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Bone Sialoprotein and Osteopontin in Bone Metastasis of Osteotropic Cancers

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Abstract

The mechanisms underlying malignant cell metastasis to secondary sites such as bone are complex and no doubt multifactorial. Members of the small integrin-binding ligand N-linked glycoproteins (SIBLINGs) family, particularly bone sialoprotein (BSP) and osteopontin (OPN), exhibit multiple activities known to promote malignant cell proliferation, detachment, invasion, and metastasis of several osteotropic cancers. The expression level of BSP and OPN is elevated in a variety of human cancers, particularly those that metastasize preferentially to the skeleton. Recent studies suggest that the “osteomimicry” of malignant cells is not only conferred by transmembrane receptors bound by BSP and OPN, but includes the “switch” in gene expression repertoire typically expressed in cells of skeletal lineage. Understanding the role of BSP and OPN in tumor progression, altered pathophysiology of bone microenvironment, and tumor metastasis to bone will likely result in development of better diagnostic approaches and therapeutic regimens for osteotropic malignant diseases.

Keywords

Bone Sialoprotein; Osteopontin; Bone metastasis; Malignancy

1. Introduction

Primary bone cancers such as osteosarcoma, chondrosarcoma, or Ewing sarcoma family of tumors are quite rare comprising < 1% of all cancers with only 2,300 new cases of primary bone cancer in the U.S. each year [1]. However, bone is one of the most common sites for metastasis of other cancers, particularly but not exclusively, those with epithelial cell

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origins. The worldwide incidence of bone metastasis reveals several malignancies have propensities to metastasize to bone including multiple myeloma and breast, thyroid, prostate and lung cancers [2] (Table 1). Under normal circumstances, bone is constantly undergoing continuous remodeling wherein osteoblasts contribute to bone deposition and osteoclasts mediate bone resorption thereby maintaining appropriate bone structure and Ca^{++} homeostasis. Osteotropic malignancies that metastasize to bone upset this balance causing lesions that are either osteoblastic, osteolytic, or both [3, 4]. Regardless of the type of lesion, the patient outcome is usually the same and may include pathologic fractures, bone pain, hypercalcemia, anemia, spinal instability, spinal cord and nerve compression, and decreased mobility [2].

The mechanisms underlying malignant cell metastasis from primary sites to secondary tissues such as bone are complex and poorly understood. Malignant cells must be able to detach from their primary tissues, evade the host immune system, cross the walls of the vasculature, penetrate through extracellular matrix in tissue, and finally take up residence and survive in tissues quite different from their origins. Studies over recent years suggest that small integrin binding ligand N-linked glycoproteins (SIBLINGs) may mediate many of the activities necessary for bone metastasis of osteotropic malignancies. In fact, the expression of SIBLINGs by malignant cells of osteotropic cancers may be intimately associated with their ability to metastasize to bone.

The SIBLINGs are primarily involved in bone morphogenesis and include bone sialoprotein (BSP), osteopontin (OPN), matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein 1 (DMP1), and dentin sialophosphoprotein (DSPP). The genes that code for them (*IBSP* for BSP, *SPP1* for OPN, *MEPE*, *DMP1*, and *DSPP*, respectively) are clustered on the long arm of chromosome 4 as a tandem array [5]. Originally thought to be expressed exclusively within mineralized tissue such as bone and dentin, SIBLINGs are now known to be produced by epithelial cell tumors that are osteotropic and in some cases produce microcalcifications [6]. These soluble secreted glycoproteins undergo extensive post-translational modifications including glycosylation, sulfation, phosphorylation, and sialylation, which in part, may confer their bioactivities. The five members of this family exert their activities in both paracrine and autocrine fashion and through multiple functional domains share the ability to bind similar proteins and exert similar activities. For example, all of the SIBLINGs bind integrins via both classical RGD motifs as well as cryptic binding sites [5]. The siblings OPN and DMP1 also bind CD44, a cell surface polymorphic hyaluronate receptor that participates in numerous cellular functions including lymphocyte activation, recirculation, homing, hematopoiesis, and tumor metastasis. SIBLINGs may regulate cell adhesion, motility, and survival of tumor cells by binding to integrins and/or CD44 expressed on tumor cells. Also, SIBLINGs bind and activate specific matrix metalloproteinases (MMPs) which may promote angiogenesis, tumor progression, and metastasis [7]. In addition, SIBLINGs bind compliment factor H (CFH) which blocks antibody-complement mediated cell lysis [8]. When these moieties are bound by SIBLINGs to the cell surface via integrins or CD44, these activities are conferred to the cell, facilitating trans-migration through tissue or extracellular matrix as well as escaping complement-mediated cell lysis. SIBLINGs also regulate cell proliferation and differentiation by activation of NF-κB [8]. Thus, SIBLINGs appear to provide most, if not all, of the activities required for tumor cell progression including the ability to metastasize to secondary sites such as bone. The involvement of BSP and OPN in tumor growth and metastasis has been more extensively studied than for the other SIBLINGs. This review article summarizes recent studies on the association of BSP and OPN with tumor progression and bone metastasis.

2. Bone sialoprotein

Human bone sialoprotein (BSP), a 33 kDa glycoprotein, is a major non-collagenous extracellular protein of mineralized tissues such as bone, dentin, cementum, and calcified cartilage [9]. BSP has an apparent molecular weight of 60–80 kDa due to extensive post-translational modifications including N-and O-linked glycosylation, serine and threonine phosphorylation, tyrosine sulfation, and sialylation. BSP is produced by osteoblasts, osteoclasts, osteocytes, and hypertrophic chondrocytes during bone morphogenesis [5, 10]. The high glutamic acid content of BSP (22%) suggests it is the focal point for mineralization of hydroxyapatite during bone formation [11–14]. The activity of BSP in bone homeostasis may be dependent on additional regulatory factors in the bone microenvironment. For example, Xu and colleagues report that BSP-collagen implants placed into surgically created rat calvarial defects stimulate osteoblast differentiation and bone repair [15]. Conversely, BSP contributes to receptor activator of nuclear factor-κB ligand (RANKL)-mediated bone resorption by inducing osteoclastogenesis and promotion of osteoclast survival [16]. Also, BSP increases survival of bone marrow derived monocyte/macrophages by enhancing NF-κB activation and diminishing apoptosis in these cells [16]. Like other SIBLINGs, BSP binds to integrins, specifically $\alpha v\beta_3$ and $\alpha v\beta_5$. The interaction of BSP with $\alpha v\beta_3$ integrin, which is up-regulated on activated endothelial cells, promotes human endothelial cell migration, attachment, and angiogenesis [17, 18]. BSP binds type 1 collagen, binds and activates MMP2, and binds CFH thereby protecting cells from complement mediated cell lysis [19].

While BSP was once thought to be produced exclusively by cells involved with bone morphogenesis, recent studies demonstrate that osteotropic malignancies such as multiple myeloma and breast, prostate, lung, thyroid, and cervical cancers, may express BSP [20–25]. Furthermore, the transcription factors Runx2 and Msx2, which regulate BSP production in cells of skeletal lineage, mediate the expression of BSP in human metastatic breast and prostate cancer cells [26, 27]. BSP production in malignant cells may be stimulated by fibroblast growth factor 2 (FGF2). For example, treatment of the human breast cancer cell line MCF7 with FGF2 *in vitro* results in increased *IBSP* transcription through activation of CRE2 and AP1 elements in the *IBSP* promoter [28]. Similar studies demonstrate FGF2 and forskolin (an activator of adenylate cyclase) stimulate *in vitro IBSP* transcription and BSP protein expression in DU145 human prostate cancer cells [29]. In both of these studies, FGF2 treatment *in vitro* caused increased expression of the Runx2 gene [26, 27].

The expression of BSP in these malignancies may underlie events related to tumor progression such as adhesion, proliferation, invasion, angiogenesis, evasion of host immune defense mechanisms, and ultimately metastasis. For example, breast cancer cells expressing $\alpha v\beta_5$ bind recombinant BSP and BSP enriched bone *in vitro* [30]. Also, BSP stimulates increased adhesive, proliferative, and migratory properties of breast cancer cells *in vitro* [31]. Endogenous production of BSP by cancer cells also promotes these pro-metastatic activities. Transfection and subsequent expression of BSP in breast cancer (MDA-MB-231, Hs578T) and prostate cancer (PC3) cells *in vitro* results in up-regulation and expression of integrin subunits αv, β_3, and β_5 (except for β_5 in Hs578T), increased expression of mature focal adhesions and their signaling pathways, and increased migration in response to exogenous transforming growth factor β1 (TGF-β1) and epidermal growth factor (EGF) [32]. Furthermore, breast cancer cells transfected with IBSP cDNA show increased capacity for migration and invasion *in vitro* [33]. BSP binding of $\alpha v\beta_3$ on several osteotropic cancer cell lines, and subsequent cell surface binding and activation of MMP2, also promotes increased invasive properties of those cell lines *in vitro* [34, 35].

In vivo studies suggest BSP is involved in tumor progression and metastasis. For example nude mice challenged in the mammary fat pad with IBSP cDNA transfected breast cancer cells show increased primary tumor growth *in vivo* [33]. Additional studies report forced expression of BSP in human breast cancer cells enhances *in vivo* bone metastasis after placement into a murine model [36] and BSP appears to mediate, in part, the pro-metastatic effects of TGF-β both *in vitro* and *in vivo* [37]. Interestingly, inoculation of IBSP cDNA transfected *non-bone-seeking* breast cancer cells (MDA-231BR) results in bone metastasis in nude mice, while no bone lesions occur in control animals receiving non-transfected MDA-231BR cells [38]. Also, targeted overexpression of osteoclast-derived BSP increases bone metastasis of murine 4T1 breast cancer cells in transgenic mice [39]. Taken together, these data suggest a major role for BSP in the processes underlying tumor progression and bone metastasis (Figure 1).

3. Osteopontin

Human osteopontin (OPN) is a 33 kDa (apparent M.W. up to 75 kDa) extracellular matrix glycoprotein that plays a major role in bone morphogenesis, immunoregulation, and inflammation. During bone remodeling, OPN helps anchor osteoclasts to the mineral matrix of bone [40]. OPN is an important cytokine in the immune system where it enhances both specific immune responses and inflammatory responses during wound healing. OPN enhances Th1 activity by inhibition of Th2-dependent interleukin (IL)-10 production, promotes B-cell proliferation and immunoglobulin production, stimulates mast cell migration and degranulation, and increases macrophage activity [41–46]. Also, OPN has anti-apoptotic activity in macrophages, T cells, fibroblasts, and endothelial cells [45, 46]. OPN is produced by cells involved in bone morphogenesis such as preosteoblasts, osteoblasts, osteoclasts, osteocytes, odontoblasts, and hypertrophic chondrocytes [5]. In addition, other sources of OPN include bone marrow myoblasts, dendritic cells, epithelial cells (breast, skin, kidney), immune cells (T-cells, B-cells, natural killer cells, macrophages, Kupffer cells), neural cells (glial cells, Schwann cells, neurons), vascular smooth muscle cells, skeletal muscle myoblasts, fibroblasts, endothelial cells, and extraosseous cells of the inner ear, brain, kidney, deciduum, and placenta [47–54]. Osteopontin undergoes significant post-translational modification and like BSP is one of the major non-collagenous proteins in extracellular matrix of mineralized tissue such as bone and dentin. OPN binds to integrins like other SIBLINGs, particularly $\alpha v\beta_1$, $\alpha 8\beta_1$, $\alpha v\beta_3$, $\alpha v\beta_5$ via the classical RGD motif, and $\alpha 9\beta_1$, $\alpha 4\beta_1$, $\alpha 9\beta_4$ via the enzymatically generated cryptic binding site SVVYGLR [8, 52, 55]. OPN also binds CD44 splice variants CD44v6 and CD44v3, binds and activates MMP3 [34], and binds CFH. The activation of NF-κB by OPN enhances survival of endothelial cells, dendritic cells, dopaminergic neurons, and activated T cells [56–59].

The multifunctional activity of OPN is also seen in cancer cells through enhanced adhesion, migration, proliferation and invasion, tumorigenesis, and metastasis. OPN enhances adhesion of human breast cancer cells *in vitro* [60]. The binding of OPN to CD44v6 on colon HT29 cells stimulates integrin activation and subsequent migration [61]. OPN stimulates increased migration of breast, melanoma, and multiple myeloma cells *in vitro* [62–64]. Over-expression of OPN in prostate cancer cell lines increases proliferation, invasiveness, and the ability to intravasate blood vessels [65]. Induction of proteases by OPN enhances tumor cell migration through extracellular matrix and tissue thereby increasing the invasiveness and metastatic properties of malignant cells. For example, Tuck and colleagues reported that OPN increases transcription of the urokinase plasminogen activator (uPA) gene and subsequent urokinase expression in breast cancer cells resulting in increased migration *in vitro* [66]. OPN binding of integrin receptors on murine mammary epithelial cancer cells and subsequent induction of uPA and MMP2 activity confers a metastatic phenotype to these cells [67]. Increased uPA and MMP2 activity in these cells

was mediated through integrin–linked kinase dependent AP1 activity [67]. Also, non-small cell lung carcinoma (NSCLC) and pancreatic cancer cells show OPN-mediated increases in invasiveness [68, 69]. Models exploiting gain and loss of function parameters show enhanced OPN expression results in metastasis of cancer cells. For example, forced expression of OPN in *non*-metastatic rat mammary tumor cells results in lung metastasis in half the animals that develop primary tumors [70]. Overall, aberrant expression of OPN is associated with tumor invasiveness and metastasis in breast, lung, prostate, and colon cancers [53, 71–74].

OPN is a substrate for thrombin, Bone Morphogenic Protein 1 (BMP1), MMP 2, 3, 7, and 9 and the fragments generated therein retain biologic activities and promote events that underlie metastasis [75–77]. For example, thrombin cleavage of OPN separates amino terminal integrin binding activity, which promotes cell adhesion, from a carboxy terminal CD44v6 binding domain which promotes invasion and tumorigenesis [52, 55, 78]. Inhibition of thrombin in breast cancer cells that express OPN decreases tumor cell growth, colony formation, adhesion and migration *in vitro* as well as decreased tumor growth and metastasis *in vivo* [79]. Interestingly, cleavage of OPN by thrombin and MMP9 generates fragments that increase hepatocellular carcinoma invasiveness [77]. Cleavage of OPN by MMP3 and MMP7 generates fragments that contain the RGD binding site and promote *in vitro* cell migration and adhesion through binding and activation of β_1 integrins [75]. Cleavage of OPN by MMP9 generates 5 fragments, one of which (5 kDa) binds to CD44, promoting tumor cell invasiveness [77]. Taken together these data demonstrate that OPN, and proteolytic fragments of OPN, promote tumor cell adhesion, migration, and survival.

4. OPN isoforms and their implication in malignancy

Investigators have identified different splice variants or isoforms of OPN. The OPN gene is comprised of 7 exons where exon 1 and 2 contain the 5' untranslated region (5' UTR), and the remainder of exon 2 and exons 3–7 contain coding sequence [80–82]. In addition to full length OPN (OPN-a), there are two splice variants, OPN-b (missing exon 5) and OPN-c (missing exon 4) [80]. Exon 5 of OPN contains a cluster of phosphorylated serine/threonine residues [83]. Exon 4 contains two glutamine residues critical for transglutaminase crosslinking of OPN. Thus, unlike OPN-a and b, OPN-c cannot form polymeric OPN complexes which have been shown to have altered functional activities [84, 85]. Investigators have begun to examine OPN-splice variant function in numerous malignancies and the diversity of these activities is significant. Therefore, this review will only discuss the evidence in those malignancies that have a high rate of bone metastasis (for review of OPN splice variants see [86]).

Different malignancies express different OPN-splice variants and the bioactivity of OPN-a, b, or c in these malignancies may be a function of the cell-type of origin. An examination of OPN splice variants in breast cancer reveals OPN-c expression is greater than OPN-a or b and is associated with tumor grade, poor prognosis, and increased recurrence [87–89]. Interestingly, the level of OPN-c expression increased in tumors as they progressed from grade I to grade 3 [88]. Normal breast tissue and tissue surrounding the tumor did not express OPN-c, whereas OPN-a and b were expressed at low levels in normal tissue, tissues adjacent to the tumor, and within the tumor itself [87, 88]. OPN-a promotes cell adhesion in non-invasive breast cancer cells MFC-7 whereas OPN-c strongly promotes MCF-7 anchorage-independent growth [87]. In 21T mammary epithelial and MDA-MB-468 cells, over-expression of OPN-b increases cell adhesion, migration, invasion, and metastasis [66, 79, 90, 91]. In lung cancer, OPN-a and b, but not OPN-c, are the predominant isoforms expressed [86]. Over-expression of OPN-a occurs in tumors from patients with NSCLC relative to normal lung tissue, whereas OPN-c is found in normal lung tissue but not tumor

tissue [92]. In another study, over-expression of OPN-a in human lung cancer cells potentiated angiogenic activity and vascular endothelial growth factor (VEGF) secretion *in vitro*, OPN-b had low angiogenic activity but exhibited no effect on VEGF secretion, while OPN-c had inhibitory effects on both angiogenesis and VEGF secretion [93]. In addition, increased cell proliferation, migration, invasion, and anchorage-independent cell growth was observed in NSCLC cell lines A549, H358, and H460 following forced expression of OPN-a [92]. In these studies, OPN-b exerted less activity on these parameters than OPN-a, whereas OPN-c had no effect [92]. Similar results have been seen in prostate cancer. For example, OPN-a, b, and c expression is significantly elevated in prostate cancer (PCa) as compared to benign prostate hyperplasia (BPH) where OPN-c expression levels are the highest of the OPN isoforms [94]. Further, OPN-c and to a lesser extent OPN-b, promotes a more aggressive phenotype in PCa cells such as enhanced cell proliferation, migration, invasion, soft agar colony formation, and tumor formation *in vivo* [94, 95]. Additionally, over-expression of OPN-c and OPN-b in PC-3 cells stimulates MMP2, MMP9 and VEGF expression [95]. Finally, OPN-c was as reliable, if not more reliable, than prostate-specific antigen (PSA) as a marker of prostate cancer progression [94].

Taken together, the data demonstrates that OPN and OPN splice variants a, b, c have pronounced effects on the processes that underlie malignancy and metastasis such as adherence, proliferation, migration, invasion, anchorage independent growth, and angiogenesis. The effects of OPN splice variants on these processes may be determined by malignancy type, cell origins, or possibly the microenvironment of the tumor. OPN and OPN-splice variants seem to have prognostic value and may represent a potential target for therapies.

5. Post-translational modifications of BSP and OPN and their relevance to cancer biology

Both BSP and OPN undergo extensive post-translational modification including N- and O-linked glycosylation, sulfation, phosphorylation, and sialylation which can contribute as much as 50% to their predicted molecular weights. Post-translational modifications influence the neoplastic activity of OPN, particularly adhesion and/or migration, in different cancer cells. For example, unphosphorylated recombinant OPN increases adhesion, migration, and invasion of both human and murine breast cancer cells [60, 66, 96, 97]. Highly phosphorylated OPN exerts less pro-adhesive activity on MDA-MB-435 breast cancer cells than OPN with a lower level of phosphorylation [60]. Most tumor cells express hypophosphorylated OPN, however it is uncertain whether total phosphorylation and/or site-specific phosphorylation is responsible for the alterations in tumor cell adhesion/migration [86]. Also, diminished phosphorylation of BSP and OPN reduces osteoclast adhesion, whereas de-phosphorylation of OPN increases osteoclast migration [98, 99]. Variations in the glycosylation of OPN may also influence the neoplastic activity of this protein. In a murine model of breast carcinoma, OPN has been identified as bearing a sialyl-Thomsen-nouveau (STn) antigen (CD175s) which has been linked to diminished response to chemotherapy and diminished survival in human breast cancers [100, 101]. Thus, post-translational modifications may be important to the tumor-specific activities of BSP and OPN in different osteotropic malignancies.

6. BSP and OPN promote pro-metastatic events through integrin binding

Integrins, a family of non-covalently associated heterodimeric transmembrane receptors, bind many different ligands including serum proteins, extracellular matrix proteins, and cell-surface proteins, thereby regulating cell adhesion, differentiation, motility, growth, gene expression and apoptosis in response to extracellular stimuli [102, 103]. While activation of

second messenger/kinase cascades has been elucidated (in part) for OPN, intracellular signaling elicited by BSP-integrin interaction has not been well characterized. Binding of OPN (and potentially BSP) to integrins induces kinase cascades such as the nuclear factor inducing kinase/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (NIK/MEK1/ERK) pathway and mitogen-activated protein kinase kinase kinase 1/ mitogen-activated protein kinase kinase 4/c-Jun N-terminal kinase 1 (MEKK1/MKK4/ JNK1) pathway subsequently promoting gene transcription through activation of nuclear transcription factors such as NF-κB and AP1 (Figure 2). Alternatively, binding of CD44 by OPN (and possibly DMP1) induces activation of NF-κB through the phospholipase C_γ (PLC_γ), protein kinase C (PKC), and phosphoinositide 3 kinase (PI3K) pathways (for review see [52]).

The binding and activation of integrins by SIBLINGs promote changes in malignant cells that favor metastasis. For example, OPN may stimulate cancer cell motility, tumor growth, and metastasis by $\alpha v\beta_3$ integrin binding and activation which stimulates PI3K/Akt-dependent NF-κB activation and uPA secretion in tumor cells [38]. Also, OPN can stimulate uPA-dependent MMP9 activation by inducing both IKK/ERK pathways and subsequent NIK-dependent NF-κB activation [38]. OPN binding of $\alpha v\beta_3$ integrin may transactivate epidermal growth factor receptor (EGFR) through activation of c-Src, a member of the non-receptor protein tyrosine kinase family that is directly associated with focal-adhesion proteins involved in cell attachment, migration, and turnover of focal adhesions [104, 105]. The binding of $\alpha v\beta_3$ in breast cancer cells (MDA-MB-231, MCF-7) by OPN and subsequent transactivation of EGFR by c-Src stimulates activation of signaling cascades such as PI3K, RAS-MAPK, PLC, and PKC in these cells [96, 106]. OPN binding of $\alpha v\beta_3$ integrin and EGFR transactivation by c-Src leads to ERK phosphorylation, AP-1 activation, and cross regulation between NIK/ERK and MEKK1/JNK1 pathways, all of which enhance malignant cell motility, invasiveness, and ability for metastasis [38]. OPN may promote cell survival and motility of malignant cells by binding CD44 and CD44 splice variants with subsequent activation of PLC-γ, PKC, and PI3K pathways. The binding of CD44 by OPN augments the pro-survival activities of IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF) through PI3K-Akt signal pathways in murine pro-B cells BA/F3 [107]. Activation of CD44 on breast cancer cells up-regulates integrin expression, stimulates integrin-mediated adhesion, and intravasation [108]. Thus, binding of integrins (or CD44) on malignant cells by OPN and BSP results in activation of signaling cascades within the cell that promotes metastasis.

7. BSP and OPN in tumor prognosis and therapy

SIBLING expression in different osteotropic cancers may be useful for establishing the risk of bone metastasis in cancer patients. For example, increased expression of BSP in breast, lung, prostate, and thyroid cancers may predict bone metastasis in these malignancies [20, 21, 23, 109–114]. Studies examining BSP levels in primary breast cancer tissue suggest elevated levels of this SIBLING are prognostic for shorter survival and correlate with the development of bone metastasis [109, 114]. In a retrospective study assessing BSP levels in tumor tissue from 454 individuals it was found that only 8% of BSP negative patients developed bone metastasis whereas 22% of BSP positive patients developed bone metastasis [109]. Expression levels of BSP, but not OPN, in primary resected lung tissues from NSCLC patients are associated with bone metastasis and may identify high risk patients [113]. Similarly, elevated levels of BSP in the blood correlate with, and may be predictive of, bone metastasis in several osteotropic malignancies including breast, lung, prostate, and multiple myeloma [114–122]. Serum BSP levels in prostate cancer increase only in the later stages of the disease bringing into question the prognostic value of BSP in prostate cancer [123]. In addition, the expression of OPN in breast cancer tissue and the elevation of OPN in patient

sera predict a poor prognosis for breast cancer patients [89, 124]. Thus, SIBLINGs may have excellent prognostic value in certain osteotropic malignancies.

SIBLINGs may represent valuable targets for therapeutics since they play a role in many aspects of tumor progression including bone metastasis. Several studies have targeted OPN and BSP utilizing antibodies, small interfering RNAs (siRNA), antisense oligonucleotides (ASOs), and short hairpin RNAs (shRNA) to block the tumor progression and metastasis induced by these SIBLINGs in animal models [125–128]. Rat monoclonal antibodies to human BSP (hBSP) reduced tumor growth and osteolytic lesion formation in nude rats receiving MDA-MB-231 breast cancer cells [129]. In this study, complete remission was observed in 75% of rats who were treated with these inhibitory antibodies against hBSP [129]. Further, many investigators have demonstrated the potential use of siRNAs as anti-SIBLING therapeutic agents in animal models [130, 131]. Reufsteck *et al* demonstrated a significant reduction in proliferation of MDA-MB-231 breast cancer cells in a nude rat model after treatment with siRNA against hBSP. Additionally, while siRNA against OPN, BSP, Runx2 and Integrin β_3 diminish migration of these cells *in vivo*, siRNA against BSP produces the greatest anti-migratory effect. Finally, siRNA against BSP reduces osteolytic bone lesions when compared to controls; however, the use of a nanoparticle delivery system lowers the effective dosage required for this reduction by approximately 25 times when compared to systemic dispersion using minipumps [132]. The use of ASOs to partially silence BSP and OPN expression in the human breast cancer cell line MDA-MB-231 reduces the ability of these cells to cause osteolytic bone metastasis in xenografted nude rats [133]. The use of shRNA against OPN diminishes tumor growth and lymph node metastasis of human esophageal squamous carcinoma cells *in vitro* [134]. While the use of small interfering or shRNA treatment reveals promising potential, the use of these methods in treatment of human malignancy is currently unavailable.

8. Conclusions and future perspectives

Taken together, current evidence demonstrates that SIBLINGs, particularly BSP and OPN, play significant roles in bone metastasis of osteotropic malignancies derived from breast, prostate, lung, thyroid, and multiple myeloma. SIBLINGs seem to directly or indirectly mediate most, if not all, of the requirements for development of metastasis including detachment of neoplastic cells from their primary site, migration, invasion, cell adhesion, proliferation, enhanced survival, angiogenesis, escape from immune surveillance, and altered gene expression. In fact, it is suggested that OPN confers the activities required for all of the “Six Hallmarks of Cancer Progression” [91]. Further studies examining BSP's role in osteotropic metastasis will determine BSP-mediated mechanisms that underlie metastatic processes.

A receptive bone microenvironment is necessary for bone metastasis, proliferation, and survival of osteotropic malignancies [135, 136]. For example, stress-induced endogenous beta 2 adrenergic receptor (β2AR) activity alone stimulates bone marrow osteoblast production of RANKL and subsequent bone metastasis of MDA-MB-231 cells [136]. Additional studies demonstrate breast cancer cell production of parathyroid hormone-related peptide (PTHrP) in the bone microenvironment stimulates osteoblast and stromal cell production of RANKL and subsequent osteoclastogenesis followed by osteoclast-mediated bone resorption. Bone degradation/resorption releases transforming growth factor β (TGF-β) and insulin like growth factor-1 (IGF-1) from bone and stimulates increased breast cancer cell production of PTHrP and proliferation (respectively), thus setting into motion the “vicious cycle” of bone resorption [137]. In fact, breast cancer cells produce many osteoclastogenic factors including tumor necrosis factor alpha (TNF-α), macrophage colony-stimulating factor (M-CSF), interleukin (IL)-6, IL-11, and prostaglandin E2 (for

review see [138, 139]). Future studies examining the changes in bone (e.g. osteoclastogenesis, osteoblastogenesis etc.) elicited by malignant cells will provide new insights into mechanisms that permit survival of malignant cells in the bone microenvironment and the mechanisms of bone pathology associated with these malignant diseases.

The mechanisms that underlie the ability of BSP and OPN to confer an “osteomimetic” phenotype to malignant cells are no doubt complex and likely multifactorial. It does not seem likely that an osteomimetic phenotype is conferred to malignant cells simply by “expressing” a SIBLING such as BSP or OPN. Rather, it seems likely that the “osteomimicry” of malignant cells is not only conferred by surface bound SIBLINGs, but more importantly includes the “switch” in the gene expression repertoire typically expressed in cells of skeletal lineage during bone morphogenesis (e.g., IBSP, SPP1, Runx2, Msx2, core-binding factor β (CBFβ), cadherin 11 (CDH11), etc. [140, 141]. This might suggest that malignant cells with an “osteomimetic” phenotype express other, as yet unidentified, cell surface moieties characteristically associated with cells of skeletal lineage. Understanding what causes the “switch” in gene expression in malignant cells of epithelial cell origins to a repertoire associated with cells of a skeletal lineage should help unlock some of the mechanisms required for malignant cell metastasis to bone. Continued examination of the mechanisms underlying the role of BSP and OPN in tumor promotion, progression and metastasis, and the altered physiology of the bone microenvironment, will likely result in development of better diagnostic approaches and therapeutic regimens for osteotropic malignant diseases.

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Biography

Thomas E. Kruger received his Ph.D. in Microbiology from the University of Texas Medical Branch in Galveston Texas, where his research focused on Neuroimmunoendocrinology. He completed two post-doctoral fellowships, one at the Marine Biomedical Institute, Galveston TX (*neuroendocrine receptor physiology*) and one at LSU Health Sciences Center, Shreveport LA (*alcohol-mediated bone marrow pathophysiology*) before joining the faculty as a Research Assistant Professor in the Department of Pediatrics where his research examined 1) cytokine-mediated mechanisms underlying Bronchopulmonary Dysplasia (BPD) in the preterm infant and 2) the role of cytokine gene polymorphisms in PTFE graft rejection. Thomas joined the Harrington Lab for Molecular Orthopedics in the Department of Orthopedic Surgery at the University of Kansas Medical Center in 2012, where his research has focused on regulatory mechanisms of osteoblast differentiation, osteogenesis, and bone metastasis of osteotropic cancers.

Andrew H. Miller is a research assistant in the Harrington Laboratory for Molecular Orthopedics at the University of Kansas Medical Center. His current projects include investigating the role of bone sialoprotein (BSP) in osteoblast differentiation and bone formation and exploring pathogenic mechanisms and novel therapeutics for osteoarthritis. He received his B.S. degree in 2011 from the University of Wisconsin-Madison.

Andrew K. Godwin is the Chancellor's Distinguished Chair in Biomedical Sciences Endowed Professor and the Director of Molecular Oncology in the Department of Pathology and Laboratory Medicine at the University of Kansas Medical Center. He is the Deputy

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Jinxi Wang is the Mary A. and Paul R. Harrington Distinguished Professor of Orthopedics and Professor of Biochemistry & Molecular Biology at the University of Kansas Medical Center in the United States. He received his medical degree from Suzhou Medical College in China and his PhD degree from a joint program of Suzhou Medical College and Loma Linda University in California, followed by postdoctoral research training in molecular and cellular biology of the skeletal system at Harvard University Medical School. His major research interests include: 1) Bone sialoprotein (BSP)-mediated bone formation and bone metastasis of osteotropic cancers, 2) regulatory mechanisms of osteoblast differentiation and bone mineralization, and 3) pathogenetic mechanisms and novel therapies for osteoarthritis. These research projects are supported by a U.S. Department of Defense (DoD) grant and National Institutes of Health (NIH) R01 grants. Currently, he serves as a grant reviewer for two NIH study sections and a DoD grant review panel; he also serves as an editorial board member for several national and international biomedical journals.

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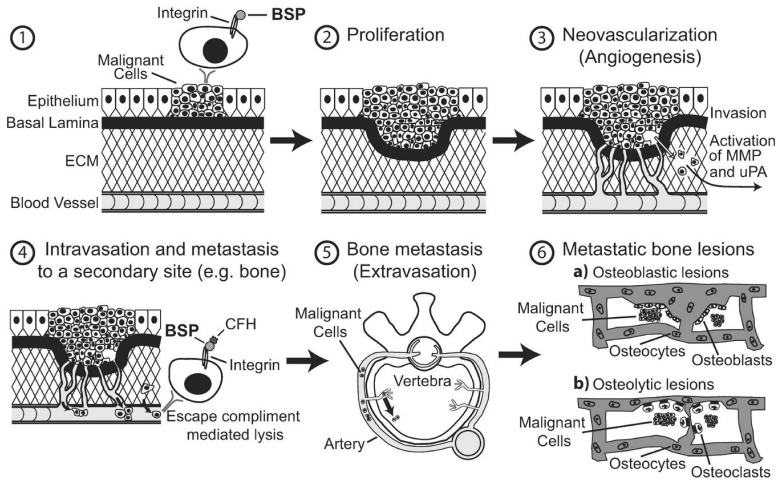

Fig. 1. Proposed steps in BSP-mediated bone metastasis

1) BSP binds to integrin receptors (e.g., $\alpha_v\beta_3$) on malignant epithelial cells. **2**) Activation of the integrin signaling by BSP stimulates malignant cell proliferation. **3**) BSP stimulates angiogenesis and neovascularization of the tumor. Activation of urokinase-type plasminogen activator (**uPA**) and matrix metalloproteinase (**MMP**) allows invasion of malignant cells directly into the extracellular matrix (ECM). **4**) BSP-stimulated angiogenesis allows intravasation of malignant cells into the circulatory system and subsequent metastasis of malignant cells to secondary sites (e.g., bone). Inset: BSP bound to integrin also binds compliment factor H (CFH) allowing malignant cell escape from compliment-mediated cell lysis in the blood. **5**) Malignant cell(s) lodge or bind endothelium in bone capillaries. BSP-stimulated angiogenesis allows malignant cell extravasation into bone tissue. **6**) Survival and proliferation of malignant cells in bone resulting in stimulation of osteoblastogenesis and bone formation causes osteoblastic bone lesions (**a**), stimulation of osteoclastogenesis and bone resorption causes osteolytic lesions (**b**).

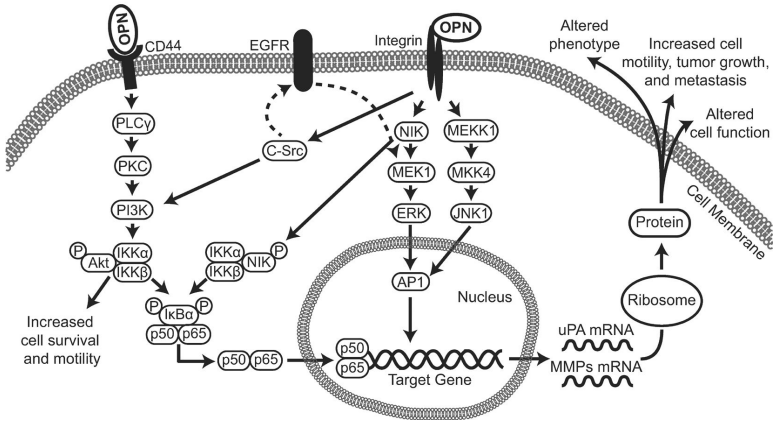

Fig. 2. Second messenger cascades associated with integrin and CD44 binding by OPN

OPN binding of $\alpha_v\beta_3$ or CD44 may activate transcription factors NF-κB and AP-1 through multiple pathways ultimately leading to expression of target genes (e.g. uPA, MMPs) that increase tumor cell survival, motility, tumor growth and metastasis. OPN binding to CD44 may also stimulate protein kinase B (Akt)-dependent activation of anti-apoptotic mechanisms enhancing tumor cell survival, as well as enhancing tumor cell motility. OPN binding to $\alpha_v\beta_3$ may transactivate (dotted line) the epidermal growth factor receptor (**EGFR**) via c-Src activation. Abbreviations: Phospholipase C-γ (**PLCγ**), protein kinase C (**PKC**), phosphatidylinositol 3 kinase (**PI3 kinase**), protein kinase B (**Akt**), IKappaB Kinase alpha (**IKKα**), IKappaB Kinase beta (**IKKβ**), NF-κB inhibitor (**IκBα**), NF-κB heterodimer [**p50** (NFKB1)/**p65** (RelA)], cellular sarcoma proto-oncogene (**c-Src**), nuclear factor-inducing kinase (**NIK**), mitogen-activated protein kinase (*MAPK*/**ERK**), MAPK kinase (*MAP2K1*/**MEK1**), activator protein 1 (**AP1**), mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*) (**MEKK1**), mitogen-activated protein kinase kinase 4 (*MAP2K4*) (**MKK4**), c-Jun N-terminal kinase 1 (**JNK1**), urokinase-type plasminogen activator (**uPA**), matrix metalloproteinase (**MMP**).

Table 1

Incidence of Bone Metastasis Associated With Various Malignancies

Primary tumor	Bone metastasis (%)	Lesion type(s)
Multiple myeloma	95–100	Osteolytic
Prostate cancer	65–75	Osteoblastic
Breast cancer	65–70	Mixed: osteolytic > osteoblastic
Thyroid cancer	60	Osteolytic
Lung cancer	30–40	Mixed: osteolytic > osteoblastic
Renal cancer	20–25	Mixed: osteolytic > osteoblastic