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REVIEW

Pre- and post-translational regulation of osteopontin in cancer

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Abstract Osteopontin (OPN) is a matricellular protein that binds to a number of cell surface receptors including integrins and CD44. It is expressed in many tissues and secreted into body fluids including blood, milk and urine. OPN plays important physiological roles in bone remodeling, immune response and inflammation. It is also a tumour-associated protein, and elevated OPN levels are associated with tumour formation, progression and metastasis. Research has revealed a promising role for OPN as a cancer biomarker. OPN is subject to alternative splicing, as well as post-translational modifications such as phosphorylation, glycosylation and proteolytic cleavage. Functional differences have been revealed for different isoforms and post-translational modifications. The pattern of isoform expression and post-translational modification is cell-type specific and may influence the potential role of OPN in malignancy and as a cancer biomarker.

Keywords Osteopontin · Cancer · Metastasis · Matricellular protein · Post-translational modification · Alternative splicing

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Abbreviations

ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
HCC	Hepatocellular carcinoma
HNSCC	Head and neck squamous cell carcinoma
MM	Malignant mesothelioma
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NSCLC	Non-small cell lung cancer
OPN	Osteopontin
PDA	Pancreatic ductal adenocarcinoma
Q	Glutamine
qRT-PCR	Quantitative real time polymerase chain reaction
RT-PCR	Real time polymerase chain reaction
RGD	Arginine-Glycine-Aspartic acid
siRNA	Small interfering ribonucleic acid
shRNA	Short hairpin ribonucleic acid
SVVYGLR	Serine-Valine-Valine-Tyrosine-Glycine-Leucine-Arginine
TG2	Tissue transglutaminase
uPA	Urokinase plasminogen activator

Introduction

Osteopontin (OPN) is a matricellular protein that is involved in both physiological and pathological processes (Giachelli and Steitz 2000; Kyriakides and Bornstein 2003; Rangaswami et al. 2006; Weber 2001). It is a secreted, integrin-binding glycophosphoprotein, involved in inflammation, wound healing, bone formation and remodelling, as well as atherosclerosis and cancer (Cho et al. 2009; Sodek et al. 2000; Tuck et al. 2007; Weber 2001). A wide variety of cell types express OPN, including osteoclasts, osteo-

blasts, endothelial cells, vascular smooth muscle cells, epithelial cells (such as kidney, breast, and skin), neural cells (neurons, glial cells and Schwann cells) and activated immune cells (such as T-cells, B-cells, macrophages, natural killer (NK) and Kupffer cells) (Kunii et al. 2009; Rangaswami et al. 2006; Wai and Kuo 2004; Wai and Kuo 2008). OPN is found as both an immobilized extracellular matrix (ECM) molecule in some (eg. mineralized) tissues and as a secreted protein in body fluids such as milk, blood, urine, saliva, seminal fluid and bile (Rangaswami et al. 2006; Wai and Kuo 2004). It signals through different integrin receptors including $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_9\beta_1$ and CD44 variant receptors, through RGD-dependent and independent mechanisms, leading to a wide variety of effects (Rodrigues et al. 2007; Wai and Kuo 2004).

OPN is heavily post-translationally modified with serine/threonine phosphorylation, glycosylation and tyrosine sulfation, which allows for a monomeric molecular weight ranging from 41 to 75 kDa (Christensen et al. 2008). These modifications can be cell type-specific (Christensen et al. 2007), may depend on other physiological and pathological factors and may impact both OPN structure and function (Anborgh et al. 2009; Kazanecki et al. 2007; Zhang et al. 2007). This review will focus on OPN's role in cancer and how splice variants and post-translational modifications may influence its effects in cancer cells.

OPN and cancer

OPN has been clinically and functionally associated with cancer for many years (reviewed in (Furger et al. 2001; Rittling and Chambers 2004; Tuck and Chambers 2001; Tuck et al. 2007; Weber 2001)). Clinically, there are numerous studies showing OPN expression in both tumour cells and cells found within the tumour microenvironment (reviewed in (Anborgh et al. 2010)). OPN expression in tumour cells has been shown in a variety of cancer types (Brown et al. 1994; Coppola et al. 2004) including carcinomas of breast (Tuck et al. 1998), prostate (Forootan et al. 2006; Hotte et al. 2002), colon (Agrawal et al. 2002), ovary (Bao et al. 2007), stomach (Imano et al. 2009), liver (Chen et al. 2010; Lin et al. 2010; Ye et al. 2003) and lung (Chambers et al. 1996; Zhang et al. 2001; Zhao et al. 2011), mesotheliomas (Pass et al. 2005), squamous cell carcinomas (Chien et al. 2009), sarcomas (Bramwell et al. 2005; Sulzbacher et al. 2002) and multiple myeloma (Saeki et al. 2003). OPN produced by other cells in the tumour microenvironment, such as macrophages and stromal cells, has been seen in a number of different cancer types as well (Reinholt et al. 1990). For example, in a cohort of lymph node negative breast cancer patients, OPN mRNA and protein were detected in both tumour cells and tumour infiltrating inflammatory cells (Tuck et al. 1998). Tumour cells and macrophages were determined to be OPN-positive in a sample of pulmonary artery sarcomas, where OPN had also been incorporated into the extracellular matrix (ECM) (Gaumann et al. 2001) and Chang et al. (2008) found similar results in cutaneous squamous cell carcinoma, as most cases showed the presence of OPN in both cancer cells and in ECM adjacent to the tumour.

Functionally, OPN alters the behaviour of cancer cells both *in vitro* and *in vivo,* in most cases in a manner that promotes malignancy. For example, adding OPN (exogenous/recombinant or transfected) to breast cancer cells *in vitro* can increase their adhesion and migration abilities, and has also been shown to change breast cancer cells' gene expression profiles, affecting genes involved in all six hallmarks of cancer (Allan et al. 2006; Cook et al. 2005; Hanahan and Weinberg 2000). In colon cancer cells, both endogenous OPN and exogenous OPN increased motility and invasive abilities of cells *in vitro* (Irby et al. 2004). Knockdown models emphasize the effects OPN has on cancer cell behaviour *in vitro*. For example, in human hepatocellular carcinoma cells, siRNA specific for OPN decreased colony formation and invasion (Lin et al. 2010) and in PC-3 prostate cancer cells, OPN specific shRNA decreased proliferation, migration and invasion (Liu et al. 2010). Similarly, in the highly tumourigenic and metastatic breast MDA-MB-435 cell line, OPN knockdown significantly decreased invasion, migration, and colony formation in soft agar (Shevde et al. 2006). Comparable results are seen in breast cancer cell lines when OPN is blocked with either an anti-OPN antibody (Dai et al. 2010) or with RNA aptamers (small structured single stranded antisense RNA) (Mi et al. 2009).

In vivo studies also illustrate the pro-malignant effects of OPN. Breast cancer cells transfected to over-express OPN had increased tumour growth, lymphovascular invasion, lymph node metastases, and earlier occurring lung micrometastases in a xenograft tumour model (Allan et al. 2006). Weakly tumourigenic colon cancer cells showed enhanced tumourigenicity after stable transfection with OPN (Irby et al. 2004). Similarly, human lung cancer cells transfected to overexpress OPN had increased tumour growth and neovascularisation (Cui et al. 2007). As expected, decreasing the expression of OPN has malignancy-inhibiting effects in mouse models. OPN knockdown via shRNA, siRNA or treatment with an anti-OPN antibody decreased tumour take, tumour progression, metastases and expression of OPN-mediated signaling proteins of breast cancer cells (Chakraborty et al. 2008; Dai et al. 2010; Mi et al. 2009; Shevde et al. 2006). Anti-sense oligonucleotide targeting of OPN in hepatocellular cancer decreased lung metastases

(Chen et al. 2010) and OPN specific shRNA decreased prostate tumour growth (Liu et al. 2010). Thus, OPN promotes tumour growth and metastases in experimental models in a variety of cancer types.

OPN as a biomarker in cancer

There have been numerous studies determining how OPN levels in tumour tissue and patients' plasma/serum correlate with prognosis and survival in a variety of cancers (reviewed in (Terpos et al. 2009; Tuck et al. 2007; Wai and Kuo 2008; Weber et al. 2010)). For example, in breast cancer, elevated OPN expression, measured both in plasma and in tumour tissue, has been associated with decreased survival. Tuck et al. (1998) showed that OPN immunopositivity of tumour cells correlated with decreased disease-free and overall survival in a group of lymph node negative breast cancer patients. Similarly, in another study of 333 breast cancer patients (lymph node negative and positive), OPN immunostaining was negatively correlated with survival (Rudland et al. 2002). Singhal et al. (1997) also found significantly higher levels of plasma OPN in a group of women with metastatic breast cancer, compared with a group of healthy women and a group of breast cancer survivors. Furthermore, within the group of metastatic cancer patients, increased OPN levels were correlated with poorer prognosis and decreased survival (Singhal et al. 1997).

OPN has also been shown to be a potentially valuable biomarker in other cancer types. In advanced gastric cancer, OPN expression was the most significant predictor of poor prognosis, as patients with OPN-positive tumours (as determined by IHC) had decreased 5-year survival compared to those with OPN-negative cancer (Zhang et al. 2009). Elevated OPN serum levels were negatively correlated with survival in glioblastoma (Sreekanthreddy et al. 2010) and in soft tissue sarcoma patients (Bache et al. 2010). OPN levels were also associated with increased stage and grade and larger tumour size in soft tissue sarcomas (Bache et al. 2010). In colorectal cancer patients, increased OPN mRNA was significantly correlated with stage, lymph node metastasis and lymphatic or venous invasion, as well as shorter disease-free and overall survival rates (Likui et al. 2010). Thus, OPN levels, whether in tumour tissue or patient plasma, may provide useful prognostic information.

Additionally, OPN levels may be useful to monitor progression of some cancers. When measured throughout breast cancer disease progression, increases in plasma OPN levels over time are strongly correlated with decreased survival (Bramwell et al. 2006). In early stage NSCLC patients, plasma OPN levels decreased after tumour resection surgery compared to pre-surgery values, whereas in patients showing recurrence of NSCLC, OPN levels at the time of recurrence were elevated compared to values immediately post-surgery (Blasberg et al. 2010). Thus, in a number of cancers, OPN may be a good biomarker to monitor disease progression, in addition to providing useful prognostic information.

OPN splice variants/isoforms

In addition to full length OPN (OPN-a), there are two other known splice variants (OPN-b, OPN-c). Full length OPN pre-mRNA consists of 7 exons (Rodrigues et al. 2007) while OPN-b lacks exon 5 and OPN-c lacks exon 4 (Hijiya et al. 1994; Kiefer et al. 1989; Saitoh et al. 1995; Young et al. 1990) (Fig. 1). Limited information is available about

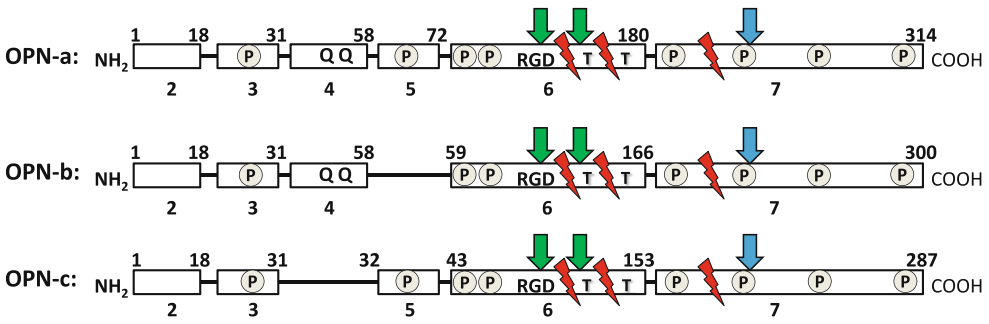

Fig. 1 Schematic of three OPN splice variants, OPN-a, OPN-b and OPN-c, highlighting important functional domains and the differences between isoforms. Full length OPN pre-mRNA is 7 exons in length, with 6 translated exons. Integrin binding (*green arrow*) occurs at RGD and cryptic SVVYGLR sequences, while CD44 variant receptor binding (*blue arrow*) occurs near the C-terminus. Thrombin cleavage (T) and MMP cleavage (red zig zag) sites are present in all three isoforms. Serine/threonine phosphorylated residues occur in clusters (circled P) throughout and are modified based on origin and function of OPN. Transglutaminase cross-linking occurs at glutamine residues (Q Q). OPN-b lacks exon 5 and is therefore missing certain phosphorylated sites, while OPN-c lacks exon 4 and is unable to undergo transglutaminase cross-linking. Clinical and functional differences do exist between OPN isoforms, however, these differences appear to be cancer cell type specific

the function of the N-terminal region of OPN where alternative splicing occurs. However, certain inferences can be made on differential effects of the three splice variants, as a result of what has been shown regarding function of the exons they contain. For example, the N-terminal region of OPN contains two conserved amino acid sequence motifs (QLYxxYP and WLxPDP), which promote migration and survival of lymphocytes via activation of MAPK/WEK/AP-1 pathways (Cao et al. 2008; Dai et al. 2009). Sequences corresponding to exon 5, absent in OPN-b, contain one of several clusters of phosphorylated serine/threonine residues (Christensen et al. 2005). Also, two glutamine residues essential for transglutaminase cross-linking are present in exon 4 (Sorensen et al. 1994), thus OPN-a and OPN-b, but not OPN-c, are able to form polymeric OPN complexes which have altered functional properties (Higashikawa et al. 2007; Sorensen et al. 1994), as discussed in greater detail below.

Little is known about the regulation of the alternative splicing of the OPN message. Venables et al. (2008) observed a shift towards expression of splice variant OPN-b in three cell lines (HeLa, PC-3 and BJT) upon siRNA knockdown of the splicing protein hnRNP K, although secondary effects of this knock-down on the expression of other hnRNP family members could not be excluded (Venables et al. 2008). A change in the splicing pattern of a protein is one of the mechanisms that can alter its function and lead to malignancy-promoting effects (Brinkman 2004). While most clinical and experimental studies regarding OPN have not distinguished between splice variants, a number of recent studies have begun to examine their relative expression, at both mRNA and protein level, as well as to assess what different effects, if any, the OPN splice variants play in cancer and metastasis. In the following section we will review the (sometimes contrasting) findings of these studies.

Expression of OPN isoforms in human tumours

Recently, a number of studies in various cancer types have sought to identify expression patterns of OPN isoforms in an effort to determine if clinical and functional differences exist (Chae et al. 2009; Goparaju et al. 2010; He et al. 2006; Ivanov et al. 2009; Mirza et al. 2008; Patani et al. 2008; Sullivan et al. 2009; Yan et al. 2010). These studies are summarized in Table 1. It appears that OPN isoforms are differentially expressed and may have diverse effects, and this may be cancer type specific. Conflicting evidence exists and much remains unclear regarding which isoform is most clinically relevant, or if all three splice variants are important. For example, in the work of He et al. (2006) and Mirza et al. (2008), using both RT-PCR and immunohistochemistry, OPN-c was detected in breast carcinomas and invasive ductal carcinomas, while normal breast tissue surrounding the tumour or normal breast tissue (obtained by reduction mammoplasty) was devoid of OPN-c (He et al. 2006; Mirza et al. 2008). OPN-a was detected in both tumour tissue and at variable levels in normal tissue (He et al. 2006; Mirza et al. 2008), while OPN-b was expressed at low levels in tumour, tumour-adjacent tissue and normal breast tissue (Mirza et al. 2008). Mirza et al. (2008) also found that the intensity of OPN-c staining increased from tumour grade 1 to grade 3 (Mirza et al. 2008). High OPN-c levels were also associated with tumour grade, poor prognosis, increased recurrence rates and poorer disease-free survival in another breast cancer study (Patani et al. 2008). These studies suggested that OPN-c may be the most clinically relevant isoform, as it was found to be the predominant form expressed in tumour tissue. However, the majority of studies published regarding OPN expression in breast cancer have not differentiated between isoforms and have still identified OPN as a strong clinical prognostic factor (Bramwell et al. 2006; Rudland et al. 2002; Singhal et al. 1997; Tuck et al. 1997; Tuck et al. 1998), and studies examining function of transfected OPN have indicated functional effect of OPN-b as well (Allan et al. 2006; Tuck et al. 1999). Therefore, while OPN-c may indeed provide clinically useful information, further studies must be done to clarify the relative contributions of the three different OPN isoforms in breast cancer.

Isoform-specific studies have also been done in glioma (Yan et al. 2010), lung (Goparaju et al. 2010), liver (Chae et al. 2009), mesothelioma (Ivanov et al. 2009) and pancreatic cancers (Sullivan et al. 2009) (Table 1). Again, these studies found isoform specific differences. However, which OPN isoform is most clinically relevant depends on cancer type, with OPN-a and OPN-b, not OPN-c, predominating in lung cancer, liver cancer and mesotheliomas. Goparaju et al. (2010) found that OPN-a was most tumour associated in a cohort of non-small cell lung cancer (NSCLC) patients as OPN-a was over-expressed in most tumour samples compared to normal lung tissue, whereas OPN-c was detected in normal lung tissue but not in patients' tumour tissue (Goparaju et al. 2010). Similar results were reported in another cohort of NSCLC patients, head and neck squamous cell carcinomas (HNSCC) and soft tissue sarcomas (Courter et al. 2010). Studies in hepatocellular carcinoma (HCC) and malignant mesothelioma (MM) also found that tumour tissue predominately expressed OPN-a, compared to control tissue (surrounding non-tumour liver or normal liver samples and normal peritoneal tissue) (Chae et al. 2009; Ivanov et al. 2009). Both of these studies speculate that OPN-a and OPN-b may be associated with poor prognosis. In HCC, the ratio

Table 1 Summary of clinical studies which have looked at differential splice variant expression/levels

Cancer Type	Reference	Method	Splice Variants[a]	Results[b]
Breast	He *et al.* (2006)	RT-PCR	OPN-a, OPN-c	Normal: OPN-a > OPN-c
				Tumour: **OPN-a ~ OPN-c***
Breast	Patani et al. (2008)	RT-PCR	OPN-a,OPN-b, OPN-c	Normal: OPN-a ~ OPN-b > OPN-c
				Tumour: OPN-a ~ OPN-b > OPN-c
Breast	Mirza et al. (2008)	RT-PCR, IHC	OPN-a,OPN-b, OPN-c	Normal: OPN-a > OPN-b > OPN-c
				Tumour: **OPN-c** > OPN-a > OPN-b
Lung	Goparaju et al (2010)	RT-PCR	OPN-a,OPN-b, OPN-c	Normal: OPN-a ~ OPN-c
				Tumour: OPN-a > OPN-c
Lung	Courter et al (2010)	qRT-PCR	OPN-a, OPN-b	Normal: OPN-a ~ OPN-b
				Tumour: OPN-a ~ OPN-b
Liver	Chae et al (2009)	RT-PCR	OPN-a,OPN-b, OPN-c	Normal: OPN-a ~ OPN-b ~ OPN-c
				Tumour: OPN-a > OPN-b > OPN-c
Glioma	Yan et al (2010)	RT-PCR	OPN-a,OPN-b, OPN-c	Normal: OPN-a ~ OPN-b ~ OPN-c
				Tumour: **OPN-b > OPN-a > OPN-c**
Head & neck	Courter et al (2010)	qRT-PCR	OPN-a, OPN-b	Normal: OPN-a ~ OPN-b
				Tumour: OPN-a ~ OPN-b
Mesothelioma	Ivanov et al (2009)	RT-PCR	OPN-a,OPN-b, OPN-c	Normal: OPN-a > OPN-b > OPN-c
				Tumour: OPN-a > OPN-b; no OPN-c
Pancreas	Sullivan et al (2009)	RT-PCR, IHC	Total OPN, OPN-c	Normal: Low total and OPN-c
				Tumour: **Total OPN ~ OPN-c**
Soft tissue sarcoma	Courter et al (2010)	RT-PCR	OPN-a, OPN-b	Tumour: OPN-a ~ OPN-b
Colon	Mirza *et al.* (2008)	qRT-PCR	OPN-a, OPN-c	Normal: OPN-a ~ OPN-c
				Tumour: OPN-c > OPN-a

a. Indicates splice variants assessed in each study

b. > greater expression than; ~ equivalent expression

* **Bold face** indicates tumour tissue OPN values were found to be significantly higher than for normal tissue

of OPN-a and OPN-b to OPN-c increased as the tumours developed (Chae et al. 2009) and Ivanov et al. (2009) found that OPN-a and OPN-b (but not OPN-c) increased substantially in one patient measured before and after MM recurrence (Ivanov et al. 2009). All three OPN splice variants showed increased expression in high grade gliomas with respect to low grade gliomas and normal brain tissue, with OPN-b being the dominant isoform (Yan et al. 2010).

In contrast, similar to the breast cancer studies discussed above, tumour tissue from patients with invasive pancreatic ductal adenocarcinomas (PDA) revealed high OPN-c mRNA and protein expression in 72% of smokers and 36% of the non smokers compared to benign tumours (premalignant intrapapillary mucinous neoplasms), in which OPN-c mRNA or protein was rarely detected (Sullivan et al. 2009). Similarly, OPN-c was expressed at high levels in a small number of colon carcinoma samples and at moderate levels in tumour-adjacent tissue, while OPN-a was expressed at very low levels in tumour tissue (Mirza et al. 2008). These studies emphasize the variability amongst OPN isoform expression in different types of cancer.

While keeping in mind that most of the above studies on OPN splice variant expression in tumours were based on small numbers of patients, it appears that differential expression of OPN splice variants occurs in tumour versus normal tissues and that in breast cancer this may be associated with tumour grade, malignancy and patient prognosis (Mirza et al. 2008). However, which one of the OPN isoforms is dominantly expressed appears to depend upon the origin of the tumour. In breast, pancreatic and colon cancer, OPN-c and/or OPN-b may be good candidates both as biomarkers and as therapeutic targets. In other types of cancer such as lung cancer, liver cancer, malignant mesothelioma and glioma the role of OPN-c is less clear, as OPN-a and OPN-b seem to be the predominant isoforms expressed by these types of tumours. It is also not known if differential OPN expression at the mRNA level or protein level in tumours is associated with a difference of OPN isoforms present in plasma, as currently available OPN ELISA systems do not distinguish between isoforms.

OPN isoform expression and functional differences in cell lines

Similar heterogeneity exists for OPN splice variant expression in tumour cell lines and for the functional effects of different OPN isoforms on the behavior of these cells. In one study using breast cancer cells, He et al. (2006) reported that recombinant OPN-a, whether bacterially expressed or over-expressed and secreted from non-invasive breast cancer MFC-7 cells, is more prone to form aggregates in the presence of calcium, and is more active in supporting cell adhesion compared to OPN-c (He et al. 2006). In contrast, OPN-c was found to be soluble and to strongly support anchorage-independent growth of these cells (He et al. 2006). Very little OPN-b was secreted, as it was degraded by the proteasome when over-expressed in HEK293 cells or MCF-7 cells (Mirza et al. 2008). In contrast, several other studies have shown that in OPN-b over-expressing breast cancer cells (21T mammary epithelial cell series and MDA-MB-468 cells transfected to over-express OPN-b), OPN-b was both secreted into media and increased cell adhesion, migration, invasion and metastasis (Allan et al. 2006; Cook et al. 2005; Schulze et al. 2008; Tuck et al. 1999). Also, the malignant breast cancer cell line MDA-MB-231 was reported to express each of the 3 OPN splice variants, as were a number of other invasive cancer cell lines of breast and other origins (He et al. 2006). On the other hand, there is evidence that non-invasive breast epithelial cell lines express either no OPN, or low levels of OPN-a mRNA, without any OPN-b or OPN-c mRNA (He et al. 2006). Therefore, OPN processing and utilization differs depending on cell type, and as suggested from the above, differences in isoform function may exist even when of the same tissue origin (i.e. breast).

Conflicting evidence also exists regarding OPN isoform function in lung cancer. OPN-a was the dominant splice variant found in lung cancer cell lines that endogenously express OPN (A549, H460, H157, H1299, and Calu-3), with less OPN-b expressed and no endogenous OPN-c expression in all cell lines tested (Blasberg et al. 2009). Blasberg et al. (2009) suggested that OPN-a and OPN-b promote angiogenesis in lung cancer, as both isoforms stimulate tubule formation in bovine capillary endothelial cells and increase vascular endothelial growth factor (VEGF) expression, while OPN-c may actually inhibit angiogenesis (Blasberg et al. 2009). In addition, over-expression of OPN-a, and to a lesser extent OPN-b but not OPN-c, resulted in increased cellular proliferation, migration, invasion and anchorage independent growth of NSCLC cells, including the cell lines H358, A549 and H460 (Goparaju et al. 2010). However, Zhao et al. (2011) reported contrasting results for the NSCLC cell line A549, as overexpression of OPN-c, but not OPN-a or OPN-b, resulted in increased invasion (Zhao et al. 2011).

A number of studies have also explored OPN splice variants in other tumour types, including liver cancer (Chae et al. 2009; Takafuji et al. 2007), mesothelioma (Ivanov et al. 2009) and glioma (Yan et al. 2010). In HCC cell lines, OPN splice variant expression correlated with tumourigenecity, where OPN-a and OPN-b were mainly expressed by more migratory and invasive cells (SK-Hep1 and HepG2) and OPN-c was the dominant form expressed in non-malignant cells (Hep3B, PLC/PRF/5 and CHANG) (Chae et al. 2009). Functionally, OPN-a and OPN-b induced cell migration of non-malignant cells (Hep3B) and activated metastasis associated signaling pathways (urokinase plasminogen activator and MAPK) while OPN-c reduced migration in malignant cells (Chae et al. 2009). Surprisingly, OPN-c was more effective in stimulating anchorage-independent growth of the Hep3B cells (Chae et al. 2009). This unexpected malignancy-promoting effect of OPN-c in HCC was supported by Takafuji *et al.* (2007) who determined that over-expression of OPN-c in Hep3B cells increased cellular invasion, potentially due to the formation of a 5 kDa OPN fragment (aa167-210), generated by cleavage of OPN-c by MMP-9, and mediated by CD44 (Takafuji et al. 2007). In mesothelioma cell lines, functional effects differ between OPN isoforms, as OPN-a, but not OPN-b or OPN-c, stimulated cell proliferation and cell migration, OPN-a and OPN-b, but not OPN-c, stimulated cell invasion, and all three isoforms stimulated anchorage independent growth (Ivanov et al. 2009). In glioma cells, it was OPN-a and OPN-c, not OPN-b, that were able to promote invasiveness, by inducing the expression of proteins such as uPA, MMP-2 and MMP-9 (Yan et al. 2010). These studies again emphasize that OPN splice variants do in fact appear to have differing expression and functional effects, however, it is highly dependent on cell type, and much remains to be learned about which functional roles may be variant-specific.

Post-translational modifications of OPN

Post-translational modifications help to regulate OPN's function in physiological and pathological processes. OPN can be highly phosphorylated and glycosylated, may contain a sulfated tyrosine residue, be subject to proteolytic cleavage and can be cross-linked by transglutaminase 2 (Fig. 1).

Phosphorylation of OPN occurs primarily by intracellular Golgi kinase and casein kinase II (Christensen et al. 2005; Lasa et al. 1997; Weber 2001). Most phosphorylated residues in OPN appear in groups of 2–5, with stretches of unphosphoryated residues in between the

groups (Christensen et al. 2005; Christensen et al. 2007). Phosphorylation patterns have been described for OPN isolated from human and bovine milk (Christensen et al. 2005; Sorensen et al. 1995), human urine (Christensen et al. 2008), rat bone (Keykhosravani et al. 2005) and chicken osteoblasts (Salih et al. 1997), as well as from Ras-transformed mouse fibroblasts and differentiated mouse osteoblasts (Christensen et al. 2007). In most of these cases, OPN phosphorylation status was found to be heterogeneous and complex (Zhang et al. 2007). Only in OPN from bovine milk were all 28 phosphorylation sites completely occupied by phosphate groups (Christensen et al. 2005). OPN isolated from human milk can be phosphorylated at 36 different serine/threonine residues, although the average number of phosphate groups per OPN molecule was found to be 32 (Christensen et al. 2005). In human urinary OPN, 30 potential phosphorylation sites have been found, of which on average only 8 are actually phosphorylated (Christensen et al. 2008). Chicken osteoblast OPN contained an average of 6 phosphate groups per molecule (Salih et al. 1997). In OPN expressed by murine ras-transformed fibroblasts, 16 potential phosphorylation sites were found, however, on average only 4 phosphate groups per OPN molecule were identified. OPN from a non-transformed parental mouse fibroblast line, as well as OPN from a differentiated mouse osteoclast cell line, appeared to be highly phosphorylated (Christensen et al. 2007; Kazanecki et al. 2007).

Functionally, phosphorylation has been found to increase or decrease adhesion and/or migration, depending on the cell type. For example, it has been shown that native human milk OPN must be highly phosphorylated in order to stimulate the migration of human choriocarcinoma cells (Al-Shami et al. 2005). In contrast, dephosphorylation of bovine milk or rat bone OPN increased osteoclast migration at the expense of adhesion (Ek-Rylander et al. 1994; Ek-Rylander and Andersson 2010). In both murine and human breast cancer cells, unphosphorylated human recombinant OPN increased adhesion, migration, and invasion (Christensen et al. 2007; Tuck et al. 1999; Tuck et al. 2000; Tuck et al. 2003; Xuan et al. 1994) and OPN with low phosphorylation status promoted adhesion of human tumour (MDA-MB-435 breast cancer) cells to a greater extent than a highly phosphorylated form of OPN (Christensen et al. 2007). Although in general, for most "normal" fibroblasts, osteoclasts and osteoblasts, higher level phosphorylation of OPN appears to be associated with increased adhesion and decreased migration (Christensen et al. 2007), most tumour cells appear to express hypophosphorylated OPN and there is conflicting literature on how the degree of phosphorylation affects their behavior. Indeed, it is possible that total protein phosphorylation is not as important biologically as phosphorylation at specific sites. Now that there is knowledge of the positions of the various phosphorylations in OPN, studies will be possible to more clearly determine the effect of site specific phosphorylation.

Native OPN isolated from human milk contains five O-glycosylated threonine residues, located in one region in the N-terminal half of the molecule (Christensen et al. 2005), that appear to be fully occupied by oligosaccharides. Variation exists in the type of glycan structures which may consist of different combinations of N-acetylhexosamine, hexose, and sialic acid residues (Christensen et al. 2007; Christensen et al. 2008; Keykhosravani et al. 2005). Corresponding regions in OPN from other sources likewise contain O-glycosylated threonines and serines. In a murine breast cancer model, OPN has been identified as one of the proteins bearing the Sialyl-Thomsen-nouvelle antigen (Julien et al. 2009). This glycan is expressed in about 30% of human breast cancers and is associated with decreased survival and lack of response to chemotherapy (Cazet et al. 2010). In keeping with the importance of OPN glycosylation to function, it has been found that a generalized reduction of sialylation may prevent OPN from binding to cell surface receptors (Shanmugam et al. 1997). N-glycosylation, on the other hand, is a post-translational modification that has so far only been reported for OPN isolated from human bone (Masuda et al. 2000).

Sulfation of tyrosine residues is a modification that is found in many proteins that are membrane bound or secreted into the extracellular matrix and is thought to play an important role in controlling protein-protein interactions (Stone et al., 2009). In OPN, tyrosine sulfation has been linked to tissue mineralization (Nagata et al. 1989), and has been found in OPN isolated from rat bone (Keykhosravani et al. 2005), mouse osteoblasts (Ecarot-Charrier et al. 1989) and also from human urine (Christensen et al. 2008), but not in human milk OPN (Christensen et al. 2005). To our knowledge, there is at present no literature regarding the role of OPN sulfation on malignant properties of cancer cells.

Proteolytic processing

Proteolytic processing by both thrombin and MMPs is another important post-translational modification which helps to regulate and alter OPN's function. OPN contains two highly conserved thrombin cleavage sites (Fig. 1), one is six amino acids from the RGD domain at Arg^{169}-Ser^{170}, and the second one is within the RGD domain, at Arg^{160}-Gly^{161} (Senger et al. 1989; Smith et al. 1996). Thrombin cleavage results in two major fragments, an N-terminal fragment and a C-terminal fragment which differ in function from each other and full length OPN (Schulze et al. 2008; Smith et al. 1996; Takafuji et al. 2007; Takahashi et al. 1998). Thrombin cleavage has been shown to enhance OPN-mediated cell attachment and spreading, possibly because OPN fragments provide increased accessibility to

the RGD binding site to cell surface receptors (Senger et al. 1994). In addition, a cryptic binding site, SVVYGLR, becomes accessible after thrombin cleavage of OPN, allowing $\alpha_9\beta_1$ integrin receptors to bind to N-terminal OPN (Yokosaki et al. 1999). Neutrophils, smooth muscle cells and some epithelial cells express $\alpha_9\beta_1$ integrin, and activation of $\alpha_9\beta_1$ integrin leads to cell migration and proliferation (Yokosaki et al. 1999). Thus, thrombin cleavage of OPN can help to regulate migration of certain cell types to desired areas (Grassinger et al. 2009; Yokosaki et al. 1999), a useful function in inflammation and tissue remodeling, and at times a pathologic function in cancer. The C-terminal fragment of thrombin cleaved OPN is able to bind CD44 variant receptors, leading to activation of downstream signaling pathways to promote cell survival, cell migration and cell adhesion (Kazanecki et al. 2007; Wai and Kuo 2004) and is able to activate Akt1/2 and MMP-2, upon binding to cyclophilin C, via interaction with the CD147 glycoprotein surface receptor (Mi et al. 2007). It also contains a calcium binding domain and two heparin binding domains (Tuck et al. 2007). Inhibition of thrombin in OPN-expressing breast cancer cells results in decreased cell growth, colony formation, adhesion and migration *in vitro* and decreased primary tumour growth and lymphatic metastasis *in vivo* (Schulze et al. 2008). Therefore, thrombin cleavage is an important regulator of OPN function in certain tissues and situations and may represent a therapeutic target.

OPN is also a substrate for cleavage by matrix metalloproteases (MMPs), including MMP-2, -3, -7 and −9 (Agnihotri et al. 2001; Dean and Overall 2007; Takafuji et al. 2007). MMP-3 and −7 cleave OPN between G^{166}-L^{167}, leading to N-terminal fragments containing the RGD site (Agnihotri et al. 2001). Similar to thrombin cleaved OPN, MMP cleaved OPN also stimulates increased adhesion and migratory abilities in a variety of cell types (Agnihotri et al. 2001). MMP-9 cleaves OPN into 5 fragments, predominately at residues G^{166} and D^{210} (Takafuji et al. 2007). A 5 kDa fragment released after MMP-9 cleavage was found to induce cell invasion in HEK-293, SMMC-7721 and Hep3B cells through the CD44 receptor (Takafuji et al. 2007). In addition, OPN is able to induce MMP-2 and −9 expression in cancer cells (Liu et al. 2010; Rangaswami et al. 2004), thus increasing cells' invasion abilities and increasing its own proteolytic processing. Therefore, MMP cleavage also appears to activate function of OPN, such that blocking MMP activity may be a potential way to target and decrease OPN-mediated cell aggressiveness.

Transglutaminase cross-linking

Osteopontin is also a substrate of tissue transglutaminase (TG2) (Kaartinen et al. 2002; Prince et al. 1991). TG2 is a widely distributed intra- and extracellular calcium dependent enzyme which leads to polymerization by cross-linking of its substrate proteins (Higashikawa et al. 2007; Kaartinen et al. 2002; Kaartinen et al. 2007). OPN contains two highly conserved transglutaminase-reactive glutamines, Gln-50 and Gln-52 (Sorensen et al. 1994), with which TG2 can form isopeptide cross-links between OPN monomers or fragments and between OPN and ECM components (Higashikawa et al. 2007). These glutamines are found in exon-4, present in OPN-a and OPN-b but not in OPN-c (Sorensen et al. 1994). In at least some instances, polymeric OPN has increased integrin-mediated cell adhesion, spreading and migration stimulating abilities, in addition to an altered conformation which enhances its collagen binding properties (Higashikawa et al. 2007). It has been speculated that OPN polymerization leads to concentration of integrin binding sites, which may enhance OPN-integrin signaling in addition to potentially exposing new integrin binding sites due to the altered conformation of OPN polymers (Higashikawa et al. 2007). TG2 cross-links are resistant to proteolytic cleavage and increase the stability of protein aggregates (Collighan and Griffin 2009), enhancing their potential functional effects. In breast cancer, TG2 expression is often increased in lymph node metastases compared to primary tumours (Mehta et al. 2004) and is thought to play an important role in the development of the metastatic phenotype (Mangala et al. 2007). For example, in MDA-MB-231 breast cancer cells, TG2 associates with integrin receptors and promotes motility, invasion and survival, compared to cells with minimal TG2 levels (Mangala et al. 2007). Also, siRNA inhibition of TG2 in these cells decreased their invasive abilities and survival in serum-free conditions (Mangala et al. 2007). While there are no clinical studies which specifically examine the relationship between OPN and TG2 in cancer, both have been independently identified as increased in metastatic breast cancer (Bramwell et al. 2006; Mehta et al. 2004; Singhal et al. 1997), and experimental evidence suggests that TG2 crosslinking of OPN promotes aggressive cancer cell behaviour (Higashikawa et al. 2007). The possibility that OPN polymers formed due to TG2 cross-linking may have different and/or enhanced functional effects in cancer cells is thus a subject worthy of further, more direct investigation.

Conclusion

OPN is a multifunctional protein that plays an important role in cancer progression and metastasis. It shows promise as a useful prognostic biomarker in a variety of cancer types, as well as a potential therapeutic target. More research is required regarding the expression of its three isoforms, OPN-a, OPN-b and OPN-c in different benign

and malignant situations, and their functional consequences. From what we have learned so far, expression of a specific OPN isoform may have different, and sometimes opposite, effects in cancer cells of different origins. Also required is a better understanding of how post-translational modifications affect OPN function and which modifications are most common and important in cancer. OPN regulation is complex, much like the many functions of this protein, and improved understanding will better allow us to appropriately utilize OPN as a biomarker and as a possible therapeutic target.

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