

Prostaglandin E₂ EP receptors as therapeutic targets in breast cancer

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Abstract Prostaglandins are lipid compounds that mediate many physiological effects. Prostaglandin E₂ (PGE₂) is the most abundant prostanoid in the human body, and synthesis of PGE₂ is driven by cyclooxygenase enzymes including COX-2. Both elevated expression of COX-2 and increased PGE₂ levels have been associated with many cancers including breast cancer. PGE₂ exerts its effect by binding to the E series of prostaglandin receptors (EP) which are G protein-coupled receptors. Four EP receptor subtypes exist, EP1–4, and each is coupled to different intracellular signaling pathways. As downstream effectors of the COX-2 pathway, EP receptors have been shown to play a role in breast and other malignancies and in cancer metastasis. The role of each EP receptor in malignant behavior is complex and involves the interplay of EP receptor signaling on the tumor cell, on stromal cells, and on host immune effector cells. While preclinical and epidemiological data support the use of nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors (COXibs) for the prevention and treatment of malignancy, toxicities due to COXibs as well as less than promising results from clinical trials have laboratories seeking alternative targets. As knowledge concerning the role of EP receptors in cancer grows, so does the potential for exploiting

EP receptors as therapeutic targets for the treatment or prevention of cancer and cancer metastasis.

Keywords Prostaglandin · Cyclooxygenase · Cancer · EP receptors

1 Introduction

Eicosanoids, which include prostaglandins and leukotrienes, are potent lipid mediators that have been connected to many pathological processes such as inflammation and cancer [1, 2]. Prostaglandin E₂ (PGE₂) is the most abundant prostanoid in the human body and exhibits the most versatile actions ranging from reproduction to neuronal, metabolic, and immune functions [1, 3]. Prostaglandin synthesis is driven by cyclooxygenases (COX) which exist in three isoforms: constitutively expressed COX-1, inducible COX-2 and COX-3, the latter is a splice variant of COX-1 [1]. COX-2 is normally absent from most cells; however, its expression can be induced by cytokines and growth factors, and it is involved in the regulation of inflammatory responses. Furthermore, COX-2 can be highly induced during tumor progression. Overexpression of COX-2 is detected in premalignant and malignant tissues and tumor cell lines including but not limited to breast, colon, biliary, skin, lung, and liver [4, 5]. PGE₂ has been implicated in various tumorigenic processes as well along with the involvement of specific PGE₂ receptors [1, 2, 6].

2 Eicosanoid biosynthesis pathway and cyclooxygenases

Eicosanoid biosynthesis begins with the mobilization of arachidonic acid (AA) from the plasma membrane by phospholipase A₂ (PLA₂) and, once free, COX enzymes

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convert AA to the precursor molecule prostaglandin H₂ (PGH₂). PGH₂ can then be converted to one of five primary prostanoids prostaglandin D₂, prostaglandin E₂, prostaglandin F_{2α}, prostaglandin I₂, and thromboxane A₂ through specific synthase molecules PGDS, PGES, PGFS, PGIS, and TXAS, respectively [2, 7, 8]. There are two classifications of PGES: cytosolic (cPGES) and microsomal or membrane-bound (mPGES). cPGES is predominantly coupled to COX-1, and mPGES is preferentially linked to COX-2 and exists in two isoforms, mPGES-1 and mPGES-2 [2, 7, 9]. The expression of mPGES-1 can be induced by proinflammatory signals, similar to COX-2, and mPGES-1 is the synthase that is primarily responsible for increasing the PGE₂ levels during inflammation and tumorigenesis [9]. Once PGE₂ is produced, it is exported into the extracellular microenvironment by a specific multidrug resistance-associated protein (MRP), MRP4, where PGE₂ then exerts its biological effects in an autocrine or paracrine manner through binding to its cognate cell surface receptors, the E series of prostaglandin receptors (EP) (Fig. 1). After binding its receptor, PGE₂ is metabolized in a two-step process in which the prostaglandin is transported into the cytoplasm through a passive mechanism or actively by prostaglandin transporter followed by inactivation by 15-hydroxyprostaglandin dehydrogenase [1, 7].

3 The role of COX-2 in breast cancer

Epidemiological data demonstrate a strong correlation between chronic inflammation and developing cancer. COX-2 can be rapidly induced by mitogens and proinflammatory cytokines, is an early response gene, and is an important component of the inflammatory response linked to carcinogenesis [4, 10]. COX-2 expression is not detectable in most healthy tissues while upregulation of COX-2 has been identified in many human cancers and precancerous lesions [11]. Initially recognized in the context of colorectal cancer, COX-2 has been shown, both experimentally and epidemiologically, to be involved in mammary carcinogenesis [11–13]. Experimentally COX-2 protein is present in rat mammary tumors induced by various carcinogens [12]. In addition, more than 85% of transgenic mice overexpressing COX-2 in mammary tissue, through the use of the mouse mammary tumor virus (MMTV), developed mammary tumors [14, 15]. This finding indicates that COX-2 overexpression alone is sufficient to cause breast carcinoma.

Clinical data also broadly support a protumorigenic role for COX enzymes in breast cancer. Elevated COX-2 protein levels have been detected immunohistochemically in approximately 40% of invasive breast carcinoma (as

reviewed in [11]). Increased expression of COX-2 is more common in breast cancers with poor prognostic characteristics and is associated with an unfavorable outcome as well as worse survival independent of known prognostic factors [13, 16]. Specifically, overexpression of COX-2 was associated with large tumor size, high histological grade, negative hormone receptor status, high proliferation rate, ductal type histology, high p53 expression, HER-2 oncogene amplification, and axillary node involvement [13]. Progression-free survival may be better in patients with breast tumors that convert from COX-2 positive to COX-2 negative through treatment with neoadjuvant chemotherapy since reduction in COX-2 expression was mainly observed in clinical responders [17].

COX-2 has also been associated with breast cancer metastasis. Expression of COX-2 is correlated with the presence of lymph node metastases and distant metastasis [12, 18]. Likewise, COX-2 expression is positively correlated with tumorigenic and metastatic potential in a murine model of breast cancer [5, 19, 20]. Gene expression analyses and mouse model systems have also associated COX-2 with breast cancer metastasis to the lung, bone, and brain [14, 21, 22]. COX-2 overexpression increases motility and invasion of breast cancer cells [23]. Migration is a key functional activity of cancer cells and is associated with their metastatic potential. Silencing of COX-2 inhibits migration of human breast cancer cell line MDA-MB-231 *in vitro* and metastasis *in vivo* [24].

4 Nonsteroidal anti-inflammatory drugs and cancer

The involvement of COX-2 in tumorigenesis was revealed by complementary observations pertaining to colorectal cancer. Multiple epidemiological studies reported an inverse correlation between colon cancer incidence and regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin [12, 25–28]. NSAIDs are known to function by inhibiting cyclooxygenase enzyme activity; therefore, these observations suggested that aberrant prostaglandin biosynthesis may contribute to colorectal cancer. In addition, overexpression of COX-2 was detected in precancerous adenomas and colon carcinomas [12, 29–32]. These data suggest that COX-2 could be a useful target for chemoprevention providing impetus for clinical trials exploring the effect of NSAIDs, which inhibit COX-1 and COX-2, on colon cancer. The results from these clinical trials showed that COX inhibitors could decrease the size and number of polyps, but adverse side effects from the use of NSAIDs including peptic ulcer disease occurred [12].

Like colon cancer, experimental breast cancer can be suppressed by inhibiting COX activity with either NSAIDs

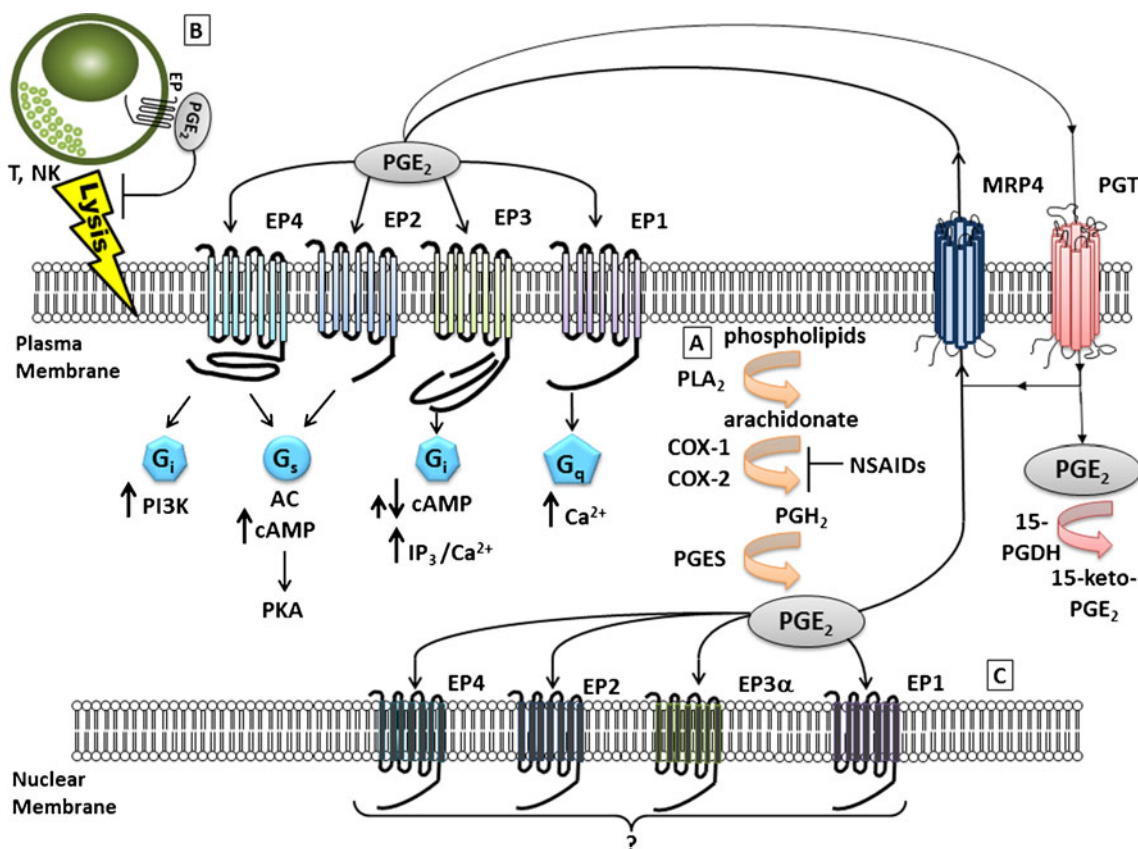


Fig. 1 Eicosanoid biosynthesis and EP receptor signaling pathway. *A* phospholipids from the plasma membrane are mobilized and converted to arachidonic acid by phospholipase A2 (*PLA*₂). COX enzymes convert AA to prostaglandin H2 (*PGH*₂) precursor molecule which is then converted to prostaglandin E2 (*PGE*₂) by the synthase molecule PGES. Once produced *PGE*₂ can exert its effects in one of two ways: (1) *PGE*₂ can be exported into the extracellular microenvironment by multidrug resistance-associated protein four (*MRP4*) where *PGE*₂ can bind to its cognate receptors, the E series of prostaglandin receptors (*EP*) on the plasma membrane of a tumor cell, stromal cell, or immune effector cell such as a T or natural killer (*NK*) cell and (2) after being synthesized by PGES, *PGE*₂ can directly act on EP receptors located on the nuclear membrane. After binding its receptor, *PGE*₂ can be transported back into the cytoplasm through a passive mechanism or actively through a prostaglandin transporter (*PGT*). *PGE*₂ is inactivated by 15-hydroxyprostaglandin dehydrogenase (*15-PGDH*) and converted to 15-keto-*PGE*₂. *B* EP receptors are G protein-coupled receptors of which four subtypes exist: EP1, EP2, EP3 and EP4. Each receptor is coupled to a different intracellular signaling pathway. The EP2 and EP4 receptors are linked to stimulation of cyclic AMP (*cAMP*) and protein kinase A (*PKA*)

signaling through sequential activation of G α s and adenylate cyclase (*AC*). EP4 can also activate phosphoinositide-3-kinase (*PI3K*) through G α i. The EP1 receptor leads to elevation of intracellular calcium through G α q. EP3 exists in multiple isoforms which are generated through alternative splicing and differing at their carboxy terminal tail. These isoforms are capable of eliciting different intracellular responses through multiple signal transduction pathways. The majority of the isoforms act to inhibit cAMP generation via G α i. Ligand binding can also lead to an increase in IP₃/intracellular calcium. Another isoform can act to stimulate adenylyl cyclase leading to an increase in cAMP. On immune effector cells, *PGE*₂ acting through the EP receptors can modulate the function of various immune effector cells. *PGE*₂ acting through its cognate receptor can inhibit NK cell activity and cytotoxic T-cell proliferation leading to a decrease in target cell lysis. There is now growing evidence to support perinuclear and/or nuclear localization of functional EP receptors. To date EP1, EP2, EP3 α , and EP4 have been shown to be colocalized at the nuclear membranes of a variety of cell types and tissues. Nuclear EP receptors could exert different effects from their plasma membrane counterparts; however, the signaling pathways for nuclear receptors have yet to be determined.

or selective COX-2 inhibitors (COXibs) [11, 12, 20, 33]. The suppression of mammary tumor formation by COXibs applies to both chemically induced breast tumors, which tend to be hormone-dependent as well as hormone-independent models [11]. COX-2 expression is associated with breast cancer metastasis. Antagonism of COX-2 decreases breast cancer cell invasion and motility [34]. COX-1 or COX-2 inhibition decreases metastatic disease in a murine mammary carcinoma model

system [20]. In a murine model of metastatic breast cancer, inhibition of COX-2 reduces the frequency of pulmonary metastases [12, 20, 35].

While the results from animal studies demonstrating the chemopreventative or chemotherapeutic efficacy of COXibs are promising, epidemiologic studies correlating breast cancer risk and NSAID use have been inconsistent. Several studies have reported reduced breast cancer incidence in association with NSAID use [11, 36–42]. Most recently,

Holmes *et al.* reported that aspirin use was associated with a decreased risk of distant recurrence and breast cancer death [41]. Meta-analyses of either NSAID or aspirin use have found a 9% to 30% reduced risk of breast cancer incidence [41, 43–46]. Additionally, patients who take COX-2 inhibitors had a reduced risk of bone metastasis [47]. Conversely, multiple studies have shown no protective association between NSAID use and breast cancer incidence [48–51]. Various factors can contribute to the inconsistencies between these studies including differences in the quality and completeness of data on NSAID use.

Despite inconsistencies in the results of the epidemiological studies, there is evidence that COXibs could reduce the incidence of both familial and sporadic malignant disease, indicating that COX and prostaglandin signaling is a viable target; however, some clinical trials identified increased cardiovascular risk associated with COXib use [11, 52, 53]. The cardiovascular toxicity of COXibs has been attributed to their selective depression of prostacyclin (PGI₂) levels which have a cardioprotective function [11, 54]. The cardiovascular toxicity of COXibs decreases the desirability of using this class of drugs in cancer prevention; therefore, alternative components of the COX signaling pathway that would offer a safer target need to be identified.

5 Functioning of G protein-coupled receptors in the cell

Prostaglandin cell surface receptors belong to the G protein-coupled receptor (GPCR) family. GPCRs have seven transmembrane-spanning α helices, an extracellular N terminus, an intracellular C terminus, and three interhelical loops on each side of the membrane. These receptors are coupled to heterotrimeric G proteins which initiate various intracellular signaling cascades in response to GPCR activation by extracellular stimuli [55]. Heterotrimeric G proteins are composed of three subunits, α , β , and γ and in their inactive state exist as a G $\beta\gamma$ monomer and a guanine diphosphate-bound G α subunit. Heterotrimers are divided into four families based on α subunit sequence identity and signaling activity: G α_s , G α_i , G $\alpha_q/11$, and G $\alpha_{12/13}$. Following ligand activation, GPCRs catalyze the exchange of GDP for GTP on the G α subunit, leading to decreased affinity of G α for G $\beta\gamma$. The resulting dissociation of the heterotrimer allows the GTP-bound G α and free G $\beta\gamma$ to interact with several downstream effectors (Table 1) [55, 56].

6 The E series of prostaglandin receptors

PGE₂ can bind any of four of the E series of prostaglandin EP receptor subtypes specified as EP1, EP2, EP3, and EP4

Table 1 G protein subunit effectors [56]

Subunit	Effectors
G α_s	Adenylyl cyclase, increase cAMP, PKA
G α_i	PI3K, adenylyl cyclase, decrease cAMP
G α_q	PLC β , increase calcium, PKC, rho GTPases
G α_{12}	Rho GTPases
G $\beta\gamma$	PI3K, PLC β , ion channels

Conformational change initiated by ligand binding of the G protein-coupled receptor results in dissociation of the G α subunit from the G $\beta\gamma$ subunit which allows for the separate subunits to activate downstream signaling events.

cAMP cyclic adenosine monophosphate, *PKA* protein kinase A, *PI3K* phosphoinositide-3-kinase, *PLC β* Phospholipase C beta, *rho GTPases* rho guanosine triphosphatases.

[2, 3, 7, 9, 57]. Each receptor has distinct biochemical properties, tissue and cellular localization and is coupled to different intracellular signaling pathways [3]. In addition to the different EP subtypes, isoforms generated through alternative mRNA splicing have been identified for two EP receptor subtypes, EP1 and EP3. The EP2 and EP4 receptors are linked to stimulation of cyclic AMP (cAMP) and protein kinase A (PKA) signaling through sequential activation of G α_s and adenylyl cyclase [58–60]. However, functional coupling of the cAMP pathway seems to be more efficient for EP2 compared to EP4 [61]. EP4, unlike EP2, activates phosphoinositide-3-kinase (PI3K) through G α_i [61]. The coupling of EP4 to G α_i may in part explain the decrease in efficiency for the EP4 receptor to couple to the cAMP/PKA signaling pathway.

The EP1 receptor elicits the elevation of intracellular calcium through two distinct pathways coupling with G α_q , specifically Gq and/or G11, and receptor-activated Ca²⁺ channels (RACC) [62, 63]. Using CHO cells transfected with mouse EP1 cDNA, Katoh *et al.* demonstrated that PGE₂ activation of the EP1 receptor elicits a large influx from extracellular calcium inducing PI hydrolysis and a very small Ca²⁺ mobilization from internal stores, most likely the endoplasmic reticulum, as a result of phospholipase C activation via Gq [62]. Further investigation of EP1-induced calcium mobilization by Tabata and colleagues identified transient receptor potential 5 as a possible candidate for the RACC coupled to EP1 and involved in Ca²⁺ influx [63]. In addition to coupling to Gq/11, utilizing human EP1 expressed in HEK cells, EP1 has been shown to couple to G $\alpha_{i/o}$ resulting in activation of PI3K [64]. A number of GPCRs that were traditionally considered to couple exclusively to Gq/11 have now been found to couple to G $\alpha_{i/o}$ and to activate PI3K [65].

As mentioned previously, isoforms for two EP receptor subtypes, EP1 and EP3, have been identified. Okuda-Ashitaka, *et al.* identified a splice variant for the EP1

(EP1v) receptor from rat uterus cDNA [66]. This variant differs from EP1 from the middle of transmembrane segment VI to the carboxy terminus and results in a receptor with a transmembrane segment VII-like structure lacking an intracellular COOH terminal tail. The EP1v receptor retained ligand-binding capability but is no longer coupled to signal transduction systems. EP1v may affect the efficiency of EP1 and EP4 signal transduction since overexpression of the EP1v in CHO cells expressing EP1 or EP4 attenuates intracellular signaling mediated by EP1 and EP4 [66]. In addition to rat, EP1v has also been identified in murine cell lines including mast cell line MC/9 and mammary epithelial cell lines; however, functional characterization has yet to be carried out in a murine model system [67].

The EP3 receptors, due to the presence of multiple isoforms, are also capable of coupling to multiple G proteins. These isoforms, which are generated by alternative mRNA splicing, differ in their cytoplasmic carboxy terminal tail and signal transduction pathways [60, 68]. In humans, ten splice variants coding for eight different isoforms have been identified [68]. Previous studies have shown that alterations in the carboxyl tail impart differences in constitutive activity, G protein coupling and agonist-induced internalization [69–72]. EP3 receptor isoforms have been identified that couple to $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12/13}$ [69, 73, 74]. Functionally, the majority of the isoforms (EP3-I, EP3-II, EP3-III, EP3-IV, EP3-e, and EP3-f) act to inhibit cAMP generation via $G\alpha_i$. Ligand binding of EP3-I, EP3-II, and EP3-III could also increase IP3/intracellular calcium [68, 70, 75].

In the mouse, three EP3 receptor isoforms, EP3 α , EP3 β , and EP3 γ have been identified and, similar to the human isoforms, are generated through alternative splicing, differ in their C-terminal tail, and couple to multiple G proteins [76–78]. The EP3 receptor signals are primarily involved in inhibition of adenylyl cyclase via G_i activation and Ca^{2+} mobilization through $G\beta\gamma$ from G_i [76, 78]. EP3 γ is also coupled to G_s and stimulates adenylyl cyclase [78]. EP3 β has the ability to superactivate adenylyl cyclase via the G_q /PLC/ Ca^{2+} pathway in a lipid raft-dependent manner [79, 80]. In addition to affecting adenylyl cyclase, all three isoforms are capable of Ca^{2+} mobilization mediated by $G\beta\gamma$ subunits from the G_i/o protein leading to activation of the PLC β isoform [76, 81].

6.1 Subcellular localization of EP receptors

GPCRs are typically thought to work at the cell surface recognizing and responding to extracellular ligands at the plasma membrane; however, there is now growing evidence supporting perinuclear and/or nuclear localization of functional GPCRs, including the EP receptors (as reviewed in [82–84]). Enzymes essential for prostanoid

biosynthesis and signaling are also located at the nucleus including PLA₂, mPGES-1, COX-2, and G proteins which suggests that eicosanoids such as PGE₂ can be produced directly at the nucleus and act through cognate receptors at the nuclear membrane [82]. Gobeil *et al.* has demonstrated that isolated nuclei with intact envelopes from porcine endothelial cells are capable of producing PGE₂ which suggests a potential role for intracellular signaling for prostanoids [82, 85]. To date EP1, EP2, EP3 α , and EP4 have been shown to be colocalized at the nuclear membranes of a variety of cell types and tissues [82, 84–87]. The plasma membrane and the nuclear receptors exhibit almost identical features based on: virtually indistinguishable kinetic profiles, immunoreactivity, molecular weight, and they arise from the same gene [82]. However, there is the possibility for differences in posttranslational modifications for the same receptor in different subcellular locations [82].

It has been suggested that plasma membrane GPCRs could exert different effects compared with GPCRs at the nucleus. In endothelial cells, there is a distinct signaling pathway and function for the EP3 receptor such that the plasma membrane receptor elicits immediate physiological actions (vasomotor effects with a decrease in cAMP); whereas, the nuclear EP3 receptor conveys gene regulation changes (induction of eNOS and nuclear calcium signals) without generation of second messengers such as cAMP or IP3 [82, 84, 85]. In breast cancer, nuclear EP1 expression has been identified via immunohistochemistry. In malignant cells, nuclear EP1 expression was correlated with good prognosis markers such as node-negative disease and PR expression and the absence of nuclear EP1 expression was correlated with worse survival [88, 89]. Conversely, in cholangiocarcinoma, nuclear EP1 was shown to play a role in the activation of signal transducer and activator of transcription-3 (stat 3) and induces tumor cell growth [90]. Considering the differences in the plasma *versus* nuclear membrane function of EP3 and the prognostic significance of nuclear EP1 and the potential role of nuclear EP1 in cholangiocarcinoma, nuclear EP receptors could play an important role in the function of normal and malignant cells.

7 PGE₂ and the role of EP receptors in breast cancer

The relationship between elevated COX-2 expression and cancer was first suggested by reports of elevated prostaglandin levels in breast tumors especially from patients with metastatic disease [12, 91–93]. COX-2-derived prostanoids promote angiogenesis, induce invasion, and increase metastasis [13]. PGE₂ is the principle COX-2 product in tumors and plays a predominant role in promoting cancer progression through

its cognate receptors [57]. As mentioned previously, the cellular effects of PGE₂ are mediated through four receptors, EP1, EP2, EP3, and EP4 that are coupled to different intracellular signaling pathways [57]. As downstream targets of the COX-2 pathway, experimental evidence has also indicated that EP receptors can play a role in cancer including breast cancer and breast cancer metastasis; however, the precise role of each EP receptor in malignant behavior has not been determined.

EP receptors play diverse roles in normal and malignant tissues, and each receptor may have a unique function in tumor behavior that can vary based on cell and tissue type and model system. In normal murine mammary gland, all four EP receptors are expressed at various points during gland development [94]. EP2 and EP4 receptors are induced during the proliferative phase of mammary gland development (pregnancy and lactation) and subsequently downregulated during the involution phase. EP3 is downregulated during the proliferative stage, and its expression is returned to high levels in the involuted mammary gland. In contrast, the EP1 receptor is expressed only in the involuted mammary gland [94]. The expression profile of the EP receptors changed in COX-2-induced mammary tumors. In these tumors, EP1, EP2 and EP4 receptors are strongly induced relative to normal mammary gland; whereas, the EP3 receptor is downregulated. The downregulation of the EP3 receptor would suggest that EP3 could have a protective role in mammary tumor development in this model system. Chang *et al.* suggested that EP2 and EP4 receptor subtypes are most likely to be involved in mammary tumor progression and angiogenesis since indomethacin, a nonselective COX-1 and COX-2 inhibitor, suppressed expression of EP2 and EP4 receptors [94]. In follow-up studies, Chang and colleagues focused on the role of EP2 specifically and determined that in COX-2-induced mammary tumors, EP2 was required for mammary epithelial hyperplasia and EP2 overexpression in mammary tumor cells mediates increased VEGF production via a cAMP/PKA-dependent pathway [95, 96]. Furthermore, EP2 plays a role in the oncogenic activities of transforming growth factor- β (TGF- β) during mammary tumorigenesis [97].

Estrogen plays a significant role in the development and progression of breast cancer. Cytochrome P450 (aromatase), encoded by the *CYP19* gene, catalyzes the synthesis of estrogen from androgens [98]. Aromatase, located primarily in the adipose stromal cells of breast tumors, catalyzes estrogen biosynthesis and is fundamental to hormone-dependent growth of breast cancer [98, 99]. In a study conducted by Richards and Brueggemeier in normal nonneoplastic breast adipose stromal cells, PGE₂ regulation of aromatase gene expression and activity involved EP1, EP2, and EP3 signaling pathways [99]. Stimulation of EP1

and EP2 with receptor agonists resulted in an increase in aromatase gene expression, and stimulation of EP2 resulted in an increase in aromatase activity most likely utilizing a PKA/cAMP mechanism. Stimulation of the adipose cells with the EP3 agonist sulprostone resulted in an inhibitory effect whereby EP3 activation blocked the PGE₂-mediated increase in aromatase gene expression and activity. Zhao *et al.* also demonstrated upregulation of the aromatase gene through activation of EP1 and EP2 via the PKC and PKA pathways in human adipose tissue [100]. Conversely, Subbaramaiah and Dannenberg published results indicating that the EP2 and EP4 receptors act to regulate aromatase expression in human adipocytes and breast cancer cells through the cAMP protein kinase A pathway that resulted in an enhanced interaction between P-CREB, p300, and the aromatase promoter 1.3/II [98]. Reduction of the EP1 and EP3 receptors in adipocytes had no effect on PGE₂-mediated increase in aromatase activity or expression. Neither EP1 or EP3 agonists were utilized in this study; therefore, the apparent discrepancy between these studies could be due to differences in the source and type of tissues used to study the effect of specific EP receptors on aromatase expression and activity [98, 99].

In another study, EP2 and EP4 have also been implicated in playing a role in increasing aromatase activity and expression in breast cancer cells after exposure to environmental toxicant *o,p'*-dichlorodiphenyltrichloroethane [101]. While some differences in experimental outcomes are present for EP1 and EP3, it would appear that there is a consensus that EP2 and EP4 receptors are involved in the PGE₂-mediated increase in aromatase expression and activity in adipose stromal cells and breast cancer cells and could play a role in the development of hormone-dependent breast cancer. Recently, Subbaramaiah and Dannenberg reported a linkage between obesity, inflammation, and aromatase activity in the murine mammary gland [102]. Obesity leads to inflammation in the mammary gland resulting in increased levels of COX-2-derived PGE₂, which could drive aromatase activity.

EP receptors have also been shown to play a role in inflammatory breast cancer. Inflammatory breast cancer (IBC) is the most aggressive form of locally advanced breast cancer and deviates from the phenotypic characteristics of either ductal or lobular breast tumors [103]. COX-2 is upregulated in primary IBC and metastatic lesions. Using human IBC tumor cell line SUM149, Robertson and colleagues demonstrate that stimulating the EP4 receptor with EP4 agonist PGE₁ alcohol increased proliferation and invasion and conversely antagonizing the EP4 receptor, by pharmacologic or genetic means, inhibited proliferation and invasion [103]. Robertson then expanded on this work confirming that EP4 plays a role in regulating invasion of IBC cells, and, furthermore, the EP3 receptor was shown to

regulate the ability of SUM149 cells to undergo vasculogenic mimicry [104]. Vasculogenic mimicry, a characteristic of tumor cells from aggressive tumors, occurs when tumor cells are able to form matrix rich capillary-like networks when placed in a three-dimensional culture in the absence of endothelial cells and fibroblasts [104]. EP3 stimulation with sulphostone inhibited the capacity of SUM149 cells to undergo vasculogenic mimicry, but antagonizing EP4 did not affect this response.

While activation of EP3 in inflammatory breast cancer and in adipose tissue appears to be beneficial, by inhibiting both the vasculogenic mimicry process and aromatase expression, respectively, there is limited additional information about the role of EP3 receptor in breast cancer [99, 104]. In CHO cells overexpressing the human EP3 receptor, stimulation with EP3 agonist M&B 28.767 can induce migration in a dose-dependent manner [105]. Much of the literature suggested that the EP3 receptor could play a role in angiogenesis. In murine mammary tumors from the MMTV-COX-2 transgenic mouse, EP2 may play a role in angiogenesis since EP3 was downregulated in the tumors [94]; however, in a sponge-induced granulation assay in *ddy* mice, EP3 agonist ONO-AEI-248 increased angiogenesis in a dose-dependent manner [106]. Furthermore, in EP3 knockout mice, using the same sponge model approach, angiogenesis was significantly reduced compared to wild-type mice. Tumor-associated angiogenesis and VEGF expression, induced in sarcoma-180 and Lewis lung carcinoma models, were reduced in EP3 knockout mice resulting in reduced tumor growth [106]. EP3-I isoform expressed in HEK-293 cells can induce the expression of vascular endothelial growth factor (VEGF) and VEGF receptor-1 mRNA via activation of PI3K and ERK [69, 107]. In Lewis lung carcinoma cells, EP3 receptor signaling was shown to regulate tumor-associated lymphangiogenesis through upregulation of VEGF-C and its receptor VEGFR-3 in tumor stromal tissues [108]. Whether EP3 plays a role in angiogenesis in breast cancer specifically has yet to be determined; however, Timoshenko *et al.* have demonstrated in several human breast cancer cell lines that antagonizing EP1 and EP4 resulted in inhibition of VEGF-C production [109].

Limited information is available about the potential role for EP1 in breast cancer. However, EP1 has been shown to play a role in other cancers. For example, EP1 has been implicated in UVB-induced inflammation and skin tumorigenesis [110] and signaling through the EP1 receptor can upregulate survivin expression in hepatocellular carcinoma [111]. Nuclear EP1 has been shown to play a growth-promoting role in cholangiocarcinoma [90]. Stimulation of EP1 can affect the transcription of aromatase in adipose cells of abdominal and breast origin

and contribute to VEGF-C production in human breast cancer cell line MDA-MB-231 potentially playing a role in angiogenesis [99, 100, 109]. In a rat model of chemically induced breast cancer, an EP1 antagonist significantly inhibited breast cancer development [112]. In a murine mammary model of metastatic breast cancer, EP1 functioned as a suppressor of breast cancer metastasis [89]. Treatment of murine mammary metastatic cell lines with pharmacological antagonists against EP1 (SC19220) or EP1/EP2 (AH6809) or reducing EP1 gene expression with shRNA resulted in an increase in the number of lung tumor colonies. Manipulation of EP1 only affected the metastatic potential of the cells with no effect on the primary tumor. In the same study, Ma and colleagues investigated the relationship between EP1 expression and survival in invasive ductal carcinomas and determined that nuclear EP1 expression correlated with improved survival compared to women with no nuclear EP1 expression [89]. This result correlated with Thorat's study which stated that nuclear EP1 expression in primary human breast tumors was correlated with good prognostic markers of progesterone expression and lymph node-negative status [88, 89]. Based on the disparate results concerning EP1 and cancer, it is possible that EP1 could play a protumorigenic role in the primary tumor in some cancers but an antimetastatic role in breast cancer.

The role of EP4 receptor in cancer appears to be clearer in comparison to the other EP receptors. EP4 has been shown to be involved in multiple cancers including colon, gastric, prostate, and lung cancer [113–119]. In metastatic murine (C3L5) and human (MDA-MB-231) breast cancer cells, EP4 plays a role in mediating autocrine PGE₂-mediated migration [120]. In a follow-up study, Timoshenko *et al.* provided an additional role for EP4 in cancer progression in C3L5 cells wherein activation of EP4 results in an increase of inducible nitric oxide synthase [121]. Tumor-derived nitric oxide has been shown to promote tumor growth and metastasis in a murine breast cancer model [122]. Additionally, EP4 is upregulated in castration-resistant hormone-naïve prostate cancer [117]. An EP4 antagonist inhibits growth in two xenograft models of castration-resistant disease.

In addition to migration, EP4 has been implicated in other aspects of metastasis. Osteolysis due to bone metastasis of breast cancer is linked to EP4 activation [123]. The stimulation of EP4 via an autocrine/paracrine mechanism results in an increase in RANKL in osteoblasts which leads to the induction of osteoclastogenesis and osteolysis in the bone. EP4 can act to enhance lymphatic invasion of breast cancer cells [124]. CCR7 is a chemokine receptor that plays an important role in the mediation of migration of leukocytes and dendritic cells toward lymphatic endothelial cells (LECs) that express the CCR7

ligand CCL21. Pan *et al.* demonstrated that in human breast cancer tissues, CCR7 expression in COX-2 overexpressing tumors was significantly correlated with lymph node metastasis. In addition, stimulation of the EP4 receptor in MDA-MB-231 cells lead to an increase in CCR7 expression through the PKA pathway which suggests that EP4 could play a role in lymphatic invasion in breast cancer. Since EP2 can activate the same pathway as EP4, EP2 often demonstrates functional similarities in regards to breast cancer. EP2 can also increase CCR7 expression in breast cancer cells leading to enhancement of migration towards LECs and promoting lymphatic invasion [124]. EP4, through activation of Erg-1 pathway through EGFR and ERK1/2, has also been connected to increasing transcription of inhibitors of DNA binding (Id-1) and cell invasion which can drive breast cancer metastasis [125].

In a murine model of metastatic breast cancer, antagonism of the EP4 receptor inhibited tumor metastasis *in vivo* [126]. Pretreatment of metastatic murine mammary tumor cell lines 410.4 and 66.1 with EP4 antagonist AH23848 or ONO-AE3-208 significantly inhibited the ability of these cells to colonize the lung. Additionally, EP4 antagonists also inhibited migration of 66.1 to PGE₂ and modestly inhibited cell growth of 410.4 cells. The chemotactic response of 66.1 cells and proliferation of 410.4 cells was also inhibited by the EP1/EP2 antagonist AH6809. In a follow-up study, reduction of EP4 expression via shRNA led to a decrease in the ability of 66.1 cells to form lung colonies or to spontaneously metastasize from a primary tumor [127]. However, EP4 gene silencing did not inhibit local tumor growth which indicates that in this model system, the role of EP4 seems to be directed to processes unique to metastasis rather than to expansion of the primary tumor.

8 The role of EP receptors in the immune system

PGE₂ has the ability to regulate the immune system. Within the immune system, PGE₂ modulates the functions of different cell populations [*i.e.*, natural killer (NK) cells, T lymphocytes, B lymphocytes, macrophages, dendritic cells (DC), and myeloid-derived suppressor cells (MDSC)]. For example, PGE₂ inhibits the proliferation of CD4⁺ T cells via decreases in intracellular calcium release and activity of p59 protein tyrosine kinase [128–130]. PGE₂ also has profound inhibitory effects on T cell apoptosis and decreases production of interferon gamma (IFN γ) and interleukin (IL)-2 [131, 132]. PGE₂ affects the development and activity of B cells in a complex manner. PGE₂ suppresses proliferation and induced apoptosis of immature B cells [133, 134]. On the other side, PGE₂ does not induce cell death or inhibit proliferation in mature B cells but regulates their activity by enhancing Ig class switching

[135]. In macrophages, PGE₂ inhibits production of cytokines such as tumor necrosis factor α (TNF α) and IL-12, suppressing type-1 immune responses [136].

The concept that PGE₂ inhibits NK cell function arises from both *in vitro* exposure of NK cells to PGE₂ and *in vivo* administration of PGE₂ inhibitors [137]. Initial work in the 1980s showed that prostaglandins inhibited NK cell activity and PGE₂ had greater suppressive effects than other prostaglandins (PGF_{2 α} and PGD₂) in both human and murine NK cells [138, 139]. Early studies observed the inability of NK cells to exert cytotoxic effects in patients with breast cancer or in animal models of breast cancer [140, 141]. The high levels of PGE₂ produced by suppressors of NK function (*i.e.*, macrophages, tumor cells) contribute to the inhibition of NK cells. With increasing tumor burden, host NK cell activity declines. Therapy with indomethacin, a dual COX-1 and COX-2 inhibitor, in tumor-bearing animals showed appreciable restoration of the NK cell activity. In one study, normal NK activity at maximum effector to target ratio was 26% specific cytotoxicity, but in tumor-bearing mice, NK cells were only able to lyse 6% of their targets; with indomethacin treatment, cytotoxicity was restored to 10–18% [138].

It is known that PGE₂ downregulates IL2-activated lymphokine-activated killer (LAK) cell cytotoxicity through EP2 receptors [142, 143]. LAK cells (activated NK cells) are generated from adherent splenocytes cultured in IL2. In a study by Su *et al.* [142], LAK cells were treated with various EP receptor agonists and antagonists to identify which were involved in the immunosuppressive effects of PGE₂. They show that an EP2 agonist significantly inhibits LAK cytotoxicity and that the inhibitory effects of PGE₂ can be blocked using an EP2 antagonist. In contrast, neither an EP1/EP3 agonist nor EP1 or EP4 antagonists modulate LAK cell cytolytic activity. Thus, PGE₂-mediated inhibition of LAK cytotoxic activity is through the EP2, but not the EP1, EP3, or EP4 receptors. We have recently investigated the role of individual EP receptors in regulating activities of endogenous, resident splenic NK cells [144]. Like PGE₂, the EP4 agonist PGE₁-OH blocked NK cell migration to a panel of chemokines. In contrast to the inhibitory actions of PGE₂, the EP1/EP3 agonist sulprostone increased migration. Unlike the opposing effects of EP4 *versus* EP1/EP3 on migration, agonists of each EP receptor were uniformly inhibiting to NK-mediated cytotoxicity. The EP4 agonist PGE₁-OH inhibited IFN γ production from NK cells. Agonists for EP1, EP2, and EP3 were not as effective at inhibiting IFN γ . Agonists of EP1, EP2, and EP4 all inhibited TNF α ; EP4 agonists were the most potent. Thus, the EP4 receptor consistently contributed to loss of function. These results, taken together, support a mechanism whereby inhibiting PGE₂ production or preventing signaling

through the EP4 receptor may prevent suppression of NK functions that are critical to the control of breast cancer metastasis.

The EP4 and/or EP2 receptors have also been identified as managing inhibitory and suppressive properties of other immune cells (T cells, B cells, macrophages, *etc.*). CD4⁺ T cells have been divided into four distinct subtypes (Th1, Th2, Tfh, and Th17) based on the help they provide to other effector cells. Proliferation of Th1 T cells, also known as the cytotoxic T cells, is inhibited through EP2 [145]. T cells deficient in EP1 and EP3 were susceptible to PGE₂ inhibition, while only at the highest concentration of PGE₂ were T cells resistant to the inhibitory effects of PGE₂ through EP4 receptor. This suggested that the EP2 and maybe the EP4 receptors mediated the suppressive effects of PGE₂ on T cells. The generation of the Th17 subset of CD4⁺ T cells by TGF- β and IL-6 is suppressed by PGE₂. The suppressive effects of PGE₂ were mediated by both the EP2 and EP4 receptors [146]. PGE₂ can exert opposing effects by enhancing Th17 cell expansion in human T cells. Using human peripheral blood T cells, Boniface *et al.* [147] reported that PGE₂ drove the development of human Th17 cells in the presence of IL-23 and IL-1 β through the EP2 and EP4 receptors. This shows that the combination of specific inflammatory cytokines during differentiation and activation determines the ultimate phenotype of Th17 cells. CD4⁺CD25⁺ T regulatory cells suppress potential antitumor responses. T regulatory cell-specific transcription factor, FOXP3, is induced by tumor-derived PGE₂. Splenocytes treated with PGE₂ had a 12-fold induction in FOXP3 gene expression which is associated with suppressed antitumor immune responses [148]. *In vitro*, the EP2/EP4 receptor agonist, 11-deoxy-PGE₁ and the EP2 agonist, butaprost, induced FOXP3 expression in Treg cells by 25- and 16-fold, respectively. This was further confirmed using knockout mice where Treg cell FOXP3 expression was reduced in EP4^{-/-} mice and ablated in EP2^{-/-} mice.

PGE₂ affects B cells differently depending on the stage of B cell development and activity. Secretion of IgG by splenic B cells was increased with the addition of PGE₂ [135]. MHC class II molecules are antigen-presenting cells that are necessary for the endocytic and endogenous pathways, while CD23, Fc ϵ RII, represents maturing B cells and is important in the regulation of IgE levels. On quiescent B lymphocytes, EP2 or EP4 agonists inhibited the expression of class II major histocompatibility complex and CD23 [149]. This shows that PGE₂ acts in an inhibitory manner on immature and developing cells. PGE₂ promotes IgE switching in IgM-positive B cells. PGE₂ promoted IL-4 and LPS stimulated B-lymphocyte Ig isotype switching from IgM to IgE mainly through the EP4 receptor [149]. Treatment of activated B lymphocytes with PGE₂ increases numbers of IgE-expressing cells,

which may lead to hypersensitivity and play a role in pathogenesis of allergy and asthma.

Macrophages are known to produce high levels of PGE₂. Released PGE₂ also acts on the macrophages themselves and exhibits inhibitory effects on early- and late-stage activation processes, producing a negative feedback. PGE₂ exerts inhibitory actions on macrophages through the reduction of cytokine production. In C3H/HeJ macrophages it is reported that PGE₂ inhibits TNF α and IL-12 through the EP4 receptor [136]. The interesting finding from this report was that stimulated macrophages undergo receptor switching from EP4 to EP2. This may be a mechanism for the macrophage to avoid EP4-mediated inhibition. It has been shown that chemokines can modulate the migratory capacity of macrophages in response to PGE₂ [150]. In response to the CCR7 ligands, CCL19 and CCL21, the EP2 and EP4 receptors regulate the migratory response of macrophages. In a chemotaxis assay, MONO-MAC-1 cells migrated efficiently toward both CCL19 and CCL21. This response was further enhanced in the presence of the EP2 agonist, butaprost, EP2/EP4 agonist, 11-deoxy-PGE₁, and the EP1/EP3/EP4 agonist, 17-PT-PGE₂. In contrast, the EP1/EP3 agonist, sulprostone, was unable to induce MONO-MAC-1 cells to migrate. Therefore, the EP2 and EP4 receptors are required for the increased migratory capacity of macrophages to CCL19 and CCL21 in the presence of PGE₂. Survivin, an inhibitor of apoptosis is increased in dendritic cells induced by PGE₂. Monocyte-DC cells treated with the EP2 agonist, butaprost, induced a significant increase in survivin expression. The EP1/EP3 agonist, sulprostone, was unable to cause survivin induction in monocyte-DC cells. Therefore, the EP2 receptor has been implicated in regulating PGE₂-mediated induction of dendritic cell survivin.

Tumor-derived PGE₂ is known to have direct inhibitory effects on immune cell functions. Monocytes from patients with breast cancer contribute to increased levels of PGE₂ produced by peripheral blood mononuclear cells in culture, and this correlates with a decrease in NK cell activity [140]. We show that NK functions (lysis, migration, cytokine production) are compromised in tumor-bearing mice and that tumor produced PGE₂ interferes with the function of NK cells ([144], unpublished data). PGE₂ inhibits the potential of NK cells to migrate, exert cytotoxic effects, and secrete IFN γ . This ability of PGE₂ to inhibit NK cells from tumor-bearing mice is through EP2 and EP4 receptors. In contrast to the inhibitory effects of PGE₂ on cytotoxicity, and IFN γ production, TNF α secretion was induced in NK cells from tumor-bearing mice. PGE₂ is uniformly inhibitory to the production of TNF α by NK cells from normal mice. Taken together these data show that NK functions are depressed in tumor-bearing hosts and this suppression

is mediated by tumor-derived PGE₂ acting on EP2 and EP4 receptors.

In the human leukemic T-cell line, Jurkat, it is postulated that an EP receptor linked to adenylate cyclase, but pharmacologically distinct from all EP receptors described, is present on the cells [151]. These findings may represent the EP receptor profile changes that can occur during tumorigenesis ([144], unpublished results) or the lack of responsiveness to selective agonists present at the time. Fedyk *et al.* [152] showed that EP1, EP3 β , and EP4 were expressed at the same level in B cells from normal splenocytes as in B cell lymphoma and myeloma cell lines. This was irrespective of the fact that PGE₂ only exerts inhibitory effects on immature and developing B cells and not mature B cells. They also showed that LPS upregulated EP4 receptor expression in the mature B cell lymphoma line, however the polyclonal activator was unable to affect EP1 and EP3 β receptor expression. This shows that the EP4 receptor may play a role in PGE₂ mediated inhibition of B cells. Myeloid-derived suppressor cells (MDSCs) are induced in cancer patients and animals and are potent suppressors of immunity. PGE₂ has been shown to promote 4T1 mammary carcinoma by inducing the accumulation of MDSC [153]. In EP2^{-/-} mice injected with 4T1 mammary carcinoma cells, the number of MDSCs was reduced and tumor growth was inhibited. This indicated that PGE₂ mediates MDSC accumulation through the EP2 receptor.

9 Summary

Elevated cyclooxygenase activity is common in many tumors. Preclinical and epidemiologic data support the use of NSAIDs and selective COXibs for the prevention and treatment of malignancy. Clinical trials with COXibs have been less successful, and significant toxicities have been associated with the use of COXibs. Many laboratories have investigated the possibility of targeting other aspects of the COX pathway. We have attempted to summarize the growing literature regarding the role of the prostaglandin E receptor family in malignant behavior. Our studies and those of many other laboratories have shown that EP receptors are expressed in malignant cells, and there is an emerging body of literature that some if not all EP receptors may be therapeutic targets. Each of the four EP receptors has distinct binding characteristics and is coupled to different intracellular signaling pathways, so it is not surprising that some receptors contribute to tumor progression and others may be protective. These different roles are tissue and cell dependent as well. Additional layers of complexity are contributed by the

multiple subcellular locations of the same receptor. While EP receptors are classical plasma membrane-expressed G protein-coupled receptors, emerging data indicate that EP receptors are expressed at other subcellular locations where they may have distinct functions. Expression of EP receptors in the stroma and by host immune effector cells also plays a role in tumor behavior. EP receptors expressed by stromal cells drive aromatase expression in breast cancer. EP receptors expressed on host immune cells mediate the marked immune suppression that characterizes tumor progression. As the reagents to study these receptors improve and as additional selective EP agonists and antagonists become available, our knowledge regarding the role of each EP receptor in cancer will grow so that we can more effectively exploit these as therapeutic targets.

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