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# Role of osteopontin in cellular signaling and metastatic phenotype

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## 1. ABSTRACT

Osteopontin (opn) is a glyco-phosphoprotein that is expressed and secreted by numerous human cancers. Opn has pivotal role in cell adhesion, chemotaxis, prevention of apoptosis, invasion, migration and anchorage-independent growth of tumor cells. Extensive research has demonstrated the pivotal role of opn in regulation of cellular signaling, which controls neoplastic and malignant transformation. The elevated expression of opn has been observed in a variety of cancers. Recently, substantial evidence has linked opn with tumor metastasis and poor prognosis. However, the understanding of the molecular mechanisms that define the role of opn in cell invasion and metastasis is incomplete. The following review will discuss the molecular structure of opn, its function role in tumor cell metastasis and its downstream target genes that activate invasive mechanisms. Understanding of the role of opn in neoplastic transformation and its cellular target genes may enable development of novel anti-cancer therapy approaches.

## 2. INTRODUCTION

Osteopontin (OPN) was first described by Senger in 1979 as a phosphoprotein secreted by transformed, malignant epithelial cells (1). In different cell models bone sialoprotein I, , secreted phosphoprotein I (Spp1), 2ar, uropontin, and early T-lymphocyte activation-1 (Eta-1) has been detected, which were then identified as identical to OPN (2). OPN is rich in aspartate and sialic acid residues and contains several functional motifs (3). OPN binds to integrin and CD44 receptors mediated cellular signaling and cell-matrix interactions (4). OPN has been identified as a key noncollagenous bone matrix protein and has a pivotal role in diverse systems such as immune and vascular systems. OPN regulates cytokine production and cell trafficking and it inhibits ectopic mineralisation and macrophage accumulation in immune and vascular systems, respectively (5). It has been shown that OPN has numerous important functions in cells. OPN mediates cell adhesion, migration (6), regulates cytokine production by macrophages and act as a survival factor (7). Recently,

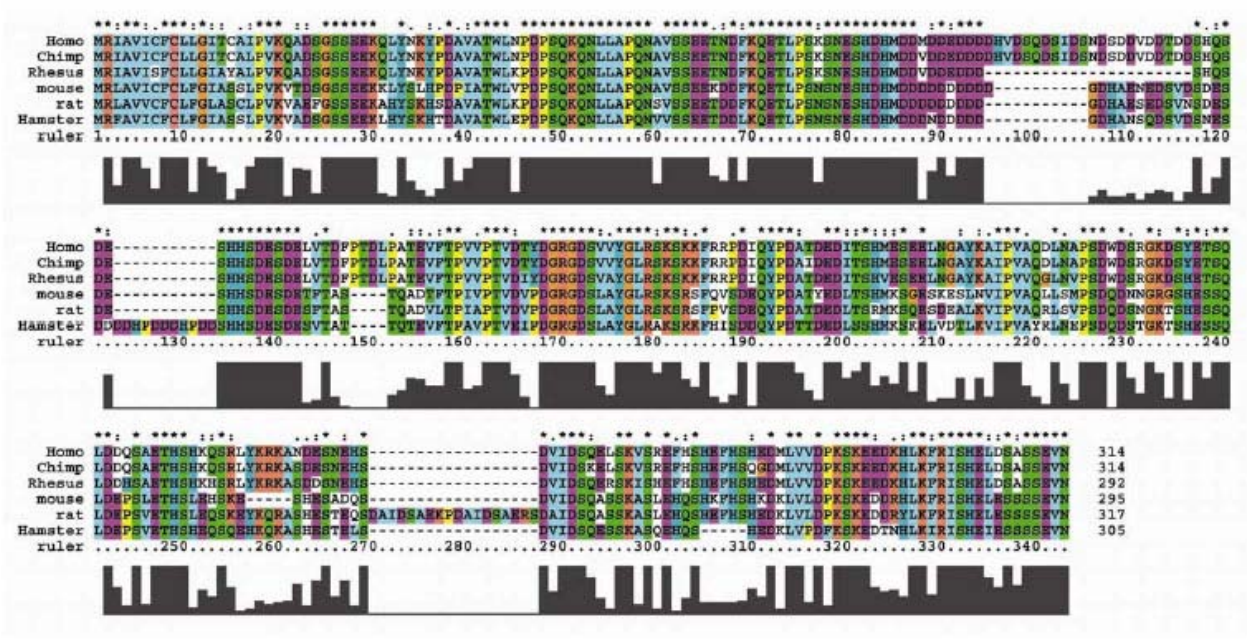


**Figure 1.** Comparison of osteopontin protein sequences from human, chimpanzee (chimp), mouse, rat and hamster. The sequences have been aligned by multiple alignment program of Clustalx software (version 1, 83).

substantial data have linked OPN with the regulation of metastatic spread by tumor cells. However, the precise molecular mechanism of OPN's action in tumor metastasis is incompletely understood. The review will discuss the molecular structure of OPN, the evidence for its functional role in tumor cell metastasis and the downstream signals that activate invasive mechanisms.

## 3. MOLECULAR STRUCTURE OF OPN

The 5' upstream sequence of the human OPN gene contains a number of potential regulatory sequences. The vitamin D responsive elements (VDREs) and an interferon regulatory factor-1 (IRF-1) and many other motifs, such as potential binding sites for the transcription factors E2A, E2BP, Myb, AP2, Ets-1 and Tcf -family (8). The OPN gene has also been identified in other species, such as rat, mouse, hamster and chimpanzee with high homology of nucleic acid sequence between these species (8) (Figure 1). The complete amino acid sequences of OPN for human, rat, mouse, pig, cow and chicken (8) have been deduced from their cDNA sequences. OPN protein consists of a single chain of 264 to 333 amino acids, rich in sialic acid, glutamic acid and serine residues, depending on species (8). OPN is a glycine-arginine-glycine-aspartate-serine (GRGDS or RGD)-containing phosphoprotein (8). The GRGDS sequence is an integrin-binding motif common to many extracellular matrix (ECM) proteins, which can mediate cell attachment. OPN also contains a polyaspartic acid motif which can bind to hydroxyapatite and calcium ions (8). Moreover, a thrombin-cleavage site is in close proximity to the GRGDS region (the GRGDS region in OPN is only six residues from the thrombin-cleavage-site) (9). In all cell lines examined, thrombin-cleaved OPN promoted greater cell attachment and spreading than uncleaved OPN (8). A motif leucine-proline-valine (LPV) occurs at the NH2-terminus of the OPN sequence (8, 9). In transformed cells of rodent tissues, both high- and low-phosphorylated forms of OPN have been identified (9).

## 4. REGULATION OF OPN EXPRESSION

Ras encodes a small GTP-binding protein. Ki-ras (Kirsten rat sarcoma viral oncogene) activates the Raf/MEK/ERK pathway, but Ha-ras (Harvey rat sarcoma viral oncogene) activates the PI3K/Akt pathway (10). Transformation by an activated ras oncogene results in constitutive activation of signal transduction pathways, which induce increased transcription of OPN in ras-transformed cells (10). Moreover, ras-transformed cells that express antisense RNA for OPN show markedly reduced ability to form tumours and metastasize in experimental animals (10). Proto-oncogene Src is a non-receptor protein-tyrosine kinase, which stimulate expression of OPN in transformed NIH 3T3 mouse fibroblasts (11). Tezuka et al. (1996) also found regulation of OPN by Src through the inverted CCAAT box in the OPN promoter (nts –52 to –46) (11). OPN expression is also enhanced by estrogen (12) .This function is postulated to be mediated by ERα and Estrogen Receptor-related Receptor α (ERRα), which can transactivate transcription through both the classical estrogen response element (ERE) and steroid factor response element (SFRE) (12). 1, 25-dihydroxy vitamin D3 is the biologically active form of vitamin D (13). After osteoblastic ROS 17/2.8 cells (a rat osteoblastic cell line) were treated with 1,25-dihydroxy vitamin D3, a less phosphorylated form of OPN was

produced and its binding to cells containing the αvβ3 integrin was reduced (13). AP-1 proteins in mammalian cells are FOS and JUN (14). AP-1 can stimulate expression of OPN by binding to the AP-1 site (15). The Ras pathway works as the main "cooperating" partner of AP-1 to support AP-1 mediated cell transformation by post-transcriptional mechanisms (16). OPN stimulates c-Fos expression, AP-1-DNA binding and AP-1 transactivation (16). It was shown recently that Wnt regulates three distinct pathways: the canonical β-catenin pathway, the planar cell polarity pathway (PCP pathway) and the Ca+2 pathway (17). The Wnt/Ca+2 pathway regulates cell adhesion and motility (18). It has been reported recently that Tcf-4 binds to A/TA/TCAAAG sequences in the OPN promoter to retard OPN transcription and protein expression (12, 19)in the absence of β-catenin. A DNA segment (designated RE 1, -94 to -24 nt) was discovered in the human OPN promoter, that is essential in maintaining OPN expression in malignant human astrocytoma cells (20). Upstream stimulatory factor (USF) is a basic helix-loop-helix (bHLH) containing transcription factor. USF has been shown to target a CCTCATGAC sequence in the mouse OPN promoter (-80 to -72 nt) (21) in rat aortic vascular smooth muscle cells. One of the major families of transcription factors enhancing OPN gene expression is that of the Ets family. The Ets family comprise 27 known genes, which encode sequence-specific transcription factors (22). The PEA3 subfamily of Ets genes, including PEA3, ER81 and ERM are coordinately overexpressed in mouse mammary tumours (23, 24). Ets binding sites have recently been identified within the OPN promoter. Ets transcription factors, particularly PEA3, upregulate OPN promoter activity. They do this by binding to, and cooperating with, other transcription factors that independently normally weakly activate the OPN promoter. PEA3 appears to be the predominant transactivation factor in combinatorial regulation of OPN transcription and gene expression of OPN. It has been shown that PEA3 and OPN were highly expressed in many human malignant breast cell lines (25). Moreover, PEA3, Ets-1, Ets-2 and OPN proteins were elevated in specimens from 29 patients with primary invasive breast carcinoma and the histochemical staining for OPN was significantly correlated with that for PEA3 (25). OPN has been identified as a direct target of p53 (TP53) (26). OPN was identified as a potential p53 target gene from differential display of mRNAs in embryonic fibroblasts from p53-deficient mice (26). Wild type BRCA1 represses OPN transcription by selective binding of its transcriptional activation complex, comprising ERα, AP-1 and an Ets family member (6). Furthermore, high levels of Mut.BRCA1 reverse wild type BRCA1 inhibition of OPN promoter activation and of OPN-mediated cellular invasion through matrigel (6). Other agents have been identified which stimulate OPN expression, e.g., TPA stimulated OPN mRNA expression in a mouse epidermal carcinogenesis model (27). Retinoic acid has been shown to induce OPN expression in the rat preosteoblastic cell line UMR 201.

## 5. OPN AND CELLULAR SIGNALING

OPN is a ligand for integrin and CD44 families of receptors. The interactions are RGD-dependent and RGD-independent. Receptor binding to OPN mediates cell-matrix interaction and activity of cellular transduction pathways.

### 5.1. Integrins

Integrins are heterodimeric adhesion receptors formed by the non-covalent association of α and β subunits which are type I transmembrane glycoproteins, and include a relatively large extracellular domain and a short cytoplasmic tail (28). Tumor cells that express a wide variety of integrins can cause constitutive activation of signaling pathways leading to increased growth of tumor cells (29). Identified OPN integrin receptors include αvβ1, αvβ3, αvβ5, α4β1, α5β1, α8β1 and α9β1 (28). It has been found that αvβ3 integrin is associated with highly metastatic compared to non-metastatic cells (28). MCF-7 cells over-expressing PKC have been shown to be resistant to apoptosis induced by phorbol esters. Addition of an anti-αvβ3 antibody to cells adhered to OPN efficiently blocked this suppression of apoptosis (30). These studies suggest that tumor cells capable of binding OPN via the αvb3 integrin may have a survival advantage. Ligation of OPN to integrin αβ provokes neovascularization by up-regulating endothelial cell migration, survival, and lumen formation during angiogenesis (28). This report supported the pro-angiogenic role of OPN and indicates that vascular endothelial growth factor (VEGF) induces OPN and $\alpha_v\beta_3$ expression in microvascular endothelial cells (28). OPN bound to integrin αvβ3 mediates activation of osteoclasts and represents a potential lytic mechanism in bone metastases (28). This interaction also leads to phosphorylation of focal adhesion kinase (FAK), paxillin, tensin, and SRC, which in turn initiate signals for proliferation, cytoskeletal organization, motility and blockage of apoptosis (28).

### 5.2. Cell differentiation antigen 44 (CD44)

CD44 is a transmembrane glycoprotein, which has extensive splicing and various types of post-translational modifications (31). It acts as a structural link from the cytoskeleton to the ECM (31). The principal ligand for CD44 is hyaluronic acid (HA), but other extracellular-matrix (ECM) proteins including serglycin, collagen, fibronectin, chondroitin sulfate, laminin, and OPN also bind CD44. OPN-CD44 interactions appear to be RGD independent (32). CD44 also acts as a signaling molecule and participates in a series of related molecular processes, such as specific adhesions (31), cell migration and signal transduction (31). CD44 expression is thought to enhance cellular migration and is associated with poor prognosis (31). It has been shown that only some CD44 variant isoforms could interact with OPN and that this interaction may be a potential basis for metastasis formation (33). It has been shown that OPN can interact specifically with CD44v6 and/or v7 and mediates chemotaxis and adhesion of fibroblasts, T cells, and of bone marrow cells (28). Binding of OPN to αvβ3 integrin can also up-regulate expression of CD44v6 (34) in a potential auto-stimulatory feedback loop. This interaction could promote cell survival, chemotaxis, homing, cell adhesion and enhance metastatic behaviour in tumors (31). CD44 expression may also play a role in growth factor receptor pathways. A recent study proposed that CD44 induces integrin expression and function by a direct pathway and also through hepatocyte growth factor (HGF) and its receptor (c-MET) (35) . This may represent an

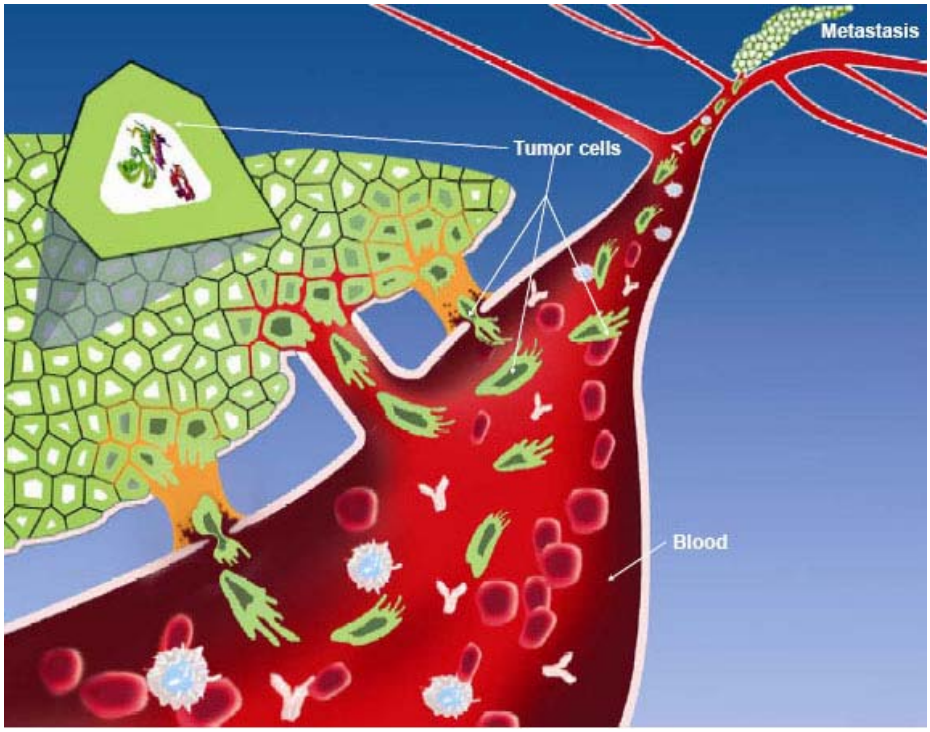


**Figure 2.** Metastasis is a complex process of genetic and phenotypic changes, which ultimately results in tumor cell dissemination, through blood circulation. Osteopontin (OPN) is an extracellular matrix glycophosphoprotein which binds to alpha v-containing integrins and has an important role in malignant transformation, malignant cell attachment and migration.

alternate pathway through which CD44 signals to activate integrin function, aiding in adhesion and migration of the tumor cells. There also is growing evidence that a cytosolic form of OPN may exist, co-localized intracellularly with CD44 and ezrin-radixin-moesin (ERM) proteins.

## 6. ROLE OF OPN IN TUMOR PROGRESSION

It has been demonstrated that OPN plays a key role in malignancy from antisense studies, in which antisense OPN cDNA or ribozymes were shown to inhibit the tumorigenic or metastatic properties of various cell types (30) . Subsequently, it has been shown that transfection of OPN cDNA in rat mammary epithelial cells increases their metastatic ability (19) . These studies support the notion that OPN is not just associated with malignancy, but may also contribute functionally to the malignant behavior of cancer cells.

## 7. OPN EXPRESSION AND METASTATIC PHENOTYPE

It was initially suggested that OPN expression in human tumors occurs in macrophages infiltrating the tumor, rather than in the tumor cells themselves (30). However, more recently some tumor cells have additionally been shown to express OPN. These include malignant cells from breast cancers and oesophageal squamous cell carcinomas (5).

*In situ* hybridization has been employed to examine OPN mRNA levels in tumors of the gastrointestinal tract (36). The OPN transcript was not detected in the tumor cells themselves, but was present in a distinct population of cells. The cells expressing OPN mRNA were identified as macrophages (36). Furthermore, direct comparison of a precursor polyp with its adjacent, associated invasive cancer demonstrates an increasing gradient of OPN expression (37).

Further investigation of 20 cases of invasive carcinoma of the colon to determine the distribution of OPN protein, using immunohistochemistry found that both macrophages and varying numbers of tumor cells stained strongly for OPN protein, even though OPN mRNA had not been detected in the tumor cells. A statistically significant correlation was demonstrated between increasing OPN protein expression and advancing tumour stage. It has been shown that 10- to 20-fold OPN induction exists in samples with liver metastases over normal mucosa (38). It has been shown that overexpression of OPN correlates with the progression of human gastric carcinoma and increases lymphogenous metastasis in poorly differentiated gastric cancer (39). In breast cancer, increased OPN expression often correlates with aggressive disease (15), indications of which include microcalcification, lymph node positivity or reduced disease-free survival (19). Tuck *et al.* have

demonstrated that elevated expression of OPN serves as a marker for malignancy in a series of 154 lymph node negative breast cancer patients, in which tumor cell expression of OPN is associated with poorer clinical outcome (40). This prognostic significance has been confirmed in a study of specimens from 333 patients with operable stage I and II breast cancer in which OPN-negative specimens correlate with a median survival of >228 months compared to only 68 months for OPN-positive specimens (41). OPN is not normally expressed in human breast cancer cell lines. However, some highly metastatic cell lines, e.g., MDA-MB-435 and LCC 115-MB, do express OPN (42). When OPN transcript levels were analysed in 14 breast carcinomas, the tumor cells of ductal carcinoma *in situ* with central necrosis or tumor cells in invasive ductal carcinomas did not label detectably for OPN mRNA, but scattered macrophages labeled strongly (36). When levels of OPN protein were examined by immunohistochemistry, tumor cells and nearby macrophages stained for OPN even though OPN transcripts were not detectable in tumor cells by *in situ* hybridization (36). Levels of OPN protein in benign and malignant human breast tumors have also been investigated (43). In invasive and *in situ* breast carcinomas, 84% expressed high levels of OPN (43). However, 72% of biopsies from invasive primary melanoma tumors expressed OPN at high levels, suggesting that OPN may be involved in the invasive process of tumor progression (44). Preliminary functional testing using siRNA ablation of OPN mRNA in a melanoma cell line (KZ-28) was found to compromise cell growth, suggesting that OPN either stimulates proliferation or inhibits apoptosis in this cell line in culture (44). When Philip *et al.* (2001) subcutaneously injected B16 melanoma cells into mice, a significant increase in tumor burden was demonstrated if the cells were treated with human OPN protein purified from milk (45). B16F10 melanoma cells, when injected in ablated bone marrow cavities, formed 5-fold fewer tumors in OPN-deficient mice than in control mice (46). It was suggested that the presence of exogenous host OPN facilitated the attachment, survival, and/or growth of the melanoma cells, therefore high-level expression of OPN by tumor cells could serve a similar purpose (47). In marked contrast to the above examples, *in situ* hybridization for OPN mRNA in carcinomas of the kidney (13 of 14 cases in renal cell carcinoma) resulted in strong labeling of tumor cells, as well as tumor-associated macrophages (36). Oates i. (1997) suggested that this distribution of OPN protein, occuring in both tumors cells and in macrophages was due to the fact that OPN may function in a paracrine manner (48).

Functional studies have provided specific evidence for OPN-mediated mechanisms in metastatic behavior. In Rama 37, a rat mammary epithelial cell line yields benign non-metastasizing adenomatous tumors in syngeneic Furth-Wistar rats (49). When genomic DNA fragments from a human metastasizing breast cancer cell line, Ca2-83, were transfected into the Rama 37 cell line, the resulting cells when injected subcutaneously into the mammary fat pads of syngeneic rats, gave rise to secondary tumors in a number of animals (50). The cell line Ca2-5-LT1 was established from a secondary lung tumor in the rat. When re-introduced into rat mammary fat pads this cell line also demonstrated the ability to metastasize (48). To examine key changes in gene expression which occur during the progression from benign Rama 37 to the metastatic derivative Ca2-5-LT1, subtractive hybridization and Northern hybridization were used. OPN mRNA was found to be expressed at a 9-fold higher level in the metastatic C2-5-LT1 cell line compared to the non-metastatic Rama 37 cell line. In primary tumors produced by these two cell lines, a similar 9-fold difference in OPN protein was observed (48). In this model system, OPN did not appear to be required for primary tumor formation and correlated more closely with metastasis (48). To show that OPN may have a direct role in metastasis, Rama 37 cells (benign tumor producing) were transfected with an OPN construct where OPN was under the transcriptional control of the constitutive cytomegalovirus promoter of the pBK-CMV vector. Rama 37 cells transfected with this vector and expressing elevated levels of OPN (R37-OPN) and lung metastasis occurred in 55% of cases when the transfected cells were injected into the mammary fat pads of Furth-Wistar rats (51). Wu *et al*. have demonstrated that OPN−/− mice show slower tumor growth after *ras* transformation (52). Antisense inhibition of OPN expression attenuates growth and colony-forming ability of human 231 LC-1 breast cancer cells (28), inhibits osteolytic metastases of human MDA-MB-231 breast cancer cells (28), reduces lung metastases by malignant B77-Rat1 fibroblasts (53), and decreases experimental metastases in *ras*-transformed NIH 3T3 metastatic mouse fibroblasts (54).

## 8. METASTATIC MECHANISMS MEDIATED BY OPN DOWN STREAM TARGET GENES

OPN has a pivotal function in physiological and pathological mineralization (55), accelerated blood vessel formation , enhanced cell survival, acute and chronic inflammation (56). OPN plays an important role in cell adhesion that confers invasiveness and metastatic capacity to tumor cells (57). OPN affects cell adhesion through integrin signaling, mediated by $\alpha v\beta 1,3,5$, $\alpha 4\beta 1$, $\alpha 9\beta 1$ and $\alpha 8\beta 1$ integrins (57). In addition, CD44, a cell surface receptor for OPN, can also mediate OPN cellular adhesion (55). Smooth muscle cells, which are $\alpha v\beta 3$ deficient retain some binding affinity to OPN via $\alpha v\beta 5$ and $\alpha v\beta 1$, but they are unable to migrate towards an OPN gradient in modified Boyden chamber assays.

Transfection of OPN into human mammary epithelial cells induced increased cell migration and

urokinase-type plasminogen activator (u-PA) mRNA (55). Mechanisms described above for OPN-mediated cell attachment are also relevant to cell migration. During OPN-mediated human mammary cancer cell migration, there is increased EGF receptor (EGFR) mRNA expression, EGFR tyrosine kinase activity, HGF receptor(MET) mRNA expression and Met kinase activity (58). Specific inhibitors of EGFR and HGFR kinase impede OPN mediated migration (58). OPN also increases cell migration via interactions between integrins and u-PA/ u-PA receptor (u-PAR) and/or integrin-mediated induction of u-PA (58).

Several important down-stream signals of OPN have been identified that regulate tumor invasive behavior. OPN regulates the activity of at least two ECM (extracellular matrix)-degrading proteins (29). It can up-regulate pro-MMP-2 (pro-matrix metal metalloproteinase-2) expression in a NF-κB dependent fashion during invasion of the ECM (45). OPN can also increase cell invasiveness in human mammary carcinoma cells through stimulation of urokinase plasminogen activator (u-PA) (58). EGFR is a downstream effector of OPN, and this receptor can also regulate cell migration and invasion (59). In fact EGFR appears to be a focal point for many cell signals, specifically G-protein coupled receptor (GPC-R) signaling, which transactivates EGFR, and promotes cell invasion (60). Studies have shown OPN target genes that regulate the neoplastic and malignant transformation. Signal transduction that disrupts the cell cycle, cell death program and enhance cell survival that are essential to neoplastic transformation (61). OPN has been shown to prevent apoptosis and promote IL-3-mediated survival on murine B cells through activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway (62). The anti-apoptotic effects of OPN on endothelial cells are also well documented and appear to be regulated through NF-κB (30). OPN also enhances tumor invasiveness and metastasis by altering host immunity (28). Several investigators have demonstrated the effects of OPN on nitric oxide (NO) production (63). NO is produced by a number of different cell types, including activated macrophages and vascular endothelial cells, and can act as a powerful signaling molecule as well as causing localized cytotoxicity. NO is a reactive nitrogen radical that can injure cells by damaging enzymes required for critical metabolic pathways in the cell, among other effects. Inhibition of inducible nitric oxide synthase (iNOS), the enzyme responsible for producing NO in response to various stimuli, by OPN may play a role in tumor defenses against the immune system (63). Interestingly, in murine macrophages, LPS-induced synthesis of NO regulates iNOS expression in a feedback fashion through OPN-dependent negative-regulatory mechanisms (64). Recently, heterogenous nuclear ribonucleoprotein A/B (hnRNP A/B) has been identified as an NO-dependent *trans*-regulator of OPN expression in murine macrophages (28). However, as there are also illustrations in which NO has been shown to have tumor-promoting effects, its role in malignancy appears to be complex (30).

The function of OPN in cell proliferation has not been clearly established and is still in debate. Angelucci *et al* (2004) reported that OPN enhanced cell proliferation induced by the epidermal growth factor in human prostate cancer cells (65). Antisense inhibition of OPN expression attenuates growth and the colony-forming ability of human 231 LC-1 breast cancer cells (52). It has also been shown that OPN can bind to insulin-like growth factor-binding protein-5 and thereby enhance the ability of this protein to stimulate proliferation of cells, presumably by making IGF-1 available to the cells (66). OPN has also been shown to promote the proliferation of cultured rat vascular smooth muscle (VSM) cells and human coronary artery smooth muscle cells (67). In contrast, another study indicated that OPN can exert a negative regulatory effect on cell proliferation (68). Overexpression of OPN in MC3T3-E1 cells (a pre-osteoblastic cell line) markedly inhibited proliferation, while overexpression of antisense RNA to OPN mRNA could stimulate cellular proliferation (68). Daniel *et al*. (2002) showed that there was no relationship between OPN and proliferation of fibroblast-like cells (69).

## 9. CONCLUSIONS

The experimental studies outlined above strongly support the idea that OPN expressed by tumor cells can alter their malignant properties. In addition, these studies indicate that OPN produced by tumor cells in vivo may have multiple effects on the ability of the tumor to grow, invade and metastasize. It has been shown that elevated OPN expression in human cancers support the hypotheses that OPN should be considered as a potential prognostic marker for a variety of human cancers. However, it is clear from the experimental and clinical findings that the role of OPN in cancer is complex and multifaceted. Understanding the function of OPN in different cell types, will be essential in developing anti-OPN therapeutic strategies.

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