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OSTEOPONTIN

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ABSTRACT: Osteopontin (OPN) is a highly phosphorylated sialoprotein that is a prominent component of the mineralized extracellular matrices of bones and teeth. OPN is characterized by the presence of a polyaspartic acid sequence and sites of Ser/Thr phosphorylation that mediate hydroxyapatite binding, and a highly conserved RGD motif that mediates cell attachment/signaling. Expression of OPN in a variety of tissues indicates a multiplicity of functions that involve one or more of these conserved motifs. While the lack of a clear phenotype in OPN "knockout" mice has not established a definitive role for OPN in any tissue, recent studies have provided some novel and intriguing insights into the versatility of this enigmatic protein in diverse biological events, including developmental processes, wound healing, immunological responses, tumorigenesis, bone resorption, and calcification. The ability of OPN to stimulate cell activity through multiple receptors linked to several interactive signaling pathways can account for much of the functional diversity. In this review, we discuss the structural features of OPN that relate to its function in the formation, remodeling, and maintenance of bones and teeth.

Key words. Sialoprotein, cell signaling, cell attachment, bone remodeling, tooth.

(1) *Introduction*

Osteopontin (OPN) was first described as a secreted, 60-kDa transformation-specific phosphoprotein (Senger *et al.*, 1979) and subsequently was rediscovered by molecular cloning of the transformation-associated gene 2ar (Craig *et al.*, 1989). Meanwhile, OPN was identified independently, together with bone sialoprotein (BSP), as a major sialoprotein in the extracellular matrix of bone (Franzén and Heinegård, 1985; Fisher *et al.*, 1987; Prince *et al.*, 1987; Zhang *et al.*, 1990) and the 2 proteins initially called bone sialoprotein I (BSP I) and bone sialoprotein II (BSP II), respectively (Franzén and Heinegård, 1985). The name "osteopontin" was introduced to reflect the potential of the bone protein to serve as a bridge between cells and hydroxyapatite through RGD and polyaspartic acid motifs discovered in the primary sequence of the protein (Oldberg *et al.*, 1986). However, the same gene product was identified as a putative lymphokine produced by activated lymphocytes and macrophages and called Eta-1 (early T-lymphocyte activation gene 1; Patarca *et al.*, 1989), and thus a more general pattern of expression for OPN was emerging. Accordingly, secreted phosphoprotein 1 (SPP 1) was introduced as an alternate name, to reflect a broader functional role of this protein. Nevertheless, the name "osteopontin" has largely been retained, in keeping with the nomenclature used for the human gene (Denhardt *et al.*, 1995).

In the past five years, several excellent reviews of OPN and its potential significance in mineralized tissues (Butler *et al.*, 1996; McKee and Nanci, 1996a; Denhardt and Noda, 1998; Gerstenfeld, 1999), the vascular system (Giachelli *et al.*, 1995), the immune system (Weber and Cantor, 1996), kidney (Rittling and Denhardt, 1999), and in cellular transformation and cancer (Oates *et al.*, 1997) have been published. Consequently, this review provides only a brief overview of the general properties of OPN and focuses on the structure-function relationships together with the implications of recent findings on the role of OPN in the formation and remodeling of mineralized connective tissues.

(i) General characteristics

OPN is expressed by a single-copy gene as a ~ 34-kDa nascent protein that is extensively modified by post-translational events. The human gene contains 7 exons, spans ~ 11.1 kb, and maps to the long arm of chromosome 4 (4q13) (Young *et al.*, 1990; Hijiya *et al.*, 1994). In comparison, the ~ 4.8-kb mouse gene is at the locus of the Rickettsia resistance gene Ric^r on chromosome 5 (Fet *et al.*, 1989; Miyazaki *et al.*, 1989), and the pig gene is on chromosome 8 (Denhardt and Guo, 1993). Whereas the mammalian and avian OPN proteins have a similar number of amino acids, the reported size of the secreted protein varies from 44 kDa to 75 kDa, due to differences in

post-translational modifications and anomalous behavior on SDS-PAGE. The protein is rich in aspartic and glutamic acid and serine, and contains a polyaspartic acid motif, through which the protein can bind to hydroxyapatite and calcium ions, and an RGD sequence which can mediate cell attachment. In addition, multiple sites of Ser and Thr phosphorylation and sites of both N- and O-linked glycosylation exist, together with a thrombin cleavage site. Variations in phosphorylation, glycosylation, and sulphation generate different functional forms of OPN which may be found in the same or different tissues.

(ii) Tissue distribution

OPN is expressed by cells in a variety of tissues, including bone, dentin, cementum, hypertrophic cartilage, kidney, brain, bone-marrow-derived metrial gland cells, vascular tissues and cytotrophoblasts of the chorionic villus in the uterus and decidua, ganglia of the inner ear, brain cells and specialized epithelia found in mammary, salivary, and sweat glands, in bile and pancreatic ducts, and in distal renal tubules and in the gut, as well as in activated macrophages and lymphocytes (Patarca *et al.*, 1989, 1993; Giachelli *et al.*, 1995; Butler *et al.*, 1996; Weber and Cantor, 1996; Denhardt and Noda, 1998; Rittling and Denhardt, 1999). In bone, OPN is produced by osteoblastic cells at various stages of differentiation (Zohar *et al.*, 1997a), by differentiated osteoblasts and osteocytes (McKee and Nanci, 1995a; Sodek *et al.*, 1995), and also by osteoclasts (Dodds *et al.*, 1995). OPN is also expressed by fibroblastic cells in embryonic stroma and in wound-healing sites (Giachelli *et al.*, 1995). Although the secreted protein is incorporated into the matrix in mineralized connective tissues, in many situations it appears in biological fluids, including blood (Bautista *et al.*, 1996), milk (lactopontin; Senger *et al.*, 1989a), urine (uropontin; Hoyer *et al.*, 1995), and seminal fluid (Cancel *et al.*, 1999). OPN is also expressed in the pre-implantation mouse embryo and during differentiation of embryonal stem cells (Botquin *et al.*, 1998) and in the rostral hind brain and notochord during neuraxis formation (Thayer and Schoenwolf, 1998). The amount of OPN in normal plasma of women ranges from 22 to 122 μg/L, with a median level of 47 μg/L (Singhal *et al.*, 1997), whereas in urine, OPN ranges from 1.9 to 4.3 μg/mL, with ~ 4 mg excreted *per* day (Min *et al.*, 1998). The concentration of serum OPN protein is markedly elevated with increased tumor burden in cancer patients (Senger *et al.*, 1989b; Singhal *et al.*, 1997) and in patients with auto-immune diseases such as systemic lupus erythematosus (Iizuka *et al.*, 1998). Recent studies have shown that OPN and bone sialoprotein in serum bind tightly to Factor H and may help protect normal and cancerous cells from complement-mediated attack (Fedarko *et al.*, 2000)

Expression of OPN is characteristically observed in pathological conditions and pathophysiological responses. Thus, OPN synthesis is induced in smooth-muscle cells and cardio-myocytes in cardiovascular diseases, including atherosclerosis (Giachelli *et al.*, 1993), restenosis (Panda *et al.*, 1997), and ventricular hypertrophy (Graf *et al.*, 1997). Similarly, OPN is induced in kidney diseases, such as interstitial nephritis (Sibalic *et al.*, 1997) and puromycin aminonucleoside nephrosis (Magil *et al.*, 1997), and in pathological mineralization such as kidney stone formation (Kohri *et al.*, 1993; McKee *et al.*, 1995). The induction of OPN in various carcinomas and adenocarcinomas has been related to the activity of various oncogenes such as Src (Tezuka *et al.*, 1996) and Ras (Chambers *et al.*, 1992), and its expression is directly correlated with the development and progression of transformation processes (Chambers *et al.*, 1996) and metastasis (Oates *et al.*, 1997), although the stage at which it functions has not been established. Synthesis of OPN by activated lymphocytes and macrophages (Patarca *et al.*, 1989, 1993) can account for the presence of OPN in diseased and traumatized tissues, OPN being one of the most abundant molecules synthesized by activated T-lymphocytes (Weber and Cantor, 1996). Studies on granulomatous inflammation have indicated that, as well as acting as a chemoattractant for T-cells, OPN can promote adhesion of T-cells and possibly amplify a CD3-mediated proliferative response (O'Regan *et al.*, 1999). The increased OPN activity following thrombin digestion observed in these studies suggests a mechanism whereby OPN and thrombin can modulate T-cell recruitment and activation. A critical role for OPN in type 1 cell-mediated immunity has been demonstrated recently in which OPN, acting through the $\alpha_v\beta_3$ and CD44 receptors, respectively, increases IL-12 and suppresses IL-10 expression by macrophages (Ashkar *et al.*, 2000). Together with its activation of B cells by OPN (Iizuka *et al.*, 1998), OPN could, therefore, contribute to the development of autoimmune diseases.

(iii) Regulation

The expression of OPN is affected by a large number of hormones, cytokines, and growth factors which can influence the rate of gene transcription, mRNA processing, stability, and translation, as well as post-translational modifications. Many of these effects have been catalogued and referenced in a recent review (Denhardt and Noda 1998) and will be only summarized here, with an emphasis on OPN expression in mineralized tissues. Although regulation is generally similar in different tissues, there are some notable differences, and, even within the same tissue, differential regulation has been observed which can be attributed, at least in part, to the target cell and its stage of maturation.

(a) Hormonal and cytokine control of OPN expression

Generally, the steroids, retinoic acid and glucocorticosteroids, and particularly the seco-steroid hormone

1,25-dihydroxyvitamin D3, increase OPN expression in bone cells (Prince and Butler, 1987; Kasugai *et al.*, 1991). The synthetic glucocorticosteroid, dexamethasone, increases OPN expression in rat periosteal and marrow bone cell cultures, and in cultures of embryonic rat bones (Chen *et al.*, 1996). Vitamin D3 is a particularly potent stimulator of OPN synthesis by bone cells and epidermal cell lines, and a marked reduction of OPN mRNA expression is observed in vitamin-D3-deficient rachitic mice (Chen *et al.*, 1999). Thus, while the increase in OPN induced by glucocorticoids is linked to the stimulation of bone formation, the vitamin D3 effect is associated with massive bone resorption, reflecting the multifunctionality of OPN in bone remodeling (Sodek *et al.*, 1992, 1995). Retinoic acid also increases OPN expression in bone (Manji *et al.*, 1998), whereas the osteotropic parathyroid hormone, PTH, has marginal effects that appear to be bone cell type-dependent (Kasugai *et al.*, 1991).

Expression of OPN is also up-regulated by various growth and differentiation factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor-βs (TGF-βs), bone morphogenetic proteins (BMPs), and inflammatory cytokines (reviewed in Denhardt and Noda, 1998). Non-physiological agents such as phorbol esters and concanavalin A also stimulate OPN expression. Increased expression of OPN induced by EGF, c-Src, and 12-O-tetradecanoylphorbol-13-acetate (TPA) appears to be mediated through protein kinase C (PKC), which activates the early response genes, Jun and Fos, which in turn can increase OPN transcription through AP-1 sites. The increase in OPN expression induced by mechanical stimuli *in vitro* (Kubota *et al.*, 1993; Klein-Nulend *et al.*, 1997; Carvalho *et al.*, 1998) and *in vivo* (Miles *et al.*, 1998) has revealed a mechano-transduction pathway, involving stress on cell adhesion sites, in which signals are mediated by protein tyrosine kinases. These signals require an intact cytoskeleton and increase OPN transcription as a primary response (Toma *et al.*, 1997).

Whereas OPN is generally up-regulated by hormones and cytokines, it is suppressed by bisphosphonates in bone (Sodek *et al.*, 1995) and kidney (Yasui *et al.*, 1998). The down-regulation of OPN by bisphosphonates in bone is consistent with the suppression of osteoclastic activity as well as the reduced OPN expression (Chakalaparampil *et al.*, 1996) and osteoclast activity in Src-/- mice (Soriano *et al.*, 1991) and the suppression of OPN synthesis and activity in osteoclasts by calcitonin (Kaji *et al.*, 1994). OPN expression is also suppressed in vascular smooth-muscle cells by cGMP-dependent protein kinase, a mediator of nitric oxide (NO), and by cGMP signaling, which inhibits cell migration (Dey *et al.*, 1998).

(b) OPN promoter and transcriptional regulation

Increased expression of OPN is frequently associated with an increase in transcription of the OPN gene, which is regulated by transactivation of cis-acting elements in the gene promoter. The promoters for the human, pig, rat, mouse, and chicken have been sequenced and show some conservation in ~ 250 bp of the proximal promoter. Within this region is a conserved (TTTAAA) TATA box (nucleotides [nts] -27 to -19), an inverted CCAAT box (nts -52 to -46), and a GC box (nts -100 to -93). One (human, mouse, and chicken: Noda *et al.*, 1990; Hijiya *et al.*, 1994; Rafidi *et al.*, 1994) or 2 (rat and pig: Ridall *et al.*, 1995; Li *et al.*, 1998) 1,25-dihydroxyvitamin D3 response elements (VDREs) that bind the vitamin D3 receptor (VDR) are present upstream of the immediate promoter region. In addition, activator protein (AP) motifs have been identified of which AP-1, a highly conserved element controlled by the proto-oncogene products Fos and Jun, mediates OPN transcription by the tumor-promoting phorbol ester, TPA, acting through PKC (Denhardt and Guo, 1993). AP-2 is also a target of PKC-mediated signaling as well as signaling through cAMP-dependent protein kinase A (PKA). There are 5 recognition sites for the polyoma enhancer activator PEA-3. These sites are the major targets of the Ets family of proto-oncogene transcription factors that are involved in T-cell activation and could, therefore, account for the up-regulation of OPN expression in activated lymphocytes and macrophages. Notably, OPN promoter activity is markedly enhanced by polyoma enhancer-binding protein/core binding factor (PEBP2αA/CBFA1), which functions as a master gene for osteogenesis. The CBFA1 acts synergistically with Ets1 in a mechanism that requires a direct interaction of the transcription factors with cognate enhancers that are critically spaced for transactivation (Sato *et al.*, 1998). OPN expression is also up-regulated during pre-implantation development by the POU transcription factor Oct-4 acting through a PORE element in the first intron and is repressed by Sox-2 acting on a closely located element (Botquin *et al.*, 1998).

Regulation of OPN by Src was shown to increase OPN expression through the inverted CCAAT box (Tezuka *et al.*, 1996), which binds nuclear factor NF-Y (Kim and Sodek, 1999). Since v-src is a transforming viral oncogene product originally identified in the Rous sarcoma virus (RSV), these observations provide a firm link between cellular transformation and induction of OPN expression. Similarly, cells transformed with v-Ras and v-Myc also increase transcription of OPN. A Ras-activated enhancer which interacts with a putative Ets-related transcription factor, the activity of which correlates with the metastatic potential of the cell, has also been identified (Guo *et al.*, 1995). Another CCAAT/enhancer binding protein family member, Nuclear Factor-IL-6 (NF-IL6), which binds to the regulatory regions of many genes induced in activated macrophages, including macrophage inflammatory protein (MIP)-1 alpha also

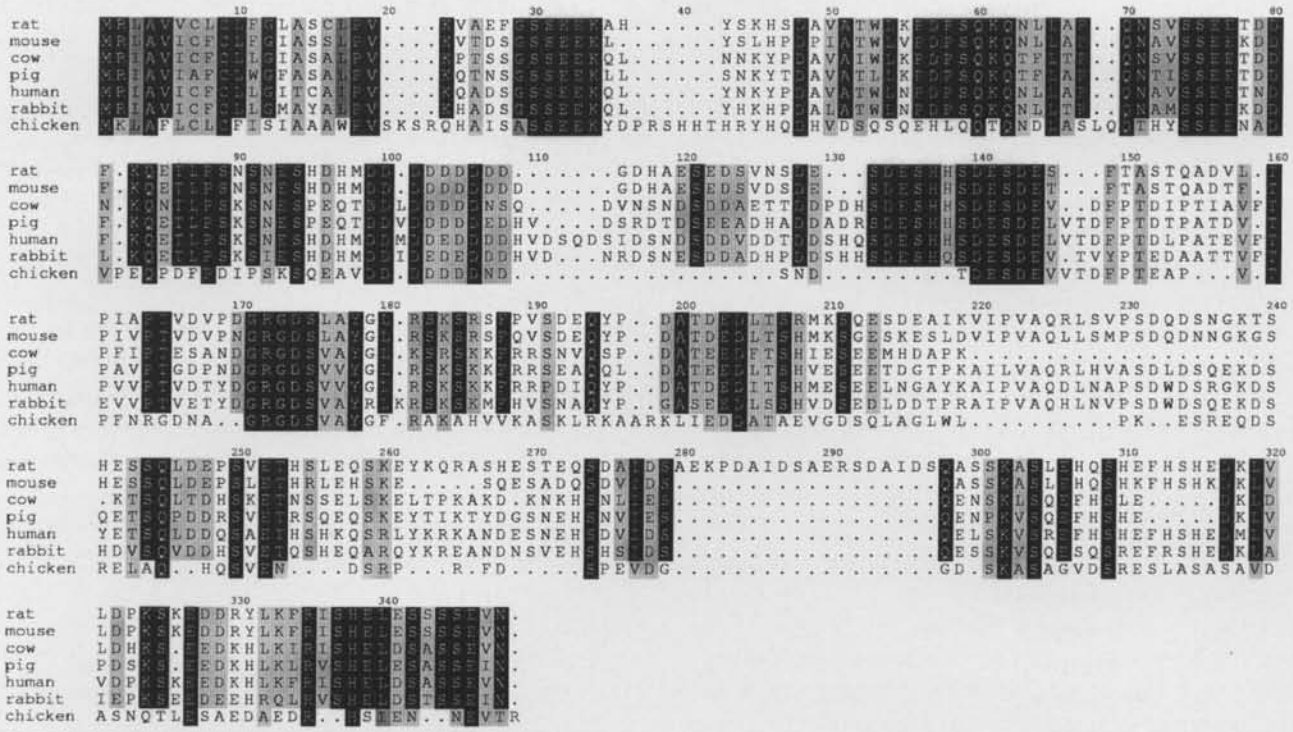


Figure 1. Comparison of osteopontin protein sequences. The amino acid sequences from six mammalian (rat, mouse, cow, pig, human, and rabbit) and one avian (chicken) species have been aligned by means of the multiple alignment program of Thompson *et al.* (1994) to maximize identical (dark shading) and similar (light shading) amino acids identified by JavaShade (Southern and Lewis, 1998; http://industry.ebi.ac.uk/JavaShade/) to reveal conserved structures. The 16-amino-acid leader sequence beginning with a methionine (position 1) is included with the first amino acid of the processed protein starting at position 17. The total number of amino acids in the sequence for each species is: chicken, 264; mouse, 294; rat, 317; rabbit, 311; cow, 278; pig, 303; and human, 300.

increases endogenous OPN gene expression (Matsumoto *et al.*, 1998), thus providing a regulatory pathway for OPN expression in inflammatory cells.

(2) *Structural Characteristics*

(i) Amino acid sequence comparisons

Insights into the potential function of proteins can be derived from the conservation of structural motifs in the amino acid sequence. The amino acid sequences of OPNs derived from human (Kiefer *et al.*, 1989), cow (Kerr *et al.*, 1991), pig (Wrana *et al.*, 1989), rabbit (Tezuka *et al.*, 1992), rat (Oldberg *et al.*, 1986), mouse (Craig *et al.*, 1989), and chicken (Moore *et al.*, 1991) cDNAs are currently available. Comparison of the mammalian OPN sequences reveals a high conservation in the amino- and carboxy-terminal regions and in the polyaspartate segment, as well as in the GRGDS and thrombin cleavage sites and in several potential phosphorylation sites (Fig. 1). Of the 333 amino acids in the consensus sequence for mammalian OPNs (CLUSTAL W program; Thompson *et al.*, 1994), 107 amino acids are identical, with 59 amino acids retaining high similarity and a further 26 with lower similarity, for a conservation of 58% of the amino acids. Notably, there is also high identity (10/21 residues identical) in a sequence (220-240) that is missing in bovine OPN and in a short sequence (312-316) that is missing in both the cow and the pig. When the avian sequence is included in the comparison, the number of perfect matches is reduced considerably, to 52, with 48 amino acids retaining high similarity and 37 with lower similarity, for a conservation of 39%. Despite the reduced conservation, the GRGDS motif, the polyaspartic acid sequence, and the thrombin cleavage sites are retained, together with several phosphorylation sites. Nevertheless, distinct differences in the developmental expression have been reported which could reflect functional differences between chicken and mammalian OPNs (Thayer and Schoenwolf, 1998).

(ii) Conserved motifs

The conserved GRGDS sequence is flanked by several highly conserved sequences, including the thrombin cleavage site, which is separated from the attachment sequence by a conserved "vaYgL" sequence. On the amino terminal side, an "fTpvvPTv" sequence is conserved in which the prolines would be anticipated to affect the structure of the cell attachment/signaling site. The thrombin cleavage site is faithfully retained in all species, although the Arg-Ser recognition site is modified to an Arg-Ala in the chicken and a Lys-Ser in the cow. Since thrombin is highly selective for Arg-Gly sites in fibrinogen, it is likely that the transition

from a β-sheet to an α-helix in this region of OPN is conducive to thrombin attack. The existence of the thrombin site is indicative of an ancestral relationship to proteins involved in hemostasis and the immune defense system, while its conservation is consistent with an important biological activity. Although the polyaspartate region is not contiguous in all species, within a stretch of 8-10 amino acids at least 7 are aspartates in all species. Four of eight (chicken) to 10 aspartates are conserved in all species, while a glutamic acid is an occasional replacement in a further three positions, producing a stretch of acidic amino acids in a region of no definitive secondary structure. However, the relative positions of the aspartates in the local coil structure are more likely to be important functionally.

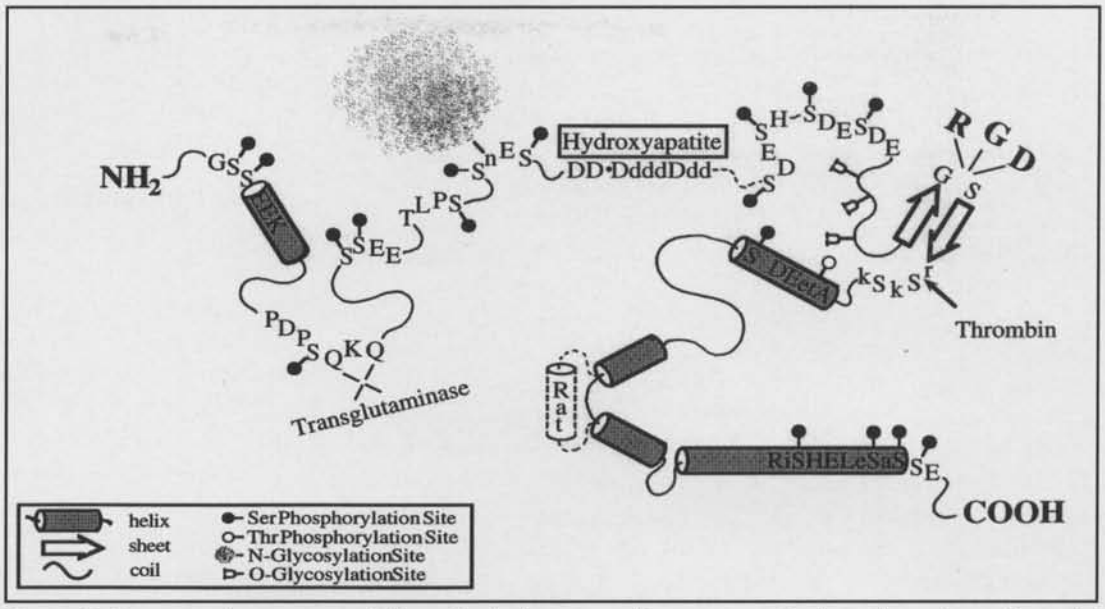


Figure 2. Osteopontin structure. A hypothetical structure for osteopontin, based on the amino acid sequence and secondary structure prediction (SOPM, Self-Optimized Prediction Method, GOR IV, and PREDATOR in Thompson *et al.*, 1994) of the mammalian proteins, is shown together with the positions of demonstrated and potential post-translational modifications. The actual amino acid sequences of the conserved regions for the GRGDS cell attachment motif, the polyaspartate sequence, sites of phosphorylation and glycosylation and thrombin susceptibility, and the transglutaminase cross-linking site are given with identical amino acids capitalized and conserved amino acids in lower case. The additional sequence present in rat OPN is shown with a dashed line.

Seven sites of casein kinase II phosphorylation are conserved in most species, with 6 of the sites involving serine phosphorylation and the other a threonine. In addition, in the mammalian sequences, 9 serines are in conserved sites (Ser-X-Glu/PSer) for Golgi-casein kinase or mammary gland casein kinase (Ser-X-Glu/PSer/Asp), with several additional sites that are conserved in some species. Potential phosphorylation sites for protein kinase C and tyrosine kinase are present in different species with no strong conservation. Although sites of N-linked glycosylation are present in all species, the conservation is generally poor, with only one Asn-X-Ser site conserved in five of the mammalian species. Two glutamines (positions 60 and 62 in the aligned sequences) separated by a lysine serve as substrates for transglutaminase cross-linking (Sorensen *et al.*, 1994) and are present in a conserved region of OPN extending from residue 56 in the mammalian sequence. Interestingly, the Gly in position 12 of the highly conserved leader sequence in the mammalian OPNs is a potential site for N-myristoylation, which can attach proteins to membrane structures.

(iii) Secondary structure predictions

Analysis of secondary structure based on the nascent protein sequences, with three different algorithms (SOPM, Self-Optimized Prediction Method, GOR IV, and PREDATOR), available in the CLUSTAL W program (Thompson *et al.*, 1994), reveals few areas with consistent secondary structure prediction through the species. Most conserved sequences are in regions predicted to form coil structures with some sheet structure between the GRGDS and thrombin cleavage site. The possibility of helical structure is increased toward the C-terminus, with a high probability of helix within the terminal 30 amino acids. Short sections of helix formation are also seen in association with sites of casein kinase II phosphorylation.

(iv) Structural model

Based on both the consensus sequence, shown in Fig. 1, and the secondary structure prediction, a model for the OPN molecule is depicted in Fig. 2. Due to the paucity of hydrophobic amino acids together with the high number of charged amino acids, including the post-translational modifications on Thr, Ser, and Asn residues, an open extended and flexible structure is anticipated, as found for the bone sialoprotein molecule (Ganss *et al.*, 1999).

(v) Post-translational modifications

Extensive alteration through post-translational modifications can have significant effects on the structure of the OPN molecule. Phosphorylation and sulphation will increase the anionic surface characteristics, while extensive glycosylation can limit flexibility. These modifications can also affect the biological properties of OPN, as discussed later.

(a) Phosphorylation

Phosphorylation of OPN, catalyzed by several kinases, can take place on tyrosine, or on serine and threonine residues (Saavedra *et al.*, 1995; Lasa *et al.*, 1997). Potential serine and threonine phosphorylating enzymes include casein kinases I and II, Golgi kinases, cAMP- and GMP-dependent kinases, protein kinase C, and ectokinases. Thus, there is considerable potential for phosphorylation reactions to modify the structure and properties of OPN, including its signaling activities. The initial characterization of rat bone OPN revealed 12 phosphoserines and a phosphothreonine (Prince *et al.*, 1987), while subsequent analyses have revealed considerable heterogeneity (Neame and Butler, 1996). Approximately 8 phosphates have been estimated in bovine bone OPN (Salih *et al.*, 1996), whereas 27 phosphorylated serines have been identified in bovine milk OPN (Sorensen *et al.*, 1995), with 19 of the phosphorylation sites being fully conserved in rat OPN (Lasa *et al.*, 1997) and retaining the consensus sequence (S-x-E/Sp) for Golgi apparatus casein kinase (G-CK). The location of G-CK and its high activity on OPN relative to the casein kinases indicate that it is the major enzyme involved in the phosphorylation of secreted OPN (Lasa *et al.*, 1997). Ectokinases on the cell surface can also phosphorylate secreted OPN (Mikuni-Takagaki *et al.*, 1995; Zhu *et al.*, 1997), and recombinant nascent OPN has been reported to catalyze autophosphorylation of tyrosines (Ashkar *et al.*, 1993). However, the lack of a kinase domain in OPN necessitates verification of the autophosphorylation activity.

(b) Glycosylation

Significant glycosylation of OPN, estimated at > 33% of the weight of the protein (Oldberg *et al.*, 1986), occurs in most tissues. Thus, treatment of OPN with glycosidases increases the relative mobility of the protein markedly on SDS-PAGE (Shanmugam *et al.*, 1997). In addition to the single Asn-X-Ser site for N-linked glycosylation, there are ~ 26 Ser-X-Glu sites for O-linked glycosylation. In bovine milk OPN, 3 O-linked oligosaccharides have been identified (Sorensen *et al.*, 1995), whereas 5 or 6 O-linked and a single N-linked oligosaccharide are present in rat bone OPN (Prince *et al.*, 1987). Glycosylation of OPN synthesized by transformed cells may prevent phosphorylation and generate non-phosphorylated OPN (Singh *et al.*, 1990). In Rous sarcoma virus-transformed Rat-1 cells, sialic acid in the OPN is removed, reducing the relative mobility from 69 kDa to 62 kDa and abrogating the cell-binding properties which may be important in the invasive behavior of OPN-expressing cancer cells (Shanmugan *et al.*, 1997).

(c) Sulfation

Studies of bone formation *in vitro* have indicated that sulphation of OPN occurs predominantly in the highly phosphorylated form of OPN, and this has been suggested as a potential marker for differentiated osteoblasts (Nagata *et al.*, 1989). Sulphate is likely to occur in the sialylated oligosaccharide side-chains. Although tyrosine can be sulphated, the tyrosines in OPN do not exist within sequences recognized by sulphotransferase enzymes.

(d) Transglutaminase cross-linking

OPN is a substrate for tissue transglutaminase, as demonstrated by Prince *et al.* (1991), indicating that polymerization of OPN occurs in connective tissues. Subsequently, 2 glutamines near the amino terminus have been identified as the transglutaminase targets (Sorensen *et al.*, 1994, 1995). Both intra- and intermolecular cross-linking to fibronectin can occur (Beninati *et al.*, 1994), and cross-linking of OPN has been reported to alter its conformation and to increase its binding to collagen (Kaartinen *et al.*, 1999). Although a pair of glutamines is also present in the avian sequence, with a second pair close by, the sequence is not as well-conserved, and it is not known whether the chicken OPN is a transglutaminase substrate.

(vi) Multiple forms of OPN

Multiple forms of OPN have been identified as products of normal (Kubota *et al.*, 1989; Kasugai *et al.*, 1991) and transformed cells derived from rodent tissues (Craig *et al.*, 1989; Nemir *et al.*, 1989; Singh *et al.*, 1990). Rat bone cells produce high- and low-phosphorylated forms of OPN, the high-phosphorylated form being associated with differentiated osteoblasts (Kubota *et al.*, 1989; Sodek *et al.*, 1995), while a low-phosphorylated form is produced in response to a vitamin-D3-induced Ca^{2+} influx (Safran *et al.*, 1998). In transformed cells, two forms of phosphorylated OPN, termed pp62 and pp69, and a non-phosphorylated form, np69, have been characterized (Nemir *et al.*, 1989). Also, a non-phosphorylated form of OPN has been reported in bovine seminal fluid (Cancel *et al.*, 1999). Although the physiological significance of these forms is not clear, it is likely that post-translational differences reflect different functional roles. Thus, sialylated forms of OPN, such as pp69, have been shown to associate with cell-surface fibronectin (Shanmugam *et al.*, 1997), whereas the non-phosphorylated, np69, but not pp62, binds to soluble fibronectin (Singh *et al.*, 1990). Moreover, a switch from the synthesis of non-phosphorylated to phosphorylated OPN in phorbol-ester-treated mouse epidermal cells (Chang *et al.*, 1995), and from a non-sialylated to a sialylated form in oncogene-transfected cells (Shanmugam *et al.*, 1997), suggests that post-translational modifications of OPN may be significant in cellular transformation.

An alternative spliced form of OPN mRNA, in which a 42-nucleotide sequence coding for 14 amino acids beginning at residue 58 is removed, has been reported in human bone and decidual cells (Kiefer *et al.*, 1989; Young

et al., 1990), and three splice variants are reported to be expressed in human glioma tumors (Saitoh *et al.*, 1995). Analysis of cDNAs prepared from Kirsten sarcoma virus-transformed normal rat kidney cells (KNRK) and rat osteosarcoma cells (ROS 17/2.8) revealed a 52-nucleotide insert in the 5'-non-coding region of the KNRK OPN cDNA (Singh *et al.*, 1992). While the differentially spliced forms expressed by the human cells could give rise to different proteins, the alternative splicing in the non-coding region of the rat transcripts would not alter the translated protein

(3) *Structure-Function Analysis*

Although the precise functions of the OPN are unknown, the unique conserved regions of the OPN molecule (*i.e.*, the RGD site, thrombin cleavage site, polyaspartic acid sequence, and serine/threonine phosphorylation sites) can provide insights into the diverse functions predicted for this protein.

(i) Regulation of the formation and growth of calcium phosphate and oxalate crystals

The association between the expression of OPN and the formation of mineral crystals is highly suggestive of a function in the regulation of mineralization in normal and pathological situations (Shiraga *et al.*, 1992; Kohri *et al.*, 1993; Bellahcène *et al.*, 1994; Goldberg and Hunter, 1995; McKee *et al.*, 1995; McKee and Nanci, 1995b; Hunter *et al.*, 1996; Mohler *et al.*, 1997; Shen *et al.*, 1997). That OPN is often enriched in biological fluids (milk, urine, bile, seminal fluid) having elevated levels of calcium salts is also indicative of a role in preventing spontaneous precipitation of calcium salts. Also, in the kidney, OPN is predominantly localized to the thin limb of the loop of Henle (Kleinman *et al.*, 1995; McKee *et al.*, 1995), upstream of a region with a high propensity for spontaneous precipitation of calcium salts. Consistent with these observations, osteopontin from bone (Boskey *et al.*, 1993; Hunter *et al.*, 1994, 1996) and uropontin (Shiraga *et al.*, 1992; Worcester *et al.*, 1995; Arsenault *et al.*, 1996; Asplin *et al.*, 1998) inhibit crystal nucleation and growth of HA and calcium oxalate (CO), respectively. This inhibition, which is believed to relate to the calcium-binding properties of the polyaspartic acid sequence and serine phosphorylation sites (Singh *et al.*, 1993), appears to involve selective binding to a specific crystal face–acidic proteins binding preferentially to the {100} face of HA, whereas phosphorylated proteins bind to the {010} face (Fujisawa and Kuboki, 1991; Furedi-Milhofer *et al.*, 1994). That the phosphate groups are the predominant moieties involved in the inhibition of crystal formation and growth is indicated by the low inhibitory activity of recombinant human OPN, which lacks post-translational modifications (Goldberg, personal communication), and the loss of inhibitory activity when OPN is dephosphorylated (Boskey *et al.*, 1993; Hunter *et al.*, 1994). In contrast, modification of OPN carboxyl groups has a more modest effect (Hunter *et al.*, 1994). Although polyaspartate can inhibit HA and COM nucleation and growth (Hunter *et al.*, 1994, 1996), the minimal number of Asp residues required is not known.

(ii) Cell attachment and signaling through integrins

Interaction of the GRGDS sequence in OPN with $\alpha_v\beta_{1,3,5}$ (Ross *et al.*, 1993; Liaw *et al.*, 1995), $\alpha_v\beta_1$ (Smith *et al.*, 1996), and $\alpha_8\beta_1$ integrins (Denda *et al.*, 1998) can mediate cell attachment, cell migration, chemotaxis, and intracellular signaling in various cells, including endothelial smooth-muscle cells (Liaw *et al.*, 1995), placental trophoblasts (Daiter *et al.*, 1996), kidney cells (Rabb *et al.*, 1996; Denda *et al.*, 1998), platelets and macrophages (Giachelli *et al.*, 1995), as well as osteoblasts (Oldberg *et al.*, 1986) and osteoclasts (Miyauchi *et al.*, 1991; Ross *et al.*, 1993). The different signals that OPN can elicit in the same and different cells can be explained by the existence of multiple heterodimeric combinations of integrin chains that can ligate OPN and by variant forms of OPN. Thus, an amino-terminal fragment of OPN, generated by thrombin digestion, that retains the RGD motif within 6 amino acids from the cleavage site, has been reported to increase the attachment and spreading properties of OPN, possibly by facilitating accessibility of the RGD (Senger and Perruzzi, 1996). However, the thrombin effects are likely to be receptor-specific, since other studies (Xuan *et al.*, 1994) have reported that thrombin digestion reduces the ability of OPN to promote cell attachment while the migration and adhesion activities of OPN mediated by interaction of the RGD sequence with $\alpha_v\beta_3$ are observed only with the thrombin-cleaved amino-terminal fragment (Smith and Giachelli, 1998). That the enhanced adhesion of cells mediated by the N-terminal fragment is suppressed by the presence of the C-terminal fragment also suggests that the C-terminal domain may regulate OPN functions by suppressing RGD-dependent cell adhesion (Takahashi *et al.*, 1998). In addition to the RGD interactions, OPN has also been shown to mediate cell attachment through non-GRGDS sites that are located in the amino- and carboxy-terminal parts of the molecule (Katagiri *et al.*, 1996) and in a 28-kDa fragment of OPN isolated following proteolytic digestion (van Dijk *et al.*, 1993). Attachment of activated leukocytic cell lines, such as HL-60 and Ramos, by OPN has also been localized to the amino-terminal half of OPN. This interaction involves the $\alpha_4\beta_1$ integrin which recognizes a sequence that is in competition with a leu-asp-val (LDV) peptide (Bayless *et al.*, 1998).

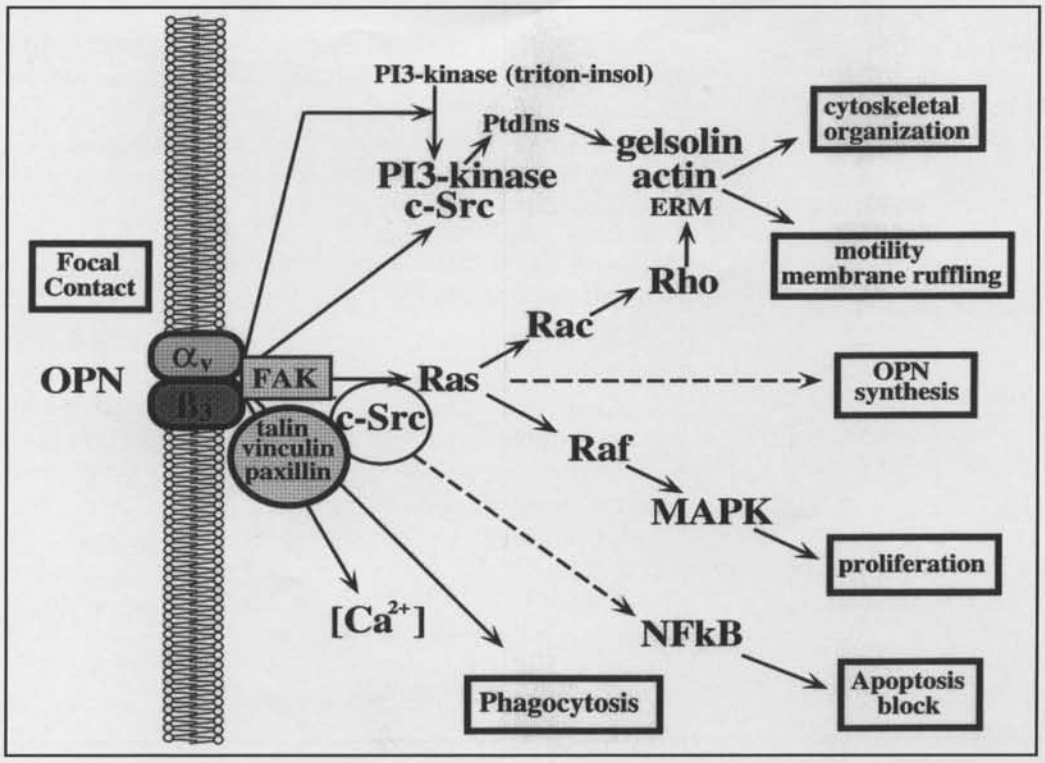


Figure 3. Osteopontin signaling through the $\alpha_v\beta_3$ integrin. A summary of potential signaling pathways for OPN that are mediated through the $\alpha_v\beta_3$ integrin, which can influence cell proliferation, cytoskeletal organization, motility, apoptosis, and phagocytosis in different cells. Both soluble and immobilized forms of OPN can interact with the $\alpha_v\beta_3$ integrin. The evidence for these pathways is discussed in the text. Pathways that may be cell-type-specific or are not well-established are shown with dashed lines.

The $\alpha_v\beta_3$ integrin, which can be co-ordinately regulated with OPN (Liaw *et al.*, 1995; Srivatsa *et al.*, 1997; Ellison *et al.*, 1998), is believed to be primarily responsible for the adhesion and migratory properties of OPN and can also generate intracellular signals through autocrine and paracrine mechanisms. Notably, activation of $\alpha_v\beta_3$ through inside-out signaling may be needed to stimulate adhesion and migration induced by OPN (Faccio *et al.*, 1998). OPN is more effective than other ECM ligands in promoting haptotaxis through the $\alpha_v\beta_3$ integrin, the thrombin fragment being twice as effective as the intact OPN molecule (Senger and Peruzzi, 1996; Senger *et al.*, 1996). OPN ligation to $\alpha_v\beta_3$ can also modulate intracellular Ca^{2+} by stimulating release of Ca^{2+} from intracellular compartments and by regulating extracellular Ca^{2+} influx *via* calmodulin-dependent, Ca^{2+}-ATPase (Miyauchi *et al.*, 1991; Zimolo *et al.*, 1994). Possible pathways for OPN signaling through integrins such as $\alpha_v\beta_3$ are depicted in Fig. 3. Ligation of integrins such as $\alpha_v\beta_3$ by OPN leads to phosphorylation of focal adhesion kinase (FAK) (Frisch *et al.*, 1996: Craig and Johnson, 1996), paxillin, tensin, and Src (Lopez *et al.*, 1995), which initiates signals for proliferation, cytoskeletal organization, motility, and blockage of apoptosis. By utilizing distinct effectors of Ras activation, discrete signaling pathways have been revealed for activation of MAP kinase and cell ruffling, both pathways being required for mitogenic activity of Ras (Joneson *et al.*, 1996). The proliferative effects of OPN have been demonstrated in a subpopulation of prostate epithelial cells which would proliferate only under growth-restricted conditions when grown on an OPN substratum (Elgavish *et al.*, 1998). Ras, PI3-kinase, and Akt pathways activated by $\alpha_v\beta_3$-mediated cell attachment have also been implicated in cell survival (Khwaja and Downward, 1997), as have MEKK-1/JNK (Cardone *et al.*, 1997) and Bcl-2 (Stromblad *et al.*, 1996). That OPN can prevent cell death was first indicated in studies of kidney proximal tubule epithelial cells exposed to hypoxia and re-oxygenation (Denhardt *et al.*, 1995). A potential mechanism whereby OPN can prevent apoptosis has been demonstrated in endothelial cells in which OPN binding to $\alpha_v\beta_3$ increases NFκB activity through Ras and Src (Scatena *et al.*, 1998). Integrin engagement can regulate the anti-apoptotic gene Bcl-2 in COS (Zhang *et al.*, 1995) and endothelial cells in which selectivity for $\alpha_v\beta_3$ has been shown (Stromblad *et al.*, 1996), while anti-$\alpha_v\beta_3$ antibodies, specifically anti-β_3, may inhibit angiogenesis by blocking the anti-apoptotic effect (Scatena *et al.*, 1998). Notably, actin re-arrangement correlates with the ability of Ras to activate PI3-kinase which acts through Rac and Rho (Rodriguez-Viciana *et al.*, 1997), and OPN can stimulate gelsolin-associated PtdIns through PI3-kinase (Chellaiah and Hruska, 1996). Moreover, c-Src has been shown to be required for stimulating the gelsolin-associated PI3-kinase in osteoclasts (Chellaiah *et al.*, 1998).

(iii) Cell attachment and signaling through CD44

OPN is also an extracellular ligand for CD44 (Weber *et al.*, 1996), which is the main cell-surface receptor for hyaluronate. CD44 and its various isoforms have been extensively studied as a cell-surface marker for immune cells, including myeloid cells, lymphocytes, and macrophages. However, CD44 is also expressed in osteoblasts, osteocytes, osteoclasts, fibroblasts, smooth-muscle cells, and epithelial

and endothelial cells. The CD44 family of receptors mediates cellular responses similar to those of integrins, including adhesion, migration, and stimulation of both normal and transformed cells. Indeed, recent studies indicate that CD44 variants co-operate with β1-containing integrins to bind multiple domains in OPN to stimulate motility and chemotaxis (Katagiri *et al.*, 1999). Expression of CD44 and its interaction with hyaluronate occur in early development of tissues as well as in repair and regenerative processes, while its expression in migrating immune cells is linked to tumor growth and progression, although the exact role of CD44 in this process is unknown. While OPN and hyaluronan can both mediate cell attachment through CD44, the aggregation of homotypic cells is observed only for hyaluronan, whereas OPN selectively exerts chemotactic activity. Thus, the up-regulation of CD44 and OPN in immune and tumor cells may relate to the directed migration of these cells induced by OPN ligation.

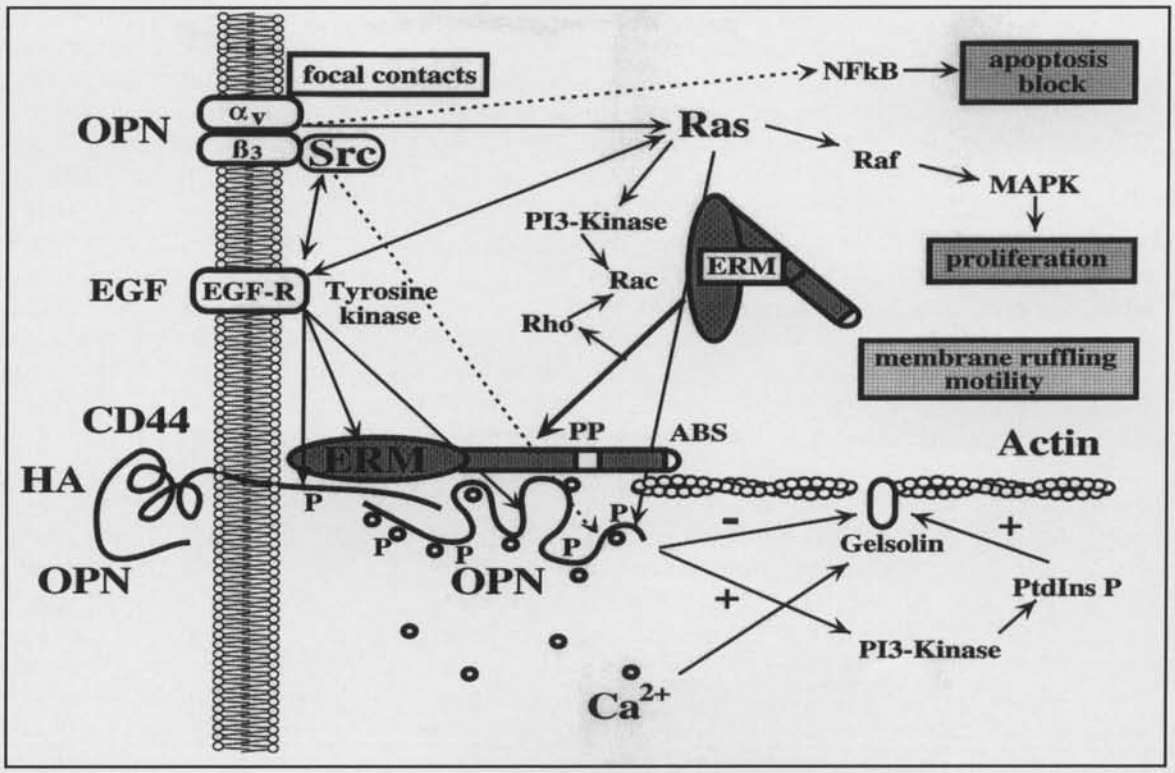


Figure 4. Association of intracellular osteopontin with the CD44-ERM complex. Although a cytosolic form of OPN, distinct from the secreted protein, is yet to be confirmed, a hypothetical interaction of intracellular OPN with components of the CD44-ERM and potential signaling pathways, involving cross-talk with focal adhesions and the epidermal growth factor receptor (EGF-R), that are involved in cell migration is shown. Evidence for this model is discussed in the text.

(iv) Intracellular OPN

Recently, immunocytochemical studies with confocal microscopy have revealed an intracellular protein with a peri-membranous distribution that is immunoreactive with several OPN antibodies and which co-localizes with CD44 and ezrin-radixin-moesin (ERM) proteins in migrating embryonic fibroblastic cells, activated macrophages, and metastatic breast cancer cells (Zohar *et al.*, 1998a; Zohar, 2000). Because of concerns regarding the specificity of OPN antibodies (Rittling and Feng, 1998), the identity of the immunoreactive protein as OPN must be confirmed. Nevertheless, there is substantial evidence to support the presence of OPN in this attachment complex. For example, expression of both OPN and CD44 is up-regulated in migratory cells, including extravasating immune cells (Weber and Cantor, 1996) and metastatic cells (Oates *et al.*, 1997; Naot *et al.*, 1997), while synthesis of OPN, CD44, and ERM proteins is increased by EGF (Zhang *et al.*, 1997), the over-activity of which is frequently linked with cell transformation. Moreover, co-expression of OPN and CD44 is seen in differentiating monocytic HL-60 cells (Atkins *et al.*, 1998), while co-immunoreaction has been reported in gastric cancer cells (Ue *et al.*, 1998). Although a possible intracellular function for OPN may appear surprising, there are similarities with calreticulin, a calcium-binding ER protein that acts as a molecular chaperone, but which also has a multiplicity of functions, including the regulation of cellular signaling through integrins (Dedhar *et al.*, 1994; Coppolino *et al.*, 1997).

A possible association of OPN with the CD44-ERM complex is depicted in Fig. 4, and the inter-relationship with OPN:$\alpha_v\beta_3$ interactions in cell signaling and motility is indicated. The formation of the hyaluronan:CD44:ERM complex is regulated by various phosphorylations mediated by serine/threonine kinases (Nakamura *et al.*, 1995) associated with Rac and Rho (Mackay *et al.*, 1997), which are downstream mediators of Ras, and by the tyrosine kinase activity of the EGF receptor. Moreover, hyaluronan binding to CD44 is dependent on the phosphorylation of the cytoplasmic tail of CD44 (Pure *et al.*, 1995). ERM and CD44 can associate in the presence of phosphoinositides

(PtInds) generated by phosphotidylinositol 3-hydroxyl kinase (PI3-kinase), which is stimulated by OPN in cell lysates (Chellaiah and Hruska, 1996). In this complex, OPN can also be phosphorylated at Ser/Thr sites and, through its Ca^{2+}-binding abilities, can modulate local intracellular Ca^{2+} levels, thereby regulating the binding of gelsolin to actin, which modulates actin-severing activity (Jamney *et al.*, 1990).

In addition to a role in linking CD44 complexes with actin filaments, ERM proteins have also been implicated in the formation of stress fibers associated with focal adhesions, both systems being regulated through Rac and Rho (Mackay *et al.*, 1997). Thus, the CD44-ERM-OPN complex may represent a novel adhesion complex that forms close contacts, as described by Abercrombie and Dunn (1975), that are transiently formed in rapidly migrating cells, consistent with the co-localization of CD44, ERM, and OPN in the leading edges of migrating cells (Zohar *et al.*, 1998a). The extracellular OPN can provide temporary (CD44) or more substantial ($\alpha_v\beta_3$) attachment complexes required for motility as well as function in the chemotaxis of the migrant cells. Moreover, the extracellular OPN can regulate the migratory processes by activating intracellular signaling pathways through these receptors. In this manner, the involvement of OPN as an attachment protein, chemoattractant, and signaling molecule in diverse biological activities involving developmental, regenerative/repair processes, immune defense, and tumor cell survival and metastasis, and its relationship to integrin and CD44 receptors, can be more readily appreciated.

(v) Potential functions

A broad range of activities involving the conserved motifs has been reported for OPN. The functionality of the polyaspartic acid sequence and the presence of phosphate groups are clearly related to the binding of OPN to mineral crystals in normal and pathological states. Additionally, the phosphate groups can also modulate the attachment activity of OPN in osteoclasts, as will be discussed. OPN appears to prevent both nucleation of mineral crystal formation and the growth of pre-existing crystals. The RGD motif, together with other sites on the OPN molecule, can mediate cell attachment, migration, and signaling. In combination with its hydroxyapatite and calcium oxalate binding properties, OPN can also mediate attachment of cells to mineral crystal structures, as originally suggested for this protein (Oldberg *et al.*, 1986). OPN expressed by activated macrophages (Patarca *et al.*, 1989, 1993) and other phagocytic cells, such as absorptive epithelial cells (Qu-Hong *et al.*, 1997), has opsonizing activity (McKee and Nanci, 1996b), which likely involves attachment to cell-surface receptors in these cells. OPN is also a chemoattractant for macrophages (Singh *et al.*, 1990; Giachelli *et al.*, 1998), as well as for smooth-muscle (Liaw *et al.*, 1995) and tumor (Senger and Peruzzi, 1996; Weber *et al.*, 1996) cells. Moreover, OPN produced by immune cells can stimulate B-cells and antibody production (Weber and Cantor, 1996; Iizuka *et al.*, 1998). Expression of OPN can suppress the cytotoxic effects of macrophages by blocking production of reactive oxygen species (Weber and Cantor, 1996). Thus, OPN can inhibit the induction of inducible nitrogen oxide synthase (iNOS) mRNA synthesis by lipopolysaccharide and interferon-gamma in macrophages (Rollo *et al.*, 1996) and kidney proximal tubule epithelial cells (Hwang *et al.*, 1994), which is also blocked by OPN in vascular tissues (Scott *et al.*, 1998). OPN has been shown to prevent apoptosis by activating NFκB in endothelial cells (Scatena *et al.*, 1998), preventing the cellular redistribution of Bcl-x, a member of the Bcl-2 family that is believed to inhibit activation of procaspases, and maintaining membrane integrity (Denhardt and Noda, 1998).

Conservation of the thrombin cleavage site and the ability of OPN to serve as a substrate for plasma transglutaminase (Factor XIIIa) also indicate functional significance and a relationship to the vascular system. Thrombin can regulate potential adhesive and migratory functions of OPN by exposing cryptic cell-binding sites and facilitating access to others, including the RGD site (Senger and Peruzzi, 1996; Senger *et al.*, 1996; Smith and Giachelli, 1998). Also, osteocalcin, which is related to coagulation factors through the vitamin-K-dependent formation of γ-carboxylic acid groups and is reported to bind to OPN (Butler *et al.*, 1996), is a competitive inhibitor of tissue transglutaminase-mediated cross-linking of OPN *in vitro* (Kaartinen *et al.*, 1997).

Despite the potential for a broad range of functions for OPN in development, bone remodeling, calcification, homeostasis, and immunoprotection, ablation of the OPN gene in two transgenic mouse lines has not revealed an obvious phenotype that can be clearly connected to any specific function (Liaw *et al.*, 1998; Rittling *et al.*, 1998). While compensatory mechanisms may mask OPN functions, embryological development is not affected in double knockouts of the OPN and vitronectin genes, indicating that vitronectin, which shares the same cell-surface receptors (most notably $\alpha_v\beta_3$), is not compensating for OPN in early development (Liaw *et al.*, 1998). However, defects in wound healing have been observed in both mouse lines. In addition, effects on osteoclast formation and bone resorption (Rittling *et al.*, 1998), as well as significant effects on tumorigenesis (Crawford *et al.*, 1998), have been observed.

By assimilating the properties of OPN outlined above and considering these in the context of the information gleaned so far from the manipulation of OPN expression

in vivo, one can formulate multi-functional roles for OPN in bone remodeling, wound healing, and tumorigenesis. Here, paradigms for the role of OPN in wound healing and tumorigenesis will be discussed, while the roles of OPN in mineralization and mineralized tissues will be discussed in more detail in Section 4 below.

(a) OPN and wound healing

Although there is no obvious impairment of wound healing in OPN-null mice, the formation of smaller, more irregularly organized collagen fibrils and the slower removal of tissue debris have been reported (Liaw *et al.*, 1998). It is conceivable that expression of OPN, which is elevated at diseased and traumatized sites (Carlson *et al.*, 1997; Nau *et al.*, 1997), attracts macrophages, the N-formyl-met-leu-phe (FMLP)-induced accumulation of which can be blocked by anti-OPN antibodies (Giachelli *et al.*, 1998). Expression of OPN by activated macrophages and lymphocytes occurs as part of the host defense mechanism, in which OPN acts as a cytokine that stimulates the cellular and humoral branches of the immune system (Weber and Cantor, 1996). That OPN has a role in resistance to bacterial infection is indicated by its relationship to Ric resistance in mice (Weber and Cantor, 1996). Moreover, secretion of OPN by phagocytic cells such as macrophages (McKee and Nanci, 1996b), osteoclasts (Dodds *et al.*, 1995), and absorptive epithelial cells (Qu-Hong *et al.*, 1997) indicates an opsonizing function in which OPN coats foreign particles and particulate debris for subsequent receptor-mediated phagocytosis. OPN secreted by macrophages and/or derived from blood also coats exposed fracture surfaces of mineralized tissues of bones and teeth and biomaterials (McKee and Nanci, 1996a,b; Lekic *et al.*, 1996) following wounding or surgical implantation. In these circumstances, OPN appears to mediate the attachment of cells as a prerequisite to tissue repair and implant integration. Intracellular OPN in macrophages and in fibroblasts populating a wound site may also be involved in the migratory activity of these cells. It is also notable that OPN and $\alpha_v\beta_3$ integrin expression in endothelial cells are up-regulated by vascular permeability factor/vascular endothelial cell growth factor (VPF/VEGF). VEGF can also influence microvascular permeability through the extrinsic coagulation pathway, which stimulates thrombin digestion of the OPN. This, in turn, can promote platelet aggregation (Bennett *et al.*, 1997) and increase the OPN-stimulated migration and survival of the endothelial cells involved in vascular repair and neo-vascularization (Senger *et al.*, 1996).

(b) OPN and tumorigenesis

Early in the development of cancerous cells, local stromal cells express OPN that acts as a signal to attract macrophages and perhaps also lymphoctyes. The expression of OPN by local cells also provides protection against the cytotoxic products of macrophages, while abnormal cells will be killed and removed. However, OPN expression is frequently induced early during the initiation and progression of cancerous growth by transforming agents. High expression of OPN is observed in high-grade metastatic malignant human gliomas (Tucker *et al.*, 1998) and in the highly invasive and metastatic human breast cancer cell lines LCC15-MB and MDA-MB-435 (Sung *et al.*, 1998). Also, transfection of OPN-negative breast cancer cells with an OPN expression vector has been shown to increase the tumorigenicity of the cells and their metastasis to bone, through a mechanism that appears to involve a promotion of angiogenesis (Yoneda *et al.*, 1998). The production of OPN can also contribute to the metastatic phenotype through autocrine and paracrine effects. Thus, autocrine effects can influence cell proliferation and survival, converting benign tumor cells into highly metastatic cells (Oates *et al.*, 1996), whereas paracrine effects can provide protection from cytotoxic macrophages, possibly by inhibiting the production of nitric oxide (Denhardt and Chambers, 1994). Collectively, these mechanisms could explain how tumor cells not expressing OPN are eliminated, while providing a mechanism of tumor cell escape and selection of OPN-producing clones in secondary metastases (Crawford *et al.*, 1998). In this regard, higher median levels of plasma OPN in metastatic breast cancer have been associated with increased tumor burden and decreased survival (Singhal *et al.*, 1997).

(4) *Function of* OPN *in Bones and Teeth*

(i) Distribution of OPN in the Cellular Matrix of Bones and Teeth

(a) The interfacial distribution of OPN in bone

Since its original identification in bone by biochemical means (Franzén and Heinegård, 1985; Fisher *et al.*, 1987; Prince *et al.*, 1987), OPN in hard tissues has been consistently localized to specific regions of the extracellular matrix (ECM) in bones and teeth by immunohistochemical and immunocytochemical methods (reviewed in McKee and Nanci, 1993). Importantly, whereas most non-collagenous proteins are more or less homogeneously dispersed throughout bone, ultrastructural immunocytochemical studies have consistently highlighted the unique, interfacial distribution of OPN relative to other ECM proteins found in bone–the most prominent accumulation being at cement lines in remodeling bone, and at laminae limitantes at bone surfaces (McKee and Nanci, 1996b,c). These two planar accumulations of non-collagenous protein define matrix-matrix and cell-matrix boundaries, respectively, and therefore represent important locations in the formative history of the bone.

Cement lines (or layers/planes) indicate sites where bone formation has been quiescent (resting line) or where a major reversal event in cell activity has occurred

between the formative and resorptive phases of this tissue (reversal line). Initially, at reversal sites, osteoclastic activity results in the enzymatic degradation of the organic bone matrix, together with the dissolution of the inorganic phase by cell-mediated acidification of the mineral-containing extracellular space (Baron *et al.*, 1993). In this manner, bone is asynchronously lost at different anatomical sites by an excavation process that results in resorption cavities that are continuously refilled with new bone such that overall bone mass is maintained (Parfitt, 1994). Cement layers demarcate the boundary between the older bone and newer bone, and characteristically contain a high content of OPN. The cellular origin of the OPN in the cement layer has been controversial (Dodds *et al.*, 1996; McKee and Nanci, 1996d), yet it is likely that both osteoclasts and osteoblasts contribute (albeit unequally) to the formation of this interfacial structure. Whereas OPN production by osteoclasts is well-documented (Tezuka *et al.*, 1992; Merry *et al.*, 1993; Dodds *et al.*, 1995), the majority of which likely binds in an autocrine/paracrine fashion to integrin receptors $\alpha_v\beta_3$ and/or CD44 on the basolateral aspect of the osteoclast (Horton *et al.*, 1995; Helfrich *et al.*, 1996; Nakamura and Ozawa, 1996; Duong and Rodan, 1999), vectorial secretion of OPN and its assembly into the cement layer have been convincingly demonstrated for cells of the osteoblastic lineage (McKee and Nanci, 1996a). In the latter case, relatively undifferentiated, early-stage osteoblastic cells migrate to the resorbed bone surface and commence matrix secretion and assembly as a cement layer on the exposed bone surface. The cement layer is deficient in collagen fibrils but rich in OPN, and sometimes contains bone sialoprotein. Initially, OPN in the cement layer may be utilized for osteoblast cell adhesion, or to guide early calcification events at this junction. Here, OPN immobilized onto pre-existing mineral and/or organic moieties of the underlying bone may act to initiate a new cycle of mineralization at this site. Studies are ongoing to determine the macromolecular interactions spanning this critical interfacial region that allow for calcification, cell attachment, and adhesion of the nascent bone matrix to its underlying, pre-existing substratum. Following cement layer deposition, bone matrix proper accumulates in the resorption lacunae from the net secretory activity of cohorts of fully differentiated osteoblasts. Notably, reversal lines (cement layers) are prominent matrix structures in regions of high bone remodeling, such as observed in the alveolar bone of the periodontium.

(b) OPN as an interfibrillar protein in the collagenous matrix of bone

OPN is particularly prevalent within the interfibrillar (with respect to type I collagen fibrils) compartment of the extracellular matrix of woven (non-lamellar) bone. OPN first appears at small calcification foci within the generally unmineralized osteoid seam, with high levels being observed at the mineralization front and extending into the mineralized bone matrix proper (McKee and Nanci, 1995b). By way of analogy, it has been proposed that OPN incorporated into bone beyond the mineralization front, together with other non-collagenous proteins, acts as the "mortar between the collagen bricks", imparting molecular cohesion to the matrix as a whole, while simultaneously guiding mineralization events within this matrix compartment. With regard to its cohesive/adhesive properties, OPN serves as a substrate for transglutaminase (Prince *et al.*, 1991; Beninati *et al.*, 1994; Sorensen *et al.*, 1994), whose covalent cross-linking activity provides for homotypic OPN-OPN binding and heterotypic binding to collagen (Kaartinen *et al.*, 1999). Additionally, the mineral-binding domains of OPN may be functional in linking matrix to mineral, and in precisely guiding and regulating hydroxyapatite crystal growth within the interfibrillar spaces of the bone matrix. Importantly, the physical properties of the bone matrix are in large measure defined by the orientation of mineral both within and between the collagen fibrils. Where the collagen fibrils of lamellar bone are more highly ordered and closely packed, non-collagenous protein content is diminished, and OPN likely plays a more subtle role in defining the biomechanical properties of the bone. In addition to mineral regulation and cohesive functions, the more diffuse distribution of OPN throughout the bone matrix may be of some importance in influencing osteoclast activity during resorption into woven and lamellar bone.

(c) OPN as a bone-surface protein: cell-matrix relationships *in vivo*

In addition to its accumulation in the cement layer, OPN is a prominent component of the laminae limitantes that line both internal and external bone surfaces (McKee and Nanci, 1996c). These surfaces are critical in that they define the interface between bone cells and matrix/mineral, and thus are strategically positioned to influence cell attachment and signaling. The OPN-rich lamina limitans is a thin organic structure that is readily apparent only by electron microscopy after decalcification of the bone, and which exists at the very margins of calcified bone interfacing with osteocytes, with bone-lining cells, and, where remodeling occurs, with osteoclasts. Such an important location implies a role in terminating mineralization at the bone surface, and where mineral encroaches upon osteocytes in their lacunae, or cell processes in their canaliculi. Concomitant with its binding mineral, OPN at these sites would be available for ligation with the cell-surface receptors of bone cells such as integrins and/or CD44 (Nakamura and Ozawa, 1996; Weber *et al.*, 1996). For osteocytes and their cell

processes within the lacunar and canalicular system emanating throughout bone, such receptor occupancy might provide a means by which these strategically located and most numerous cells detect and respond to bone tissue strain, thereby acting as mechano-sensors capable of initiating signaling cascades to the bone surface and activating the bone remodeling cycle (McKee and Nanci, 1995a, 1996a). Perhaps most importantly, surface-located OPN subjacent to bone-lining cells would be exposed upon retraction or death of these cells, thereby being available for the initial events of osteoclast attachment, migration, and resorption at the bone surface.

(d) OPN in teeth

OPN present in tooth extracellular matrices resides principally within the cementum that lines root surfaces and which serves as the tooth-related attachment point for periodontal ligament fibers (McKee *et al.*, 1996; D'Errico *et al.*, 1997; Bosshardt *et al.*, 1998). Both acellular and cellular cementum types contain abundant OPN at levels which are substantially higher than that in bone, particularly for the acellular cementum variety. OPN accumulation in cementum typically occurs between collagen fibrils and is thought to guide and stabilize the interfibrillar mineralization pattern within this tissue. An additional accumulation of OPN is frequently observed at the dentino-cementum junction, a site where non-collagenous cementum proteins accumulate in a variable pattern according to the surface topography and packing of the underlying mantle dentin collagen fibrils (McKee *et al.*, 1996; Bosshardt *et al.*, 1998). At the dentino-cementum junction, cementoblasts secrete OPN and coordinate the integration of cementum collagen fibrils with those of dentin in a manner that may be likened to the extracellular matrix assembly events occurring at cement lines in bone. While a role for OPN in cell adhesion is less obvious at the tooth root surface, its ability to act in this manner has been suggested (Somerman *et al.*, 1990).

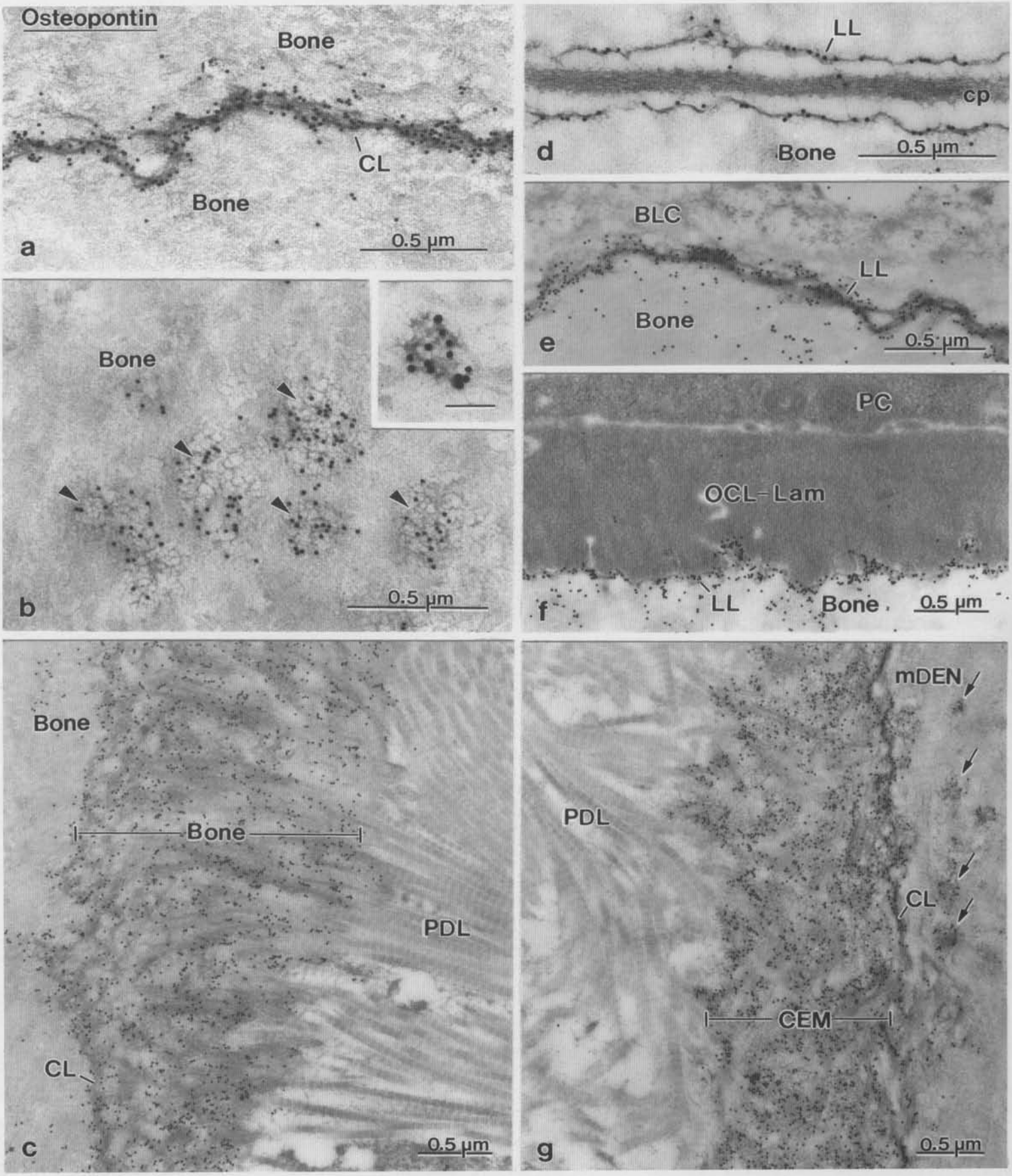
In dentin, the large majority of OPN appears to be associated with the initial sites of calcification that occur within the so-called mantle dentin of the developing root (McKee *et al.*, 1996). Additional, moderate amounts of OPN appear diffusely distributed throughout the thin layer of mantle dentin, with very little being present in the peritubular dentin itself. This OPN may originate cellularly from both the odontoblasts and the cementoblasts. The implication of this distribution is that while OPN may regulate early calcification events in root dentin, its role in the ongoing mineralization process in the bulk of this tissue is less obvious, and other matrix constituents in dentin may function in a similar manner

(ii) Temporal expression of OPN in bone formation

Studies on the temporal expression of OPN during the formation of bone *in vitro* and during the formation of intramembranous and endochondral bone *in vivo* have revealed a biphasic pattern in which OPN is produced early in the differentiation of bone cells, with higher levels expressed after mineralization has been initiated (McKee and Nanci, 1995b; Sodek *et al.*, 1995). The high levels of expression continue in association with the remodeling of bone. Studies of OPN expression in populations of fetal rat calvarial cells have revealed a high proportion of cells expressing OPN through the various stages of differentiation (Zohar *et al.*, 1997a,b, 1998a,b), with different, post-translationally-modified forms of OPN being expressed by undifferentiated and differentiated osteogenic cells (Kubota *et al.*, 1989; Nagata *et al.*, 1991). In addition to OPN-secreting cells, proliferating osteogenic cells express intracellular OPN associated with cell migration (Zohar *et al.*, 2000). Although the nature of the intracellular OPN has not been characterized, OPN secreted at this stage of osteogenic differentiation is less phosphorylated than the protein secreted by osteoblasts; it also migrates more slowly on SDS-PAGE and may have a lower affinity for hydroxyapatite. Compared with embryonic calvariae-derived cells, marrow-derived stromal cells stimulated to differentiate along the osteogenic pathway produce much higher levels of OPN during the early proliferative stage. The high expression of OPN by the marrow cells is associated with the formation of a collagen-free cement layer *in vitro* (Davies, 1996) upon which bone is subsequently deposited, following the differentiation of osteoblasts (Sodek *et al.*, 1995). The cement layer formed *in vitro* is thought to correspond to the cement (reversal) line structure formed during bone growth and remodeling (Zhou *et al.*, 1994; McKee and Nanci, 1995a, 1996a), and on implant surfaces prior to the formation of bone (Nanci *et al.*, 1994). Since this OPN co-migrates with highly phosphorylated OPN produced by osteoblasts and is associated with mineral deposits, it is clearly different from the OPN produced by undifferentiated calvarial cells. Following the formation of bone and the initiation of mineral deposition, production of the highly phosphorylated OPN is increased with osteoblast maturation. When bone formation is completed, OPN forms a surface coating, the lamina limitans, residing between the mineral at the bone surface and the quiescent bone-lining cells. The temporo-spatial expression of OPN and its different forms during the formation of bone is depicted in Fig. 5.

(iii) Role of OPN in the formation of mineralized tissues

Expression of intracellular OPN, together with CD44, in proliferating osteogenic cells appears to be related to the



migratory properties of these cells. In comparison, the low-phosphorylated form of extracellular OPN could conceivably have regulatory functions involving integrin ligation, as depicted in Fig. 3. Also, OPN expressed during cement layer formation is likely to regulate hydroxyapatite crystal growth, which would be consistent with the demonstrated ability of phosphorylated OPN to be a potent regulator of hydroxyapatite crystal growth (Hunter *et al.*, 1994, 1996; Wada *et al.*, 1999). However, in the absence of collagen and BSP in the cement layer, OPN could conceivably act as a nucleator, especially if it were immobilized on the substratum or the cell surface. Similarly, the highly phosphorylated OPN expressed following the initiation of mineralization is thought to control the sizes and shapes of mineral crystals. Whether it is the same OPN that forms the lamina limitans layer is unknown. However, the ability of OPN, which is prominent in the lamina limitans, to mediate cell attachment is well-established. Although the expression of OPN is clearly not essential for cement layer formation and tissue integrity, as evident in *Opn-/-* mice, its absence in the lamina limitans structure may contribute to impaired osteoclast activity.

(iv) OPN IN BONE RESORPTION

Remodeling of bone, which occurs throughout life, requires the maintenance of a careful balance between formation and resorption. Although the coupling of osteoclast and osteoblast activities in the homeostasis of bone mass has been questioned by recent studies in which selective ablation of osteoblasts failed to affect bone mass significantly (Corral *et al.*, 1998), an influence of osteogenic cells on the formation and function of osteoclasts is, nevertheless, a likely scenario. Moreover, OPN appears to be an important component of the communication between these cells, and there is strong evidence for the involvement of OPN in the formation, migration, and attachment of osteoclasts and for their resorptive activity. The perceived role of OPN in osteoclast formation and function is supported by the increased capacity of spleen cells from *Opn-/-* mice to generate osteoclasts (Rittling *et al.*, 1998). This may reflect compensation for a deficiency in osteoclastic resorption (Noda *et al.*, 1998) associated with increased osteoprotegerin ligand (TRANCE/RANKL/ODF) and its receptor in the bones of *Opn-/-* mice (Yoshitake *et al.*, 1998). Impaired bone resorption has also been observed in *Opn-/-* mice treated with IL-1, an inflammatory cytokine which, like TNF-α, strongly stimulates OPN expression and bone resorption in normal animals (Takazawa *et al.*, 1998). Similarly, the enhanced bone resorption due to the effects of estrogen deficiency on osteoclastic activity in ovariectomized mice, which is also mediated by inflammatory cytokines, is not evident in the *Opn-/-* mice (Yoshitake *et al.*, 1999). Thus, while it is clear that OPN is not essential for normal bone resorption, it appears, nevertheless, to be required for stimulating osteoclast generation, as illustrated in Fig. 6.

(a) Attraction, migration, and maturation of osteoclast precursors

Although OPN is not essential for osteoclast activity, impaired recruitment of osteoclasts to ectopically implanted bone from *Opn-/-* mice (Noda *et al.*, 1998) is indicative of the requirement of OPN expression at targeted sites of bone resorption to attract osteoclast precursors. This can occur through a combination of a chemotactic response involving the CD44 receptor (Weber *et al.*, 1996) and by haptotaxis through the integrin $\alpha_v\beta_3$ (Senger *et al.*, 1996), both receptors being expressed at high levels in osteoclasts. In contrast to the $\alpha_v\beta_3$ receptor, the CD44 receptor does not appear to be involved in cell attachment (Katayama *et al.*, 1998) but may be more important in the migratory response, in which OPN can function as an extracellular ligand, and also possibly inside migrating cells, as discussed earlier. High levels of OPN are expressed by differentiated osteoblasts, including bone-lining cells and osteocytes, as well as by osteoclasts themselves, and, in both osteogenic and osteoclastic cell lineages, OPN expression is strongly stimulated by vitamin D3, which promotes osteoclast differentiation

Figure 5. Post-embedding, colloidal-gold immunocytochemistry for OPN in bone and teeth. Electron micrographs illustrate the most prominent sites of OPN accumulation in the calcified extracellular matrices of mineralized tissues where OPN predominantly resides at various cell- and matrix-matrix interfaces, and in the bulk of the matrix. (a-d) Within the bone, OPN accumulates primarily in cement (reversal) lines (CL) and in patches (arrowheads) of non-collagenous proteins found among the collagen fibrils and in the osteoid at small calcification foci (inset in b). In the alveolar bone of the periodontium, a frequent observation is the presence of a seam of bone rich in OPN (bracket in c) at sites of periodontal ligament (PDL) insertion. Osteocytes and their cell processes (cp) are found within lacunae and canaliculi, respectively, both of which are lined by a thin, OPN-containing coating termed the lamina limitans (LL). (e-f) At external bone surfaces, bone-lining cells (BLC) and osteoclasts (OCL) are typically observed in direct apposition to the OPN-rich lamina limitans (LL). More specifically, in the case of osteoclasts, well-developed lamellipodia (OCL-Lam) extend beneath periosteal cells (PC) to follow the contour of the lamina limitans at the bone surface. (g) In the tooth, immunolabeling for OPN is particularly abundant in cementum (CEM, bracket) that lines the root surface and serves as the tooth attachment site for the periodontal ligament (PDL). At certain locations along the root, an OPN-containing cement line (CL), but not a reversal line in this case, is present at the dentino-cementum junction. The mantle dentin (mDEN) of the root, immediately adjacent to the cementum, also exhibits small patches (arrows) of non-collagenous matrix containing OPN that represent the initial sites of calcification that occur along the advancing root edge during tooth development. All electron micrographs are taken from thin sections of aldehyde-fixed, decalcified rat bone embedded in LR White acrylic resin, immunolabeled with OPN antibodies and colloidal-gold conjugates by means of the post-embedding approach, and conventionally stained with uranyl acetate and lead citrate.

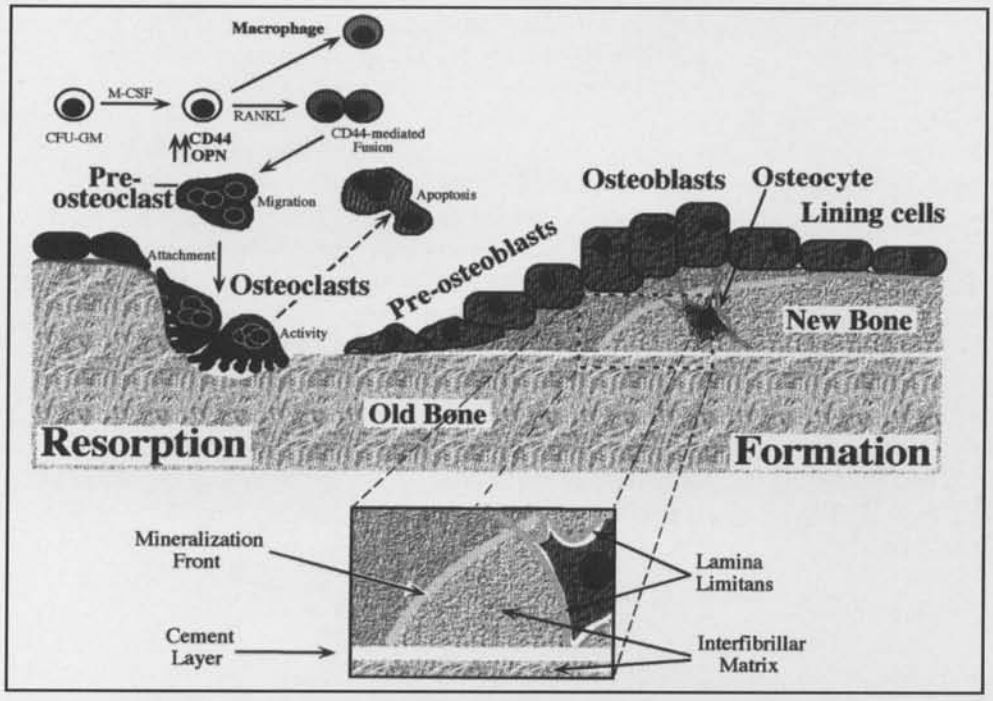


Figure 6. Temporal expression of osteopontin during bone remodeling. The resorption of bone by osteoclasts, and the subsequent replacement by new bone, is shown in this Fig. Osteoclasts are formed from pluripotential mononuclear precursors (CFU-GM) stimulated by monocyte-macrophage colony-stimulating factor (M-CSF). Expression of OPN, which is associated with the early development of osteoclasts, is up-regulated with CD44 in differentiating osteoclast/macrophages and can regulate osteoclast formation, activity, and survival (see Fig. 7). Osteogenic precursors express intracellular OPN, associated with cell motility, and secrete a low-phosphorylated OPN which may act as a cytokine in osteodifferentiation. Osteogenic cells express OPN that associates with the non-collagenous cement layer, while osteoblasts produce a highly phosphorylated OPN which regulates hydroxyapatite crystal growth and which is enriched at the mineralization front and becomes incorporated into the interfibrillar matrix. OPN is also deposited as a lamina limitans layer between the mineralized bone surface and both osteocytes and bone-lining cells. The major sites of OPN deposition in bone, including the mineralization front, the cement layer, the interfibrillar matrix, and the lamina limitans, are shown in the enlarged section.

and bone resorption. The expression of OPN is associated with the early development of osteoclasts (Yamate *et al.*, 1997) and macrophages (Krause *et al.*, 1996), and together with CD44, OPN synthesis is increased in promyelocytic leukemia HL-60 cells stimulated by PMA to differentiate along the monocyte/macrophage pathway (Atkins *et al.*, 1998). Notably, macrophages and osteoclast precursors demonstrate strong similarities in their chemoattractive responses to OPN and the expression of OPN. Moreover, the expression of OPN can regulate the activity of the macrophages/osteoclasts at different developmental stages as well as provide a substratum for osteoclast attachment and opsonin activity in phagocytic mechanisms. In the subsequent fusion of pre-osteoclasts to form multi-nucleated osteoclasts, the non-ligated CD44 receptor may have an important role (Sterling *et al.*, 1998).

(b) Regulation of osteoclastic activity

Since an OPN-deficient bone matrix is poorly resorbed and has a diminished capacity to support cell attachment to bone (Noda *et al.*, 1998), OPN in the lamina limitans appears to be important for attachment of osteoclasts through the $\alpha_v\beta_3$ receptor *in vivo*. Moreover, phosphorylation of OPN has been shown to be important in mediating osteoclast, but not osteoblast, cell attachment through $\alpha_v\beta_3$ (Ek-Rylander *et al.*, 1994; Katayama *et al.*, 1998), and it has been suggested (Dodds *et al.*, 1995) that the tartrate-resistant acid phosphatase (TRAP) characteristically expressed by osteoclasts may control osteoclast attachment and motility by dephosphorylating OPN. Regulation of osteoclast activity by OPN appears to occur primarily through the highly expressed $\alpha_v\beta_3$, which mediates intracellular signaling pathways (Yamate *et al.*, 1997; Duong and Rodan, 1999), shown in Fig. 7. Moreover, an autocrine pathway is implicated by the inhibition of osteoclast activity by OPN anti-sense deoxyoligonucleotides (Tani-Ishii *et al.*, 1997). The activation of the $\alpha_v\beta_3$ receptor is Ca^{2+}-dependent and is thought to provide a regulation point for osteoclasts during bone resorption (Faccio *et al.*, 1998). Thus, OPN can modulate intracellular Ca^{2+} in osteoclasts through ligation to $\alpha_v\beta_3$, which stimulates release of Ca^{2+} from intracellular compartments (Zimolo *et al.*, 1994) *via* a calmodulin-dependent, Ca^{2+}-ATPase (Miyauchi *et al.*, 1991). Ligation through the β subunit of $\alpha_v\beta_3$ also activates the FAK-related PYK2 (and p130cas) through c-Src, which binds PYK2 through the SH2 domains. Upon activation, PYK2 translocates to the Triton X-100 insoluble cytoskeletal compartment and, together with p130cas, is found in the sealing zone required for osteoclastic bone resorption (Duong *et al.*, 1998: Duong and Rodan, 1999). In addition, OPN can potentially stimulate other signaling pathways in osteoclasts. For example, activation of NFκB by OPN, which mediates anti-apoptotic effects of OPN in endothelial cells (Scatena *et al.*, 1998), may also contribute to osteoclast survival as well as providing a link with the osteoprotegerin regulation of osteoclast formation (Kong *et al.*, 1999) and the effects on pro-inflammatory cytokines (Franzoso *et al.*, 1997). Moreover, OPGL has recently been shown to activate osteoclasts directly (Burgess *et al.*, 1999). In addition, OPN can stimulate matrix

metalloproteinase (MMP) production through the ERK pathway, which stimulates Fos expression through an SRE, as well as the activation of MMP-2, which has been shown to be mediated by the $\alpha_v\beta_3$ integrin in GTC23 giant tumor cells (Teti *et al.*, 1998). These pathways are also relevant to phagocytosis by macrophages.

(5) *Summary*

The functional diversity of OPN in bone formation and remodeling appears to relate to fundamental roles of this protein in host defenses and tissue repair, the bone remodeling sequence having many features of repair processes involving inflammatory responses. Thus, a primary role of OPN appears to be that of facilitating recovery of the organism after injury or infection, which generally causes an increase in its expression. OPN stimulates cellular signaling pathways *via* various receptors found on most cell types, including the cells of mineralized tissues. It can regulate cell proliferation and phagocytic activity, and can both promote and participate in cell migration. Moreover, its apparent ability to enhance cell survival by inhibiting apoptosis may explain why the metastatic proficiency of tumor cells increases with increased OPN expression. Modulation of immune and inflammatory responses by OPN is reflected in the formation and activity of macrophages and also osteoclasts. As an extracellular matrix component, it can regulate mineral crystal formation and growth and can form supramolecular structures by covalent interactions with other matrix macromolecules. However, more detailed studies are needed to determine precisely how OPN is able to perform these diverse functions.

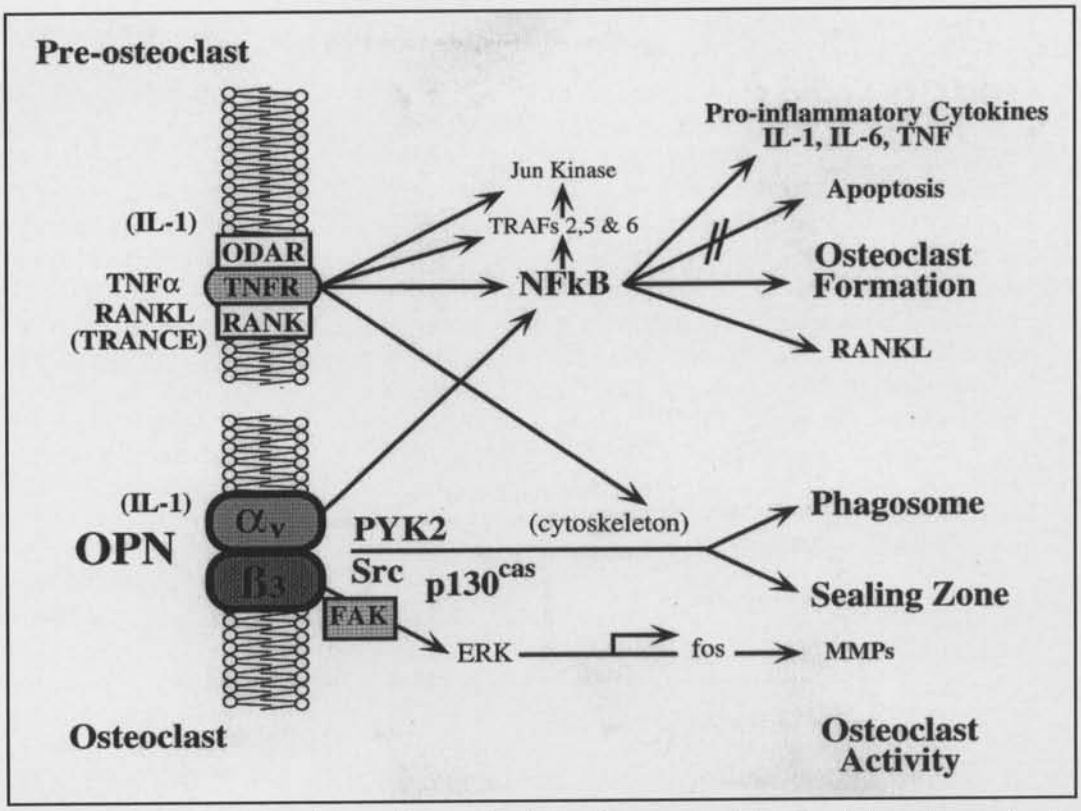


Figure 7. Osteopontin regulation of osteoclast formation and activity. Possible pathways for the regulation of osteoclast formation and activity by OPN, as a matrix protein or as a cytokine, are shown in this diagram. Together with CD44, the $\alpha_v\beta_3$ integrin is highly expressed in osteoclasts and osteoclast precursors, both receptors being a primary target for OPN in signaling, cell attachment, and also possibly for osteoclast chemotaxis (haptotaxis) and migration. Ligation of OPN to pre-osteoclasts could modulate osteoclast development through the OPG/OPGL/RANKL regulatory pathway, which is also influenced by inflammatory cytokines, including TNF-a and IL-1. Ligation of OPN to osteoclasts through the ß subunit of the $\alpha_v\beta_3$ integrin can activate the FAK-related PYK2 (protein tyrosine kinase 2) through c-Src, which binds PYK2 through the SH2 domains. Upon activation, PYK2 translocates to the Triton X-100 insoluble cytoskeletal compartment and, together with $p130^{cas}$, is found in the sealing zone required for osteoclastic bone resorption. Abbreviations: OPG, Osteoprotegerin (decoy receptor for RANK); also known as OCIF, Osteoclast Inhibitory Factor: RANK, Receptor Activator of NFκB, also known as ODAR, Osteoclast Differentiation and Activation Receptor, and as TNFR, Tumor Necrosis Factor Receptor: RANKL, Receptor Activator of NFκB Ligand, which is identical to TRANCE, Tumor Necrosis Factor (TNFα)-Related Activation-induced Cytokine; and ODF, Osteoclast Differentiation Factor; and TRAIL, TNF-Related Apoptosis-Inducing Ligand: TRAF, TNFα-Receptor-Associated Factor.

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