

Review

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Review

Osteopontin, a Key Multifaceted Regulator in Tumor Progression and Immunomodulation

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Abstract: Tumor microenvironment (TME) is composed of various cellular components such as tumor cells, stromal cells including fibroblasts, adipocytes, mast cell, lymphatic vascular cells and infiltrating immune cells, macrophages, dendritic cells and lymphocytes. The intricate interplay between these cells influences tumor growth, metastasis and therapy failure. Significant advancements in breast cancer therapy have resulted in a substantial decrease in mortality. However, existing cancer treatments frequently result in toxicity and nonspecific side effects. Therefore, improving targeted drug deliveryand increasing the efficacy of drugs are crucial for enhancing the treatment outcome and reducing the burden of toxicity. In this review, we provided an overview of how tumor and stroma-derived osteopontin (OPN) plays a key role in regulating the oncogenic potential of various cancers including breast. Next, we have dissected the signalling network by which OPN regulates tumor progression through interaction with selective integrins and CD44 receptors. This review addresses the latest advancement in role of various splice variants of OPN in cancerprogression and OPN-mediated tumor-stromal interaction, EMT, CSCs enhancement, immunomodulation, metastasis, chemoresistance, metabolic reprogramming and further suggest that OPN might be a potential therapeutic target and prognostic biomarker for the evolving landscape of cancer management.

Keywords: breast cancer; osteopontin (OPN); tumor associated macrophages; cancer associated fibroblast; immune modulation

1. Introduction

An estimated new cases of cancer incidences are 19.3 million and cancer related deaths are 10.0 million according to GLOBOCAN 2020 [1]. Recent data showed that female breast canceraccounts for the second most commonly occurring cancer after lung globally. Tumor microenvironment (TME) consists of heterogenous cell subpopulations including infiltrating immune cells like tumor-associated macrophages (TAMs), dendritic cells (DCs), lymphocytesand other stromal cells like cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, and extracellular matrix (ECM) [2]. All these components form a complex network through cell-cell and cell-matrix interactions to shape the microenvironment conducive to tumor progression and metastasis. These cells enhance several hallmarks of cancer via secreting a richarray of cytokines and chemokines [2].

Osteopontin (OPN), a sialic acid rich, non-collagenous, chemokine like, glycosylated, phosphoprotein is expressed in various cell types such as osteoblasts, osteoclasts, epithelial cells, endothelial cells and immune and stromal cells [3]. OPN has emerged as a pivotal mediator facilitating intracellular crosstalk within the breast TME. Due to variations in post-translational modifications (PTMs) and proteolytic cleavage, the molecular weight of OPN ranges from 44 to 75

kDa [3]. OPN plays a crucial role in several normal physiological processes including vascularisation, bone remodelling and immune-regulation as well as pathological processes including tumor progression, metastasis, immunosuppression, angiogenesis and chemoresistance [4,5]. The overexpression of OPN has been linked to poor prognosis in various malignancies, including breast, lung cancer, glioblastoma, colorectal cancer, hepatocellular carcinoma, bladder cancer, melanoma, and acute myeloid leukemia [6]. It also serves as diagnostic as well as prognostic biomarker in different cancers [6].

In the present scenario, the diverse function attributes to OPN in tumor progression are directly linked to its structural features binding to specific integrins and CD44. Moreover, OPN-mediated signalling activates various oncogenic molecules and promotes tumor growth as well as metastasis [3]. Interestingly, OPN also governs immunosuppression by activating macrophages and suppressing T-cell activation in various cancers [7]. Single cell and spatial transcriptomics analyses, revealed a correlation between interactions of CAF and OPN+ TAM with the progression of cancer [8].

This review comprehensively covers recent progress in the field of OPN with a focus on elucidating its structural features, immune modulatory properties, its role in regulating CAFs, influencing epithelial-mesenchymal transition (EMT), contributing to the enrichment of cancer stem cells (CSCs), promoting metastasis, fostering therapy resistance, and influencing metabolic regulation. The goal of this review is to provide the latest progress in elucidating the role of OPN in shaping the TME. We also discuss recent treatment strategies to target OPN and its receptors for the management of cancers using small molecules inhibitors, monoclonal antibodies and aptamer-based approaches.

2. OPN Structure and Function

Structural Architecture and Splice Variants of OPN

OPN is a member of SIBLING (small integrin binding ligand N-linked glycoprotein) family of ECM-associated chemokine like aspartic acid rich, glycosylated phospho-sialoprotein [9]. It consists of various domains such as arginine-glycine-aspartic acid (RGD) containing integrin binding, two calcium binding, MMP and thrombin cleavage and CD44 binding site [10].

The $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 1$ and other integrins bind OPN through RGD motif whereas $\alpha 9\beta 1$ and $\alpha 4\beta 7$ integrins interact with OPN through SVVYGLR motif. The C-terminal region of OPN is responsible for the interaction with CD44 [11] (**Figure 1**). Moreover, OPN also manifests in five structural isoforms, distinguished by exon deletions, resulting from alternative splicing, exon shuffling, and other PTMs [12]. PTMs including Ser/Thr phosphorylation, tyrosine sulfation and glycosylation, contribute to the molecular weight variation of OPN, ranging from 44-75 kDa. These modifications induce both structural alterations and functional modifications deepening our understanding of the diverse roles of OPN [3]. Moreover, these five isoforms such as OPN-a, OPN-b, OPN-c, OPN-4 and OPN-5 are mainly involved in various tumorigenic events [13] (**Figure 2**). OPN-a, the full-length isoform consists of seven exons whereas OPN-b and OPN-c lacks exon 5 and exon 4 respectively. In addition, OPN-4 lacks both exon 4 and 5 while OPN-5 contains seven exons along with a translation start sequence positioned between the third and fourth exons. More recently, additional splice variants of OPN-5 have been reported which are denoted as OPN-5b, OPN-5c, OPN-5d and OPN-5e [13] (**Figure 2**). However, OPN-5c and OPN-5d have additional 9 bp insertion into the extra exon in between exon 3 and 4 [13]. Apart from secreted OPN (OPN-s), intra cellular OPN (OPN-i) is also found to orchestrate various hallmarks of cancer. Translation of OPN-s originates at the 5' AUG start codon while OPN-i translation begins at the downstream of non-AUG codon. In addition, PTMs in OPN-i deletes the 16-aa signal sequence from the N-terminus which allows it to localize within the cytoplasm [14].

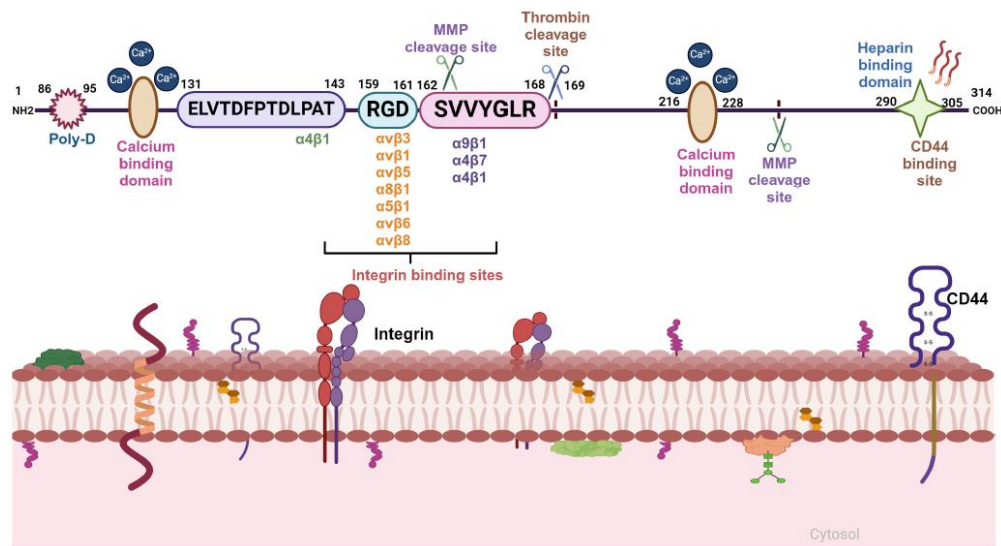


Figure 1. Structural domains of full-length OPN and its receptors. N-terminus of OPN consists of poly-D region, calcium binding domain, and ELVTDFPTDLPAT sequence motif which interacts with $\alpha 4 \beta 1$ integrin. The central region consists of RGD domain, binds with other integrins such as $\alpha v \beta 3$, $\alpha v \beta 1$, $\alpha v \beta 5$, $\alpha v \beta 6$, $\alpha v \beta 8$, $\alpha 5 \beta 1$ and $\alpha 8 \beta 1$; the SVVYGLR sequence binds to $\alpha 9 \beta 1$, $\alpha 4 \beta 1$ and $\alpha 4 \beta 7$ integrins. The C-terminal region includes another calcium-binding domain, MMP-cleavage site, and heparin-binding domain which facilitates the interaction of OPN with CD44. Adopted from Mirzaei et al, Asian Pacific J of Cancer Prevention, 19 (3), 2018 and Bandopadhyay et al, EOTT, 18 (8), 2014 with modifications.

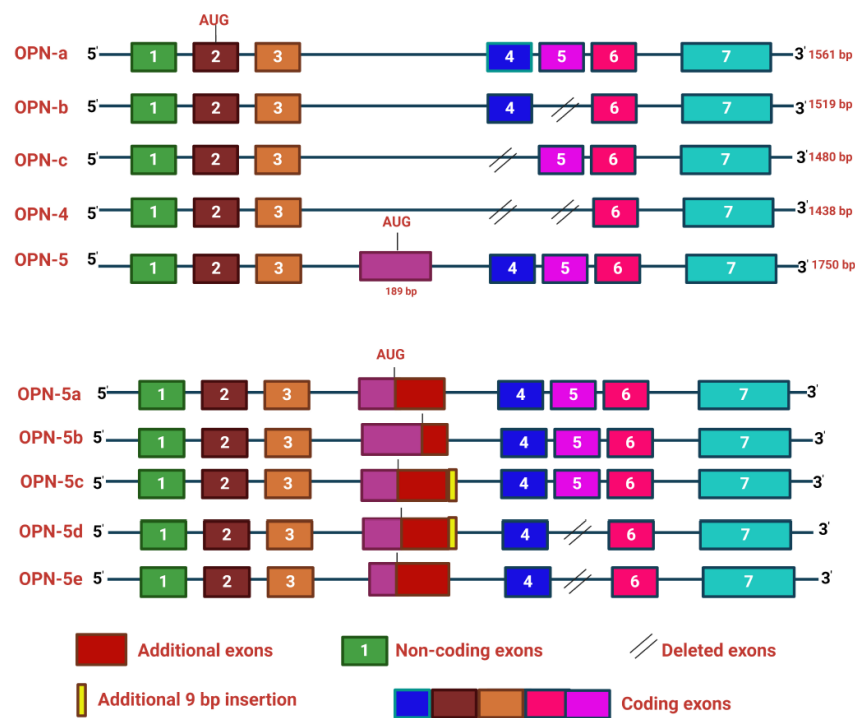


Figure 2. Schematic illustration of the OPN gene and its splice variants. Alternative splicing of OPN transcript results in five splice variants, which are denoted as OPN- a, OPN-b, OPN-c, OPN-4, and OPN-5. OPN-a is a full-length variant that consists of 7 exons; OPN-b lacks exon 5; while in OPN-c exon 4 is absent. In OPN-4, both exon 4 and exon 5 are missing; whereas OPN-5 is the longest variant which consists of an additional exon, generated from a portion of intron 3. Additionally, four new sub-variants of OPN-5 (OPN-5b, OPN-5c, OPN-5d, OPN-5e) have been identified. OPN-5a is the same as OPN-5; OPN-5b has the extra shortened exon while in OPN-5c has additional 9 base pairs in the 3' region of extra exon. In OPN-5d, there is deletion of exon 5 with the addition of 9 base pairs in the 3'

region of the extra exon. OPN-5e lacks exon 5. Adopted from Bastos et al, Intl J of Mol Sci, 24, 2023 with modification.

3. OPN Expression in Various Cancers

OPN exhibits high levels of expression in a wide range of tumor types, such as breast, ovarian, cutaneous, head and neck, thyroid, lung, liver, esophageal, gastric, pancreatic, colorectal, kidney, prostate, bladder and glioblastoma [15]. Tumor, stromal, and tumor- infiltrating myeloid cells express high levels of OPN within the heterogeneous TME [6]. Clinical research has demonstrated a correlation between OPN expression in various tumor tissues, plasma, serum and has been shown to be correlated with an advanced tumor stage, grade, size, invasiveness, metastasis, poor survival rate of patients in a variety of human malignancies [6]. Furthermore, the differential expression level of OPN isoforms vary depending upon the cancer types as shown in **Table 1**. In general, OPN-a is expressed in various malignancies and is involved in promoting cancer progression, metastasis, angiogenesis, immunosuppression and drug resistance. In breast cancer, OPN-a and OPN-c are expressed at higher levels whereas high level of OPN-b is expressed in lung cancer and are associated with poor survival and relapse [16]. However, the correlation of OPN expression with its clinical implications in various cancers remain to be explored.

Table 1. Expression of OPN in various cancers.

Cancer type	Pathway	Function	References (PMID)
Breast Cancer	Akt/Erk-1 Pathway	EMT, Metastasis, Angiogenesis	33603163
	Brk /NF-κB)/ (ATF-4)	Angiogenesis	18172307
	JAK2/STAT3	Apoptosis and migration	19926637
Prostate Cancer	Akt/mTOR and MNK/eIF4E	Restoration of immune surveillance in prostate cancer	37460872
Bladder cancer	JAK1/STAT1 pathway	Apoptosis, Proliferation	32088853
Melanoma	ERK-1/Akt/AP-1 Pathway	Macrophage polarisation, Metastasis, Angiogenesis,	23728342
Glioblastoma	JAK/STAT3 pathway	Angiogenesis	33544472
Gastric Cancer	PI3-K-Akt and NF-κB pathways	ECM degradation, migration and cell proliferation	22912540
	MAPK, PI3K and NF-κB	ECM degradation, metastasis and apoptosis	24756245
Hepatocellular carcinoma (HCC)	JAK2/STAT3/NOX1	Cell proliferation and migration	35418176
Colorectal Cancer (CRC)	PI3K-Akt-GSK/3β-β/catenin pathway	Cell proliferation, migration and invasion	31017126
Oral Cancer	PI3K/AKT/mTOR pathway	Cell proliferation, invasion, metastasis and angiogenesis	33188646
Ovarian cancer	PI3K/AKT signalling	Chemoresistance	34396988
Head and Neck Cancer (HNC)	Integrin αvβ3-NF-kappa B pathway	Cell proliferation, migration, invasion and stemness	29463991
Non-small cell lung cancer (NSCLC)	OPN/integrin αvβ3/FAK signalling	promote cell proliferation in NSCLC	33287873
	NF-κB pathway	metastasis, proliferation and immunosuppression	34423566

4. Role of OPN in Tumor Progression

OPN regulates cancer progression by influencing several hallmarks of cancers through the interaction with its receptors. This section mainly focuses on the mechanism by which tumor and stroma derived-OPN promotes the tumor growth.

4.1. OPN Receptors

4.1.1. Integrin Receptors

Integrins, a heterodimeric cell surface receptor consisting of α and β subunits, are a cell adhesion molecule that bind with matricellular and extracellular matrix (ECM) proteins [15]. Mammals have been shown to possess 18 α and 8 β subunits which constitute 24 different integrins. Both the integrin α and β subunits are type I transmembrane proteins with a short (~30-40 aa) cytoplasmic domain, substantial extracellular domain, and single transmembrane domain [15]. OPN interacts with integrins $\alpha v \beta 3$, $\alpha 8 \beta 1$, $\alpha 5 \beta 1$, $\alpha v \beta 1$, $\alpha v \beta 6$ and $\alpha v \beta 5$ through GRGDS motif whereas binds with integrins $\alpha 9 \beta 1$, $\alpha 4 \beta 1$ and $\alpha 4 \beta 7$ via the ELVTDFPTDLPAT and/or SVVYGLR sequences [11]. The expression of integrins are low in normal adult epithelia whereas significantly high in most of the cancer cell types and tumor tissues [17]. The $\alpha v \beta 3$ integrin not only binds to vitronectin but also interacts with other ECM proteins such as OPN, fibronectin and laminin in various cell types [18]. Expression of $\alpha v \beta 5$ is high in non-small cell lung cancer (NSCLC), prostate cancer, lung cancer, gastric cancer as well as in associated stromal cells [19]. The $\alpha 5 \beta 1$ integrin is an essential angiogenic marker correlating with tumor malignancy, invasiveness and development of metastasis [19]. $\alpha 8 \beta 1$ integrin is mostly expressed in smooth muscle cells [20]. The $\alpha 4 \beta 1$ integrin is overexpressed in leukocytes including eosinophils, lymphocytes, monocytes, macrophages, NK cells, basophils, and mast cells whereas $\alpha 9 \beta 1$ integrin is over expressed in macrophages and other immune cells [21]. OPN is involved in the activation of several signal transduction pathways via integrins that promote tumor metastasis, migration, adhesion and survival [22].

4.1.2. CD44 Receptors

Apart from integrins, OPN also binds to CD44, a type I transmembrane glycoprotein composed of intracellular, extracellular and transmembrane domains [23]. Alternative splicing of CD44 genes result in the generation of two isoforms: CD44s (standard) and CD44v (variant) [23]. CD44v isoforms can have a single variant exon as seen in CD44v6 and CD44v7 or multiple variants as observed in CD44v4-v5 and CD44v3-v10 [24]. Thrombin cleavage of OPN results in two fragments that interact with CD44 independent of RGD sequence [25]. One of which is situated downstream of the RGD motif and shown to overlap with SVVYGLR domain, because binding of OPN and CD44 competes with $\alpha 9 \beta 1$ integrin but not with $\alpha v \beta 3$ [26]. Interaction of OPN with CD44 activates multiple signalling pathways which in turn promote tumor growth. OPN is reported to enhance the radiation resistance by maintaining stemness in the adjacent cells through activation of CD44 in glioma [27]. In addition, OPN secreted by macrophages bind to CD44 and promote invasiveness by activating the Rac-specific guanine nucleotide exchange factor, TIAM1 in bladder cancer [28]. It has been reported that OPN-CD44 interaction activates c-Jun-NH (2)-kinase (JNK) signalling cascade which drives the tumorigenicity in colorectal cancer [29]. Interestingly, binding of OPN with CD44 leads to cleavage of CD44 intra-cellular domain by γ -secretase, which in turn governs the stemness characteristics in glioma [27]. Thus, targeting the OPN-CD44 axis might open a new dimension for cancer therapy.

4.1.3. Receptor-Mediated Signalling

OPN interacts with various integrins as well as other co-receptors collectively and activates downstream complex signalling cascade such as PI3K/Akt, p38/MAPK/ERK, JNK, Ras/Raf/MEK/ERK, JAK/STAT and TIAM1/Rac1 pathway thereby inducing the oncogenic gene expression in various cancer cells [15]. The activation of these pathways in cancer cells regulate several pathological processes such as cell adhesion, migration, invasion, metastasis, proliferation, tumor growth, survival, chemoresistance, stemness, angiogenesis, and immune suppression. In the following section, we highlighted the OPN-mediated activation of PI3K/Akt and p38/MAPK/ERK signalling pathways in various cancer cells [15]. Moreover, we depicted such OPN-mediated signalling pathways in **Figure 3**.

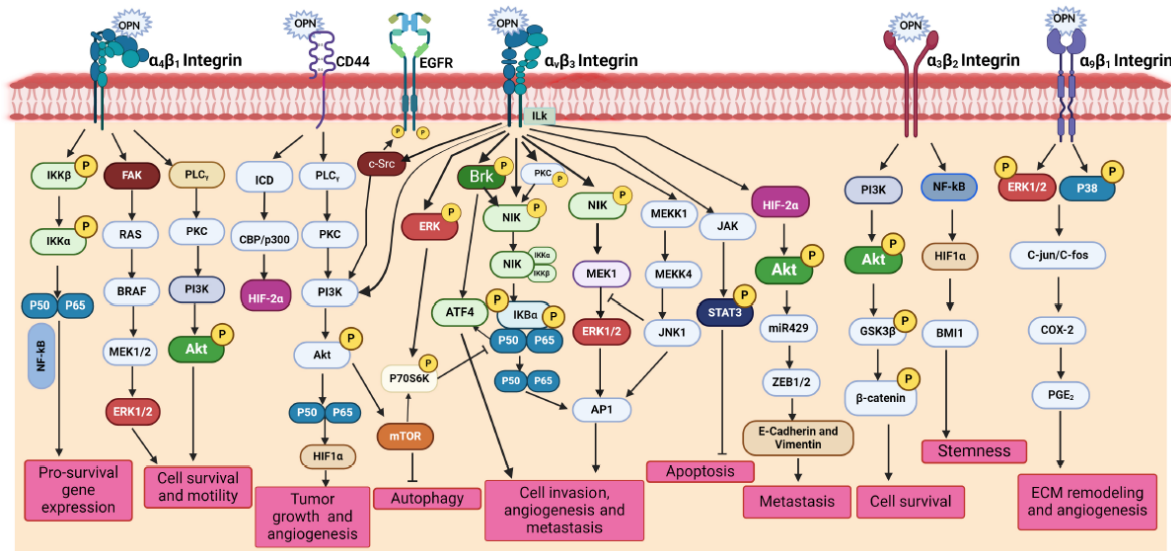


Figure 3. Role of OPN in the regulation of various signalling pathways. OPN through its interaction with $\alpha v\beta 3$, $\alpha 4\beta 1$, $\alpha 3\beta 2$, and $\alpha 9\beta 1$ integrins, and CD44 receptor transduces multiple signalling pathways and their cross-talks such as FAK/MEK/ERK, PKC γ . /PKC/PI3K/Akt/mTOR, NIK/I κ B α /NF κ B, JAK/STAT3, PI3K/Akt/ β -catenin, NF κ B/HIF1 α /BMI1, c-Src/EGFR/MEK/ERK and MAPK pathways. These signalling cascades induce the activation of various tumor-promoting genes such as VEGF, MMPs, and COX-2, thereby inducing the tumor growth at the primary sites, angiogenesis, metastases at the distance sites, ECM remodeling, immune suppression, stemness, immune evasion, chemoresistance, migration, and survival. Adopted from Han et al, Oncology Letters, 17, 2019 and Ahmed et al EOTT, 15 (9), 2011 with modifications.

PI3K/Akt Signalling

The PI3K/Akt signalling cascade plays crucial role in regulating several cellular processes associated with tumorigenesis. Dysregulation of this pathway has been linked to tumor growth, metastasis, EMT, immunosuppression and drug resistance. In colorectal cancer cells, OPN has been identified as a key factor that induces stem cell-like properties and cell invasion via the PI3K-Akt-GSK/ β -catenin pathway [30]. Silencing of OPN downregulates migration and invasion but induces apoptosis and autophagy via inactivating the PI3K/Akt/mTOR pathway [31]. OPN promotes tumor progression and angiogenesis in oral cancer through the activation of PI3K/Akt/mTOR signalling cascade [32]. Moreover, OPN- $\alpha v\beta 3$ interaction increases HIF-1 α expression, which in turn transactivates TCF12 gene expression [33]. TCF12 engages in transcriptional repression of the VE-cadherin gene by interacting with histone deacetylases and EZH2, promoting the endothelial-mesenchymal (EndoMT) transition [33]. OPN expression is regulated by the EGF/PI3K signaling pathway in HepG2 cells [34]. Additionally, OPN upregulates angiogenesis via activation of PI3K/Akt and ERK1/2 pathways in breast cancer whereas promotes tumor progression via PI3K/Akt/Twist signalling axis in HCC [35]. OPN knockdown reduced Akt phosphorylation and down-regulated the expression of VEGF and MMP-2 in gastric cancer [36]. Silencing calpain subunit 4 (Capn4) downregulates OPN expression and suppresses the migration of ovarian cancer cells [37]. Interestingly, OPN has also been reported to modulate drug resistance through PI3K/Akt pathway in several cancers. For example, OPN overexpression increased PI3K, p-ERK1/2, and ERCC1 expressions in lung cancer and caused cisplatin resistance, but OPN silencing decreased this effect [38]. Similarly, blocking OPN-mediated PI3K/Akt signalling pathway reversed OPN-induced cisplatin resistance in HCC cells [39]. Similarly, OPN is upregulated in EGFR-TKI resistant NSCLC cells thereby activating PI3K/Akt pathway leading to downregulation of EGFR-TKI-induced apoptosis while augmenting EMT [40]. Moreover, epoxyazadiradione, a limonoid inhibits the growth of breast tumors by depolarizing the mitochondria and inducing caspase-dependent apoptosis through the inhibition of PI3K/Akt

pathway [41]. A splice variant of OPN, OPN-c has been reported to be involved in promoting tumorigenesis and proliferation by activating PI3K/Akt pathway in ovarian cancer [42].

p38/MAPK Signalling

The binding of OPN to its receptors activates MAPK pathway which in turn regulates several vital processes like EMT, chemoresistance and senescence [15]. It is observed that OPN-induced the PI3K/Akt and MAPK/Erk1/2 cascade promotes EMT which in turn is associated with increase cell migration, invasion, and proliferation in lung cancer [43]. OPN-mediated stimulation of the MAPK pathway is essential for the cell growth and metastasis in HCC [44]. The binding of OPN to $\alpha v \beta 3$ stimulates VEGF expression via MAPK pathway leading to increased prostate cancer cell invasion [45]. However, OPN stimulates NF- κ B nuclear translocation through the PI3K/Akt and MAPK pathways, which in turn increases HIF-1 α to support the growth and survival of gastric cancer cells [46]. It was revealed that OPN-knockout breast cancer cells exhibit increased levels of cyclophosphamide-induced apoptosis when compared with control [47]. The expression of Immediate Early Response 2 (IER2) triggers senescence in melanoma cells via p53/MAPK/Akt-dependent manner and results in poor prognosis in melanoma patients [48]. Doxorubicin-treated breast cancer cells inhibit caspase-3-induced apoptosis via activating the MAPK pathways in response to OPN [49].

Other Signalling

Many other signalling pathways are also involved in OPN-mediated regulation of tumor progression, angiogenesis and metastasis. For instance, OPN interacts with CD44 and integrins and mediates several signalling networks such the JAK/STAT and NIK pathway to trigger gene expression that mediates invasion, metastasis, and angiogenesis. Intracellular signalling pathways can be triggered by the c-Src-dependent transactivation of EGFR through interaction with OPN and integrin via the Brk/NF- κ B signalling pathway that ultimately regulates VEGF expression in breast cancer cells [50]. Under hypoxic conditions, OPN triggers integrin-linked kinase (ILK)/Akt-mediated NF- κ B activation, which results in HIF-1 α -dependent VEGF production in breast cancer cells and subsequent angiogenesis [15]. NF- κ B and HIF-1 α are involved downstream of OPN signalling and induce a CSC-like phenotype in HCC [6].

4.2. Multifaceted Functions of OPN in Tumor Progression

OPN has been shown to stimulate the progression of cancer by activating specific signalling pathways. These components collectively create an intracellular "signalling traffic network" that controls the expression of various oncogenic molecules which are essential for initiating tumorigenesis, regulating EMT, stemness, angiogenesis, metastasis and drug resistance. The OPN-regulated various oncogenic functions are depicted in **Figure 4**.

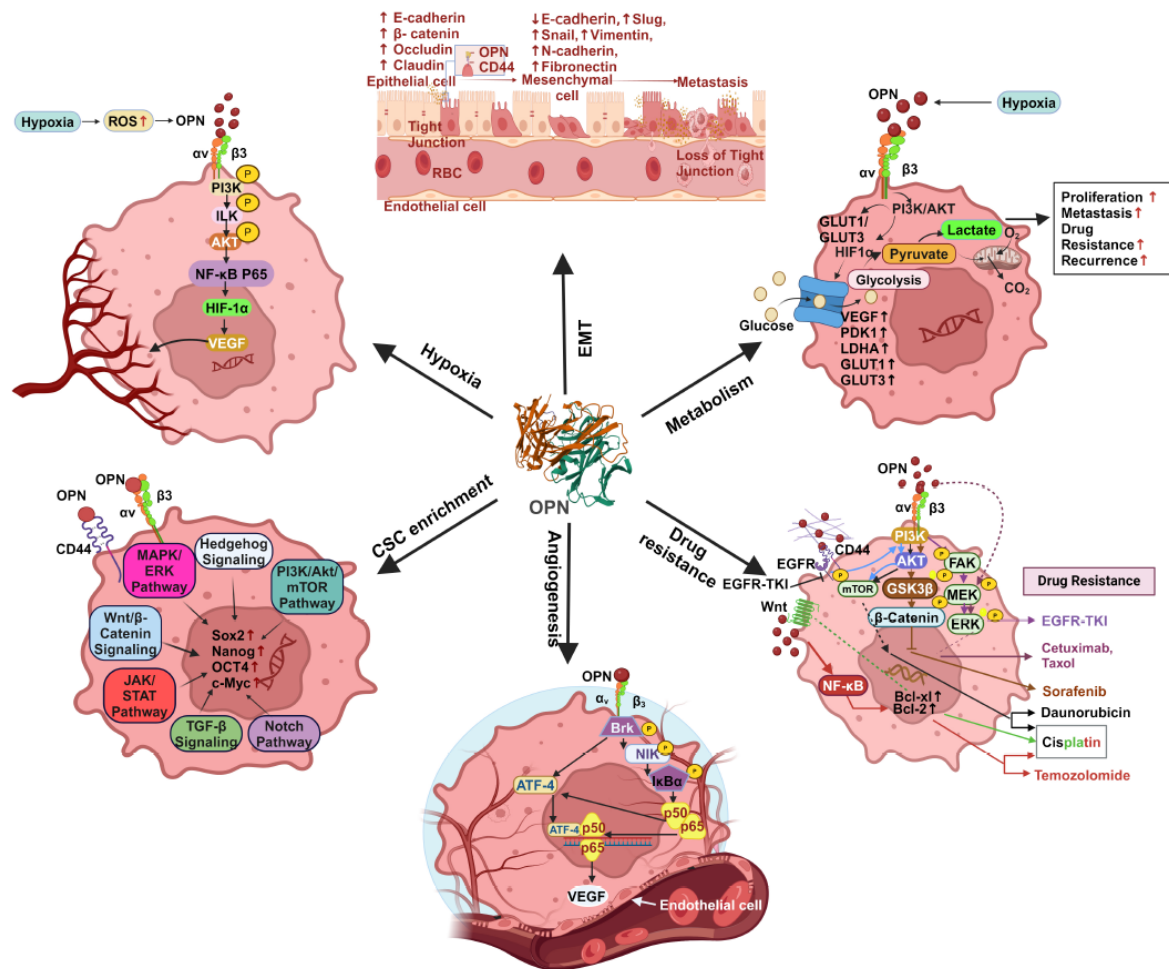


Figure 4. Diagrammatic representation of multifaceted function of OPN in various tumors. OPN regulates the epithelial to mesenchymal transition (EMT) resulting in the loss of tight junctions, thereby enhancing metastasis, with the high expression of vimentin, slug, snail and fibronectin. Under hypoxic conditions, OPN induces activation of PI3K leading to phosphorylation of Akt, thereby upregulating VEGF dependent angiogenesis. OPN is responsible for metabolic function by activating HIF1α, under hypoxic conditions which further aids in the glycolytic process with high expression of VEGF, PDK1, LDHA, iNOS, GLUT1 and GLUT3. OPN further regulates CSC enrichment by activating a cascade of signalling pathways involving PI3K/Akt/mTOR, Hedgehog, MAPK, Wnt/β-catenin, JAK/STAT and Notch signalling. OPN-activated signalling pathways result in drug resistance. The interaction between OPN, αvβ3, and CD44 results in the activation of PI3K/Akt, FAK/MEK/ERK, EGFR, and Wnt/NFκB signalling cascades thereby aiding therapeutic resistance.

4.1.1. EMT

EMT is a multifaceted process that occurs when epithelial cells lose their adhesion capacity and acquire a mesenchymal phenotype. The loss of epithelial integrity is characterized by downregulation of E-cadherin and upregulation of N-cadherin, fibronectin, vimentin and [11]. OPN modulates many EMT-related transcription factors such as Twist, Snail, and Slug in various solid cancers including breast. OPN also plays a critical role in initiating EMT through activation of Twist in breast cancer [51]. Interestingly, Butti et al have demonstrated that OPN-educated fibroblasts induce EMT in breast cancer cells through the secretion of CXCL12 and promotes breast cancer progression [52]. Moreover, OPN triggers an autocrine MAPK intracellular signalling cascade which leads to activation of Twist and upregulation of Bmi1 in MDA-MB-231 cells [53]. Similarly, Snail is also involved in the upregulation of genes linked with mesenchymal and invasive characteristics [51]. OPN interacts directly with Runx2 resulting in induction of Snail-dependent EMT in mammary epithelial cells [54]. OPN-specific aptamer reduces Snail expression thereby abrogating EMT using *in*

vitro breast cancer models [51]. In addition, OPN induces NF- κ B activation and increases the expression of both ZEB1 and ZEB2 resulting in the acquisition of an EMT-like phenotype in breast cancer cells [55]. The intricate interplay between the tumor and its surrounding environment is a crucial regulatory component of EMT and OPN is proven to be a key player in the tumor-stroma interaction. Tumor derived OPN upregulates Cox-2 expression in TAMs leading to an enhancement in angiogenesis and melanoma growth [56]. Myofibroblasts produced directly by OPN or by other mechanisms secrete a variety of chemokines, TNF β , TGF β , IL-6, sphingosine-1-phosphate (S1P) and angiotensin II (Ang II) which promotes EMT [57]. In both *in vitro* and *in vivo* breast cancer models, tumor-derived OPN have been shown to regulate the transformation of tissue-resident normal mammary fibroblasts into tumor-supporting CAFs [58].

Both tumor-derived and exogenous OPN can trigger the transition of mesenchymal stem cells (MSCs) to CAFs in breast cancer models. This is accomplished by activating MSCs to generate TGF β , thereby initiating a feedback loop that drives the CAF phenotype [59]. OPN is found to upregulate HIF-1 α which leads to Twist 1 activation followed by EMT. Remarkably, OPN-s initiates cancer metastasis by inducing the EMT, whereas OPN-i activates the MET to promote metastatic formation [60]. Therefore, targeting OPN/ α v β 3 integrin and OPN/CD44 signalling cascade may control epithelial-mesenchymal plasticity in various cancers.

4.1.2. Enrichment of CSC

CSCs, also known as tumor-initiating cells, are a subpopulation of cells within a neoplasm that exhibit distinctive characteristics, including self-renewal capacity, pluripotency, and the ability to generate distinct progeny, mirroring the heterogenous inherent in the primary tumor [61]. These cells possess a unique ability to sustain tumorigenesis by maintaining their undifferentiated state, resisting conventional anti-cancer therapies, and orchestrating the hierarchical organization of the tumor mass [61–63]. CSCs are majorly characterized by the expression of CD44, CD24, ALDH1 and CD133 in breast cancer [61].

Several factors secreted by tumor and stroma, cell-cell contacts or cell-matrix interactions are reported to impact the stemness of cancer cells through autocrine, paracrine and juxtacrine mechanisms [11]. OPN emerges as a critical player in the enrichment of CSCs for its attribute to interact with its receptor and a recognized stem cell marker, CD44 [64]. OPN along with the stem cell marker CD44, shapes a perivascular niche that promotes CSCs phenotype and radiation resistance in glioma. The γ -secretase-cleaved intracellular domain of CD44 interacts with OPN that enriches stem cell phenotype and glioma growth through CBP/p300-dependent activation of HIF-2 α [27]. Furthermore, ALDH^{hi}CD44⁺CD24⁻ stem cells successfully metastasize to bone, where bone-derived OPN is implicated in promoting stem-like phenotype in breast cancer cells, thereby influencing metastasis. The mechanism underlying OPN-induced CSC-facilitated metastasis involves CD44 and RGD-dependent cell surface integrins, which augment the functional response to bone-derived OPN, potentially through activation of WNK-1 and PRAS40-related pathways [65]. Thus bone metastasis can be targeted by disrupting this dynamic interaction. However, another study reported the contrasting role of osteoclast-derived OPN in bone metastasis, suggesting a counterintuitive option for the treatment of breast cancer-associated bone metastasis [66]. Mechanistically, the Lrp5-overexpressing osteoclast-derived OPN regulates Hsp90ab1 (Hsp90 beta) and moesin (MSN). Importantly, Hsp90ab1 immuno-precipitated latent

TGF β and inactivated TGF β , whereas MSN interacted with CD44 thereby inhibiting CD44 pathway [66]. This interaction suggests a potential impact on CSCs highlighting the complexity of the regulatory network established by OPN. The dichotomous role of OPN signalling may arise from the existence of multiple isoforms of both OPN and its receptors, each exhibiting context-dependent functions. Hu et al demonstrated that CD44v exhibits significantly higher lung metastatic potential as compared to CD44s in CD24/CD44⁺ CSC population [67]. Modulating the CD44v/CD44s ratio through epithelial splicing regulatory protein 1 (ESRP1) expression influenced lung metastasis without affecting the stemness. CD44v, responsive to OPN in the lung environment, enhances cancer cell invasiveness and promotes lung metastasis, distinguishing it from CD44s [67]. Their findings

identify a subset of metastatic breast CSCs marked by CD44v expression and respond to OPN, suggesting CD44v and OPN as key regulators of CSCs and metastasis.

4.1.3. Chemoresistance

The primary contributor to the ineffectiveness of chemotherapy in treating cancer stemness from the emergence of drug resistance against conventional therapeutic interventions. Chemoresistance can be induced by the interplay between the heterogeneous cell population within the TME [68]. This interplay between several intrinsic factors such as tumor mutation load, heterogeneity, ECM and epigenetic modifications, along with extrinsic factors such as pH, hypoxia, paracrine signalling, and other stromal cells which triggers chemoresistance [69]. Mechanistically, chemoresistance results due to upregulation of various multidrug resistance efflux pump such as ABCC 1-9, ATP binding cassette (ABC) transporters and P-glycoprotein (P-gp) [70]. Elevated level of OPN mRNA expression is associated with poor disease free survival (DFS) and overall survival (OS) in a large cohort of breast cancer patients treated with adjuvant chemotherapy in clinical trials [71]. Overexpression of ABC transporters reported to be a key factor in drug resistance as it can mediate the efflux of various drugs thereby decreasing the intracellular concentration of the drug. Stromal OPN increases ABCG2 expression and enriches the side population (SP) through ERK2-dependent pathway in melanoma [72]. In prostate cancer, it has been shown that the activation of FAK leads to increased expression of P-gp upon the binding of secreted OPN to $\alpha v \beta 3$ integrin [73]. Furthermore, Yi et al has shown that binding of OPN to $\alpha v \beta 3$ through activating the PI3K/Akt/GSK3 β / β -catenin signalling cascade results in cell survival and sorafenib insensitivity in FLT3-ITD mutant AML cells [74]. OPN causes aberrant activation of the PI3K/Akt signalling pathway via CD44 and $\alpha v \beta 3$ integrin while blocking OPN could reverse the chemoresistance of cisplatin in HCC [39]. Upon binding to $\alpha v \beta 3$, OPN induces autophagy thereby maintaining Foxo3a stability, which increases tumor growth and resistance to epirubicin and cisplatin in HCC cells [75]. Hyperactivated EMT has also been shown to be linked with increased treatment resistance in cancer cells and this may be induced by abnormal activation of multiple signalling pathways that drive the EMT phenotype [76]. Moreover, OPN induces the expression of N-cadherin, vimentin, Twist, Slug, MMP9 by activating the GLI-dependent Hedgehog signalling pathway in breast cancer. In this study, they have also reported that OPN upregulated the expression of drug resistant related proteins like ABCB1 and ABCG2 which in turn promote the EMT and induces efflux of therapeutic drugs such as paclitaxel, cisplatin and doxorubicin [77].

4.1.4. Angiogenesis

Angiogenesis, a pivotal process in tumor growth, is intricately regulated by the VEGF. It has been shown that OPN secreted by the tumor cells has the ability to enhance the metastatic potential and angiogenesis by regulating VEGF [78]. For example, upregulation of OPN by TBX3iso1 in breast cancer cells leads to angiogenesis using *in vivo* mice models [79]. ADAM8 activates OPN expression through the JAK/STAT3 pathway thereby enhancing angiogenesis in U87 cells and primary macrophages [80]. In addition, high levels of ADAM8, a proteolytically active member of the ADAM family is found in several malignancies contribute to tumor cell migration and invasion and are associated with poor patient prognosis [80]. Chakraborty et al. have shown that OPN promotes VEGF-dependent angiogenesis via the activation of the Brk/NF- κ B/ATF-4 signalling pathway in paracrine, autocrine and juxtacrine manner in breast cancer [81]. Moreover, under hypoxic condition, OPN modulates HIF1 α -induced VEGF expression via ILK/NF- κ B signalling cascade which ultimately culminates in breast cancer progression and angiogenesis [82]. OPN induced ICOSL promotes angiogenesis and cell migration in breast cancer [83]. The interaction between SPP1+ macrophages and endothelial cells promotes angiogenesis through VEGF-A-VEGFR1/R2 [84].

4.1.5. Metastasis

In several cancers, there is a correlation between high level of OPN expression and metastasis. OPN facilitates the molecular process that dictate the development of metastatic lesions, including inhibition of apoptosis, ECM degradation and remodeling, cellular migration, host immune cell

evasion and neovascularization [85]. Breast cancer cells spontaneously metastasized to lung in mouse model and the level of OPN is high in lung lesion [86]. The level of serum OPN is closely correlated with lymph node metastasis in small cell lung cancer suggesting that OPN acts as predictor of OS [87]. OPN secreted by tumor-associated cells, increased the expression of CD44v6 in colon cancer stem cells through Wnt/ β -catenin pathway thereby promoting migration and metastasis [88]. In gastric cancer, increased OPN expression is linked to lymph node and distant metastasis [89]. The SNP at locus -443 of OPN promoter and related haplotypes (Ht2 and Ht3) considerably boost the activity and expression level of OPN promoter which significantly increased tumor growth and lung metastasis in HCC [90]. Lysosomal-associated membrane protein 3 (LAMP3) may control the invasion and metastasis by regulating downstream signalling of OPN in osteosarcoma [91]. OPN acts as diagnostic predictor with specificity and sensitivity of 96.15% and 52.94% respectively in bone metastasized breast cancer patients [92]. The osteolytic bone metastasis in breast cancer is significantly inhibited in conditional knocked down of OPN in nude rat models [93]. The neutralization of OPN effectively mitigated the enhanced osteoclast development and bone metastasis induced by Fam20C deficiency [94]. Combination of erufosine with OPN knockdown enhances anti-metastatic effect for the control of skeletal metastases using breast cancer cells [93]. Breast cancer bone metastasis is thought to be significantly influenced by RUNX2 which is regulated by OPN- α v β 3/CD44 axis [95]. The premetastatic niche in bone marrow requires OPN-dependent migration of CAF to promote the stemness using *in vivo* breast cancer model [96]. Therefore, OPN is a vital regulator of bone and lung metastases in breast cancer.

4.1.6. Cancer Cell Metabolism

Through metabolic alterations linked to carcinogenesis, transformed cells can persist in abnormal growth and invade various tissues by avoiding tissue homeostasis and utilizing an array of internal signalling pathways along with a variety of local tissue and whole-body resources. Significantly, the stromal cells in the TME, and the transformed cells themselves, all undergo metabolic remodelling in various cancers [97]. This promotes the accumulation and dissemination of cancer cells, weakens the immune system to prevent tumor growth, and increases the lethality associated with cancer [97]. This can be accomplished by rewiring the glucose metabolic pathways, which confer drug resistance and facilitate metastasis [98]. Tumor cells require glucose as a metabolic energy source for survival and proliferation. Glucose transporters (GLUTs) facilitate aerobic glycolysis, commonly called the Warburg effect, by delivering glucose into the cytosol. The class I glucose transporters GLUT1 and GLUT3 are sensitive to hypoxia and have a strong affinity for glucose [99]. Numerous genes including OPN, VEGF, PDK1 (pyruvate dehydrogenase kinase 1), iNOS, LDHA (lactate dehydrogenase A), EPO, GLUT1 and GLUT3 are regulated by HIF-1 under hypoxic conditions [100,101]. It has been reported that GLUT1 and GLUT3 upregulation is linked to a poor prognosis in breast cancer [102]. Additionally, poor survival in NSCLC is associated with overexpression of GLUT1 [103]. OPN is upregulated in hypoxic environments, that leads to increased expression of GLUT1 and GLUT3 via α v β 3 integrins mediated PI3K/Akt and p38 MAPK pathways. This process ultimately results in the development of osteosarcoma [99].

5. OPN-Mediated TME Regulation

The ECM, stromal cells, immune cells, extracellular proteins, fibroblasts, cytokines and growth factors define the complex microenvironment surrounding the tumors. Recent evidence suggests that tumor cell-TME interaction modulates tumorigenesis, tumor cell invasion, metastasis, chemoresistance, and immune response (Figure 5).

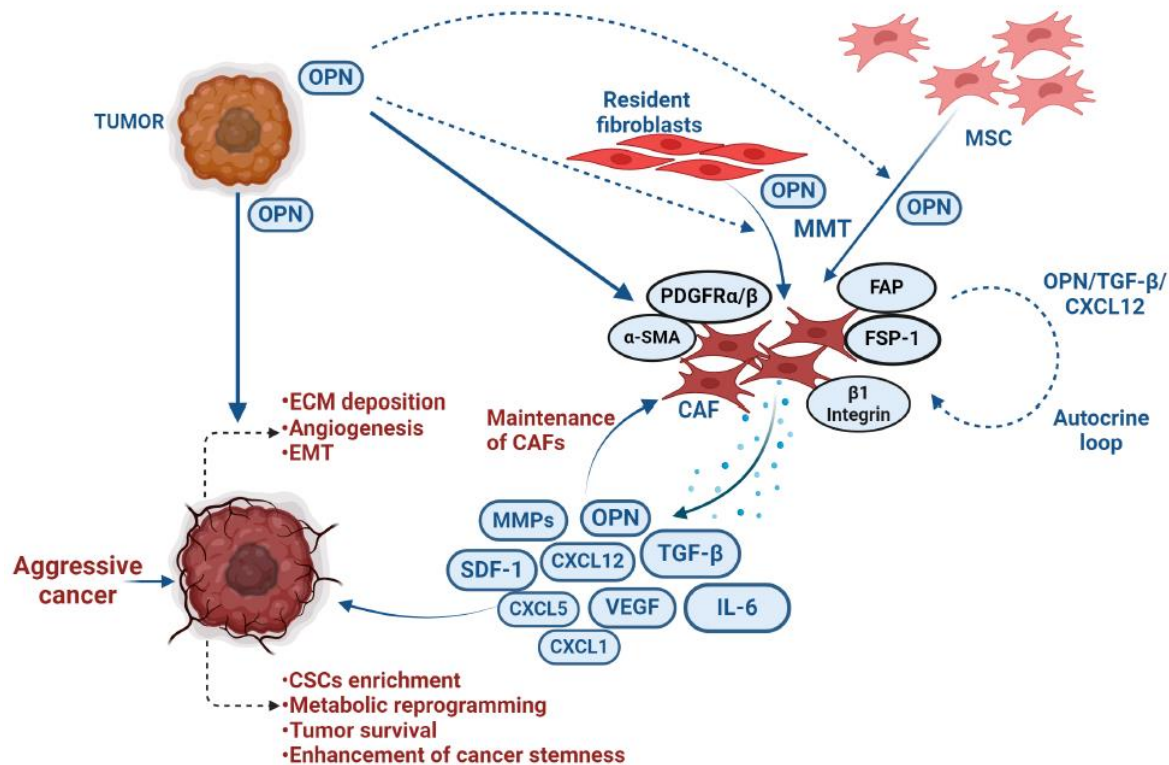


Figure 5. Myofibroblast differentiation of resident fibroblasts and MSCs by tumor-derived OPN. Tumor-derived OPN is involved in the transition of resident fibroblasts and MSCs into myofibroblast or CAFs. CAF-derived factors induce ECM deposition, EMT, angiogenesis, CSC enrichment, metabolic reprogramming, and tumor survival, resulting in the enhancement of tumor progression.

5.1. CAF

The reciprocal interaction between tumor cells and fibroblasts plays a crucial role in the progression of breast cancer [104,105]. OPN emerges as a key regulator in facilitating the crosstalk between tumor and stromal fibroblasts, contributing to breast cancer advancement [62,104,106]. Sharon et al demonstrated that OPN has the ability to reprogram normal fibroblasts, inducing a proinflammatory state that supports the growth of breast cancer. In this study, the authors have revealed that the OPN-reprogrammed fibroblasts secrete various proinflammatory cytokines, including CXCL1, CXCL2, COX-2, and IL-6. The ability of OPN to reprogram mammary fibroblasts is found to be reliant on signalling through CD44 and $\alpha\beta3$ integrin [107]. Butti et al have reported that tumor cell-derived OPN, triggers the differentiation of fibroblasts into CAFs through activation of Twist1 [52]. Mechanistically, OPN induces Twist1-dependent myofibroblastic gene expression such as α -SMA, FSP, FAP, SDF1 and PDGFR β by binding to CD44 and $\alpha\beta3$ integrins, activating Akt and ERK signalling pathways. OPN-driven CAFs then release CXCL12, inducing EMT and angiogenesis. OPN and CXCL12 are identified as crucial components perpetuating this crosstalk [52]. Costa et al have identified FAP+ CAFs (CAF-S1) subset enriched mainly in TNBC and it induces the immunosuppressive microenvironment by secreting CXCL12 [108]. The induction of FAP and CXCL12 in CAFs by OPN suggested that the reprogramming of CAFs may play an immunosuppressive role through CXCL12 secretion. Moreover, studies also implicated the involvement of OPN autocrine signalling in the generation of CAFs using the loss of function studies in fibroblasts [52,107]. To support this observation, it has been indicated that CAFs actively secrete OPN while its silencing in α -SMA+ CAF attenuated the growth of colonies using breast cancer cells [109]. The 3D cell culture and animal model data revealed that blocking CAF-derived OPN effectively prevented lung metastasis in breast cancer. Intriguingly, this study also highlighted a correlation between OPN expression and tumor invasiveness in patient specimens [110]. Collectively, the studies

demonstrate that OPN plays an important role in CAF and cancer cell interaction leading to breast cancer progression.

The senescent fibroblasts exhibit characteristics reminiscent of myofibroblasts (CAFs), serving as potential regulators of senescence-associated cancers. Senescent fibroblasts induce preneoplastic growth through OPN [111]. Interestingly, the reduction of OPN levels through RNAi did not affect the induction of senescence in fibroblasts, however, it had a profound impact on diminishing the growth-promoting activities of senescent fibroblasts [112]. Another study has shown that altering Tiam1 expression in senescent fibroblasts induces the invasive and migratory potential, EMT, and CSC characteristics by upregulating OPN in breast cancer cells [110].

Although resident fibroblasts contribute majorly to CAF population, generation of CAFs from different cellular sources including MSCs have been well documented [113]. Tumor-derived OPN has also exhibited a very significant role in the generation of CAFs from MSCs in breast cancer. OPN induces expression of various myofibroblast markers including α -SMA, FSP-1, CXCL12. OPN-educated MSCs also produce higher levels of CXCL5 through integrins and activate c-Jun and OPN-interacted MSCs induce breast cancer metastasis [114]. Moreover, OPN instigates integrin-dependent MSC expression via TGF- β 1 to facilitate acquisition of CAF phenotype. OPN activates the TGF- β expression transcriptionally via MZF-1 transcription factor. This study concluded that tumor-derived OPN induces MSC-CAF trans-differentiation to enhance tumor growth and metastasis via the OPN–MZF1–TGF- β 1 signalling pathway [59]. These studies show that OPN may be a potential fibrogenic factor in breast tumor microenvironment to facilitate growth and metastasis of cancer cells.

5.2. Adipocytes

Adipose tissue (AT) is the site of inflammatory responses linked to obesity, which triggers a range of cytokines and modify metabolic regulation [115]. It has been demonstrated that OPN causes inflammatory signalling in adipocytes and is primarily produced by macrophages in obese AT [116]. Obese humans and mice exhibit elevated levels of OPN in plasma circulating levels as well as in AT macrophages [117]. MMP-2 and MMP-9 are highly expressed in various cancer cells and targeting MMPs with its inhibitor may act as important therapeutics in cancer [118].

5.3. Osteoclast

Osteoclastic cells have originated from monocytic lineage involved in bone remodelling, bone resorption and ossification [119]. About 15 to 30% of breast cancer patients are prone to metastasized to bone [94]. Zuo et al have shown that neutralization of OPN downregulates *Fam20C* deficiency which reduces differentiation of osteoclast along with bone metastasis [94]. This study has established a correlation between OPN and osteoclastogenesis and suggested that OPN may act as a potential therapeutic target for breast cancer bone metastasis.

6. OPN in Immunomodulation

Within the heterogeneous TME, tumor immune microenvironment (TIME) comprises spatially distributed immune cells such as lymphocytes, monocytes, dendritic cells and macrophages. These cells play a critical role in supporting tumor progression and metastasis via crosstalk with the cancer cells. The role of OPN in immunomodulation has been greatly appreciated in mediating tumor-immune cell interaction and creating an immunosuppressive TIME. OPN can reprogram immune cells such as macrophages to induce tumor growth, angiogenesis and metastasis by secreting pro-tumorigenic cytokines and growth factors. Further, these re-educated immune cells may also secrete OPN which further aids in tumor progression [11]. Besides this, OPN can also induce an immunosuppressive effect in TME by promoting anti-inflammatory macrophages and inhibiting T cell activation via various mechanisms [11].

6.1. OPN Modulates Macrophages into TAMs

Various experimental studies have identified a population of macrophages in TME known as TAMs linked with drug resistance and poor prognosis in various cancers. TAMs are categorized as

proinflammatory M1 and anti-inflammatory M2 phenotypes. M1 macrophages are known to induce inflammatory cytokines for the anti-tumor Th1 cell response, whereas M2 macrophages are involved in promoting tumor growth, angiogenesis, metastasis, CSC regulation and immune suppression in cancer by secreting anti-inflammatory cytokines [120].

The unique structure and ability of OPN to bind integrins make it an efficient signalling molecule and predominantly promotes tumor progression by governing macrophage polarisation, activation, migration and immunosuppression [121]. Owing to the evidence that OPN+ (SPP1+) macrophages are critical determinant of tumor progression within TME but its comprehensive regulatory principles underpinning the tumor regulation is remain unclear. OPN induces polarization of macrophages into M2 type, characterized by CD163, CD206 and CD209 expression, to promote tumor progression. It also induces immunosuppressive cues in TME- mediated through the TAMs [6]. The monocytes treated with OPN rich conditioned media from cancer cells leads to an increase in M2 macrophages and co-implanted xenograft of OPN expressing tumor cells and monocytes leads to high tumor growth and poor survival in gastric cancer mouse model [122]. However, this effect of OPN in promoting M2 macrophages may not be universal as treatment of monocyte from healthy donor with recombinant OPN could not enhance the M2 population but it helped in the maintenance of M2 phenotype [123]. Besides promoting M2-type macrophages, OPN also plays a crucial role in recruitment of TAMs in TME by acting as a chemoattractant and inducing TAM migration. A study using OPN KO mice demonstrated that absence of OPN reduced the infiltration of macrophages in tumor while there was no effect in normal tissue [56]. Another report suggested that reduced macrophage infiltration and enhanced T cell activity are observed in OPN deficient *in vivo* glioma model. OPN deficiency also reduced immune-suppressive regulatory T cells in blood and sensitized glioma cells to direct CD8+ T cell cytotoxicity [123]. Tumor-derived OPN stimulates CSF-1 through activation of PI3K/Akt/p65 signalling leading to the infiltration of macrophages in HCC [124]. Hence, these reports suggested that OPN may induce or maintain M2-type macrophages depending upon pathological conditions and tissue type in cancer.

Single-cell RNA seq (scRNA seq) analyses reported that SPP1 is highly expressed in monocyte-derived TAMs compared to resident macrophages in breast cancer [125]. Moreover, TAM subpopulations were divided into two subtypes (Group 1: high expression of CD204, APOE, C1QA, TREM2, CADM1 and SPP1; Group 2: strong expression of CD206, S100A9 and FCN1) [126]. Similarly, pan cancer scRNA-seq data revealed that SPP1+ TAMs were predominantly enriched in association with EMT, hypoxia, angiogenesis and enhances tumor metastasis [127]. Additionally, single-cell and spatial analyses suggest that FAP+ fibroblasts and SPP1+ macrophages cooperate to create a desmoplastic milieu that hinders lymphocytes from penetrating the tumor core, hence decreasing the effectiveness of PD-L1 therapy [8]. At single cell level, SPP1+ TAM subset regulates gene signatures and serve as a novel characteristic markers for M2 TAM. It is associated with a worst prognosis, poor immune cell infiltration, and decrease immune checkpoint expression.

Lipid-associated macrophages, also known as foamy macrophages, are TAMs that are linked to breast cancer and display an M2-like gene profile, such as CD163 expression, and release different pro-tumor secretory factors [128]. Moreover, OPN has been reported to modulate the expression of IL6 and IL12, suppresses the expression of IL27 in dendritic cells, downregulate the expression of IL10 in monocytes, and function as a chemoattractant cytokine that recruits neutrophils and macrophages [6]. Furthermore, a different study found that the NLRP3 TAM, SPP1 TAM, and IL4I1 TAM niche are strongly linked with tumor nests that includes acute inflammation, hypoxia and diffuse tissue necrosis. Bill et al recently demonstrated the synergistic ratio of CXCL9 and SPP1 in TAMs. This ratio is not only defines TAM (beyond its M1 and M2) polarity but also collectively dictates patient outcomes, anti- tumor potential, immunosuppressive behaviour of TME [129] (**Figure 6A**).

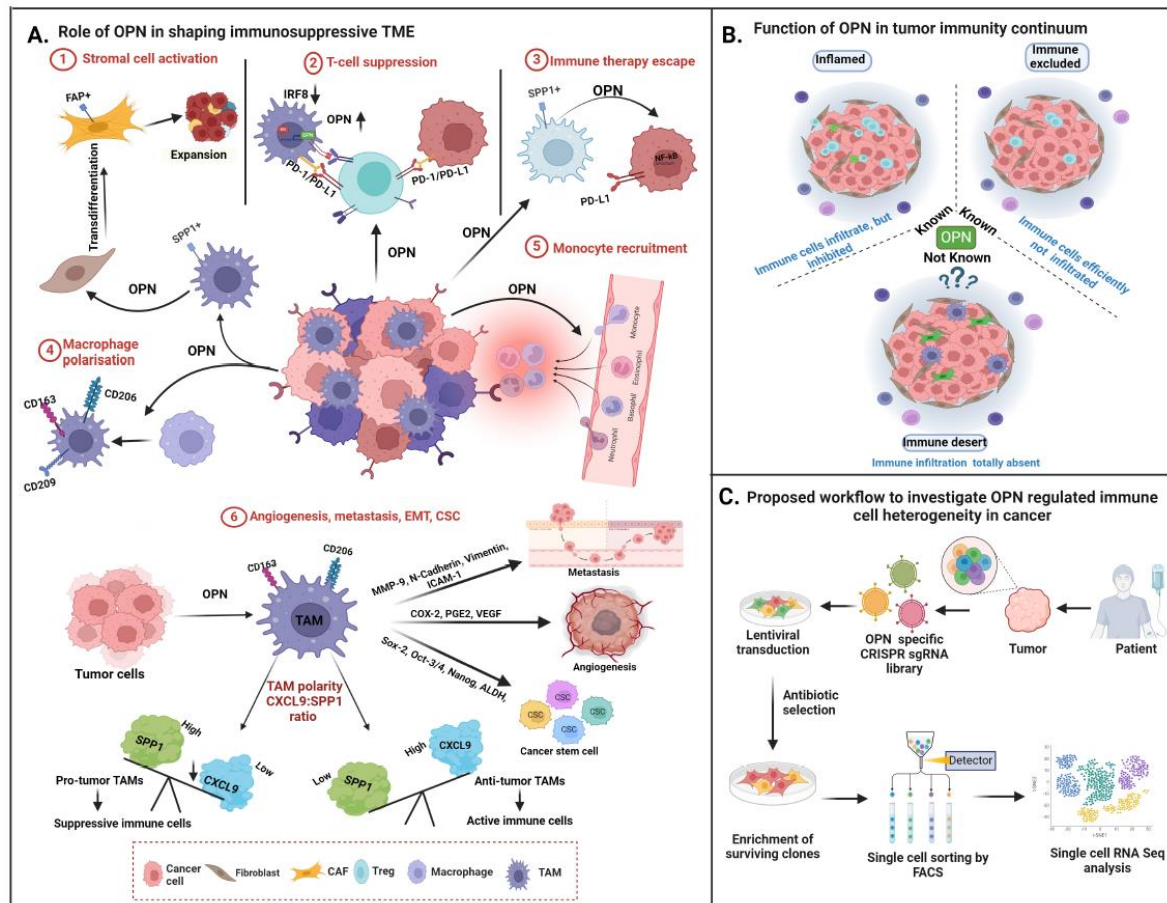


Figure 6. Model depicting the role of OPN in tumor immune microenvironment (TIME): A. Role of OPN in shaping immunosuppressive TME: Tumor-derived OPN activates stromal cells by transdifferentiation of fibroblasts to myofibroblasts resulting in expansion of tumor. OPN-regulated PD-1/PD-L1 interaction inhibits T-cell activation. Further, tumor cells induce lower expression of IRF8, resulting in overexpression of OPN thus leading to T-cell suppression. OPN via the NF-κB pathway, upregulates PD-L1 expression aiding in immune therapy escape. OPN induces the polarization of macrophages and the recruitment of monocytes. OPN activates TAM leading to angiogenesis, metastasis and enrichment of cancer stem cells via upregulation of various tumor-promoting factors like MMP-9, N-Cadherin, Vimentin, ICAM-1, COX-2, PGE-2, VEGF, Sox-2, Oct-3/4, Nanog and ALDH. The polarity ratio of CXCL9 and OPN (SPP1) determines the anti- and pro-tumorigenic properties of TAMs. B. Involvement of OPN in tumor immunity continuum: OPN is primarily associated in inflamed and immune excluded tumors whereas its role in immune desert tumor is remains elusive. C. Schematic representations to identify OPN regulated immune cell heterogeneity in cancer: OPN regulated immune modulatory genes may be identified in TIME by CRISPR technology in breast cancer using scRNA seq. based platform.

6.2. Role of OPN in TAM-Mediated Tumor Progression

OPN-mediated interaction between tumor and macrophages in TME plays an important role in the promotion of various hallmarks of cancer including proliferation, angiogenesis, metastasis, evading immune destruction and CSC enrichment. OPN induces tumor fibrosis by acting as a promoter of both TAMs and CAFs [52]. CAFs recruit monocytes in tumor and help in polarization into TAMs whereas TAMs promote activation and proliferation of fibroblasts by secreting TGF-β and PDGFs [130]. OPN acts as a facilitator of these processes in TME. Tokuda et al reported that TAM-derived OPN activates hepatic stellate cells into CAFs and promotes malignancy in HCC [131]. Further, crosstalk between tumor cells and TAMs via OPN leads to tumor growth, angiogenesis and metastasis. The reciprocal crosstalk between TAMs and cancer cells via OPN/CD44 axis advances the tumorigenicity through activation of JNK pathway in colorectal carcinoma [29]. Similarly, Nakajima

et al showed that TAM derived OPN acts as a key regulator of cancer progression through interacting with CD44v6 in colorectal cancer [132]. Kaleet al demonstrated that tumor-derived OPN promotes macrophage-dependent tube formation ability of HUVEC by inducing Cox-2 expression in macrophages via ERK/p38-dependent signalling pathway in melanoma [56]. Furthermore, ADAM8 enhances angiogenic potential of macrophages by inducing OPN expression via JAK/STAT3 and NF- κ B signalling in glioblastoma [133]. TAMs also function as regulators of stem cell enrichment and maintenance and thus contribute to chemotherapy resistance and tumor relapse. Radharani et al demonstrated that macrophages activated by treatment with cancer cell condition media positively regulate CSC-mediated tumor progression by IL-6-dependent activation of Jak/STAT pathway in breast cancer cells [134]. OPN acts as a mediator of crosstalk between TAMs and CSCs and play a significant role in maintenance of stemness in cancer. It has been also demonstrated that TAMs interact with CD44 in CSCs through OPN to regulate CSC-mediated tumor progression by activating the PI3K/Akt in colorectal carcinoma [88].

6.3. Role of OPN in Immune Evasion

Immune evasion is a key hallmark of cancer. Tumors employ various mechanisms to evade immune attack including restricting antigen recognition, inhibiting the immune system, inducing T cell exhaustion and reprogramming immune cells from tumor suppressor to promoter type. Cancer cells hijack inflammatory mechanisms to convert anti-tumor to tumor promoting immune cells that secretes pro-tumor factors that supports tumor growth and metastasis. Cancer cells also express immune checkpoint proteins to induce inhibitory signals leading to suppression of T-cell activity [135]. Cancer cells utilise different molecules and signalling pathways to modulate immune response in TIME. OPN has been identified as one such regulatory molecule which can modulate immune response in favour of tumor promotion. It is predominantly expressed in tumors and perform an important role in immune evasion in cancer. OPN plays a regulatory role in T cell activation, conversion of macrophages from M1 to M2 type and expression of immune checkpoint proteins [136].

6.4. OPN Inhibits T Cell Activation

OPN was initially recognized as a regulator of T cell activation and called an early T cell-activated gene (Eta-1) [3]. OPN modulates the adhesion, migration, and activation of inflammatory cells along with T-cell differentiation to govern the immune response against infection [6]. However, various reports have demonstrated the role of OPN in suppression of T-cell response in cancer. Myeloid-Derived Suppressor Cells (MDSCs), an immature granulocytic or monocytic myeloid cell population are known to suppress both adaptive and innate immune systems by altering T and NK cell functions. Granulocytic MDSCs are predominantly found in tumor and associated with downregulation of interferon regulatory factor-8 (IRF-8) in tumor [137]. OPN is highly expressed in MDSCs and suggested that IRF-8 negatively regulates the expression of OPN in MDSCs. MDSCs and tumor-derived OPN have been shown to suppress T-cell activation and T-cell mediated IFN- γ secretion by binding to CD44 on T-cells leading to poor patient survival [7]. Further, tumor and host-derived OPN rendered an immunosuppressive effect in the lung metastatic model of breast cancer. Interestingly, monocytic MDSCs were found to be the primary source of host-derived OPN [138]. Tumor-derived OPN is also associated with the recruitment of MDSCs at tumor site which might lead to MDSC-mediated suppression of T-cell activity. Allegrezza et al indicated that trametinib, a MEK inhibitor, induces anti-tumor T-cell activation by inhibiting tumor-derived OPN-mediated MDSC recruitment in breast cancer [139].

Further, researchers demonstrated that silencing OPN in colon cancer cells lead to a significant increase in the efficacy of the tumor-specific cytotoxic T cells in killing the target cells suggesting an immunosuppressive role of OPN [140]. Macrophage-mediated immunosuppressive effect of OPN is determined in the lung adenocarcinoma model where co-culture of macrophages with cancer cells led to diminished activity of CD4⁺ T-cells which was rescued upon OPN depletion [141]. TIME may be categorized into three different categories: (i) inflamed type, (ii) the immune-excluded type, and (iii) the immune-desert type [142]. The role of OPN and its function has been explored in inflamed and

immune- excluded TIME where as its function in immune-desert need to be explored further (**Figure 6 B**).

6.5. OPN Regulates Immune Checkpoints

Immune checkpoint molecules are ligand-receptor complex that exhibit inhibitory or stimulatory effect upon immune responses [143]. Tumor cells regulate immune checkpoints and evade host immune surveillance leading to tumor progression. PD-L1 is one such immune checkpoint molecule on tumor cells which binds with its receptor, PD-1 on T cells and induces Tcell dysfunction. It has been demonstrated that TAM derived OPN increased the expression of PD-L1 in NSCLC via NF- κ B signalling resulting in suppression of anti-tumor immune response[144]. In another reports, PD-L1 expression was upregulated on HCC by OPN through induction of CSF1-CSF1R pathway in macrophages [145]. Zhang et al showed that OPN upregulates PD- L1 in macrophages to facilitate their polarization and immune escape in lung adenocarcinoma [141]. CRISPR based approaches may be utilised to identify the OPN-driven immune-modulatorygenes in breast cancer using scRNA seq platform [129] (**Figure 6C**).

7. Osteopontin as a Therapeutic Target

Several therapeutic approaches have been reported in targeting OPN including blocking the upstream and downstream pathways, inhibition of OPN expression, immune checkpoint blockade and OPN inhibitors (**Figure 7**).

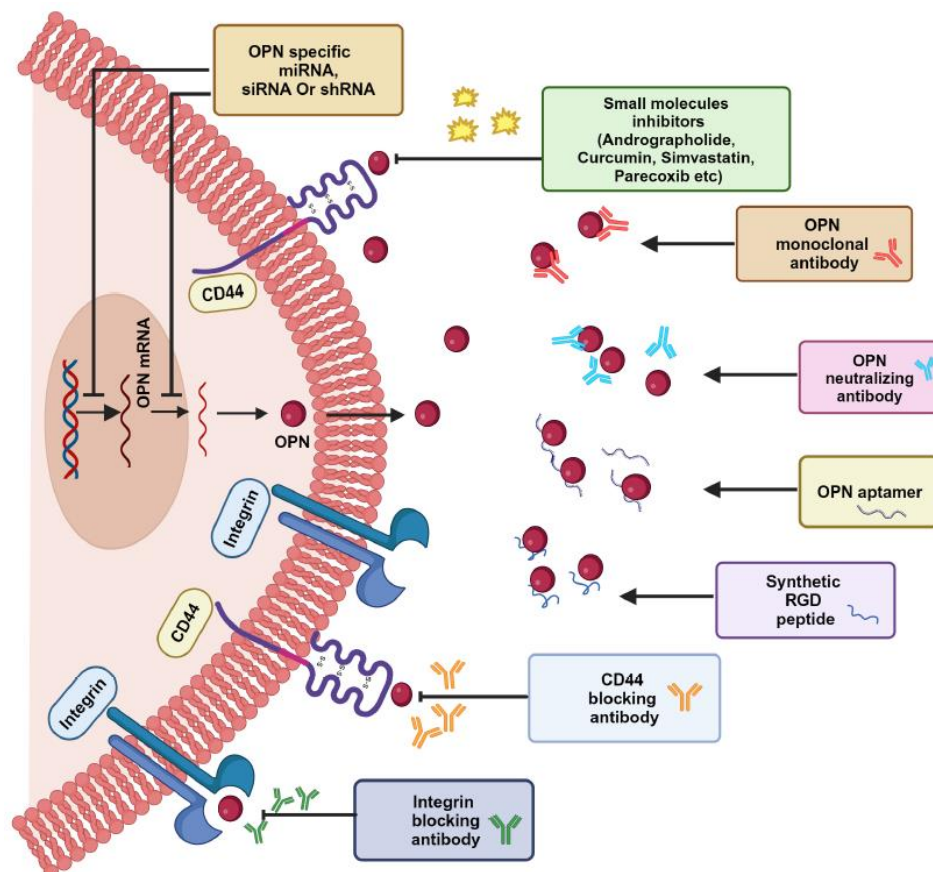


Figure 7. OPN targeted novel therapeutic strategies. Small molecule inhibitors (andrographolide, curcumin, etc), OPN-specific siRNA, miRNA, shRNA, OPN aptamers, synthetic RGD peptides, OPN neutralizing antibodies, CD44 or integrin blocking antibodies, have been used recently as therapeutic approach to target OPN-integrin/CD44axis which leads to downregulation of various oncogenic molecules and suppression of tumor progression by disrupting OPN regulated various signalling pathways in cancers.

7.1. OPN Neutralizing Antibody-Mediated Cancer Therapy

OPN neutralizing antibody or synthetic peptide binds with OPN or its receptor CD44 or $\alpha\beta3$ to inhibit the OPN-mediated cellular function [146]. A study in breast cancer cohorts showed a reduction in bone metastasis and osteoclast differentiation after the treatment with OPN neutralizing antibodies along with decreased precursors of osteoclast [94]. A humanized OPN antibody, hu1A12 recognizes the epitope N²¹²APSD²¹⁶ in full-length OPN adjacent to the calcium-binding domain and inhibits cell adhesion and migration in breast cancer [147]. The antibody also exhibited efficacy in reducing the primary tumor growth and spontaneous metastasis *in vivo* lung metastatic mouse models [147]. The OPN neutralizing antibody attenuates Slug-mediated tumor-enhancing ability in CRC patients [148]. The anti-OPN monoclonal antibody, AOM1 blocks the $\alpha\beta3$ binding site as well as the thrombin cleavage site of OPN, thus effectively inhibiting OPN- $\alpha\beta3$ integrin interaction and reducing cell migration in colon cancer [140]. Similarly, AOM1 is shown to prevent the tumor growth in metastatic lesions of NSCLC mouse model while no effect is observed at primary sites [149]. OPN neutralizing monoclonal antibodies (100D3 and 103D6) showed reduced tumor growth by attenuating the interaction of OPN with T-cells in colon cancer model [140]. Blocking the interaction of OPN with $\alpha\beta3$ integrin resulted in decreased expression of MMP-2, ILK and uPA whereas blocking CD44 interaction resulted in decreased tumor growth in mouse mammary epithelial cancer cells [11]. However, it has been reported that OPN undergoes frequent turnover in healthy human cohorts. The high concentration of OPN in plasma, coupled with its rapid turnover, underscores the demand for enhanced therapeutic antibodies targeting OPN. These antibodies should facilitate high-dose administration within short time interval and exhibit extended pharmacokinetics compared to conventional antibodies. Thus, antibodies targeting OPN receptors, CD44 or integrin can be administered for OPN-targeted cancer therapy [146].

7.2. Small Molecule Inhibitors as a Potential Therapeutic Agent

Owing to the small volume and ease in accessibility to tumor sites, small molecule inhibitors have gained much importance in this current era of cancer therapeutics. One of the well-known small molecules, andrographolide exhibits its activity via suppressing c-Jun and down-regulating the PI3K/Akt signaling pathway thereby abrogating the OPN expression and reducing the breast tumor growth [150]. Bandopadhyay et al have reported that the expression of OPN is inhibited by usage of small molecules, 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) inhibitor and parecoxib, a Cox-2 inhibitor in the treatment of ovarian cancer and colorectal cancer respectively [11]. It has been demonstrated that luteolin and curcumin suppresses the OPN expression in HCC and prostate cancer models respectively, where curcumin attenuates the VEGF expression through OPN/ $\alpha\beta3$ pathway [45,151]. Additionally, administration of bisphosphonate resulted in decreased expression of CD44/MMP-9 as well as reduced migration in prostate cancer cells. Bisphosphonate inhibits the Rho GTPase activity through disrupting the interaction of OPN with $\alpha\beta3$ integrin [152].

7.3. Epigenetic Approaches

Epigenetic alterations can influence the growth and development of healthy cells, leading to neoplastic transformation. Interestingly, WDR5-H3K4me3 epigenetic axis modulates OPN expression leading to tumor immune evasion and anti-PD-1 immunotherapy escape in pancreatic cancer [153]. In addition, OPN induces DNA methylation via DNMT1 and renders the CD133+/CD44+ CSC subpopulation more sensitive to 5-azacytidine in HCC. Thus, the OPN-DNMT1 axis promotes aberrant DNA methylation while inhibiting CD133+/CD44+ sphere formation and migration. These findings suggest that OPN could be an appealing target for HCC resistance through methylome reprogramming. [154]. Bromodomain and extra-terminal domain (BET) inhibitors impeded cell proliferation, invasion and migration via decreased OPN expression through transcriptional inactivation of NF- κ B2 [155]. It is also observed that miR-181c downregulates the expression of OPN thereby enhancing the chemosensitivity to adriamycin and decreased chemoresistance in breast cancer cells [156]. OPN siRNA encapsulated nanoparticle significantly downregulated OPN mRNA level along with enhanced inhibition of tumor growth in mouse mammary carcinoma model [157].

miR-196a knockout showed decreased expression of OPN and reduced lung metastasis in HCC [158]. Therefore, the identification and combination of epigenetic modulators of OPN with the conventional chemotherapeutic agents pose great potential in preventing tumor growth and recurrence.

7.4. OPN Aptamer

Aptamers are highly stable, 12-30 short ssRNA nucleotide sequences that are capable of adopting 3D structure of the target molecule so as to precisely bind with the protein ligands or small molecules [159]. The well-known OPN aptamer, OPN-R3 is tailored to bind with OPN and exhibited decrease in the cellular migration, invasion and adhesion in MDA-MB-231 cells [160]. In addition, the modified OPN-R3 aptamer has shown to reduce tumor growth using *in vivo* model [161].

7.5. Biomarker

Biomarkers constitute an evolving and dynamic strategy within the realm of cancer research. Numerous discoveries in biomarker identification have significantly contributed to the realms of cancer diagnosis, assessment of cancer progression and monitoring of recurrence risk post-treatment.

Interestingly, it is found that expression of OPN is a predictable biomarker not only for breast cancer but also for NSCLC, HCC, gastric, prostate and colorectal cancer. Levels of OPN expression can be effectively used to evaluate the histological grade of tumor, clinical stage, response to treatment, risk of recurrence after surgery, overall survival, and disease-free survival [10]. OPN as a prognostic and diagnostic biomarker in various cancer types are summarized in **Table 2**.

Table 2. OPN as early prognostic & diagnostic biomarker in various cancers.

Cancer type	Biomarker	Clinical significance	Prediction type	Reference (PMID)
Breast (HER2 type)	OPN+COX-2	Overexpression of OPN and COX-2 indicates poor prognosis	Prognostic	35951454
Breast Cancer	OPN-c + E-cadherin+β-catenin	OPN-C expression correlated with TNM staging & histological grading	Diagnostic (staging & grading)	35951454
NSCLC	OPN+VEGF	OPN & VEGF positive shows worse prognosis	Prognostic	35951454
NSCLC	OPN+CD44v6+MMP-2	Over Expression of OPN+CD44v6+MMP-2 associated with staging & histology	Diagnostic	35951454
Gastric Cancer	OPN+E-cadherin+β-catenin	Overexpression of OPN+E-cadherin+β-catenin serves as prognostic factor	Prognostic	35951454
Gastric Cancer	OPN	High OPN expression associated with lymph node metastasis, TNM stage, depth of invasion, tumor size and distant metastasis	Prognostic	27626167
Renal cell carcinoma	OPN (with PAZ treatment)	High concentrations of six CAFs signature IL-6, IL-8, HGF, Osteopontin, VEGF-A, and TIMP-1	Prognostic	24398141
Malignant pleural mesothelioma (MPM)	OPN	Osteopontin pooled diagnostic sensitivity and specificity for MPM was 0.65 and 0.81, respectively.	Diagnostic	32699075
Melanoma	OPN	OPN overexpression associated with poor prognosis	Prognostic & Diagnostic	33052224
Colorectal cancer (CRC)	OPN	Associated with high tumor grades & metastasis	prognostic	26234583
Hepatocellular carcinoma (HCC)	OPN	Serum/plasma-based OPN have significant predictive ability & diagnostic value	Prognostic & Diagnostic	24247813
Cervical Cancer	high OPN, low E-cadherin or both	Resistant to radiotherapy, negative prognostic factor for patient survival	Prognostic	25380749

8. Conclusion and Future Perspective

OPN emerges as a pivotal regulator in the intricate interplay between cancer cells and the TME, exerting influence across various facets of cancer progression. The abundance of OPN within the TME is responsible for modulating the fate of tumor and stromal cells but yet its clinical relevance remains to be a major limiting factor. Our earlier reports have dissected several OPN- modulated fundamental signalling pathways in breast and other cancers [3,11,52,81]. Furthermore, its multifaceted role encompasses angiogenesis, CSCs, bone metastasis, cancer cell metabolism, and modulation of the TME, involving interactions with CAFs, adipocytes, osteoclasts and immune cells. In addition, OPN intricately regulates cancer cell metabolism, particularly in glucose metabolism, influencing glucose transporters and contributing to the Warburg effect [98]. Moreover, OPN plays a central role by affecting the bone microenvironment, promoting bone metastasis, and contributing to the formation of premetastatic niches [85]. Thus, OPN acts as a potential therapeutic target for mitigating osteoclast development and controlling skeletal metastases.

In TME, the influence of OPN spans interactions with CAFs, adipocytes and osteoclasts. OPN-reprogrammed CAFs contribute to proinflammatory states, angiogenesis, and EMT, emphasizing its role in cancer progression [52]. OPN significantly impacts immunomodulation within the TME, influencing macrophage polarization towards a tumor-promoting phenotype (M2 type) and contributing to an immunosuppressive microenvironment. Moreover, OPN is implicated in immune evasion mechanisms, including the inhibition of T-cell activation and the regulation of immune checkpoint molecules such as PD-L1. CAF and TAM interaction are majorly regulated by OPN and its associated signalling events causing upregulation of various tumor events. For example, scRNA-seq revealed that OPN mediates the interaction between OPN-PTGER4 and OPN-CD44 stimulating the crosstalk between HCC cells and macrophages [162]. Similarly, single-cell transcriptome sequencing identified three TAMs subgroups: C1Q⁺, FCN1⁺ and OPN (SPP1)⁺ TAMs whereas OPN (SPP1)⁺ TAMs modulate TIME via interaction with CAFs [163]. Zhang et al reported that OPN⁺ TAMs are tightly associated with CAFs and endothelial cells in modulating TME [164]. However, the complex molecular mechanism of OPN mediated TAM subset enrichment, regulation of metabolic switch and intricate crosstalk between CAF-TAM is poorly elucidated. Although, multiple studies have demonstrated the function of CAFs in regulation of TAM, comprehending the influence of TAM in controlling CAF phenotypes warrant further investigation.

Using single-cell and spatial transcriptomics, immunofluorescent labelling, and other methods, have advanced our understanding the role of OPN in inflamed and immune-excluded TME. However, its role in immune-desert needs further exploration [129]. Detailed mechanistic studies on OPN-regulated networks and core regulatory transcription factors governing OPN expression in tumor and immune cells are warranted.

Considering the central role of OPN in cancer progression, targeted therapies aimed at inhibiting OPN expression or its downstream signalling pathways hold promise. However, current interventions lack efficacy in both preclinical and clinical trials, necessitating further development of small molecules or antibodies to neutralize the effect of OPN. Stratifying breast cancer patient based on expression levels of OPN and associated signalling pathways could tailor treatment strategies and identify the patient subgroups more responsive to OPN-targeted therapies. Understanding the immunomodulatory role of OPN suggests exploring combination therapies involving OPN inhibition and immunotherapeutic agents, potentially enhancing the effectiveness of immune checkpoint inhibitors and promoting antitumor immune responses.

In summary, the diverse function of OPN in breast and other cancers underscore its significance as a potential therapeutic target and diagnostic marker. Thus, the continued research on role of OPN and its associated signalling networks will unravel new insights in developing innovative therapeutic strategies for precision medicine, ultimately improving patient outcomes in various cancers including breast.

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