Prostaglandin E2 in neuroblastoma: Targeting synthesis or signaling?

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Abstract

Neuroblastoma (NB) is the most common pediatric extracranial solid tumor arising from neural crest cells of the developing sympathetic nervous system. Despite marked advances in cancer treatment, the survival rate of high-risk NB remains unsatisfactory. As a key pro-inflammatory mediator regulating tumor microenvironment, prostaglandin E2 (PGE2) promotes NB proliferation, angiogenesis, and immune evasion via acting on four G protein-coupled receptors, particularly the EP2 subtype. Recent studies have been vigorously focused on developing and evaluating compounds targeting PGE2-regulated tumor inflammation in animal models of NB. In this review, we revisit these translational efforts and examine the feasibility of pharmacological inhibition of enzymes responsible for PGE2 biosynthesis or its signaling receptors as emerging therapeutic strategies for NB. We also explore the potential downstream oncogenic pathways upon the activation of PGE2 receptors, aiming to bridge the knowledge gap between tumorigenesis and the role of elevated PGE2/EP2 signaling, which is widely observed in high-risk NBs.

Keywords

Childhood cancer; COX; EP2; mPGES-1; Tumor inflammation; Tumor microenvironment

1. Introduction

Neuroblastoma (NB) is a malignancy of the sympathetic nervous system that sprouts during the embryonic stage [1]. Annually, there are more than 600 children diagnosed

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Contributions

R.H. and J.J. conceptualized the manuscript. R.H. drafted the manuscript. Y.Y. and J.J. discussed contents and edited the manuscript.

All authors have read and approved the final version of the manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

CRediT authorship contribution statement

Ruida Hou: Conceptualization, Writing – original draft. Ying Yu: Writing – review & editing. Jianxiong Jiang: Conceptualization, Writing – review & editing.
with NB in North America, making it the most prevalent extracranial solid tumor and the fourth most common pediatric cancer only after leukemia, lymphoma, and brain tumors [2]. Developed from the primitive neural crest cells, NBs are highly heterogeneous. The most malignant NBs are mainly composed of undifferentiated neuroblasts that might carry amplified oncogene MYCN, segmental chromosome aberrations (SCA) and few Schwannian stroma cells, while the intermediate malignant NBs comprise an increasing portion of stroma cells along with less unfavorable genetic features [3, 4]. Therefore, NB exhibits remarkably divergent clinical presentations and outcomes, which give rise to the risk-stratification system that helps to make therapeutic decisions and prognostic evaluations. Based on clinical stages, ages at diagnosis, histological and genetic characteristics, NBs are classified into low-risk, intermediate-risk, and high-risk tumors [3, 4]. For low- and intermediate-risk tumors, patients can achieve a promising over 95% long-term survival after surgery and chemotherapy alone. However, for high-risk tumors, which account for 50% of all NB cases, multimodally based treatment including chemotherapy induction, stem-cell transplant consolidation, and immune therapy maintenance is usually applied [5]. Currently, with the clinical application of monoclonal antibody (mAb), the 5-year survival for high-risk NB has been greatly improved from an abysmal less than 20% to approximately 60% [6]. Nevertheless, around 20% of the high-risk NB patients are refractory to the induction phase of chemotherapy [7], meaning that these patients may not even survive to have the chance for the mAb therapy. Moreover, mAbs and stem-cell transplant are with limit access in low- and middle-income countries [8]. Hence, there is an unmet need for more efficient and affordable pharmacotherapies for the treatment of NB.

Inflammatory response, hallmarked by the infiltration of innate immune cells and the release of cytokines, chemokines, and other inflammatory mediators including prostaglandins, is an essential mechanism for the innate immune system to respond to deleterious stimuli. It is well known that the chronic inflammation often fosters a tumor microenvironment (TME) that can facilitate immune evasion and treatment resistance [9]. Although the mechanisms whereby inflammatory pathways transform the TME to favor tumor growth are not fully understood, mounting evidence suggests that cyclooxygenase (COX), mainly via producing prostaglandin E2 (PGE2), plays an essential role in triggering the inflammatory storm within the TME of multiple types of cancers including NB [10–13]. As such, targeting the COX-governed inflammatory cascade has been proposed to treat NB and other malignancies as monotherapy or more widely as adjunctive/secondary treatment to the current standard therapies [14–16].

2. PGE2 in neuroblastoma

Metabolic and hormonal stimuli associated with tumor development can induce the release of arachidonic acid (AA) from the cell membrane-derived phospholipids, a process catalyzed by phospholipase A2 (PLA2). Freed AA is then converted to an intermediate prostaglandin H2 (PGH2) by the COX, which has two isoforms – constitutive COX-1 and inducible COX-2. Short-lived PGH2 is further catalyzed to five different prostanoids, i.e., prostaglandin PGD2, PGE2, and PGF2α, thromboxane TXA2 and prostacyclin PGI2, by their respective tissue-specific synthases [17]. In NB, PGE2 is the most abundant and the only significantly elevated prostaglandin, especially in high-risk tumors [10, 11]. PGE2 exerts its
functions through acting on four G protein-coupled receptors (GPCRs), namely EP1, EP2, EP3 and EP4.

All these four EP receptor subtypes are highly expressed in NB primary tumors of diverse clinical stages and biological subsets as well as in the vasculature of the adjacent stromal tissues. In addition, the expression of EP receptors is commonly found in many NB cell lines with different genetic backgrounds at both mRNA and protein levels. Specifically, EP1 and EP4 are expressed in the cell membrane, cytoplasm, and nuclear compartment of NB cells, whereas EP2 and EP3 are mainly detected in the cell membrane and cytoplasm [10]. Likewise, all four PGE$_2$ receptors are expressed in NB tumors with 11q-deletion and MYCN-amplification. However, EP1, EP2 and EP3 receptors are ubiquitously found in both tumor cells and stromal cells, whereas EP4 is predominantly expressed in the stromal cells of NB tumors [12].

Among these four EP subtypes, EP2 and EP4 are coupled to G protein $\alpha_s$ subunit that functions to promote the intracellular cAMP signaling when activated by PGE$_2$. EP1 is linked to $\Gamma\alpha_q$ subunit which can initiate the phospholipase C (PLC)-calcium-calmodulin signaling and protein kinase C (PKC)-mediated pathways. EP3 is mainly associated with the $\Gamma\alpha_i$ subunit and plays an inhibitory role in the cAMP generation upon activation [17]. As an active immune and inflammatory response regulator, PGE$_2$ has been reported, largely by acting on $\Gamma\alpha_s$-coupled EP2 and EP4 receptors, to promote tumor growth, angiogenesis, treatment resistance, and immunosuppressive cells through a complex molecular signaling network within the TME (Fig. 1) [16,18].

2.1. Proliferation

All the four EP receptors are expressed in human NB cells and tissues [10,12]. However, which PGE$_2$ receptor subtype plays a dominant role in PGE$_2$-promoted tumor proliferation was not fully known. An early study reported that autocrine and/or paracrine PGE$_2$ promotes NB cell survival and proliferation with increased intracellular calcium concentration and enhanced cAMP signaling followed by phosphorylation of Akt [10], suggesting the involvement of both $\Gamma\alpha_s$ and $\Gamma\alpha_q$-coupled receptors. Later, it was found that in MYCN non-amplified high-risk NB, PGE$_2$ can stabilize and enhance the nuclear translocation of $\beta$-catenin, a dual functional protein that regulates cell adhesion and gene transcription, therefore promoting cancer cell survival through a cAMP-dependent mechanism [19]. More recently, we showed that pharmacological inhibition or genetic ablation of EP2 significantly suppressed NB proliferation and survival both in MYCN-amplified and MYCN non-amplified models, indicating that EP2 might be the dominant subtype of PGE$_2$ receptors involved in the NB development and progression [20].

2.2. Angiogenesis

PGE$_2$ can induce microvascular endothelial cells to express angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), to support neovascular growth [21]. We found that the expression of EP2 in NB is strongly associated with VEGF, endoglin (ENG) and platelet endothelial cell adhesion molecule 1 (PECAM-1), which are the three markers of microvascular proliferation [20]. Intriguingly, Amano et
al. reported that in EP3 knockout mice, the tumor-associated angiogenesis was reduced in both inoculated sarcoma and lung carcinoma models, indicating that EP3 might also be involved in regulating the tumor microvasculature formation [22]. However, this may not necessarily be the case in NB where EP3 does not show any significant correlation with these angiogenic factors. In fact, EP3 is considerably downregulated in nonsurvival NB patients when compared to survival cohorts, suggesting that EP3 negatively correlates with the aggressiveness of NB [20]. These findings together raise the possibility that PGE2 may engage different receptor subtypes in different tumor cells, stroma cells, and vascular endothelial cells and function synergistically with other factors such as hypoxia-inducible factor and fibroblast growth factor receptor in orchestrating the angiogenesis under physiological and pathological conditions [21,23]. However, EP2 likely is the leading receptor subtype that mediates the PGE2-promoted angiogenesis during NB progression.

2.3. Treatment resistance

About 20% of patients with high-risk NB are refractory to the standard chemotherapy induction. In addition, nearly 80% of NB patients, regardless of their initial response to the multimodal treatment, were reported to experience disease relapse within the first two years after diagnosis [24]. The therapeutic resistance of NB can be attributed to several factors, particularly the cancer stem cell (CSC) repopulation and enhanced epithelial to mesenchymal transition (EMT), which are highly correlated with the activated COX/PGE2/EP signaling axis [25,26]. CSCs are well known for their capability to asymmetrically divide into self-renewal stem cells and daughter cells that can differentiate and proliferate as tumor cells [27]. Owing to their versatile and dynamic metabolism phenotypes, CSCs are highly resistant to stressful conditions and cytotoxic effects induced by chemotherapy [27]. Within the TME, the expression of COX/PGE2 axis is upregulated concurrently with CSC markers such as CD44, which is highly associated with NB aggressiveness and poor prognosis [28]. In addition, via interacting with EP4 receptor, PGE2 can promote CSC self-renewing, expansion, and therapeutic resistance through the activation of phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway and mitogen-activated protein kinase (MAPK) signaling [25,29]. On the other hand, the EMT process can also enable tumor cells to develop multiple drug resistance, as undifferentiated mesenchymal tumor cells derived from EMT during NB development were largely found in relapse and chemoresistance cases [30]. PGE2 via EP2 and EP4 receptors downregulates E-cadherin, and thus promotes the EMT to generate refractory mesenchymal cells through the activation of signal transducer and activator of transcription 3 (STAT3) and the PI3K/Akt cascade in colorectal cancer and prostate cancer [26,31]. However, whether PGE2 promotes EMT and treatment resistance in NB through a similar mechanism remains to be determined.

2.4. Immunosuppressive cells

As an important immune response mediator, PGE2 is well known for its roles in regulating infiltration of immune cells within the TME. Myeloid-derived suppressor cells (MDSCs) are a group of immunosuppressive cells developed from bone marrow precursor cells under a variety of pathological circumstances. Recent studies suggest that both tumor paracrine and MDSCs autocrine PGE2 might be involved in the differentiation and activation of MDSCs [32]. In NB, elevated levels of MDSCs in the circulation might be associated with immune
therapy resistance and enhanced expression of tumor-promoting factors such as reactive oxygen species (ROS), arginase 1 (ARG-1), and transforming growth factor β1 (TGF-β1) [33,34].

As another group of immunosuppressive cells, tumor-associated macrophages (TAMs) are composed of M1 macrophages, which have anti-tumor effects, and M2 macrophages that can promote tumor cell survival and metastasis [35]. Within the TME, elevated PGE2 can stimulate M2 macrophage polarization and migration, most likely through the activation of Gs-coupled EP2/EP4 receptors and the cAMP-response element binding protein (CREB)-mediated induction of the downstream targets [11,32]. In NB, PGE2 can prime TAMs toward M2-like phenotype to promote tumor progression [11]. Further, hypoxia inducible factor (HIF) activated by PEG2 might also regulate VEGF and matrix metalloproteinases (MMPs) secreted by TAMs, which can promote angiogenesis and metastasis in NB [36,37].

PGE2 plays a critical role in the differentiation and maturation of dendritic cells (DCs) from bone marrow progenitor cells via acting on the EP1 receptor [38]. In addition, tumor-derived PGE2 can downregulate the chemokine receptor expression in DCs and therefore induces immune tolerance effects [39]. In NB, intratumor infiltration of mature and functional DCs along with natural killer (NK) cells is positively correlated with cytotoxic T-cell recruitment, a well-known indicator for promising clinical outcomes [40]. As such, DCs-induced NK cell activation can increase the cytotoxic functions of NK cells in NB patients receiving dinutuximab-based immune therapy [41]. On the other hand, PGE2 may also directly exert its effect on NK cells via activating EP receptors expressed on the NK membrane in a DCs-independent manner. It has been shown that PGE2 may suppress NK cytotoxicity, attenuate interferon γ (IFN-γ) production, and thus promote cancer metastasis and progression by selectively acting on EP2 and EP4 receptors of NK cells [42].

Within the TME, T cells also play dynamic roles that affect tumor progression and prognosis, and a large portion of tumor-infiltrated T cells are CD4+ helper T cells (Th) and regulatory T cells (Treg). PGE2 can elicit immunosuppressive effects by orchestrating the mature DCs enriched in immunoregulatory molecules (mregDC)-Treg axis for the recruitment and activation of Treg within the TME [18]. Moreover, via interacting with EP2 and/or EP4 receptors on T cells, PGE2 can modulate the balance of Th1/Th2 and elevate the levels of circulating Th17 and Treg, thereby fostering an immune suppressive environment [43]. In addition, tumor cells-derived PGE2 also can inhibit CD8+ T cell proliferation, attenuate its cytotoxicity, and induce exhaustion [44]. These interesting findings together suggest complex roles for PGE2 in regulating immunosuppressive cells including MDSCs, TAMs, DCs, and Tregs. These types of immune cells are key components of the TME that promote tumor growth and invasion, and there are also interactions between these cells, leading to tumor immune escape.

3. Targeting the COX/PGE2/EP axis for NB

Given that PGE2 plays a pivotal role in orchestrating the tumor-promoting inflammation in the TME, targeting PGE2 biosynthesis or its pro-inflammatory signaling has been extensively studied as new therapeutic strategies for the treatment of NB over the past
As such, a number of pharmacological agents targeting the COX/PGE\(_2\)/EP axis have recently been evaluated for \textit{in vivo} efficacy in various preclinical NB models (summarized in Table 1).

### 3.1. COX-1/2

As a key rate-limiting enzyme in the biosynthesis of PGE\(_2\), COX catalyzes the first step to convert the membrane-released AA to intermediate PGH\(_2\) (Fig. 1). Blocking COX enzymes by non-selective COX inhibitors, the conventional non-steroidal anti-inflammatory drugs (NSAIDs), or selective COX-2 inhibitors (Coxibs) has long been known as a potential anti-tumor strategy, and a number of these drugs have been evaluated in NB models (Table 1). Diclofenac, a non-selective NSAID, when supplied in drinking water, showed dose-dependent inhibition on NB xenografts developed in both athymic nude rats and mice, accompanied by enhanced apoptosis and reduced PGE\(_2\) in tumor tissues \([11,46]\). The anti-NB effect of systemic treatment with diclofenac was recapitulated in a transgenic TH-MYCN mouse model of high-risk NB where tumors are developed spontaneously \([12]\). Similarly, oral treatment with low-dose aspirin, a prototype of NSAIDs, substantially reduced the burden of spontaneous tumors developed in the transgenic NB mouse model \([47]\). Interestingly, the presence of tumor-associate cells of the innate immune system, such as DCs and TAMs, along with the intratumoral expression of TGF-\(\beta\) was also reduced by aspirin \([47]\).

Considering the potential pediatric adverse effects of non-selective NSAIDs (e.g., Reye’s syndrome), drugs selectively targeting the inducible COX-2 have also been examined in various NB animal models (Table 1). Celecoxib, when administered orally, significantly reduced xenograft growth in athymic nude rats \([46,48]\). The synergistic inhibitory and apoptotic effects of celecoxib and chemotherapy drugs, such as doxorubicin and irinotecan, on established NB tumors suggest that COX-2 inhibition should be explored as an adjunctive treatment to potentiate the anti-tumor effects of the current chemotherapeutic drugs in NB \([48,49]\). Likewise, NS-398, another selective COX-2 inhibitor, also showed promising therapeutic effects in a mouse model of high-risk NB, such as inhibiting PGE\(_2\) production, reducing NB xenografts, diminishing metastasis, and impeding angiogenesis \([50]\). These findings using different COX inhibitors in several animal models reinforce the candidacy of COX-2 as a molecular target for tumor growth, metastasis, and angiogenesis in NB.

### 3.2. mPGES-1

The microsomal prostaglandin E synthase 1 (mPGES-1) is the inducible isoform of the terminal enzyme for PGE\(_2\) biosynthesis – PGES, which also has two constitutive isoforms: microsomal PGES 2 (mPGES-2) and cytosolic PGES (cPGES). Functionally coupled to COX-2, mPGES-1 directly converts COX-2-derived intermediate PGH\(_2\) to PGE\(_2\) when both enzymes are induced at the pathological sites \([51–53]\). Unlike the COX-2 inhibitors, small-molecule compounds blocking the activity of mPGES-1 are considered less likely to cause cardiovascular adverse effects, as they hypothetically do not interfere with the biosynthesis of the prostacyclin PGI\(_2\), which is well known for its cardioprotective effects \([54]\). However, there are significant structural differences in the active site of the enzyme between human and rodent orthologs \([55]\), making it difficult, if not impossible, to evaluate...
potential inhibitors developed for human mPGES-1 in rodent models. Recent monumental efforts in medicinal chemistry campaigns led to the discovery of mPGES-1 inhibitors that overcame the cross-species differences and thus were tested in various animal models [53]. As such, intraperitoneal administration of compound III, a selective mPGES-1 inhibitor, showed marked suppression on NB xenografts in athymic nude mice and PGE$_2$ production in tumor tissues [12]. The anti-NB effects of compound III were fully recapitulated in TH-MYCN transgenic mice with spontaneous NB, accompanied by a favorable shift in the M1/M2 phenotype macrophage ratio, reduced angiogenesis, and impaired infiltration and migration of cancer-associated fibroblasts (CAFs) [12].

Moreover, mPGES-1 inhibition by compound III augmented the cytotoxic effects of chemotherapeutic drugs such as doxorubicin and vincristine in spheroids constructed by human NB cells, suggesting a possible additive/synergetic effect [13]. Intraperitoneal administration of compound III alone was able to reduce the growth of established NB allografts developed in immunocompetent A/J mice but only had marginal effects on the tumor weight and PGE$_2$ levels in tumors (Table 1). However, combined treatment with compound III and vincristine significantly reduced both tumor growth and tumor weight in the same NB model when compared to the vehicle treatment [13], confirming the additive or synergetic effect between compound III and vincristine. These findings highlight the potential of mPGES-1 inhibitors as either monotherapy or polytherapy together with tumor cells-targeting drugs like vincristine for the treatment of NB with high-risk factors (Table 1).

### 3.3. EP2 receptor

PGE$_2$ has long been known to mediate the pro-inflammatory and pro-tumoral signaling via interacting with its four EP receptors in tumors of various origins. However, by analyzing expression profiles of the EP1-EP4 receptors among the NB patient cohorts on R2 platform (http://r2.amc.nl), we found that only the expression of EP2 is significantly elevated in tumors of nonsurvival patients and correlated with the poor clinical outcomes [20]. Conversely, the expression of EP3 and EP4 was significantly decreased in the nonsurvival group, suggesting that the EP2 might be the main EP receptor subtype responsible for the PGE$_2$-mediated tumor inflammation in human NB. Indeed, EP2 was identified as the dominant G$_s$-coupled receptor that mediates COX/PGES/PGE$_2$-/cAMP signaling in human NB cells with high-risk factors, such as MYCN amplification and 11q deletion, as these cells responded to EP2 agonist butaprost but not EP4 agonist CAY10598 [20].

The genetic ablation and conditional depletion of EP2 were able to impair the development and progression of NB xenografts in athymic nude mice [20]. Systemic treatment with TG6–10–1, a first-generation EP2-selective antagonist with modest potency and short plasma half-life, only showed moderate inhibition on xenografts derived from human NB cell line SK-N-AS in nude mice (Table 1) [56]. However, daily treatment with TG6–129, a much more potent EP2 antagonist with longer in vivo half-life [17,57], substantially reduced the aggressiveness of NB cells with high-risk factors including MYCN amplification and 11q deletion. Importantly, therapeutic outcomes from studies on NB xenografts in immunodeficient mice were largely recapitulated in syngeneic NB tumors developed in immunocompetent hosts (Table 1) [20]. Interestingly, pharmacological inhibition of EP2 by TG6–129 also showed antiangiogenic, anti-inflammatory, and apoptotic effects in
these NB models, which may underlie the molecular mechanisms of its anti-proliferative actions (Table 1). These findings from studies utilizing both genetic and pharmacological approaches establish PGE₂ receptor EP2 as a promising therapeutic target for high-risk NB.

4. PGE₂ and oncogenic pathways in NB

All four PGE₂ receptor subtypes have been found in human NB cells and tissues [10,12]. Activation of these GPCRs can lead to multiple Ga/Gβγ-dependent and independent downstream signaling pathways that may contribute to tumor cell survival, migration, metastasis, and therapy resistance (Fig. 1)[17]. In addition to the conventional cAMP/PKA signaling mediated by Ga₃, the PI3K/Akt, Wnt/β-catenin, and Ras/MAPK pathways are among the most investigated NB carcinogenesis signaling cascades that can potentially be triggered by activated EP receptors (Fig. 2) [58].

4.1. PI3K/Akt pathway

The PI3K signaling pathway plays critical roles in promoting cancer cell survival and treatment resistance. Class I PI3Ks, which are subdivided into class IA and class IB, are heterodimers consisting of a catalytic subunit (CAT) and a regulatory adaptor. For receptor tyrosine kinase (RTK) activated class IA PI3Ks, the three mammalian CAT isoforms have been identified and cloned as p110α, p110β and p110δ. The class IB PI3K, on the other hand, is identified with p110γ CAT that can be activated by GPCRs. Activation of class I PI3K induces the production of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), which recruits and activates a number of proteins with Pleckstrinhomology (PH) domain, such as Akt [59]. As a pivotal downstream signaling cascade of the class I PI3K signaling for survival, the Akt/m-TOR has been found highly activated in more than 60% of NB patients and correlated with the advanced disease stages [60]. The PI3K-mTOR complex 2 (mTORC2)-induced HIF2α expression was reported to contribute to vascularization and tumor metastasis in NB [61]. Inhibition of the PI3K/Akt/mTOR complex with small-molecule inhibitors significantly impairs NB growth and survival through inducing apoptosis and enhances the efficacy of currently used chemotherapy drugs [62,63]. It is also well known that the release of Gβγ subunit upon EP receptor activation can induce the class I PI3K-mediated PI3K/Akt/mTOR signaling by its interaction with p110 (p110γ preferred) (Fig. 2) [64], and thus promotes tumor survival, possibly through the NF-κB-induced transcription activation of a variety of pro-inflammatory genes and oncogenes [17,65].

4.2. Wnt/β-catenin pathway

The wingless-related integration (Wnt) gene encodes a family of secreted glycoproteins that can bind to the N-terminal extracellular domain of the frizzled (Fzl) receptor. The latter subsequently disrupts the tertiary β-catenin destruction complex formed by axin, adenomatous polyposis coli (APC), casein kinase I (CK1) and glycogen synthase kinase 3β (GSK-3β), and therefore stabilizes the cytoplasmic β-catenin [66]. Enhanced and aberrant Wnt/β-catenin transcription activation was found in non-MYCN amplified high-risk NB and correlated with increased MYC expression [67]. The Wnt inhibitory factor 1 (WIF1) was downregulated in NB cells; however, restoration of WIF1 function with demethylating
agents significantly inhibited Wnt/β-catenin signaling and tumor proliferation, indicating the importance of Wnt/β-catenin pathway in NB carcinogenesis [68]. In addition, the overexpression of Fz11 Wnt receptor was detected in doxorubicin-resistant NB cells. Genetic ablation of Fz11 can cause the downregulation of p-glycoprotein, which is a downstream target of β-catenin as well as an important mediator in cancer chemoresistance [69]. Notably, PGE2-induced EP2/EP4 activation was reported to stimulate cancer growth through the Wnt/β-catenin signaling, which is associated with the Gβγ-triggered PI3K/Akt phosphorylation, as well as the direct interaction between the Gaα subunit with the regulator of G protein signaling (RGS) domain on axin (Fig. 2) [70,71]. Both interactions lead to the release of GSK-3β and the activation of the canonical Wnt/β-catenin pathway. Therefore, it is likely that the enhanced PGE2/EP signaling in NB contributes to tumor proliferation and survival through the canonical Wnt signaling-related mechanisms.

4.3. Ras/MAPK pathway

The Ras/MAPK pathway is among the most dysregulated signaling cascades in cancer. The three human Ras genes, known as H-Ras, K-Ras, and N-Ras, encode four highly related Ras family proteins that are anchored to the cytoplasmic membrane and function as molecular switches controlling several fundamental cellular events including proliferation, differentiation, and apoptosis [72]. By carboxyl-terminal farnesylation, Ras protein is localized to the cell membrane where it is within the proximity to the growth factor receptor-bound protein 2 (Grb2) and the guanine nucleotide exchange factor Son of the Sevenless (SOS) [73]. Grb2 and SOS mitigate the exchange of Ras bound GDP to GTP, which causes conformational change of Ras and enables it to activate Raf. Upon activation, Raf acts as a MAPK kinase kinase that subsequently phosphorylates the downstream targets including MAPK kinase 1/2 (MEK1/2) and extracellular signal-regulated kinases 1/2 (ERK1/2), which can translocate into the nucleus to trigger the expression of effector genes engaged in diverse cellular activities [73].

In NB, the activating mutation of Ras/MAPK pathway has been identified as a featured marker especially for relapsed cases [74]. By inhibiting both the Ras/MAPK signaling and the tyrosine phosphatase SHP2 (Src homology-2 domain-containing protein tyrosine phosphatase-2) that functions to dephosphorylate Ras to increase its association with Raf, synergistic growth inhibition effects have been observed in NB models [75]. Interestingly, it was reported that PGE2 can induce the activation of Ras and Ras-associated PI3K signaling through the enhanced cAMP/PKA cascade (Fig. 2) [76]. Alternatively, as a downstream target of the activated GPCR, β-arrestin directly binds and forms a complex with Src family tyrosine kinases. Subsequently, the β-arrestin/Src complex can trigger the growth factor receptor-induced Ras/MAPK signaling, which may contribute to cancer cell migration and metastasis (Fig. 2) [77]. Therefore, the PGE2 activated EP in NB may directly enhance the Ras/MAPK signaling through the interaction with cAMP/PKA cascade or via the β-arrestin mediated Ras/Raf/ERK pathway to promote cancer cell survival, metastasis, and relapse.

4.4. Other potential downstream targets

Gαs-coupled EP2 is the only highly elevated PGE2 receptor subtype in human NB tissues and contributes to PGE2-promoted NB malignancy [20]. To examine the relationships between
EP2 and other potential contributory factors involved in the carcinogenesis process of NB, we analyzed seven large NB patient cohorts on R2, including SEQC-498, Kocak-649, NRC-283, Versteeg-88, Westermann-579, Cangelosi-786, and Olsen-72606. Within each cohort, we used the integrated analysis tools on R2 to identify genes correlated with EP2 (PTGER2). The top 20 EP2-correlated genes from each cohort were compared for consistency across all these datasets (Fig. 3A). Among these, five genes, namely CCDC88C [78,79], SHISA3 [80,81], HAPLN3 [82,83], ADAMTS3 [84, 85], and SLC1A5 [86,87], were identified as the most frequently presented EP2-correlated genes among at least three datasets (Fig. 3B). Intriguingly, the functions of these genes are all related to the development of ectoderm, which is the outer germ layer formed in the early embryonic development and gives rise to primitive neural tissues where NB tumors develop. The functions of these genes and their potential interactions with the PGE2/EP2 signaling are highlighted in Table 2. The Kaplan-Meier survival analysis reveals that, resembling the EP2, all these five genes are consistently and significantly correlated with poor clinical outcomes in NB patient datasets on R2 platform (Fig. 3C). However, whether and how PGE2/EP2 signaling regulates their expression during NB development and progression remain to be determined.

5. Concluding remarks and future directions

Owing to its elevated expression in tumor tissues and correlation with tumor aggressiveness, COX was widely considered as a promising target for cancer treatment. Numerous preclinical and clinical studies have suggested the potential useful applications of selective or nonselective COX inhibitors in the treatment of various solid tumors, often yielding increased patient survival. Such therapeutic benefits are likely due to direct effects on tumor cells and combined tumor-host cell interactions including improved host metabolism [14,15,88]. However, blocking COX enzymes as a feasible strategy to treat cancers including NB has a big hurdle to overcome, i.e., the potential adverse effects on gastrointestinal tract and microvascular systems that can lead to internal bleeding, myocardial infarction, and strokes. The activation of COX produces five different prostanoids that act on nine currently known GPCRs, implementing broad physiological and pathological functions. Particularly, COX inhibition can potentially decrease the systemic levels of PGI2 that acts as a vasodilator and platelet inhibitor, thereby increasing cardiovascular risk. The untoward consequences of COX inhibition inspired us and others to seek for the next-generation therapeutic targets from the COX downstream prostanoid synthases or signaling receptors [89–95]. As such, blocking mPGES-1, the inducible terminal enzyme for PGE2 biosynthesis, arises as an alternative strategy to COX inhibition. Given that it only disrupts PGE2 synthesis without affecting COX itself or other prostanoid synthases [96], mPGES-1 inhibition is considered more specific than blocking the entire COX cascade, thereby less side effects should be anticipated.

PGE2 receptor EP2 is a key mediator of tumor-associated inflammation and can also trigger a number of conventional oncogenic signaling pathways, such as PI3K/Akt, Wnt/β-catenin, and Ras/MAPK (Fig. 2), which together potentially facilitate tumor development and progression in NB. Thus, targeting EP2 represents another emerging strategy for treatment of NB and other cancers where the receptor is a key mediator or tumor-associated
inflammation [20,97]. Blocking EP2-mediated signaling by small-molecule antagonists should not affect other PGE2 receptor subtypes (EP1, EP3, and EP4), whose expression levels do not positively correlate with the tumor aggressiveness in human NB, thereby providing likely higher therapeutic specificity than mPGES-1 inhibition. The efficacy of EP2 receptor as a feasible target for NB treatment has been validated by genetic approaches and demonstrated in both athymic nude mice and immunocompetent hosts [20]. Early studies reported some adverse phenotypes on blood pressure and fertility in global EP2 knock-out mice [98]. However, pharmacological inhibition of EP2 does not alter general well-being, cardiovascular/respiratory functions, blood cell counts, or bone morphology in rodents [99]. In contrast to the permanent genetic ablation, the inhibition by competitive antagonists is generally reversible. The overall safety profile paves the way for advancing EP2 antagonists to treat NB.

However, it should be noted that the pharmacological inhibition of COX-1/2, mPGES-1, or EP2 by small-molecule compounds can slow down the NB growth but cannot completely diminish tumors. In addition, its inhibitory effects on tumor growth in immunocompetent NB models were not as great as that was observed on xenografts in athymic nude rodents (Table 1). Therefore, it is unrealistic that targeting COX/mPGES-1/EP2 signaling axis can be used as a monotherapy for NB treatment. Mounting evidence also suggests that blocking PGE2 synthesis by COX-2 or mPGES-1 inhibitors can potentiate the cytotoxic effects of chemotherapy drugs on NB xenografts in both immunodeficient and immunocompetent hosts (Table 1). As such, future translational efforts should also be directed to evaluate the polytherapy with mPGES-1 inhibitors, EP2 antagonists, and current chemotherapy or immunotherapy drugs to determine whether inhibition of mPGES-1 and/or EP2 receptor is a feasible adjunctive strategy for the NB treatment.

Acknowledgements

This work was supported by the National Institutes of Health (NIH)/National Institute of Neurological Disorders and Stroke (NINDS) grants R01NS100947 (J.J.) and R21NS109687 (J.J.). Figures were created with BioRender.

Abbreviations:

| Abbreviation | Definition                                      |
|--------------|------------------------------------------------|
| AA           | arachidonic acid                               |
| COX-1/2      | cyclooxygenase 1/2                              |
| Coxibs       | selective COX-2 inhibitors                     |
| cPGES        | cytosolic prostaglandin E synthase             |
| CREB         | cAMP-response element binding protein          |
| EGFR         | epidermal growth factor receptor               |
| EP1–4        | PGE2 receptor subtypes 1–4                     |
| GPCRs        | G protein-coupled receptors                    |
| IFN-γ        | interferon γ                                   |
**IL-1β** interleukin 1β

**IL-6** interleukin 6

**MAPK** mitogen-activated protein kinase

**mPGES-1/2** microsomal prostaglandin E synthase 1/2

**mTOR** mammalian target of rapamycin

**NB** neuroblastoma

**NF-κB** nuclear factor κB

**NK** natural killer cell

**NSAIDs** non-steroidal anti-inflammatory drugs

**PECAM-1** platelet endothelial cell adhesion molecule 1

**PGE_2** prostaglandin E2

**ROS** reactive oxygen species

**TAM** tumor-associated macrophage

**TME** tumor microenvironment

**TNF-α** tumor necrosis factor α

**VEGF** vascular endothelial growth factor

### References

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Fig. 1. PGE$_2$-mediated signaling pathways in NB. Phospholipase A2 catalyzes the release of arachidonic acid from the phospholipid membrane. Arachidonic acid is then converted to an intermediate PGH$_2$ by the constitutive COX-1 or inducible COX-2. PGH$_2$ is further converted to PGE$_2$ primarily by mPGES-1. PGE$_2$ functions through acting on four cell membrane-bound GPCRs: EP1, EP2, EP3, and EP4. Among these, EP2 and EP4 are coupled to G$_{\alpha}$s subunit and can promote the intracellular cAMP signaling upon activation by PGE$_2$. EP1 is linked to G$_{\alpha}$q subunit which can initiate the calcium-calmodulin and PKC-mediated pathways. EP3 is mainly associated with the G$_{\alpha}$i subunit which plays an inhibitory role in the cAMP generation. As an active immune and inflammatory response regulator, PGE$_2$ has been reported to promote cancer progression and angiogenesis, facilitate treatment resistance by regulating cancer stem cell (CSC) self-renewal and epithelial to mesenchymal transition (EMT), and cause immune evasion. Currently, three different types of small molecules targeting the COX/PGE$_2$/EP signaling axis have been investigated in the treatment of NB. They are COX inhibitors (diclofenac, aspirin, celecoxib, and NS-398), mPGES-1 inhibitors (compound III and compound 934), and EP2 antagonists (TG6–10–
1 and TG6–129). Abbreviations: AA, arachidonic acid; AC, adenylyl cyclase; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; CSC, cancer stem cell; DAG, diacylglycerol; DC, dendritic cell; EMT, epithelial to mesenchymal transition; ER, endoplasmic reticulum; IP3, inositol triphosphate; MDSC, myeloid-derived suppressor cell; mPGES-1, microsomal prostaglandin E synthase 1; NK, natural killer cell; PGE₂, prostaglandin E2; PGH₂, prostaglandin H2; PKA, protein kinase A; PKC, protein kinase C; PLA₂, phospholipase A2; PLC, phospholipase C; TAM, tumor-associated macrophage.
Fig. 2.
Potential crosstalk between PGE2 signaling and tumorigenic pathways in NB. Upon activation by PGE2, the Gα subunit released from EP receptors can induce the PKA-associated phosphorylation of Ras, which subsequently activates the Ras/Raf/MEK/ERK signaling. Meanwhile, Gα may also interact with axin to activate the canonical Wnt/β-catenin pathway. On the other hand, the freed Gβγ subunits can induce the PI3K/Akt signaling, which may interact with mTOR and NF-κB to induce the transcription of pro-inflammatory genes and oncogenes. Akt can also phosphorylate GSK-3β and stabilize β-catenin signaling. In addition, β-arrestin/Src complex released upon EP receptor activation may induce the Ras/MAPK pathway, which might contribute to the progression of NB.

Abbreviations: Akt, protein kinase B; APC, adenomatous polyposis coli; CK1, casein kinase 1; EGFR, epidermal growth factor receptor; EP, PGE2 receptor; ERK1/2, extracellular signal-regulated kinases 1/2; Grb2, growth factor receptor-bound protein 2; GSK-3β, glycogen synthase kinase 3β; LRP5/6, low-density lipoprotein receptor-related protein 5/6; MEK1/2, MAPK kinase 1/2; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor
κB; PI3K, phosphatidylinositol-3-kinase; PIP3, phosphatidylinositol 3,4,5-triphosphate; PKA, protein kinase A; SOS, Son of the Sevenless.
Fig. 3. Potential targets related with PGE2/EP2 signaling in NB. (A) The heatmap of the top 20 EP2-correlated genes from seven NB patient cohorts (SEQC-498, Kocak-649, NRC-283, Versteeg-88, Westermann-579, Cangelosi-786, and Olsen-72606) was generated using Prism GraphPad. The intensity of the blue color indicates the value of Pearson correlation coefficient (r). (B) The Venn diagram reveals five genes, namely CCDC88C, SHISA3, HAPLN3, ADAMTS3 and SLC1A5, as the most frequently presented, EP2-correlated genes in at least three NB patient datasets. (C) The Kaplan-Meier survival analysis of NB patients across three NB patient datasets (SEQC, Kocak, and NRC) on R2 platform. N/A, data not available.
## Table 1

Pharmacological studies targeting PGE\(_2\) biosynthesis or signaling for neuroblastoma.

| Compounds | Targets | Animal models | Dose and route | Main outcomes | References |
|-----------|---------|---------------|----------------|---------------|------------|
| Diclofenac | COX | Xenografts derived from SH-SY5Y cell line in nude rats | 250 mg/L in drinking water for 11 days | Inhibition of tumor growth; induction of apoptosis | [46] |
| Diclofenac | COX | Xenografts derived from SK-N-AS cell line in nude mice | 250 mg/L in drinking water for 10 days | Inhibition of tumor growth | [11] |
| Diclofenac | COX | TH-MYCN transgenic mice with spontaneous NB | 10 mg/L in drinking water for 14 days | Inhibition of tumor growth | [12] |
| Aspirin | COX | TH-MYCN transgenic mice with spontaneous NB | 10 mg/kg, p.o., for 10 days | Inhibition of tumor growth and inflammation | [47] |
| Celecoxib | COX-2 | Xenografts derived from SH-SY5Y cell line in nude rats | 10 mg/day, p.o., for 10 days | Inhibition of tumor growth | [46] |
| Celecoxib | COX-2 | Xenografts derived from SH-SY5Y cell line in nude rats | 10 mg/day, p.o., combined with 2 mg/kg irinotecan or 1 mg/kg doxorubicin, i.p., for 12 days | Inhibition of tumor growth; sensitization of tumor cells to doxorubicin or irinotecan | [48] |
| Celecoxib | COX-2 | PDXs in nude mice | 5 mg/kg, combined with 5.9 mg/kg or 59 mg/kg irinotecan, i.p., for 20 days | Inhibition of tumor growth; induction of apoptosis | [49] |
| NS-398 | COX-2 | Xenografts derived from SK-N-AS cell line in nude mice | 15 mg/kg, i.p., for 5 weeks | Inhibition of tumor growth, angiogenesis, and bone metastasis | [50] |
| Compound III | nPGES-1 | Xenografts derived from SK-N-AS cell line in nude mice | 50 mg/kg, i.p., for 9 days | Inhibition of tumor growth | [12] |
| Compound III | nPGES-1 | TH-MYCN transgenic mice with spontaneous NB | 50 mg/kg, i.p., for 10 days | Inhibition of tumor growth and angiogenesis; M1 macrophage polarization; reduced infiltration and migration of CAFs | [12] |
| Compound III | nPGES-1 | Allografts derived from Neuro-2a cell line in A/J mice | 50 mg/kg, i.p., for 7 days | Inhibition of tumor growth; mild reduction in tumor weight | [13] |
| Compound III | nPGES-1 | Allografts derived from Neuro-2a cell line in A/J mice | 50 mg/kg combined with 0.15 mg/kg vincristine, i.p., for 7 days | Inhibition of tumor growth; significant reduction in tumor weight; sensitization of tumor cells to vincristine | [13] |
| TG6–10–1 | EP2 | Xenografts derived from SK-N-AS cell line in nude mice | 10 mg/kg, p.o., twice daily, for 13 days | Moderate decrease in tumor growth | [56] |
| TG6–129 | EP2 | Xenografts derived from SK-N-AS cell line in nude mice | 10–20 mg/kg, i.p., for 18 days | Inhibition of tumor growth and angiogenesis | [20] |
| TG6–129 | EP2 | Xenografts derived from BE (2)-C cell line in nude mice | 10–20 mg/kg, i.p., for 21 days | Inhibition of tumor growth and inflammation; induction of apoptosis | [20] |
| TG6–129 | EP2 | Allografts derived from NXS2 cell line in A/J mice | 20 mg/kg, i.p., for 20 days | Inhibition of tumor growth, angiogenesis, and inflammation; induction of apoptosis | [20] |

Abbreviations: CAFs, cancer-associated fibroblasts; i.p., intraperitoneal injection; NB, neuroblastoma; PDXs, patient-derived xenografts; p.o., oral gavage.
### Table 2

The functions and potential relationships of the identified five genes with the PGE$_2$/EP2 signaling.

| Genes    | Proteins encoded                                           | Functions                                                                 | Potential relationships with PGE$_2$/EP2                                                                 | References |
|----------|-----------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|------------|
| CCDC88C  | Dishevelled-associated protein with a high frequency of leucine residues | Bind with Gα subunit through the Gα-binding and activating motif to activate the non-canonical Wnt-signaling. | Binding with Gα, activating non-canonical Wnt signaling that facilitates cell migration. The releasing of Gβγ could activate PI3K/Akt and Rac/p21-activated kinase pathway that may contribute to tumor invasiveness and progression. | [78, 79]  |
| SHISA3   | SHISA3 protein                                            | Sequester Fz1 receptor trafficking to the cell membrane; induce cell cycle arrest. | Potential downstream target of CREB and PKA.                                                         | [80, 81]  |
| HAPLN3   | Hyaluronan and proteoglycan binding link protein 3        | Maintain the architecture of the extracellular matrix.                     | Elevated intracellular cAMP could induce the GSK3 phosphorylation and β-catenin mediated transcription, which subsequently may decrease the HAPLN3 protein expression, and therefore creates a disorganized matrix environment which may promote tumor metastasis. | [82, 83]  |
| ADAMTS3  | A disintegrin and metalloprotease with thrombospondin type I motifs 3 | Form and remodel the extracellular matrix; promote lymphangiogenesis.      | Proinflammatory cytokines such as IL-1β, IL-6 and TNF-α induced by the activation of PGE$_2$/EP2 regulates the expression and function of ADAMTS3, which in turn facilitates the vessel generation. | [84, 85]  |
| SCL1A5   | ASCT2                                                     | Mediate the exchange transport between Na$^+$ and glutamine; provide essential amino acid transport in rapid proliferating cells. | Gα activation could phosphorylates and activates the mTOR signaling, which in turn regulates the expression and physiological function of ASCT2. | [86, 87]  |

Abbreviations: ASCT2, Alanine-Serine-Cysteine transporter 2; CREB, cAMP-response element binding protein; GSK3, glycogen synthase kinase 3; IL-1β, interleukin 1β; IL-6, interleukin 6; PKA, protein kinase A; TNF-α, tumor necrosis factor α.