in miR-526b expression levels for MCF7-COX-2 cells (relative to control vehicle treatment), treated with a COX-2 inhibitor (NS398; 10 µM), EP4 receptor antagonist (ONO-AE3-208; 10 µM), or vehicle (DMSO).

Figure 4. PI3K Inhibition Reduces miR-526b Expression. The data are presented as a mean of triplicate experiments ± SEM. * Indicates p<0.05 compared to respective vehicle or DMSO treatment. Scale bar is 100 µm. (A) Comparison of fold change in miR-526b expression levels for MCF7-COX-2 cells treated with a reversible PI3K inhibitor (LY; 10 µM), an irreversible PI3K inhibitor (Wortmannin; 10 µM), or vehicle (DMSO). Comparison of spheroid number (B) and area (C) of MCF7-COX-2 cells treated with LY, WM, or DMSO. (D) Representative images
of MCF7-COX-2 spheroids grown on ultra-low attachment plates and treated with LY, WM and DMSO. Comparison of miR-526b expression with stimulation of the EP receptors with PGE2 or EP4 receptor with PGE1OH (10 µM each) followed by treatment with two PI3K inhibitors (Wortmannin, LY-240-002, 10 µM) in (E) MCF7 and in (F) T47D cells. (G) Comparison of fold change in miR-526b expression levels for MCF7-COX-2 cells treated with a PKA inhibitor H89 (30 µM) or vehicle (DMSO). (H) Schematic diagram demonstrating a hypothetical mechanism for miR-526b regulation, including involvement of the prostanoid pathway and the EP4 receptor. Change in miRNA expression in (I) MCF7 and (J) T47D cells treated with NF-κB inhibitor BAY-11-7082 (BAY) along with PGE2 and PGE1OH. In figure I and J, # indicates p<0.05 compared to PGE2 and PGE1OH treatments respectively.

**Figure 5. Expression of miR-526b Supports Lung Colony Formation In Vivo.** The data are presented as a mean of triplicate measurements ± SEM. * Indicates p<0.05 compared to Mock cell line. Scale bar is 100 µm. Inoculum dose 5x10⁵ cells per animal (n=5 per group) delivered via intravenous injection. (A) Comparison of lung colony number (>8 cells) of Mock and miR-526b transfected cells for MCF7 and SKBR3 cell lines. (B) Representative images of lung sections stained for HLA (red) and DAPI (blue) demonstrating lung colony formation 4 weeks post-injection.

**Figure 6. MiR-526b is Over-Expressed in Breast Tumors and Negatively Correlates with Patient Survival.** (A) Box-and-whisker plot showing over-expression of miR-526b in primary human breast tumors (n=105), in comparison to non-tumor control tissue (n=20). A more negative ΔCt value indicates a higher miRNA expression level. (B) Kaplan-Meier curve demonstrating the inverse relationship between high miR-526b expression and reduced overall survival rates in primary human breast carcinomas (n=639). A positive correlation exists between (C) COX-2 and (D) EP4 mRNA expression with miR-526 expression in primary breast cancer samples. * Indicates p<0.05.
Figure 2

A

B

Sphere Area (Units)

C

D

Sphere Area (Units)

E

MCF7

Parental

526b

F

G

Sphere Formed (#)

Sphere Formed (#)

Mock

526bKD

Mock

526bKD
Figure 5

A

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B

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Figure 6

A

B

C

D

Δ Ct miR-526b

Δ Ct COX-2

Δ Ct EP4

Survival

Days

R²=0.1048

R²=0.1288

Control

Tumor

Low miR526b Expressors

High miR526b Expressors
Table 1: Demography, Tobacco Exposures, Tumor Grade and miRNA expression

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Tumor grade and miR-526b expression in cancer samples

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<td>63 (60)</td>
<td>5 (7.94)</td>
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Molecular Cancer Research

COX-2 Elevates Oncogenic miR-526b in Breast Cancer by EP4 Activation

Mousumi Majumder, Erin Landman, Ling Liu, et al.

Mol Cancer Res  Published OnlineFirst March 2, 2015.

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