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Title: "EP4 as a therapeutic target for aggressive human breast cancer"

Authors: Mousumi Majumder [1], Pinki Nandi[2], Ahmed Omar[1], Kingsley Chukwunonso Ugwuagbo[1] and Peeyush K. Lala[2,3,*]

[1] Department of Biology, Brandon University, Brandon, Manitoba R7A 6A9, Canada

[2] Departments of Anatomy and Cell Biology, and [3] Oncology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario N6A5C1, Canada

Author Emails: MM; majumderm@brandonu.ca; PN: pnandi@uwo.ca; AO: ismailao09@brandonu.ca; KU: ugwuagkc97@brandonu.ca; PKL: pklala@uwo.ca

*Corresponding author; Tel 1-519-661-3015; email: pklala@uwo.ca

Abstract

G protein-coupled receptors (GPCRs, also called seven-transmembrane or heptahelical receptors) are a superfamily of cell surface receptor proteins that bind to many extracellular ligands and transmit signals to an intracellular guanine nucleotide-binding protein (G protein). When a ligand binds, the receptor activates the attached G-protein by causing the exchange of Guanosine-5'-triphosphate (GTP) for guanosine diphosphate (GDP). They play a major role in many physiological functions as well as in the pathology of many diseases including cancer progression and metastasis. Only a few GPCR members have been exploited as targets for developing drugs with therapeutic benefit in cancer. Present review deals with the Prostaglandin E receptor EP4, a member of the EP family of GPCR, as a promising newer therapeutic target for treating breast cancer. We show that aberrant over-expression of cyclooxygenase

(COX)-2, an inflammation-associated enzyme, occurring in 40-50% of breast cancer patients leads to tumor progression and metastasis due to multiple cellular events resulting from an increased prostaglandin (PG) E2 production in the tumor milieu. They include inactivation of host anti-tumor immune (NK and T) cells, increased immuno-suppressor function of tumor-associated macrophages, promotion of tumor cell migration, invasiveness and tumor-associated angiogenesis (due to upregulation of VEGF-A), lymphangiogenesis (due to upregulation of VEGF-C/D) and a stimulation of stem-like cell (SLC) phenotype in cancer cells. All these events were primarily mediated by activation of the PGE receptor EP4 on tumor or host cells. We show that selective EP4 antagonists (EP4A) could mitigate all these events tested with cells *in vitro* as well as *in vivo* in syngeneic COX-2 expressing mammary cancer bearing mice or immune-deficient mice bearing COX-2 over-expressing human breast cancer xenografts. We suggest that EP4A can avoid thrombo-embolic side effects of long term use of COX-2 inhibitors by sparing cardio-protective roles of PGI2 via IP receptor activation or PGE2 via EP3 receptor activation. Furthermore, we identified two COX-2/EP4 induced oncogenic and SLC-stimulating microRNAs - miR526b and miR655, one of which (miR655) appears to be a potential blood biomarker in breast cancer patients, for monitoring SLC-ablative therapies such as with EP4A. We suggest that EP4A will likely produce the highest benefit in aggressive breast cancers such as COX-2 expressing triple-negative breast cancers, when combined with other newer agents such as PD-1 or PD-L1 inhibitors.

Key words: COX-2, Breast Cancer, PGE2, EP receptors, Stem-like cells, Metastasis, Angiogenesis, Lymphangiogenesis, MicroRNAs, Triple negative breast cancer

1. Introduction

G protein coupled receptors (GPCRs) are a superfamily of receptors that transduce signals by their coupling with guanine nucleotide-binding proteins (G proteins). They include about 900 members, some with known ligands, others identified as orphan receptors. A diverse set of ligands including peptide hormones, neurotransmitters, and odor molecules bind to GPCRs. They represent the most notable family of validated pharmacological targets in a variety of diseases, including cancer. Numerous GPCRs such as receptors for chemokines, thrombin, lysophosphatidic acid (LPA), gastrin-releasing peptide, angiotensin, the sphingosine 1-phosphate, endothelin and prostaglandins have been reported to play a key role in cancer progression and metastasis [reviewed in 1, 2]. Present article will focus on prostaglandin E receptor EP4 as a therapeutic target in aggressive breast cancer including triple negative breast cancer.

2. Breast Cancer: Needs to identify novel therapeutic targets.

Breast cancer accounts for the most frequent cancer in the female globally. It represents the second highest cause of cancer-related mortality in the western hemisphere due to resistance of some 25-30% of the patients to currently practiced therapies such as surgery, radiotherapy, chemotherapy, hormone therapy and HER2-trageted drugs, necessitating the search for newer therapy targets. Recent advances in cancer genomics have formed the basis of "Personalized medicine" in identifying therapeutic target(s) appropriate for the individual patient [3]. Genomic profiling of breast cancer by gene micro-array has recently been used to predict therapeutic outcome, that forms the basis for numerous commercially developed assays for use in the clinic [reviewed in 4, 5]. The remarkable advent of current high-throughput technologies in combination with improved knowledge of the molecular basis of malignancy provides a solid base for identifying novel molecular targets. As reviewed below, we show that 40-50% of breast cancer patients including a most aggressive subset of patients identified as ER-/PR-/HER-2- or

"triple negative breast cancer (TNBC)" reveal an upregulation of the inflammation-associated enzyme cyclooxygenase (COX)-2 which drives tumor progression and metastasis, and that prostaglandin E receptor EP4, a GPCR family member, presents as a promising newer therapeutic target in these patients.

3. Cyclo-oxygenase pathway.

Molecular cascade in the COX pathway has been adequately reviewed [6, 7]. Briefly, COX family of enzymes includes three members COX-1, COX-2 and COX-3. COX-3 is an isoform of COX-1 produced by alternative splicing of *COX-1* or *PTGS-1* gene, and not present in the human. Most somatic cells constitutively express COX-1, and a small minority of cells (of the reproductive and immune systems) constitutively express COX-2. Cell membrane phospholipids, under the influence of phospholipase A2 (PLA2) produce Arachidonic acid which acts as the substrate for lipoxygenases (LOX) to produce leukotrienes and cyclooxygenases (COX) to produce prostaglandins PGE2, Thromboxane A2, PGI2, PGF2α and PGD2, all of which exert physiological functions by binding to their respective receptors (EP family for PGE2, TP for Thromboxane A2, IP for PGI2, FP for PGF2 α and DP for PGD2). PGE2 is the most abundant eicosanoid produced by the action of PGE synthase (PGES) enzymes on PGG2 downstream of COX (**Figure 1**). Secreted PGE2 is a short-lived molecule, quickly catabolized to the inactive 15-keto-PGE by the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH, also known as HPGD). PGE2 acts locally in an autocrine or paracrine manner through its four cognate G protein coupled receptors EP1 to EP4. Under physiological conditions, PGE2 mediates many biological functions, such as regulation of immune responses, blood pressure, gastrointestinal integrity, and fertility. Deregulated PGE2 synthesis or degradation is associated with many pathological conditions like chronic inflammation, Alzheimer's disease, and tumorigenesis. COX-2 is expressed constitutively

only in a small minority of cells such as macrophages and some cells in the reproductive organs. Typically, it is an inflammation-associated enzyme induced by inflammatory cytokines, mitogens and certain carcinogens. PGE2 production via COX-1 pathway occurs steadily at low local concentrations. In contrast, COX-2-mediated PGE2 production during inflammation occurs at high local concentrations and stops after withdrawal of the inflammatory stimulus. However, aberrant COX-2 activity that occurs in many epithelial cancers including breast cancer leads to persistent PGE2 production [7, 8].

3.1. EP receptors: PGE2-mediated intracellular signaling depend on its binding of target cells to one or more of the specific prostaglandin E receptors (EP1-4) which are coupled to different G proteins. [9, 10, 11] (**Figure 1**). The activation or inactivation of G proteins occurs as follows (**Figure 2**). When a ligand binds, the receptor activates the attached G-protein by causing the exchange of GTP for GDP. The activated G-protein then dissociates into an alpha (G-α) and a beta-gamma (G-β/ϒ) complex. GTP bound G-α is active, and can diffuse along the membrane surface to activate (and sometimes inhibit) target proteins, typically enzymes that generate second messengers. Similarly, the G-β/ϒ complex is also able to diffuse along the inner membrane surface and affect protein activity. Intrinsic GTPase activity is responsible for inactivation of the G-Protein. After GTP hydrolysis, GDP bound G-α will re-associate with a β/ϒ complex to form an inactive G-protein that can again associate with a receptor. Signaling mediated by the EP family depends on the coupled G-protein. As shown in **Figure 3,** EP1 couples with G_q, activating phospholipase C (PLC) that cleaves PIP2, a membrane phospholipid, to generate secondary messengers, IP_3 and diacylglycerol (DAG). IP_3 is water soluble, diffusing through the cytosol to bind to and open a ligand-gated Ca^{++} channel in the endoplasmic reticulum (or sarcoplasmic reticulum in muscle cells), leading to an increase in cytosolic Ca^{++}. Ca^{++} in the cytosol exerts its effects by binding to Ca^{++}-binding proteins such as calmodulin (**Figure 3**). EP2 and EP4

couple with Gs, which activates the enzyme adenylyl cyclase (AC**)** and catalyzes the formation of the second messenger cyclic AMP (cAMP) (**Figure 4**). An activated AC can generate many molecules of cAMP within the cell to amplify the signal. The major effect of cAMP is to bind to and activate protein kinase A (PKA; also known as cAMP-dependent kinase). PKA then phosphorylates target proteins in the cell. cAMP is rapidly broken down by phosphodiesterases, limiting the length of the signal. Additionally, in contrast to EP2, EP4 also stimulates non-canonical pathways phosphatidylinositol 3 kinase (PI3K)/protein kinase B (PKB, also known as Akt) promoting cell survival, and extracellular regulated kinase (ERK), promoting migration and proliferation. The phosphorylation of EP4 receptor recruits β-arrestin-1, which in turn activates c-Src to initiate the transactivation of the epidermal growth factor receptor (EGFR) and subsequent downstream signaling through phosphatidyl inositol 3-kinase (PI3K) and Akt [12] (**Figure 5).** The activation of this signaling cascade has been proposed to regulate the migration and metastasis of colorectal carcinomas [13]. EP3 receptor mediated signaling (**shown in Figure 6**) depends on the coupling with several G-protein isoforms. Most are coupled with Gi inhibiting cAMP-PKA; those coupled with Gs stimulate cAMP-PKA; those coupled with $G_{12/13}$ stimulate Rho family GTPases involved in cytoskeletal changes required for cellular migration**.** Exploitation of EP receptors as therapeutic targets by the development of selective agonists and antagonists has been elegantly reviewed [12, 13, 14]. Of them, the roles of EP4 receptor in health and disease have received much attention [15]. As documented below, COX-2 expressing breast cancers utilize the EP4 pathway for cancer cell survival and metastasis, making EP4 a targetable molecule for treating aggressive breast cancer patients.

4. COX2 /PGE2 mediated cancer progression.

Aberrant COX-2 expression promotes tumor initiation, progression and metastasis in most epithelial cancers [16]. This has been shown by over-expression [17] and down regulation [18] of the *COX-2* gene, and protective effects of the use of selective as well as non-selective COX-2 inhibitors from colorectal and mammary carcinogenesis [16, 19-23]. COX-2 overexpression is a phenotype shared by aggressive cancers of the colon [24], lungs [25], pharynx and larynx [26], pancreas [27], and the breast [28]. Elevated COX-2 expression noted in 40-50% of breast cancer, marks poor prognosis [29] resulting from the high levels of PGE2 in the tumor microenvironment.

4.1. Prostaglandin-inhibitors in cancer immunotherapy-- a historical perspective: We demonstrated that tumor or host macrophage -derived PGE2 in the tumor micro-environment inactivated host NK and T cells by two mechanisms: (a) an inhibition of production of IL-2 required for their activation into killer cells and (b) a down-regulation of IL2 receptors on host lymphocytes [30]. We exploited this information for devising cancer immunotherapy protocols. Chronic oral administration of indomethacin (a non-selective COX-1/COX-2 inhibitor) in mice, if started early during tumor growth, re-activated host lymphocytes and caused regression of transplanted murine mammary adenocarcinomas [31]. Chronic indomethacin therapy (CIT) also delayed the appearance and attenuated the growth and metastasis of spontaneous mammary adenocarcinomas in retired breeder C3H/HeJ female mice [32]. However, CIT alone was unable to cure advanced metastatic cancers, because of an inadequacy of endogenous IL-2 production required for killer cell activation. At this time, Dr Stephen Rosenberg's group at the surgery branch of the NCI, USA started IL-2 therapy in mice and later in humans. IL-2 therapy alone or in combination with IL-2 activated patients' lymphocytes (LAK cells) showed significant benefit in human melanoma and kidney cancer patients [33]. Our team found that IL-2 therapy alone was inadequate for optimal activation of killer cells in situ because of PGE2-mediated suppression of IL2 receptors. However, a combination of CIT with systemic IL-2 therapy was effective

in activating both NK and T cells *in situ* and curing a high proportion of mice bearing a variety of advanced syngeneic metastatic cancers, *e.g.* melanomas, mammary adenocarcinomas and fibro-sarcomas [34-36]. It was also effective in curing metastatic human melanomas grown in nude mice, which were NK cell competent [37]. Encouraged with these results, we tested this protocol in a single-center phase 2 human trial in advanced kidney cancer and melanoma patients with promising results [38-42]. However, high-dose systemic IL2 therapy soon became unpopular due to IL-2 mediated capillary leak syndrome (CLS), a major side effect leading to rapid fluid accumulation in tissues and serous cavities. We discovered that CLS resulted from IL2-mediated activation of inducible NO synthase (iNOS) leading to high Nitric Oxide (NO) production causing vascular leakage [43]. We showed that therapy with NOS inhibitors could ameliorate IL2 induced CLS in healthy mice [44] as well as in mammary tumor-bearing mice [45-47]. Interestingly, NOS inhibitors had anti-tumor effects on their own as well as in combination with IL2 [46-47]. This is because endogenous NO resulting from aberrant NOS activity in certain tumors including breast cancer, also promoted tumor progression and metastasis by stimulation tumor cell migration, invasiveness and tumor-associated angiogenesis [48,49,50]. The pro-migratory and pro-invasive role of tumor-derived NO was mediated by an elevation of cyclic GMP followed by activation of the PI3K and ERK pathways. However, a combination of IL2 with NOS inhibitors was never translated to the human, because NO was shown to have both anti-tumor and pro-tumor roles depending on the tumor genotype and NO concentrations in the tumor milieu [51, 52].

4.2. COX-2/PGE2 mediated breast cancer progression: role of EP4 receptor. Elevated COX-2 expression (noted in about half of breast cancer patients) signals poor prognosis [29]. We found that this is due to high endogenous PGE2 levels that promote breast cancer progression by multiple mechanisms: inactivation of host anti-tumor immune cells [30,31], enhanced cancer cell migration [53,54],

invasiveness [53,55], tumor-associated angiogenesis [30] due to upregulation of VEGF-A and tumor-associated lymphangiogenesis [56-59] due to upregulation of VEGF-C and -D. These events were primarily due to activation of the PGE2 receptor EP4 on tumor and host cells, as listed below. EP4 activity on tumor cells promoted tumor cell migration, invasion, angiogenesis and lymphangiogenesis [53, 54, 56-59]. We further observed that in COX-2 expressing breast cancer cells, under inductive conditions, endogenous PGE2 upregulated iNOS by activation of EP4 to promote invasive functions [55]. Others reported that EP4 on host NK cells [60, 61] and T cells [62] blocked their killer functions. We found that EP4 on tumor-associated macrophages [58] promoted their lymphangiogenic function by upregulating VEGF-C or –D. Similarly EP4 activation on host lymphatic endothelial cells (LEC) promoted lymhangiogenesis, resulting from stimulated LEC proliferation, migration and tube formation triggered by upregulation of VEGF-C or –D and VEGFR3 [59]. EP4 activation on host macrophages also promoted their immunosuppressor functions [63]. EP2/EP4 activity on dendritic cells blocked their antigen-presenting function [64]. Finally we discovered that COX-2/EP4 activities also induced and sustained stem-like cell (SLC) phenotype in breast cancer cells in a syngeneic murine breast cancer model [58] and human breast cancer cells [65]. Similar findings have been reported in a different murine breast cancer model [66]. SLCs are a minor subpopulation of cells within tumors, which have an unlimited self-renewal capacity [67, 68], and their activities regulated by the microenvironment, indicating that they have a plastic phenotype. They resist conventional chemo/radiation therapies, frequently leading to recurrence of primary or metastatic tumors, necessitating the search for SLC-specific markers and therapeutic targets [69, 70]. We suggest that EP4 is a highly suitable therapeutic target to eliminate SLCs, and therefore impact breast cancer metastasis. A schema of the cascade of cellular events in breast cancer progression and metastasis, which can be blocked with EP4 antagonists, is presented in **Figure 7**.

***4.3. Rationale for the choice of EP4 as a potential therapeutic target in breast cancer*:** While intake of COX-2 inhibitors can reduce breast cancer risk and morbidity [71-73], their reported cardiovascular side effects [74, 75] necessitated the search for alternative downstream target(s) that may spare vaso-protective functions of prostanoids. We suggest that EP4 represents as an ideal target for breast cancer to replace COX-2inhibitors for 3 reasons: (i) the primary roles of EP4 in COX-2 mediated breast cancer progression listed above. (ii) EP4 is relatively redundant for many physiological functions shared by EP2 via PKA stimulation [9,10,11] (iii) vasoprotective actions of prostanoids were shown to be mediated primarily by IP and EP3 receptors, suggested by findings in a variety of animal models of cardiac ischemia. For example, PGI2 has been reported as a cardio protective prostanoid, implicating IP-mediated action in hypoxia-induced pulmonary hypertension and intravascular thrombosis [76]. In support, using IP-/- and TP-/- mice, it was shown that IP but not TP receptor was cardio protective. PGI2, which was produced endogenously during cardiac ischemia/reperfusion, exerted a protective effect on cardiomyocytes independent of its effects on platelets and neutrophils [77]. Furthermore, PGE2 was shown to mediate cardio protective effects via EP3 receptor activation in myocardial ischemia models. Ischemic myocardial injury was attenuated in transgenic mice with cardio-specific overexpression of the EP3 receptor [78]. In support, structurally diverse EP3 agonists could reduce myocardial infarct size in rats. The therapeutic effect was mediated by PKC activation and opening of KATP (ATP-sensitive K) channels [79]. However one study also implicated EP4 receptor. An EP4-selective agonist EP4RAG attenuated myocardial dysfunction after infarction and reduced infarction size in a rat myocardial ischemia/reperfusion injury model. The effects of the EP4 agonist appeared to be indirect by suppressing monocyte chemo-attractant protein-1 (MCP-1) and the infiltration of inflammatory cells, especially macrophages [80]. While no human data are available on whether EP4 antagonists can be cause cardiovascular toxicity, an EP4 antagonist AAT-007 used in phase 1/2 trials in

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> 800 human arthritis patients was well tolerated in pharmacologically effective doses (300 mg orally twice daily) with no evidence of dose-limiting toxicity (Dr Yukinori Take, Ask/At, Japan, personal communication).

4.4. Functional roles of COX-2 in the absence or presence of HER-2 in breast cancer: Human epidermal growth factor receptor (HER) 2 expressed by approximately 20 % breast cancer patients is another major driver of breast cancer progression. HER-2 is often co-expressed with COX-2 in human breast cancer [81], although the reverse in not true. Interestingly, most HER-2 actions e.g., upregulation of aromatase [82], angiogenesis [83], lymphangiogenesis [81] and anti-apoptotic action [84] were shown to be intermediated by COX-2. To define the functional roles of COX-2 in the absence or presence of HER-2, we stably transfected *COX-2* gene into MCF-7 (COX-2-, HER-2-) and SKBR-3 (COX-2-, HER-2-high) human breast cancer cell lines [63]. Ectopic COX-2 over-expression in MCF-7 and SKBR-3 cell lines resulted in: increased migration/invasion/proliferation, epithelial-mesenchymal transition (EMT), elevated SLCs (spheroid formatting ability *in vitro*), increased ALDH activity- a recognized SLC marker [85] and co-localization of COX-2 with numerous SLC markers (ALDH1A, CD44, β-Catenin, NANOG, OCT3/4 and SOX-2) in spheroids. These changes were reversed with COX-2-inhibitor or EP4-antagonists (EP4A), indicating dependence on COX-2/EP4 activities. COX-2 over-expression or EP4-agonist treatments of COX-2-low cells caused up-regulation of stem cell related NOTCH/WNT genes, blocked with PI3K/AKT inhibitors. NOTCH/WNT inhibitors also blocked COX-2/EP4 induced SLC induction. Microarray analysis showed an up-regulation of numerous SLC-regulatory and EMT-associated genes. MCF-7-COX-2 cells showed increased mammary tumorigenicity and spontaneous multi-organ metastases in NOD/SCID/IL-2Rϒ-null mice for successive generations with limiting cell inocula, a rigorous test for testing SLC in vivo [86]. Conversely, lung colonization was abrogated with EP4 knockdown or EP4 antagonist treatment of the cells. Orthotopic mammary tumors grown with

MCF-7-COX-2 cells (as compared to control cells) showed up-regulation of angiogenic /lymphangiogenic factors VEGF-A/C/D, Vimentin (mesenchymal marker) and phospho-AKT (an EP4 signaling marker), down-regulation of epithelial marker E-Cadherin and an enrichment of SLC marker positive and spheroid forming cells. Findings in primary human breast cancer tissues were supportive of the findings in mice as noted above. Expression of *COX-2, EP4* and *ALDH1A* mRNA in these tissues were highly correlated with one other, more marked in progressive stage of disease. *In situ* immunostaining of the tissues revealed co-localization of SLC markers with COX-2, supporting SLC induction by COX-2. Finally, high *COX-2*/*EP4* mRNA expression was linked with reduced survival. These preclinical and clinical data strongly suggest that EP4 represents a novel therapeutic target to inhibit tumor growth, metastasis and eradicate SLCs in human breast cancer [63]. This contention was fully validated by us in mouse breast cancer models [57, 58] with two EP4 antagonists (ONO-AE3-208, ONO pharma, Japan; and RQ-15986, currently renamed as AAT 007, Ask/At Pharma, Japan). Treating mice bearing syngeneic COX-2 expressing, highly metastatic C3L5 mammary tumors with EP4A at non- toxic doses inhibited tumor growth, spontaneous metastasis and eradicated SLCs in residual tumors [57, 58]. This finding has been duplicated in another murine breast cancer model [66] with AAT 007. Similarly therapeutic efficacy of ONO-AE3-208 was reported in a castration-resistant prostate cancer model [87].

4.5. SLC-linked microRNAs induced by COX-2/EP4 activity as breast cancer biomarkers: There are very few reliable blood biomarkers for breast cancer that are useful to monitor the disease. Levels of specific miRNAs in blood plasma remain as a newer family of cancer biomarkers. miRNAs are single stranded non-coding RNAs (20-24 nucleotides) that down-regulate specific genes at the post-transcriptional level. There are 1881 sequences in the human miRNA registry [88], some proposed as cancer biomarkers [89, 90] which can be detected in body fluids [91] due to exosome-mediated release

and transit in the blood. Recently, levels of a panel of 7 candidate miRNAs were measured in tissue and blood specimens of 148 patients with minimally invasive breast cancer and 44 age-matched and disease free control individuals [92]. The authors found increased levels of blood miR-195 in breast cancer patients, which decreased to control levels following curative tumor resection. The circulating miRNAs correlated with certain clinic-pathological variables, namely nodal status and estrogen receptor status. We conducted differential gene and miRNA expression micro arrays using control MCF-7-Mock-tranfected vs. MCF-7-COX-2 transfected cell lines, which identified downregulation of six miRNAs and an upregulation of two miRNAs (miR-655 and miR-526b) by COX-2. Both COX-2 upregulated miRNAs were also inducible by EP4 activation by exposing MCF-7 cells to selective EP4 agonists. Both miRNAs were shown to be highly oncogenic and SLC-linked [93, 94]. Expression of both miRNAs positively correlated with COX-2 in genetically disparate breast cancer cell lines and increased in all cell lines when grown as spheroids. Spheroid assay is a vitro surrogate for measuring self-renewal of stem-like cells (SLC), indicating the link of both miRNAs with SLC activity. Ectopic miR-526b or miR-655 over-expression in MCF7 and SKBR3 cells resulted in increased proliferation, migration, invasion, spheroid formation and Epithelial to Mesenchymal transition (EMT). Conversely, knocking down either miRNA in aggressive MCF7-COX-2 and SKBR3-COX-2 cells reverted these phenotypes. MCF7-miR526b and MCF7-miR655 cells displayed upregulated *NOTCH*/ *WNT* genes; both pathway inhibitors abrogated miRNA-induced spheroid formation, linking both miRNAs with SLC-related pathways. Expression of both miRNAs was dependent on EP4 activity and EP4 downstream signaling pathways PI3K/AKT, ERK and NF-kB. Interestingly, while both miRNAs were upregulated in ectopic COX-2 expressing cells, ectopic miRNA overexpressing cells also upregulated COX-2. We suggest that this is due to targeting of NF-κB repressor genes by both miRNAs. These results indicate a positive feedback loop for COX-2/EP4/NF-κB/miRNA/COX-2-mediated SLC perpetuation (93, 94).

MiR-655 expression also led to TGFβ resistance for Smad3 phosphorylation [94]. Tail vein injection of ectopic miR-526b or miR-655 over-expressing MCF7 and SKBR3 cells into NOD/SCID/GUSB-null mice revealed increased lung colony growth and micro-metastases to other organs. Expression of both miRNAs was strongly correlated with each other in human breast cancer tissues, was higher than in non-tumor tissues, and associated with reduced patient survival [93, 94]. Thus they could serve as prognostic breast cancer biomarkers for monitoring SLC-reduction during therapies. In support, our preliminary data reveal that miR-655 levels are significantly higher in the plasma of breast cancer bearing than in patients with benign tumors (unpublished; manuscript in preparation). In summary, we found that aberrant COX-2 activity in human breast cancer leads to tumor progression and metastasis by utilizing multiple signaling pathways in which EP4 activation plays a pivotal role, and two COX-2/EP4 upregulated miRNAs are important partners (schema presented in **Figure 8**).

4.6. Triple negative breast cancers (TNBC) are mostly COX2 expressing: TNBC represents the most deadly type of breast cancer, which resist cytotoxic therapies. In an earlier study [81], designed to identify the roles of COX-2 and HER2 in VEGF-C expression and lymphangiogenesis, we used 65 human breast cancer tissue samples and multiple human breast cancer cell line genetically manipulated for COX-2 and HER2 expression. We concluded that COX-2 was a primary driver of lymphangiogenesis, and the role of HER2, if any, was intermediated by COX-2. Interestingly most HER-2 expressing tumors identified by immunohistology were also COX-2 positive. In addition, all of the 23 tumors identified as TNBC in this study also expressed COX-2 (unpublished). These findings have been fully validated by another laboratory [95] in 35 primary TNBC showing that COX-2 is over-expressed in these tumors ($p < 0.009$). Since we found that most COX-2 mediated mechanisms in breast cancer progression result from EP4 activation, we suggest that TNBCs will respond to EP4 antagonist (EP4A) therapy and miR655 could be used as a plasma biomarker for therapeutic monitoring in TNBC

patients. Our future goal is to use EP4A as an adjunct in metastatic TNBC patients. However it is currently unknown whether EP4A as a single agent will provide any benefit, as observed in our syngeneic murine breast cancer models [57, 58]. We suggest that a combination therapy with other agents such as immune checkpoint inhibitors holds a greater promise.

4.7. Proposed combination of an EP4 antagonist with an immune checkpoint inhibitor for treating TNBC. Programmed cell death (PD)-1 is a checkpoint protein on T cells that normally acts as an "off switch" preventing them from attacking other cells in the body. This is mediated by binding of PD-1 to its ligand PD-L1 produced by other cells. Some cancer cells produce large amounts of PD-L1, which helps them evade immune attack by T cells even if they can recognize tumor-associated antigen. This appears to be a defense mechanism hijacked by many solid tumors, leading to a recent renewal of interest in immunotherapy with immune checkpoint (PD-1, PD-L1) inhibitors. They have shown promise in multiple solid tumors in the human [96-99]. A recent study [100] reported a heterogeneous PD-L1 expression in primary breast cancer tissues, generally associated with the presence of tumor-infiltrating lymphocytes and poor-prognostic features such as high grade, and aggressive molecular subtypes (TNBC, basal, HER2 +). Early phase clinical trials using PD-1 or PD-L1 inhibitors alone or in combination revealed objective tumor responses and durable long-term disease control, in heavily pre-treated patients, notably in the TNBC [100]. We believe that a combination therapy using a PD-1 or PD-L1 inhibitor with an EP4 antagonist will improve the therapeutic efficacy of either drug. As summarized earlier, EP4 antagonists abrogate multiple mechanisms in breast cancer progression by binding to EP4 on multiple cell classes -- tumor cells, host immune cells and endothelial cells. EP4 on tumor cells promote tumor cell migration, invasiveness, EMT, stem cell activity, angiogenesis (by upregulating VEGF-A) and lymphangiogenesis (by upregulating VEGF-C/D); EP4 on lymphatic endothelial cells promote lymphangiogenesis by upregulating VEGF-C/VEGFR3. Furthermore, PGE2 meditated

inactivation of host antitumor immunity was shown to be due to EP4 binding on multiple immune cell classes: NK cells, T cells, macrophages, and dendritic cells. EP4 antagonists were shown to be highly effective in abrogating all these events in animal models leading to tumor cell killing [57, 58, 60]. As outlined earlier, immune check point inhibitors work via different non-overlapping mechanisms. Thus it is expected that a combination of the two should cast the net far wider to block multiple tumor and host cell mediated pathways, leading to a synergistic action. Indeed a synergistic action on tumor regression and animal survival was shown with an EP4 antagonist in combination of either of two checkpoint inhibitors, anti-CTLA4 and anti-PD-1 antibodies, in murine colon and breast cancer models [101].

4.8. EP4 antagonist in the breast cancer clinic. Recently a phase 2 human trial with the EP4 antagonist AAT 007 (AskAt, Japan) was registered by Dr Martin Edleman at the University of Maryland (currently moved to the FOX Chase Cancer Centre) in advanced solid tumors including prostate, breast or non-small cell lung cancer (Clinical Trials.gov Identifier: NCT02538432, last update posted on June 6, 2017). The trial will test (a) whether the administration of the study drug AAT 007 can decrease circulating tumor cells or myeloid-derived suppressor cells; and (b) whether the drug may improve outcome on its own in these solid tumors or when combined with a cytotoxic drug gemcitabine in patients with prostate or lung cancer, if the disease worsened with AAT 007 alone. No patient registration or outcome has yet been reported.

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MM and PN: Performed some of the cited studies and wrote parts of the Review**;** **AO and KU :** Prepared Figures; **PKL:** Wrote and finalized the Review.

Conflict of interest statement:

The authors declare no conflict of interest.

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Figure legends:

Figure 1. The pathway for the synthesis of prostaglandins, their respective receptors and signaling. (Adapted from Markovicˇ T et al 2017; ref 14). Arachidonic acid acts as the substrate for COX-1 and COX-2 to produce Prostagandins PGE2, Thromboxane A2, PGI2, PGF2α and PGD2, all of which exert functions by binding to their respective receptors.

Figure 2. Heterotrimeric G protein activation and inactivation cycle. The activation occurs by conversion of Gα-coupled GDP to GTP. The activated G-protein then dissociates into an α and

a β/ϒ complex. GTP bound Gα is active. Intrinsic GTPase activity leads to inactivation of the G-Protein. GDP bound Gα re-associates with a β/ϒ complex to form the inactive G-protein that can again associate with a receptor.

Figure 3. EP1-mediated signaling events**.** EP1 couples with G_q, activating PLC that cleaves PIP2, to generate second messengers, IP_3 and diacylglycerol (DAG). IP_3 binds to and opens a ligand-gated Ca^{++} channel in the endoplasmic reticulum leading to an increase in cytosolic Ca^{++}. Ca^{++} in the cytosol exerts its effects by binding to Ca^{++}-binding proteins.

Figure 4. Shared pathway of EP2/EP4 mediated Signaling. There is activation of adenylyl cyclase (AC) leading to a rise in the second messenger cAMP in the cytosol that activates Protein kinase A (PKA). PKA in turn activates a transcription factor CREB (cAMP response element-binding protein).

Figure 5. EP4 mediated signaling (in addition to PKA activation) not shared by EP2. (Adapted from Callaghan and Houston, 2015; reference 12) There is non-canonical activation of the PI3K-Akt and ERK pathways. Akt, also called protein kinase B (PKB) promotes cell survival by activating the transcription factor NF-κB. ERK is primarily a promoter of cell proliferation and migration. Cell proliferation depends on ERK mediated activation of the transcription factor EGR-1

Figure 6. EP3 mediated signaling (adapted from Callaghan and Houston, 2015; reference 12). EP3 has multiple isoforms, most of which are coupled with the inhibitory G protein Gi that acts by inhibiting AC-cAMP-PKA pathway. Those coupled with Gs stimulate AC-cAMP-PKA pathway. Those coupled with $G_{12/13}$ are involved in Rho family GTPase signaling utilized in cell migration by cytoskeleton remodeling.

Figure 7. Schema of cellular events in tumor progression and metastasis. Primary tumor growth depends on proliferation of tumorigenic cells, some of which adopt a stem-like cell (SLC) phenotype under the influence of genetic and epigenetic (micro-environmental) mechanisms. Local tumor growth is dependent on angiogenesis (formation of new blood vessels), which also facilitates tumor cell egress into the circulation. In addition, many epithelial tumors undergo intra-tumoral and/or peri-tumoral lymphangiogenesis (formation of new lymphatic vessels) that helps tumor cells to migrate to lymph nodes and then enter circulation. Epithelial-mesenchymal transition (EMT) is a phenotypic change in epithelial tumor cells utilized for invasion and migration out of the local confines. These cellular events are stimulated in COX-2 expressing breast tumors by activation of EP4 on tumor cells and tumor-associated host cells (immune cells, endothelial cells), so that EP4 presents as a therapeutic target to block multiple cellular events in tumor progression.

Figure 8. Schema of EP4 mediated signaling pathways in COX-2 expressing breast cancer. Aberrant COX- 2 activity leads to tumor progression and metastasis by utilizing multiple signaling pathways in which EP4 activation plays a pivotal role, and two COX-2/EP4 upregulated miRNAs (miR526b and miR655) are important partners. EP4 activation (like EP2) results in cAMP-dependent PKA activation leading to phosphorylation of the transcription factor CREB. PKA also upregulates WNT/β-catenin and NOTCH pathways by inhibiting GSK3. Furthermore, unlike EP2, EP4 also utilizes the non-canonical PI3K/AkT and ERK signaling pathways, respectively promoting cell survival and migration/proliferation. COX-2 upregulates the miRNAs miR526b and miR655 via EP4 mediated

PI3K/Akt activation and WNT/β-catenin / NOTCH pathways. While COX-2 induces these miRNAs, the miRNAs, in turn, upregulated COX-2. We suggest that these occurs via upregulation of NF-κB, a well-known upregulator of COX-2 under certain conditions. Predicted targets of these miRNAs include NF-κB repressor genes. Thus there appears to exist a positive feedback loop for COX-2/EP4/NF-κB/miRNA/COX-2-mediated SLC perpetuation.

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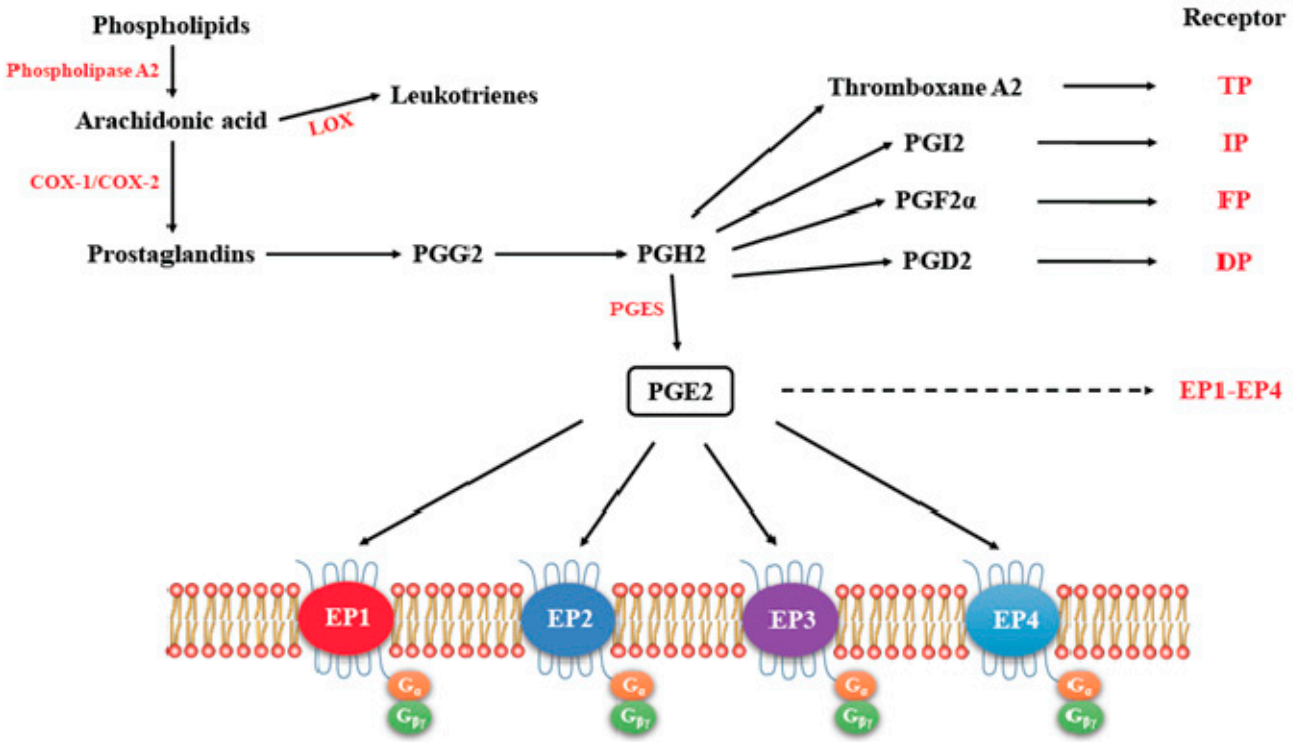


Figure 1.

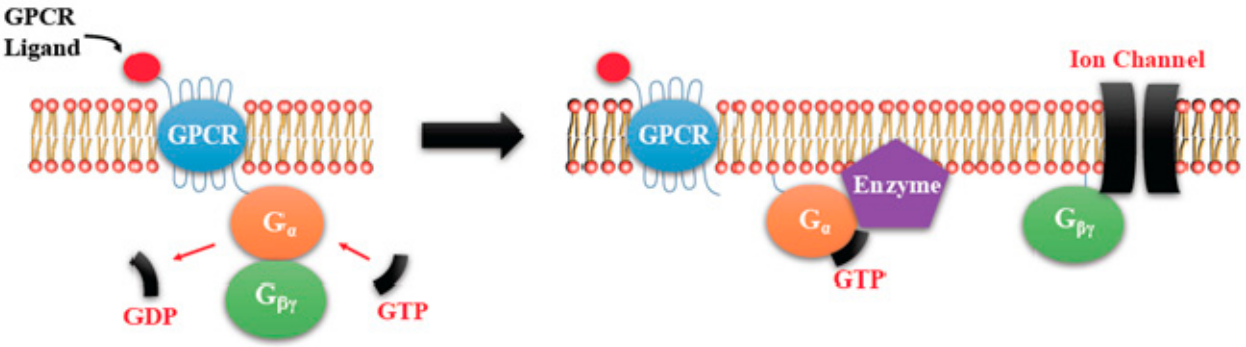
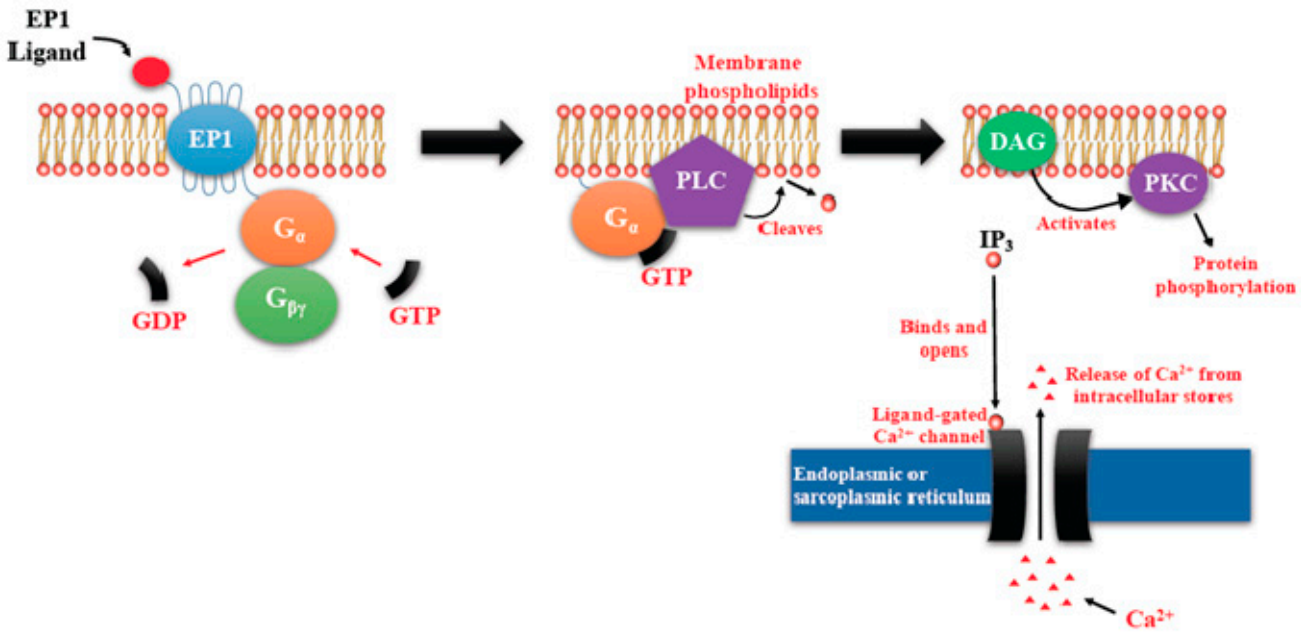


Figure 2



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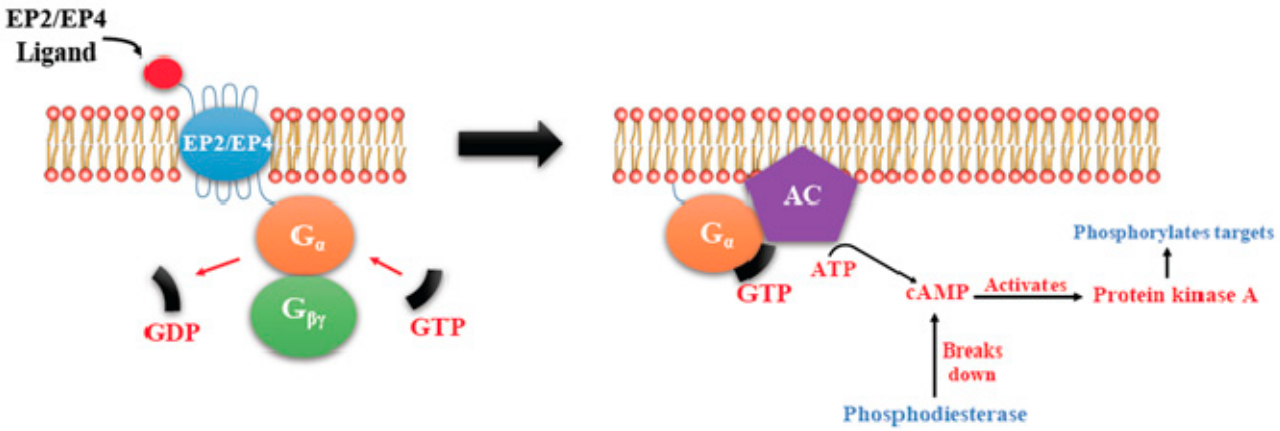

Figure 4

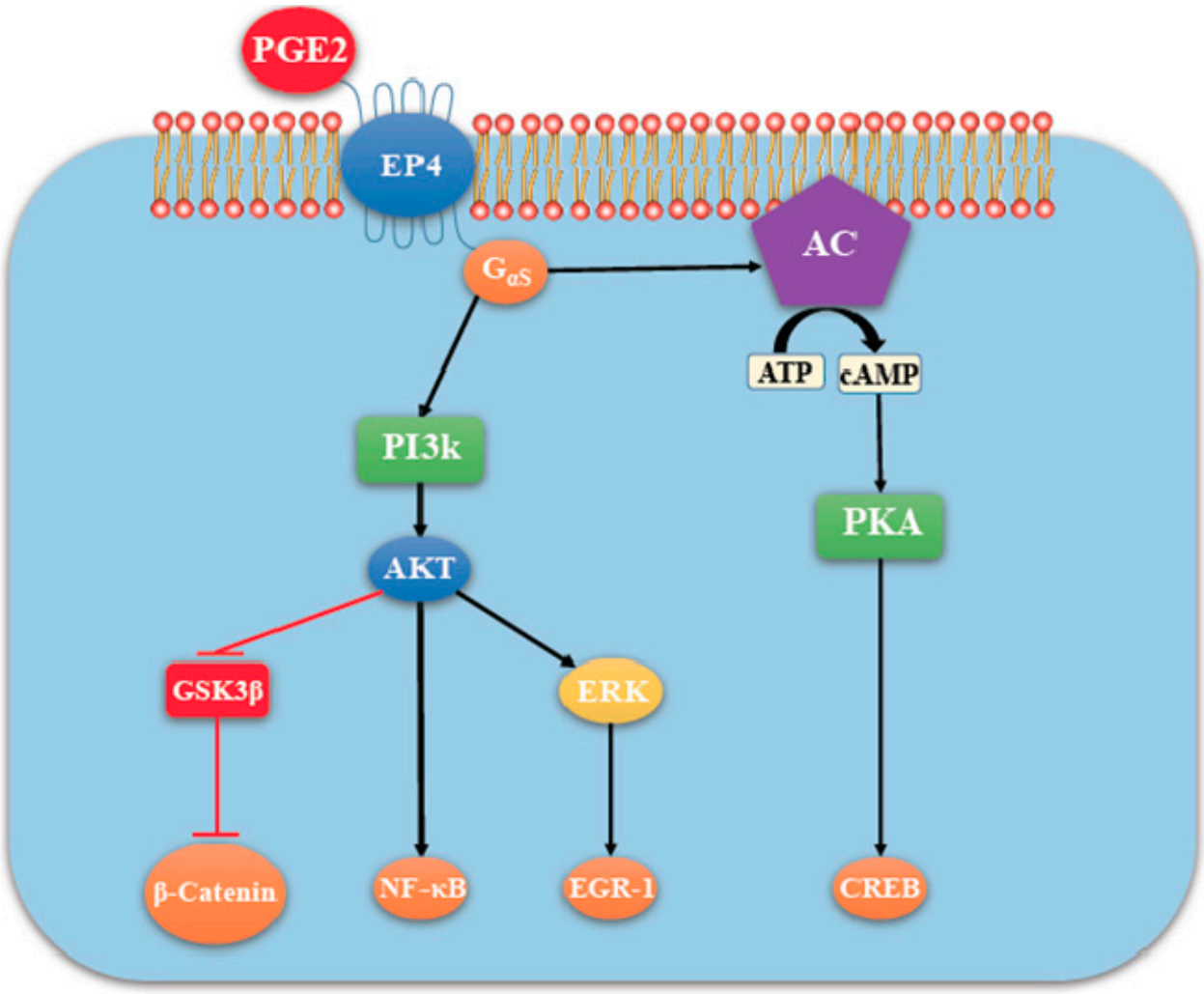

Figure 5

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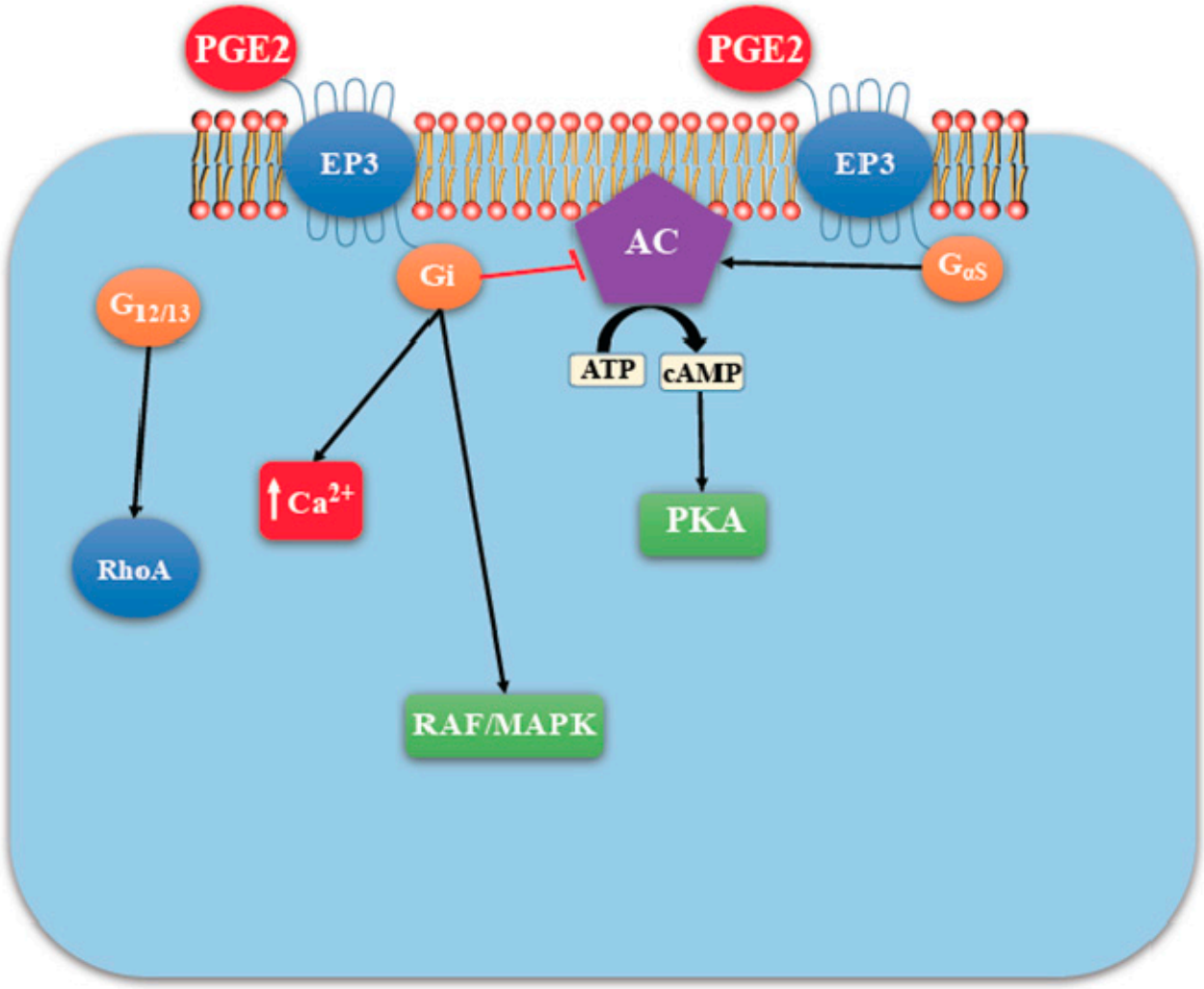

Figure 6

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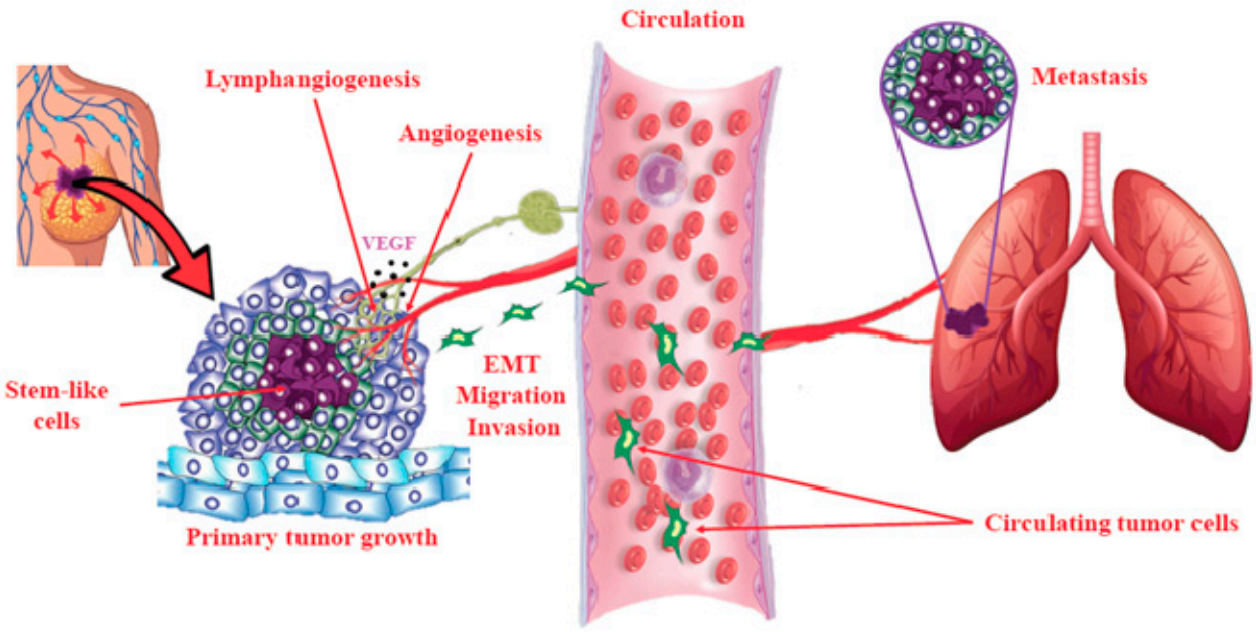

Figure 7

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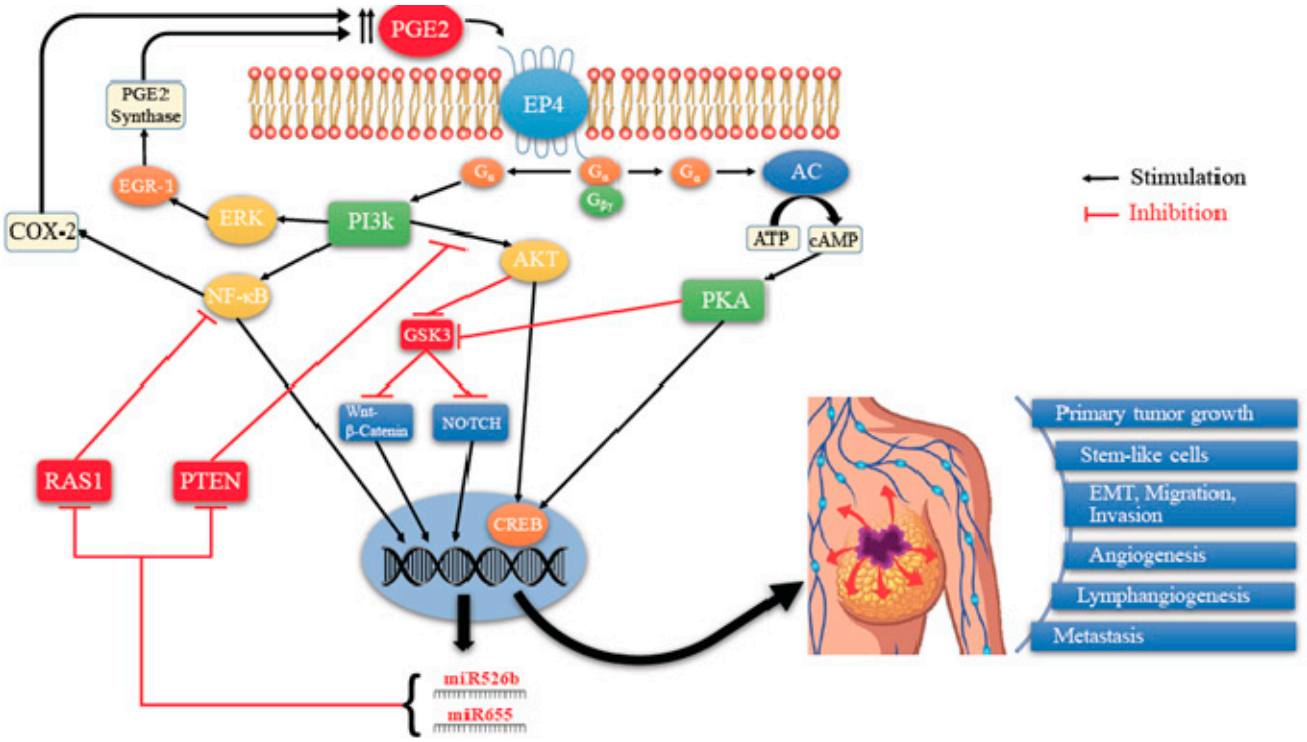

Figure 8