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

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Oncogenes and Tumor Suppressors

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 overexpression also upregulated 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 upregulation were examined. A positive correlation was noted between miR-526b and COX-2 mRNA expression in COX-2 disparate breast cancer cell lines. Stable overexpression 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 upregulation and tumorsphere formation in MCF7 and SKBR3 cells. NF-κB inhibitor abrogates EP agonist–stimulated miRNA expression in MCF7 and T47D cells, indicating that the NF-κB pathway is also involved in miR-526b regulation. In addition, inhibition of COX-2, EP4, PI3K, and PKA in COX-2–overexpressing cells downregulated miR-526b and its functions in vitro. Finally, miR-526b expression was significantly higher in cancerous than in noncancerous 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 upregulated, oncogenic miRNA promoting SLCs, the expression of which follows EP4 receptor-mediated signaling, and is a promising biomarker for monitoring and personalizing breast cancer therapy. Mol Cancer Res; 13(6); 1022–33. ©2015 AACR.

Introduction

COX-2 is an inducible inflammatory enzyme found to be upregulated in approximately 40% of primary breast cancer cases (1, 2) at both the preinvasive (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 prostanooid pathway, ultimately leading to the production of prostaglandin E2 (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++); EP2 and EP4 are coupled with Gs, stimulating the adenylyl cyclase (AC)–PKA pathway, whereas most EP3 isoforms are coupled with Gi, thus inhibiting AC (7). Unlike EP2, EP4 can additionally stimulate the PI3K–AKT–mediated cell survival pathway as well as the promigratory 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 cardioprotective prostanooids such as PGIL (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 antitumor immune cells (14, 15), stimulation of tumor cell migration (16); invasiveness (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). Although 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 antitumor, antimetastatic, 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.

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MicroRNAs (miRNAs; miRs) are short, noncoding regulatory RNA molecules that downregulate 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). Because 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 subtypes, 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).

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 upregulated in COX-2 high human breast cancer cell lines, both under natural and ectopically overexpressing 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, using 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 SCL stimulation and their respective mock cell lines maintained with Geneticin (GIBCO). Other human breast cancer cell lines, MDA-MB-231 (high COX-2, ER, and HER-2 positive) and T47D (low COX-2, ER positive, and HER-2 negative) were grown in RPMI-1640 minimal essential medium (GIBCO). All media were supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma) and maintained in a humidified incubator with 5% CO2 at 37°C. In addition, MCF7-COX-2 and SKBR3-COX-2 and their respective mock cell lines maintained with Geneticin (GIBCO) 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 miRNA Arrays 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 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. ONO-AE3-208 (selective EP4 agonist) was a gift of ONO Pharmaceuticals. 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. NF-kB 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 the miRNeasy Mini Kit (Qiagen) and reverse transcribed using the TaqMan microRNA and total RNA cDNA Reverse Transcription Kit (Applied Biosystems). The TaqMan miRNA Assay or Gene Expression Kit was used for quantitative PCR. Cq 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 analysis
Cells were treated with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with HALT Protease Inhibitor Cocktail (Thermo Fisher Scientific) and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) to extract protein. Total protein (20 μg) was electrophoresed per well on a SDS-polyacrylamide gel and transferred onto Immobilon-FL polyvinylidene difluoride membranes (Millipore). Membranes were then incubated with the following primary antibodies: E-Cadherin (Cell Signaling Technology; #24E10); Vimentin (Millipore; #AB3400); TWIST (Santa Cruz Biotechnology Inc.; #sc15393); SNAIL (C15D3) from Cell Signaling Technology (#3879), and β-actin (Santa Cruz Biotechnology; #sc47778) and probed with a mixture of IRDye polyclonal secondary antibodies (LI-COR Biosciences). Images were read with an Odyssey infrared imaging system (LI-COR Biosciences).

Stable miRNA knockdown
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 GFP marker (OriGene) using the Amaxa Cell Line Nucleofector Kit V (Lonza) and the E-009 or P-020 program for SKBR3 cells or MCF7 cells, respectively, according to the manufacturer’s protocol. Cells were treated with the antibiotic resistance selection agent Gentecin at 500 μg/mL MCF7 and SKBR3 cells stably transected with the pCMV-MIR-mock (empty) vector are referred to as MCF7-Mock and SKBR3-Mock, respectively, and cell lines stably transected with the pCMV-MIR-miR-526b expression plasmid are referred to as MCF7-526b and SKBR3-526b.

Transient miRNA knockdown
We used morpholino oligonucleotides, which provide highly specific knockdown of miRNAs (37) for the target miR-526b. Morpholino oligos specific to miR-526b (MG526b) purchased from Gene Tools LLC, at two different concentrations (10 and 20 μM/L), 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). Functional assays as described later (migration, invasion, and spheroid formation) were performed within 48 hours of transient transfection using control and MG526b (20 μM/L). For spheroid formation, data were collected after 7 days.

Transwell migration and invasion assays
Cells (2 × 10⁵ cells/mL) in 300 μL of either EMEM or McCoy’s 5A media were added to the upper chamber of a multiporous polycarbonate (8-μm pore size) insert (BD Falcon), and placed in a 24-well plate (BD Falcon). For invasion assays, cell inserts were coated with Matrigel (1:100 in basal media; BD Biosciences). The lower chamber contained 700 μL of either serum-free media or 2% FBS supplemented media as positive control. Plates were incubated for 24 hours (migration assay) or 48 hours (invasion assay) at 37°C and 5% CO2, as previously established (16, 38). Cells were subsequently fixed with pure methanol, stained with eosin, and thiazine, respectively, and washed with ddH2O. The 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 (in vitro surrogate of SLC), cells (1 × 10⁴ cells/mL) were passed through an 8-μm filter (Falcon; BD Biosciences) and a syringe fitted with a 27-gauge needle, and plated in 6-well ultra-low attachment plates (Corning), as previously described (19, 39). All tumorspheres were grown in HuMEC (GIBCO) media supplemented with EGF (20 ng/mL; Invitrogen), basic fibroblast growth factor (FGF; 20 ng/mL; Invitrogen), and B27 (1:50 dilution; Life Technologies), and allowed to grow for 7 to 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/GUSβ (Glucuronidase-beta)-null mice (Robarts Research Inst.) were maintained on standard mouse chow and tap water on a 12 hours 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 5 × 10⁵ cells, and sacrificed after 4 weeks to assess micrometastases to the lung. Lungs were harvested after inflation with PBS and flash frozen, with OCT compound. At least three 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), followed by secondary horse anti-mouse antibody (1:1,000 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/GUSβ hosts were chosen initially to identify tumor cells in the lungs by staining for the GUSβ marker, we adopted HLA staining preferred to GUSβ staining because our preliminary studies revealed that some human cancer cells lost GUSβ staining as lung colonies grew bigger.

Human tissue samples
To examine the clinical relevance of miR-526b expression in breast cancer, we obtained frozen 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. Although 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
miR-526b, COX-2, and EP4 gene-expression analysis as described before. Negative ACs values are indicative of higher miRNA expression in tissue samples.

Survival analyses

Coded patient survival data were extracted from The Cancer Genome Atlas (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 (OS) associated with miR-526b expression were conducted in 639 primary breast carcinomas. A cutoff \( P \) value (\( P < 0.05 \)) was determined using the log-rank test.

Statistical analysis

Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software). 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 nonparametric (Wilcoxon rank sum test) methods, giving same results. The Student \( t \) test was used when comparing two datasets and Pearson’s coefficient was used to assess statistical correlations. Statistically relevant differences between means were accepted at \( P < 0.05 \).

Results

Identification of upregulated miR-526b in MCF7-COX-2 cells

Using miRNA microarray to compare MCF7-COX-2 and mock-transfected cells, we identified numerous miRNAs whose expressions were up- or downregulated. However, this increased expression was statistically significant only for two miRNAs miR-526b and miR-655 (Supplementary Table S1). In this study, we investigated the role of miR-526b, upregulated 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 Supplementary Fig. S1A and S1B reveal that this was indeed the case, suggesting that, among many genes, COX-2 played a dominant role in miR-526b upregulation. That COX-2 activity was instrumental in this upregulation is shown later.

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

Stable overexpression of miR-526b in MCF7 (nonmetastatic, low COX-2) and SKBR3 (low metastatic, COX-2 negative), both low miR-526b–expressing human breast cancer cell lines were achieved using nucleotransfection as per the manufacturer’s protocol. They were, respectively, called MCF7-526b and SKBR3-526b, with their empty vector controls called as MCF7-Mock and SKBR3-Mock, respectively. Overexpression of miR-526b was confirmed using real-time RT-PCR, RNU44, and RNU48 miRNAs served as control miRNA. MiR-526b was significantly overexpressed in both cell lines, compared with empty vector controls (Supplementary Fig. S1C).

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

Morpholino-mediated knockdown 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 downregulated (more than 80%) in both cell lines at two different morpholino concentrations (Supplementary Fig. S1D), compared with Mock-transfected cells. The resulting cell lines (at 20 \( \mu \)mol/L concentrations) were termed MCF7-COX-2-526b KD, SKBR3-COX-2-526b KD, with their empty vector controls being termed 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. Overexpression of miR-526b in both cell lines resulted in a significant increase in cellular migration (Fig. 1A and C) and invasion (Fig. 1B and D), images of migrant MCF7 and SKBR3 cells are presented in Supplementary Fig. S2. Conversely knocking down miR-526b in both MCF7-COX-2 and SKBR3-COX-2 cells resulted in significantly reduced abilities to migrate (Fig. 1E and G) and invade (Fig. 1F and H).

EMT/MET. Cellular morphology changes after miR-526b overexpression (Supplementary Fig. S3), combined with increased
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Figure 1. miR-526b overexpression promotes cellular migration and invasion and EMT, and knockdown 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; \( ^* \), \( P < 0.01; \) \( ^\# \), \( P < 0.00, \) compared with parental and Mock-transfected cell lines. Comparison of migration (A) and invasion (B) of parental MCF7, empty vector (Mock)-transfected, and miR-526b-overexpressing cells. Comparison of migration (C) and invasion (D) of parental SKBR3, mock-transfected, and miR-526b-overexpressing cells. Comparison of migration (E) and invasion (F) of MCF7-COX-2-526b KD, compared with empty vector (Mock)-transfected cells. Comparison of migration (G) and invasion (H) of SKBR3-COX-2-526b KD after transient miR-526b knockdown, compared with empty vector (Mock)-transfected cells. Fold change in E-cadherin, Vimentin, TWIST, and SNAIL mRNA (I), and protein (J) in MCF7-526b cells, indicating EMT. K, MCF7-COX-2-526b KD cells showing MET with upregulation of E-cadherin and downregulation of Vimentin, TWIST, and SNAIL mRNA, compared with MCF7-COX-2.

migration may suggest epithelial-to-mesenchymal (EMT) phenotype. Although miR-526b has no binding site on E-cadherin, it binds to the 3' untranslated regions 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 overexpression in MCF7 cells induced EMT, evidenced by decrease in E-cadherin and upregulation of Vimentin, TWIST, and SNAIL (mRNA and protein, Fig. 1I and 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 (Fig. 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 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, whereas this was more pronounced in COX-2 and miRNA low cell lines (Supplementary Fig. S1E). 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 with
controls, both miR-526b–overexpressing cell lines displayed a significant increase in the number of spheroids (Fig. 2A and C), as well as spheroid sizes (given by average perimeter, Fig. 2B and D). Conversely, transient knockdown 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 (Fig. 2F and G); however, no significant difference was observed in the average size of spheroids compared with mock-transfected cells (size data not presented). These results suggest that overexpression 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 hours and quantified miR-526b expression. Although 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 (Fig. 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**

On the basis of 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 (Fig. 3B). Then miRNA was extracted. Compared with vehicle-treated cells, MCF7 cells treated with PGE2 or PGE1OH displayed notable increases in spheroid number (Fig. 3C) and size (Fig. 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 tumoursphere conditions in both low COX-2–expressing MCF7 cells and T47D cells (Fig. 3E). These results, taken together, reveal that both spheroid formation and miR-526b expression in human breast cancer cells are 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

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**Figure 2.**

miR-526b overexpression induces SLC phenotype in MCF7 and SKBR3 and knockdown of miR-526b reverses these effects. The data are presented as a mean of triplicate independent experiments ± SEM. * P < 0.05 compared with parental and mock cell lines: scale bar, 50 μm. Comparison of spheroid number (A) and size (B: perimeter) of parental MCF7; Mock-transfected, and miR-526b–overexpressing cells. Comparison of spheroid number (C) and area (D) of parental SKBR3, Mock-transfected, and miR-526b–overexpressing 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 MCF7-COX-2 or SKBR3-COX-2 cells compared with respective Mock controls.
with either agent showed a significant decrease in miR-526b expression levels (Fig. 4A) compared with vehicle treated controls. This result suggests that miR-526b expression in MCF7-COX-2 cells is reliant on PI3K–AKT signaling activity. A tumosphere formation assay conducted in the presence of two PI3K inhibitors (LY or WM) with MCF7-COX-2 cells showed significant decreases in spheroid numbers (Fig. 4B) and spheroid sizes (Fig. 4C) compared with 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 hours. Following miRNA extraction and qPCR analysis, we observed that treatment with both PI3K inhibitors significantly blocked miR-526b upregulation in a time-dependent manner in both cell lines (Fig. 4E and F). These results reveal the role of PI3K–AKT signaling, 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 μmol/L). Cells were grown in monolayer and treated with H89 for a period of 24 hours. TaqMan analysis showed that inhibiting PKA with H89 resulted in a significantly lower expression of miR-526b, compared with DMSO treated controls (Fig. 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 MCF7-COX-2 and SKBR-COX2 cells also reduced COX-2 mRNA (Supplementary Fig. S4), indicating a positive feedback loop for COX-2/EPR/miR-526b mediated SLC perpetuation. Post-study genome data mining performed in silico in our laboratory (miRanda-mirSVR software) revealed that miR-526b targets the NF-κB–negative regulator, Ras-like 1 (Ras1), a gene that downregulates NF-κB. A downregulation of Ras1 could, thus, lead to upregulation of NF-κB, and subsequent upregulation of COX-2 and EP4. In addition, miR-526b also targets PTEN, suggesting a possible mechanism of PTEN downregulation leading to upregulation of PI3K–AKT signaling via the EP4 receptor (scheme presented in Fig. 4H). To test the role of NF-κB in miR-526b regulation, we treated MCF7 and T47D cells with BAY-11-7082 (10 μmol/L), an NF-κB inhibitor along with PGE2 and PGE1OH for 24 hours. BAY-11-7082 significantly blocked PGE2- and PGE1OH-stimulated miR-526b expression in both MCF7 and T47D cells (Fig. 4I and J, respectively), indicating the positive loop indeed exist.
miR-526b promotes lung colony formation in an experimental metastasis model

To investigate the tumorigenic functions of miR-526b in vivo, miR-526b-overexpressing 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 5 x 10^5 cells) and allowed to colonize for 4 weeks. Lung sections were stained with anti-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 with Mock controls (Fig. 5). The increase in lung colonization (about 9-fold relative to Mock controls) was higher than relative tumor-sphere forming efficiency (1.5- to 2-fold, Fig. 2A and C) in vitro. This result supports the hypothesis that overexpression 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 OS

Primary data generated in our laboratory revealed significantly higher expression of miR-526b in breast cancer tissues compared with adjacent non-tumor tissues (Fig. 6A). A weak but positive correlation between miRNA-526b expression and COX-2 or EP4 expression in tumor tissues was noted (Fig. 6B).
and C). 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 histopathologic 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, whereas no expression was noted in well-differentiated grade 1 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 OS (Fig. 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 miRNAs 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...
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EP4A and COX-2 inhibitor could reduce miR-526b expression. We suggest that miR-526b is an oncogenic miRNA and a potential biomarker of SLC 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 used 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 biomarkers. For example, upregulation 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.

miRNAs can be pro- or antioncogenic, 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 using 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 downregulation of the let-7 family of miRNAs could distinguish SLC populations from non-SLC populations within primary breast tumors (35). Forced overexpression 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). Forced overexpression 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-overexpressing 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-reducing 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 upregulation of miR-526b expression, whereas MCF7-COX-2 cells treated with an EP4 antagonist, PKA inhibitor, or PI3K inhibitors resulted in downregulation 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 Supplementary Table S1. We already established the role of COX-2 in SLC induction in breast cancer (19) and based on our observation that miR-526b knockdown markedly reduced COX-2 expression in breast cancer cell lines (Supplementary Fig. S4), 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 upregulating 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), make EP4 an attractive therapeutic target in preference to COX-2 inhibitors. This is because EP4 spares cardioprotective 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, make this miRNA an attractive biomarker for monitoring therapies with EP4 antagonists as adjuvant in selected breast cancer patients.
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