OSTEOPONTIN AND CELL ADHESION: ROLE OF POST-TRANSLATIONAL

MODIFICATIONS AND THE C-TERMINAL REGION

by

CHRISTIAN CHARLES KAZANECKI

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and approved by

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ABSTRACT OF THE DISSERTATION

Osteopontin and Cell Adhesion: Role of Post-Translational Modifications and the

C-Terminal Region

By CHRISTIAN CHARLES KAZANECKI

Dissertation Director:
David T. Denhardt

Osteopontin (OPN) is an integrin-binding secreted phosphoglycoprotein found in many tissues and body fluids, where it acts as both a soluble cytokine and a component of mineralized matrices. It exhibits a broad range of functional activities in physiological and pathological processes, many of which are influenced by post-translational modifications. OPN is highly modified by phosphorylation and glycosylation that can vary depending on the source. This study identified two different forms of OPN produced by murine *ras*-transformed fibroblasts (FbOPN) and differentiating osteoblasts (ObOPN) based on the reactivity of a panel of anti-OPN monoclonal antibodies. By enzymatic dephosphorylation, mass, and sequence analyses, it was shown that FbOPN contained approximately four phosphate groups distributed over 17 potential phosphorylation sites, whereas ObOPN contained approximately 21 phosphate groups distributed over 27 sites. This is the first extensive characterization of OPN produced by transformed cells, and the FbOPN form is the least modified form of OPN that has been characterized to date.

The forms of OPN also differed in their ability to support adhesion of human MDA-MB-435 tumor cells and mouse 275-3-2 *ras*-transformed fibroblasts. The MDA-MB-435 cells bound with approximately 60% greater efficiency to the FbOPN than ObOPN, whereas the 275-3-2 cells bound the ObOPN with 35% greater efficiency than FbOPN. Inhibition of adhesion with an Arg-Gly-Asp (RGD)-containing peptide inhibited the binding of both lines to the OPN forms, suggesting that different integrins preferentially bind to OPN based upon its phosphorylation state.

This study has also identified a potential role for a novel region of OPN in cell adhesion. Two anti-osteopontin monoclonal antibodies, both of which recognize the C-terminal region of OPN, were shown to inhibit MDA-MB-435 cell adhesion to recombinant human OPN. Synthetic peptides corresponding to this region were bound by cells using a flow cytometry assay, suggesting a receptor interaction in this region of OPN. This interaction is hypothesized to be a signaling interaction because cells were not able to adhere to the synthetic C-terminal peptides and adhesion in the presence of these soluble peptides was not affected.

ACKNOWLEDGEMENTS

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ABBREVIATIONS USED

ALP - Alkaline phosphatase

BSA - Bovine serum albumin

CaOx - Calcium oxalate

CD44s - Standard version of the CD44 receptor

CD44v - Variant of the CD44 receptor containing additional variable exons

EAE - Experimental autoimmune encephalomyelitis

FbOPN - Osteopontin produced by *ras*-transformed fibroblasts

FN - Fibronectin

HA - Hydroxyapatite

IL-10, IL-12 - Interleukin-10, Interleukin-12

iOPN - Intracellular OPN

MALDI-TOF - Matrix-assisted laser desorption ionization time-of flight

MC3T3 - MC3T3-E1 subclone 4 pre-osteoblastic cells

ObOPN - Osteopontin produced by differentiating MC3T3 osteoblasts

OPN - Osteopontin

OPN-KO - Osteopontin knock-out

PTMs - Post-translational modifications

PVDF - Polyvinylidene fluoride

SDS-PAGE - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SIBLING - Small integrin-binding ligand, N-linked glycosylation

TPA - 12-O-tetradecanoyl-phorbol-13-acetate

VitD3 - $1\alpha,25(OH)_2$-vitamin D_3

Chapter 1

Introduction

Osteopontin (OPN) is a secreted phosphorylated glycoprotein expressed by many tissues and cell types and is found in all body fluids. It has a high degree of post-translational modification, which is very heterogeneous and can vary according to the cell source. OPN can exist as a soluble cytokine or a mineralized matrix-associated molecule. OPN's involvement in many systems led to the discovery of the protein by numerous laboratories, and multiple names for the gene/protein. OPN was initially described by Senger et al (1979) as a transformation associated phosphoprotein and later named secreted phosphoprotein 1 (SPP-1) (Craig et al., 1989). The protein was also described as early T-lymphocyte activation gene 1 (Eta-1) (Patarca et al., 1989) and 2ar (Smith and Denhardt, 1987). The name osteopontin, meaning 'bone bridge' was proposed by Oldberg et al. (Oldberg et al., 1986), who isolated the protein as a bone sialoprotein from a rat osteosarcoma cell line that stimulated cell adhesion via an arginine-glycine-aspartate (RGD) sequence located in the protein.

1.1 Osteopontin Gene Structure

The OPN gene has been mapped to mouse chromosome 5 (Fet et al., 1989; Patarca et al., 1989) and human chromosome 4q13 (Young et al., 1990) where it exists as a single copy gene congruent with the previously identified *Ric* locus associated with resistance to infection by *Rickettsia tsutsugamushi* (Patarca et al., 1989). The OPN gene consists of 7 exons, of which exon 1 is non-coding (Craig and Denhardt, 1991; Hijiya et al., 1994). Similarities in gene location and exon structure, but not amino acid sequence,

have led to the proposal that OPN is a member of a family of proteins termed the small

integrin binding ligand, N-linked glycosylation (SIBLING) family (Fisher et al., 2001;

Fisher and Fedarko, 2003). Other members of this family include bone sialoprotein

(BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix

extracellular phosphoglycoprotein (MEPE).

1.2 Osteopontin Gene Expression

The promoters for human, pig, rat, mouse and chicken OPN have been sequenced

and show conservation in the proximal promoter region (~250bp). Many transcription

factor binding sites exist, including a TATA box, an inverted CCAAT box, a GC box,

vitamin D3 responsive elements, and AP-1, Ets family, GATA-1 and PEA-3 binding

motifs (reviewed in Sodek et al., 2000).

Alternate splice forms, different alleles, and polymorphisms of OPN have been

reported, however the significance of these changes with regard to OPN's function have

largely not been elucidated. Young et al. (1990) described an OPN allele that differed by

42bp (14 amino acids) from the previously reported human OPN sequences (Kiefer et al.,

1989). They demonstrated that both alleles were expressed in bone and decidua cells,

although the allele containing the extra 14 amino acids was significantly more abundant

(Young et al., 1990). Evidence for cell-type specific processing of the OPN transcript

was also described by Singh et al. (1992) in which a 52bp insert was present in the 5'

non-coding region of OPN cDNA from normal rat kidney cells, but not from rat

osteosarcoma cells. The significance of this inserted non-coding sequence is unknown.

Recently, multiple splice variants of OPN expressed in human breast cancer cells have

been described. The shortest splice variant, in which exon 4 has been spliced out, was

demonstrated to enhance colony formation in soft agar and the expression of oxidoreductases, and was hypothesized to be a contributor to invasiveness (He et al., 2006).

1.2.1 Tissue Distribution

OPN is expressed in a great number of tissues and cell types including bone, dentin, mammary gland, kidney, brain, smooth muscle cells, ganglia of the inner ear, macrophages and lymphocytes (Sodek et al., 2000). In addition, OPN is secreted into many body fluids by epithelial cells, including bile, blood, urine, milk and seminal fluid. OPN is also upregulated and expressed in a great number of cancers, and the corresponding increase in serum concentration has been correlated with poor prognosis in multiple studies. Similarly, OPN expression is upregulated in numerous pathological situations and in response to injury, most likely due to its increased expression by activated macrophages and T-lymphocytes.

1.2.2 Regulation of Expression

Due to the wide distribution of expression of OPN, it is not surprising that OPN expression is regulated by a large number of cytokines and growth factors. The effects of numerous drug and cytokine treatments have been catalogued in Denhardt and Noda (1998). Only significant ones with respect to the work being presented will be discussed in this text. OPN was initially discovered as a transformation-associated protein, and early studies demonstrated that the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) increased OPN mRNA and protein levels (Smith and Denhardt, 1987, 1989). Similarly, expression of oncogenes H-*ras* and *src* were demonstrated to increase

the expression of OPN, and in the case of *ras* at a level proportional to the level of *ras*

expression (Chambers et al., 1992; Chackalaparampil et al., 1996; Tezuka et al., 1996).

Another important molecule, especially in the bone compartment, that regulates

OPN message and protein levels is $1\alpha,25(OH)_2$-vitamin D_3 (vitD$_3$). Treatment of rat

osteosarcoma cells with vitD$_3$ resulted in the increased synthesis of *phosphorylated* OPN,

whereas treatment of mouse epidermal JB6 cells with this molecule increases the

expression of *non*-phosphorylated OPN (Prince and Butler, 1987; Chang and Prince,

1991). These studies are the earliest to show changes in the post-translational state of

OPN in response to cytokine treatment. Interestingly, the induction of OPN by vitD$_3$ in

the JB6 mouse epidermal cells was not associated with transformation as observed for

TPA treatment. However, concurrent treatment with both molecules resulted in an

additive effect on OPN mRNA and phosphorylated protein levels and enhanced TPA-

induced anchorage independent growth (Chang and Prince, 1993). These results suggest

the phosphorylation state of OPN may affect the ability to support transformation and

anchorage-independent growth. Similarly, Safran et al. (1998) showed that vitD$_3$

treatment of rat osteosarcoma cells (ROS17/2.8) induced a shift in the isoelectric point

(pI) of OPN from 4.6 to 5.1 in addition to upregulating transcription.

1.3 Osteopontin Protein Sequence

The amino acid sequence of OPN is highly conserved among many species

(figure 1) including mouse, rat, human, rabbit, and pig (Oldberg et al., 1986; Craig et al.,

1989; Kiefer et al., 1989; Wrana et al., 1989; Kerr et al., 1991; Tezuka et al., 1992).

Figure 1: Alignment of mammalian OPN amino acid sequences. Amino acid sequences of bovine, pig, rabbit, human, mouse, and rat OPN were aligned using the ClustalW program (Chenna et al., 2003). The alignment was then viewed with the Jalview alignment viewer (Clamp et al., 2004) to generate the figure. The conservation bar graph displays the conservation of physico-chemical properties, whereas the consensus annotation is the percentage of the modal residue per column. Both annotations are shown because the conservation calculation is more stringent and sensitive to deletions and single amino acid changes within a column. The more divergent chicken OPN sequence was removed in order to better display areas of high conservation in the mammalian species.

The calculated molecular weight of murine OPN (peptide sequence only) is approximately 32 kDa, however many labs have reported that when analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), OPN's apparent molecular weight can range from 45-75 kDa. This is most likely due to the high and variable degree of post-translational modifications of the protein, the highly negative overall charge (pI is less than 5), as well as the gel composition used for SDS-PAGE. A more accurate molecular weight determination of rat bone OPN using sedimentation equilibrium resulted in a mass of 44 kDa (Prince et al., 1987). A more recent determination using matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectroscopy resulted in a mass of 37.6 kDa for the same OPN. (Keykhosravani et al., 2005).

1.3.1 Conserved Functional Domains

The majority of identified functional domains of OPN are well conserved among species. The central integrin attachment motif GRDGS is completely conserved, and a high degree of conservation also exists in the neighboring thrombin cleavage site and cryptic integrin attachment motif 'SVVYGLR' ('SLAYGLR' in mouse) which is revealed upon cleavage by thrombin. The mineral binding poly-aspartate region is also conserved, although the overall number and strings of consecutive aspartic acids residues does vary. As will be described below, OPN is highly post-translationally modified. Many of the phosphorylation and glycosylation sites that have been identified are well conserved (see figure 2). In addition, OPN can be crosslinked to other proteins via transglutaminase utilizing two glutamine residues conserved in all species. Other

predicted motifs include a potential calcium-binding site as well as two putative heparin-binding sites.
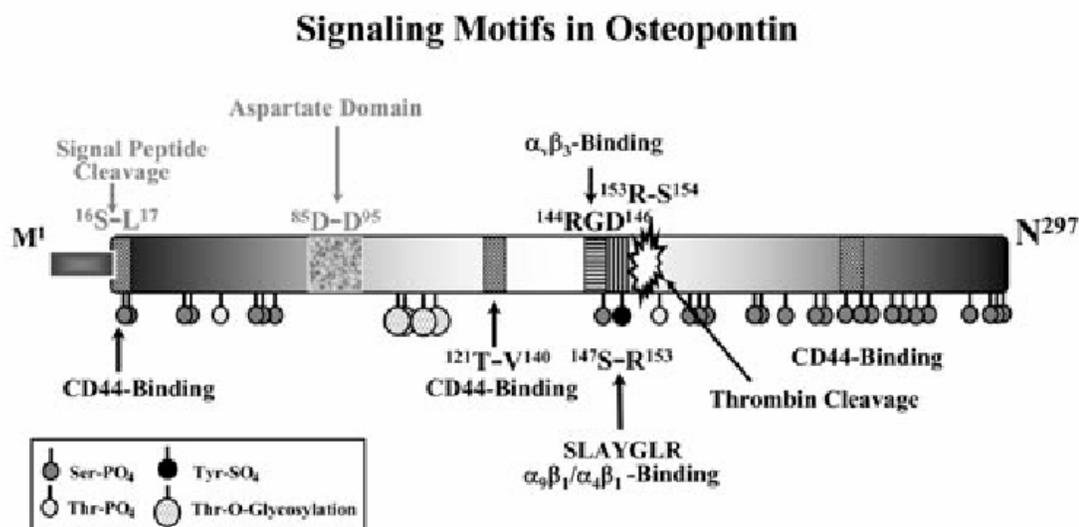
Figure 2: Signaling motifs in Osteopontin. The locations of the two integrin-binding sites are shown. Also shown are 3 CD44-binding sites (the precise location of the CD44-binding in the carboxy-terminal region has not been identified). The cryptic SLAYGLR is exposed upon thrombin cleavage of the protein. The displayed sites of post-translational modifications are adapted from rat OPN. M, methionine; S, serine; L, leucine; D, aspartate; T, threonine; V, valine; R, arginine; G, glycine; A, alanine; Y, tyrosine; N, asparagine. Taken from Sodek et al. (2006), and reprinted by permission from the Journal of Dental Research.

1.3.2 Post-Translational Modifications

1.3.2.1 Phosphorylation

Serine phosphorylation is the most abundant modification of OPN, and is also the most characterized and studied. Phosphorylation sites have been mapped for rat bone OPN, bovine, and human milk OPN. Initial characterization of rat bone OPN estimated 13 sites of phosphorylation (12 serine, 1 threonine) (Prince et al., 1987). A later more in-depth characterization of proteolytic fragments from a large portion of the molecule using Edman sequencing and mass spectrometry indicated 11 sites of phosphorylation (Neame and Butler, 1996). The phosphorylation was quite heterogeneous, with none of the peptides examined exhibiting phosphorylation 100% of the time. Recently a more

comprehensive characterization of rat bone OPN using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy was published (Keykhosravani et al., 2005). The authors found 29 sites of potential phosphorylation, however the overall degree of phosphorylation was much less and heterogeneous, with an estimated efficiency of 32%.

The phosphorylation sites of both bovine and human milk osteopontin proteins have been mapped in the laboratory of Dr. Esben Sørensen. Bovine milk OPN was shown to have 27 phosphoserines and 1 phosphothreonine (Sørensen et al., 1995). All but two of these modifications were shown to be in the recognition motif for mammary gland casein kinase (Ser/Thr-X-Glu/Ser(P)/Asp) with the other two found in the sequence: Ser-X-X-Glu/Ser(P), which is recognized by casein kinase II. The human milk protein also contained a large number of phosphorylations, with a total of 34 phosphoserines and two phosphothreonines (Christensen et al., 2005). Again the majority of phosphorylated residues (31) were located in the mammary gland casein kinase motif. Six residues were located in the casein kinase II motif and one phosphoserine was in a sequence not corresponding to either recognition motif. The phosphorylation pattern of both milk OPNs revealed that the phosphorylated residues were located in clusters of three to five residues separated by larger strings of unmodified amino acids. Milk OPN in general seems to be more highly phosphorylated and less heterogeneous than OPN from bone, although the harsh methods required for purification of OPN from bone may remove some phosphorylations. The importance of phosphorylation of OPN with respect to function will be discussed below.

1.3.2.2 Glycosylation

The studies cited above also examined glycosylation of osteopontin. Prince et al. (1987) estimated that rat bone OPN contained 1 N-linked glycosyation, 5-6 O-linked glycosylations, and approximately 16% carbohydrate. A more in-depth characterization determined rat bone OPN to have 4 O-linked glycosylations and no N-linked glycosylations (Keykhosravani et al., 2005). Bovine mammary gland OPN only contained 3 O-glycosylations and no N-glycosylations (Sørensen et al., 1995). Similar results were obtained from human milk OPN which has 5 O-glycosylations and no N-glycosylations (Christensen et al., 2005). In all studies the O-linked glycosylations were observed to contain sialic acid residues. These detailed studies of OPN suggest that OPN is primarily O-glycosylated with little or no N-linked glycosylation, bringing the SIBLING family name into question – at least for OPN. However, N-linked glycosylation has been described for normal rat kidney cells (Singh et al., 1990a) and for human bone OPN (Masuda et al., 2000).

Overall very little research focusing on glycosylation of OPN has been conducted, especially in relation to function. One study reported that altered sialylation of OPN occurred in transformed cells compared to non-transformed counterparts, and this reduction in sialic acid prevented receptor-mediated localization of OPN to the cell surface (Shanmugam et al., 1997).

1.3.2.3 Transglutaminase Crosslinking

OPN has also been observed to be a substrate for tissue transglutaminase, factor XIIIa (Prince et al., 1991). Two glutamines (Gln-34 and Gln-36), which are conserved in all known species, have been demonstrated to be substrates for transglutaminase activity

(Sørensen and Petersen, 1994). Transglutaminase-mediated cross-linking between OPN and fibronectin has been reported (Beninati et al., 1994), as well as the generation of OPN multimers, the latter of which increases OPN's collagen binding properties (Kaartinen et al., 1999). Curiously, the bone protein osteocalcin was demonstrated to inhibit transglutaminase cross-linking of OPN. These studies are highly suggestive of a role of transglutaminase cross-linking in mediating the covalent incorporation of OPN into extracellular matrices, particularly that of bone (Kaartinen et al., 1997). Indeed, recent studies have identified high-molecular weight cross-linked OPN complexes in pathological situations, usually involving calcification. High-molecular weight OPN complexes were observed in calcified arteries of matrix Gla protein-deficient mice (Kaartinen et al., 2006). Transglutaminase cross-linked OPN was found to augment the stimulation of calcium pyrophosphate dihydrate crystals commonly found in osteoarthritic joint tissues in an *in vitro* model using cultured chondrocytes (Rosenthal et al. 2007). A possible feedback loop was also discovered as osteopontin stimulated transglutaminase expression in these cells.

1.3.3 Secondary Structure

Osteopontin is generally believed to exist as an open, flexible molecule primarily devoid of typical secondary structure such as α-helices or β-sheets. Computer algorithms consistently predict the existence of some α-helical regions, especially in the carboxy-terminal portion of the molecule, and small areas of β-sheet structure on either side of the central GRDGS sequence (Craig et al., 1989; Prince, 1989; Sodek et al., 2000). However, the existence of such structures has not been supported experimentally. Analysis of bone sialoprotein and OPN structures in solution using NMR suggested that these proteins are

completely unstructured (Fisher et al., 2001). The structures of bone and milk OPN in the

presence and absence of hydroxyapaptite or calcium ions were determined by attenuated

total reflection infrared (ATR-IR) spectroscopy**.** In all cases there was a lack of

predominant structure, consistent with a random coil, although binding of milk OPN to

hydroxyapaptite slightly increased the β-sheet percent (Gericke et al., 2005). In contrast,

studies by Gorski et al. (1995) using solution circular dichroism and Fourier-transformed

infrared spectroscopic data indicated that the conformation of bone OPN is dependent

upon its concentration, and the presence of calcium ions increased the extent of

disordered structure.

1.3.4 Proteolytic Cleavage

Osteopontin can also be cleaved by thrombin, which can alter its function and

expose an additional integrin binding site. The thrombin cleavage site, located 7 amino

acids C-terminal to the RGD motif, is conserved in all species. Human OPN has also

been shown to be a substrate for matrix metalloproteinases (MMPs) MMP-3

(stromelysin-1) and MMP-7 (matrilysin) (Agnihotri et al., 2001). Cleavage of OPN

increases its adhesion and migratory signals though integrin receptors.

1.4 Interactions

1.4.1 Integrin Receptors

The primary receptor class for OPN binding is the integrin family of receptors

that bind to the centrally located RGD motif of OPN. The integrin $\alpha_v\beta_3$ was established

early on as a primary receptor for OPN expressed by numerous cell types. The OPN-$\alpha_v\beta_3$

interaction was reported to be essential for osteoclast migration and resorption (Miyauchi

et al., 1991; Ross et al., 1993), as well as smooth muscle cell migration and adhesion (Liaw et al., 1994). Additional RGD-binding integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$ were later determined to be used by cells to bind and adhere to OPN (Hu et al., 1995b). The binding of all three of these integrins was enhanced by the positive cations Mg^{2+} or Mn^{2+}, but not by Ca^{2+} which blocked the OPN-$\alpha_v\beta_3$ interaction (Hu et al., 1995a; Hu et al., 1995b). Other integrins ($\alpha_5\beta_1$, $\alpha_8\beta_1$ and $\alpha_9\beta_1$) have also been reported to bind OPN *in vitro* in an RGD-dependent manner (Nasu et al., 1995; Smith et al., 1996; Denda et al., 1998), and more recently $\alpha_v\beta_6$ (Yokosaki et al., 2005). Notably, some of these interactions ($\alpha_5\beta_1$ and $\alpha_9\beta_1$) bound more effectively to the N-terminal thrombin fragment of OPN than to the full-length protein (Smith and Giachelli, 1998; Barry et al., 2000b; Yokosaki et al., 2005).

A cryptic integrin recognition sequence that is exposed upon cleavage by thrombin is also used by integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ for adhesion (Yokosaki et al., 1999; Barry et al., 2000a). This site lies at the C-terminal end of the N-terminal thrombin fragment and comprises the amino acids SVVYGLR (SLAYGLR in mice). Additional integrin binding sites or possible synergy sites have been reported, for example peptides composed of amino acids 132-146 of the human protein supported cell adhesion via integrin $\alpha_4\beta_1$ (Barry et al., 2000a), and mutation of two aspartic acid residues (amino acids 154 & 157) upstream of the RGD site inhibited adhesion of $\alpha_v\beta_5$ or $\alpha_v\beta_6$ (Yokosaki et al., 2005).

1.4.2 CD44 Receptor

The hyaluronan receptor CD44 has also been identified as a receptor for OPN (Weber et al., 1996), however many of the details of this interaction have yet to be

elucidated. CD44 can exist in many forms due to alternative splicing of several 'variant' exons into the membrane proximal extracellular region of the protein. The standard version of the protein, 'CD44s', consists of a core set of exons found in all variants. The initial report by Weber et al. (1996) showed that a CD44 species expressing the variant exons 7-10 was able to bind OPN at a site C-terminal to the central RGD motif. Others have reported that CD44 was able to recognize both the N- and C-terminal halves of the thrombin-cleaved OPN molecule. Katagiri and colleagues (1999) have demonstrated that the standard form of CD44 was not able to bind OPN, but certain variant isoforms of CD44 (those specifically containing v6-v7) were able to independently bind both thrombin-cleaved fragments of OPN. This interaction seemed to also require binding of the β1 integrin yet occurred in an RGD-independent manner.

The interaction of CD44 and OPN has been implicated in migration of macrophages and tumor cell lines. A feedback loop may also exist as many researchers have shown that OPN increased expression of CD44, primarily using cancer cell lines such as the breast cancer cell line 21NT (Khan et al., 2005), liver carcinoma cell line HepG2 (Gao et al., 2003), melanoma cells (Samanna et al., 2006) or macrophages (Marroquin et al., 2004).

1.4.3 Non-receptor interactions

Osteopontin has also been shown to have non-receptor binding partners, many of which are extracellular matrix proteins. The formation of a complex between OPN and the bone protein osteocalcin in multiple *in vitro* assays was reported by Ritter et al (1992). OPN has also been shown to bind type I collagen (Chen et al., 1992). Cross-linking of OPN by transglutaminase was shown to increase its collagen binding

properties in a calcium dependent manner (Kaartinen et al., 1999). Others have reported an interaction of OPN with fibronectin in addition to its abovementioned cross-linking to fibronectin via transglutaminase (Mukherjee et al., 1995).

Osteopontin and two other SIBLING members, bone sialoprotein (BSP) and dentin matrix protein (DMP), have also been shown to form complexes with the serum protein complement Factor H, sequestering it to the cell surface via receptor binding and allowing evasion of complement-mediated cell lysis (Fedarko et al., 2000a; Fedarko et al., 2000b; Jain et al., 2002). These same three SIBLING members have additionally been shown to bind and activate specific matrix metalloproteinases. Osteopontin bound proMMP-3 and active MMP-3, BSP bound proMMP-2 and active MMP-2, and DMP bound proMMP-9 and active MMP-9 (Fedarko et al., 2004). These complexes are disrupted by the interaction with complement Factor H.

An intracellular form of OPN (iOPN) has also been described (Zohar et al., 1997; Zohar et al., 1998). This intracellular form of OPN colocalized with CD44 and the protein ezrin, components of the ezrin/radixin/moesin (ERM) complex. This colocalization was associated with the leading edge of migratory fibroblasts, activated macrophages and metastatic cells, and was also reported to be associated with migration and resorption in osteoclasts (Zohar et al., 2000; Suzuki et al., 2002). Recently, iOPN was reported to associate with the adaptor molecule MyD88 after Toll-like receptor-9 engagement and increase interferon-α gene expression in plasmacytoid dendritic cells (Shinohara et al., 2006).

1.5 Osteopontin Functions

Osteopontin's function in many tissues is to promote adhesion and facilitate migration of a variety of cell types through interaction with the integrin and CD44 variants; it is also a cytokine that activates many signaling pathways and supports cell survival (Denhardt et al., 2001). Its numerous functions in a wide variety of systems have led to a recent increase in OPN research, and more specialized functions of the molecule are rapidly being described.

1.5.1 Bone

Osteopontin is one of the more abundant non-collagenous proteins in bone and is localized to cell-matrix and matrix-matrix interfaces (McKee and Nanci, 1996) where it is believed to have multiple roles. The protein serves as an attachment protein linking cells to the bone mineral via its hydroxyapatite-binding poly-aspartate region and various receptor binding attachment motifs. Many *in vitro* studies have also suggested that OPN is a regulator of crystal growth and nucleation, described in more detail below. OPN is expressed by all the major bone-specific cell types: osteoblasts, osteoclasts and osteocytes.

Osteopontin has been demonstrated to have a very important role in osteoclast function. Early studies indicated that osteoclasts are able to attach and migrate to OPN via the $\alpha_v\beta_3$ integrin receptor (Reinholt et al., 1990; Miyauchi et al., 1991; Faccio et al., 1998). Interaction of OPN with this receptor on the osteoclast surface has been shown to stimulate phosphatidylinositol 3-hydroxyl kinase activity (Hruska et al., 1995). In addition, Hu et al. (1995a) have shown that this interaction is inhibited by Ca^{2+},

suggesting a mechanism of regulation because resorbing osteoclasts greatly increase the local concentration of calcium ions.

The importance of OPN's function in bone was not revealed until the development of the OPN knock-out (OPN-KO) mouse (Liaw et al., 1998; Rittling et al., 1998). Surprisingly, the OPN-KO mice developed normally and had morphologically normal bones, including at the interfaces where OPN is typically localized although there was some osteopetrosis with age. Later studies have identified differences in the ultrastructure of the bones, such as increased mineral crystallinity (Boskey et al., 2002), possibly translating into a difference in mechanical properties (Kavukcuoglu et al., 2007).

Many studies since have determined a major role for OPN in stress-induced bone remodeling, with most studies focusing on stresses that induce bone resorption by osteoclasts. Yoshitake et al. (1999) demonstrated that OPN-KO mice are deficient in ovariectomy-induced bone remodeling. Microcomputed tomography analysis of trabecular bone volume after ovariectomy revealed an approximate 60% reduction in wildtype mice compared to about a 10% reduction in OPN-deficient mice. Similarly, bone loss due to reduced mechanical stress using a tail-suspension model was also impaired in the OPN-KO mice (Ishijima et al., 2001; Ishijima et al., 2002). In addition, parathyroid-induced as well as high phosphate load induced bone resorption did not occur to the same extent in OPN-KO mice compared to their wildtype counterparts (Ihara et al., 2001; Koyama et al., 2006).

In vitro studies using osteoclasts isolated from OPN-KO mice have indicated that osteoclast motility is impaired in the absence of OPN and both the $\alpha v \beta 3$ and CD44 are involved (Chellaiah and Hruska, 2003). The hypomotility of OPN-KO osteoclasts is due

in part to the decreased expression of surface CD44; exogenous OPN stimulated CD44

expression and partially restored bone resorption (Chellaiah et al., 2003). From these

studies it is generally accepted that OPN facilitates osteoclast migration to sites of

resorption and is necessary for proper resorption and bone turnover.

Many of OPN's effects on osteoclast function require phosphorylation of the

molecule. Phosphorylation of OPN by casein kinase II increased osteoclast (but not

osteoblast) adhesion (Katayama et al., 1998), and dephosphorylation of OPN by the

osteoclast-expressed tartrate-resistant acid phosphatase (TRAP) eliminated osteoclast

binding *in vitro* suggestion a mode of regulation (Ek-Rylander et al., 1994). In addition,

native milk OPN stimulated *in vitro* bone resorption to a greater extent than recombinant

OPN (Razzouk et al., 2002). Two OPNs differing in their levels of phosphorylation also

showed similar differences, with the more phosphorylated form supporting more *in vitro*

bone resorption (Razzouk et al., 2002).

It has been suggested that the majority of OPN in bone is synthesized by

osteoblasts during the bone remodeling sequence (McKee and Nanci, 1995). Supporting

this is OPN's biphasic expression pattern during the osteoblast differentiation sequence *in*

vitro. OPN is expressed early by proliferating pre-osteoblasts, and highly expressed again

by differentiated mineralizing osteoblasts (Owen et al., 1990; Sodek et al., 1995).

Notably, the post-translational modification of the OPN secreted by these cells was

determined to be different. A 55-kDa form that contains little sulfation and

phosphorylation was secreted by pre-osteoblasts, and a 44-kDa highly phosphorylated

and sulfated form was secreted by fully differentiated OBs (Sodek et al., 1995). In

contrast to OPN's effects on osteoclast function, the protein's effects on osteoblast

differentiation and function are less well understood. *In vitro* studies of osteoblast differentiation in culture have shown that recombinant OPN can increase proliferation and differentiation (Jang and Kim, 2005), yet overexpression of OPN in MC3T3 cells decreased both proliferation and mineral deposition (Huang et al., 2004). Studies using the OPN-KO mouse have also suggested a role of OPN in regulating osteoblast function. The decrease in bone formation observed after tail suspension of wild-type mice was not observed in OPN-KO mice (Ishijima et al., 2001). Also, intermittent parathyroid (PTH) treatment, which is known to increase bone mineral density and bone mass, caused a significantly greater increase in OPN-KO mice compared to wild-type controls (Kitahara et al., 2003). This difference was determined to be solely an effect of osteoblastic cell activity, not due to the known osteoclast defect in OPN-KO mice.

In addition to its effects on the cells in bone, OPN is also a regulator of crystal growth, including hydroxyapatite (HA) - the crystal of bone. Using a gelatin gel diffusion system, Boskey et al. (1993) showed that OPN at concentrations greater than 25 μg/ml inhibited both hydroxyapatite formation and growth in a dose-dependent manner. Partial enzymatic dephosphorylation reduced the inhibitory activity. Using an autotitration system to buffer metastable solutions, Hunter et al. (1994) also showed that OPN inhibited HA formation, and again enzymatic dephosphorylation reduced the effect. In a steady state agarose gel system, nucleation of HA was unaffected by OPN (Hunter et al., 1996). The importance of phosphorylation in OPN's regulation of HA was demonstrated by Pampena et al. (2004), who used OPN phosphopeptides to show that specific phosphorylated sequences play an important role, and by Gericke et al. (2005), who compared OPN from various sources. Bone OPN inhibited HA formation and seeded

growth, whereas recombinant OPN and dephosphorylated OPN had no effect. In contrast, highly phosphorylated milk OPN promoted HA formation, as did mixtures of OPN and osteocalcin. This last result highlights the confusion regarding OPN and crystal regulation. Generally considered to be a negative regulator of crystal growth, especially in fluids such as urine and milk (see below), OPN has also been shown to induce HA growth under certain conditions. For example, OPN crosslinked to agarose beads promoted HA formation whereas OPN adsorbed to these beads did not (Ito et al., 2004).

1.5.2 Kidney and Urine

OPN is abundant in the kidney and urine (Yoon et al., 1987) and was identified as a mediator of urinary stone formation (Kohri et al., 1992; Worcester et al., 1992). OPN protein has been shown to be a component of kidney stones and its expression is upregulated in the diseased state (McKee et al., 1995; Umekawa et al., 1995; Lieske et al., 1997). Murine and human OPN has been shown to inhibit the *in vitro* growth of calcium oxalate (CaOx) crystals (Shiraga et al., 1992; Worcester et al., 1992). In constant composition seeded crystal growth assays, OPN addition was found to inhibit the aggregation of hydroxyapatite crystals, which are also present in a significant fraction of renal stones (Beshensky et al., 2001). Again there are contradictory reports suggesting that OPN facilitates the attachment of CaOx crystals to renal cells (Yasui et al., 2002) and that immobilized OPN increases crystal aggregation - adhering OPN to the surface of collagen granules caused an increase in CaOx crystal adherence and aggregation (Umekawa et al., 2001; Konya et al., 2003). Further complicating matters is the finding that phosphorylation of OPN also plays a role. Phosphorylated peptides were much more effective at inhibiting CaOx crystal growth than non-phosphorylated peptides (Hoyer et

al., 2001). It can be concluded that OPN in solution binds Ca^{2+}, thereby inhibiting crystal formation, but when immobilized on a surface it draws ions to it, thus increasing crystal adhesion and aggregation when using *in vitro* assays.

Studies using the OPN-KO have shed some light on the *in vivo* mechanism of OPN action. Wesson et al. (2003) induced hyperoxaluria in wild-type and OPN-KO mice and found intratubular CaOx crystal formations in OPN-KO mice but not the wildtype controls, which had significantly upregulated OPN expression. Analysis of the Aprt/Opn double knockout mice revealed that OPN is a major inhibitor of 2,8-dihydroxyadenine (DHA) crystal deposition in male mice but not female mice (Vernon et al., 2005).

1.5.3 Vasculature

Osteopontin is expressed by vascular smooth muscle cells and has been shown to increase proliferation, adhesion, spreading and is chemotactic for these cells (Gadeau et al., 1993; Liaw et al., 1994). These effects seem to be modulated by the αvβ3 integrin (Yue et al., 1994; Liaw et al., 1995). OPN was also identified as a component of atherosclerotic plaques (Giachelli et al., 1993; Hirota et al., 1993) and has been shown to inhibit the calcification of smooth muscle cells *in vitro* (Wada et al., 1999; Speer et al., 2005). An *in vivo* study in which aortic valve leaflets were subcutaneously implanted into OPN-KO and wild-type mice showed 4 to 5-fold greater calcification in the OPN-KO mice (Steitz et al., 2002). Interestingly, this calcification could be mitigated by either injection of soluble OPN or preadsorption of OPN onto the implants (Ohri et al., 2005).

Post-translational modifications also have a role: maximum inhibition was achieved when the OPN was phosphorylated and contained a functional RGD domain. In another study, native phosphorylated OPN inhibited calcification of human smooth

muscle cells in culture, whereas recombinant or enzymatically dephosphorylated OPN had no effect (Jono et al., 2000). In spite of these results, the precise role of OPN in atherosclerotic plaque formation remains unclear. Chiba et al. (2002) generated transgenic mice in which hematopoietic cells were engineered to express OPN, and these mice had significantly larger atherosclerotic lesions when fed atherogenic diets. The authors observed high numbers of activated macrophages and determined that the OPN in these lesions was produced by the infiltrating macrophages. Considering that OPN is a chemoattractant for macrophages (see below) this may be a unique situation not representative of the true *in vivo* scenario. It also suggests that OPN produced by macrophages may have a distinct function compared to OPN from other sources used to show inhibition of calcification.

Osteopontin also affects endothelial cells and has a role in angiogenesis and vascular injury repair. OPN is upregulated by many factors expressed after vascular injury and during *in vitro* angiogenesis, particularly vascular endothelial growth factor (VEGF). OPN protects endothelial cells from apoptosis (Scatena et al., 1998; Khan et al., 2002), and in cooperation with VEGF stimulates cell adhesion and migration (Senger et al., 1996). Notably, thrombin-cleaved OPN was more effective at promoting migration in these experiments, and an SVVYGLR peptide was also shown to cause increased adhesion, migration and tube formation (Hamada et al., 2003). An *in vivo* study of bone discs implanted intramuscularly into wild-type and OPN-KO mice revealed that differences in resorption of the disks were mainly due to reduced vascularization in the OPN-KO mice (Asou et al., 2001). OPN stimulated angiogenesis in the chick embryo chorioallantoic membrane model but it is unclear whether this was a direct effect or the

result of the recruitment of proangiogenic monocytes (Leali et al., 2003). OPN's angiogenic effects have also been established in the growth of tumors. OPN-transfected neuroblastoma cells exhibited increased neovascularization after implantation into mice (Takahashi et al., 2002; Hirama et al., 2003).

1.5.4 Injury, Inflammation, and Immune Response

Osteopontin is expressed by many cell cells of the immune system, mostly upon activation. These include macrophages, T-cells, B-cells, NK cells, and platelets (Patarca et al., 1989; Miyazaki et al., 1990; Pollack et al., 1994). OPN's role in immunity was initially identified by its abundant expression after T-cell activation and its mapping to a locus on murine chromosome 5 termed Ric that confers resistance to infection by *Rickettsia tsutsugamushi*, an obligate intracellular bacterium (Patarca et al., 1989). In a mouse model of systemic lupus, OPN produced by T-cells stimulated IgM and IgG production by B-cells (Lampe et al., 1991). Osteopontin expression is increased in response to cellular injury, attracting and supporting the infiltration of macrophages and T-lymphocytes into sites of injury and inflammation (Singh et al., 1990b; Giachelli et al., 1998).

Besides serving as a chemoattractant for macrophages, OPN has also been associated with other macrophage functions. Using OPN-KO mice, Liaw et al (1998) showed that wound repair following skin incisions was impaired, with reduced levels of debridement, reduced organization of matrix and collagen fibrillogenesis. These results could be attributed to impaired macrophage function, as increased levels of inactive, resting macrophages were observed. OPN has also been shown to reduce nitric oxide production by kidney epithelial cells and macrophages activated by interferon-γ and LPS,

which reduces the cytolytic activity of macrophages towards tumor cells (Hwang et al., 1994; Rollo et al., 1996). A recent paper using the RAW264.7 monocyte/macrophage cell line suggests that OPN expression is required for maintenance of a differentiated macrophage phenotype as well as for migration (Nystrom et al., 2007).

OPN also has a role in cell-mediated and granulomatous responses (reviewed in O'Regan and Berman, 2000). OPN induces migration and binds to activated leukocytes and macrophages, causing changes in cytokine production. One key effect of OPN is its effect on the balance of the Th1/Th2 response. OPN enhances Th1 cytokine production and inhibits Th2 cytokine expression. Ashkar et al. (2000) showed that OPN enhanced IL-12 production by peritoneal macrophages and inhibited IL-10 production. Interestingly, these effects were mediated by different receptors, as the IL-12 response was blocked by RGD peptides and an antibody to integrin subunit β_3, while the IL-10 response was blocked by an anti-CD44 antibody, but not the β_3 antibody. Also important was the observation that phosphorylation of OPN was needed for the IL-12 induction (Ashkar et al., 2000). Osteopontin stimulation was also demonstrated to augment the ability of anti-CD3 monoclonal antibody to induce CD40 ligand (CD40L) and interferon-γ expression on human T cells, resulting in CD40L- and IFN-gamma-dependent IL-12 production in vitro (O'Regan et al., 2000).

Osteopontin's functions in the cell-mediated immune response have implicated the protein in various autoimmune diseases, such as demyelinating disease, autoimmune nephritis, and rheumatoid arthritis. In the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), OPN-KO mice were more resistant and had more frequent remissions; myelin-reactive T cells in OPN$^{-/-}$ mice produced more

IL-10 and less interferon-γ than in OPN$^{+/+}$ mice, two cytokines that increase the Th1

response (Chabas et al., 2001). OPN also contributes to the progression and relapses of

EAE by increasing T-cell survival (Hur et al., 2007). Similarly, in a model of rheumatoid

arthritis using injection of lipopolysaccharide and anti-collagen type II antibodies, there

was a decrease in joint swelling, damage to surface structures, and loss of proteoglycans

in cartilage of OPN-KO mice compared to wild-type control mice (Yumoto et al., 2002).

However, OPN's role is likely more complicated as other studies have shown no role for

OPN in the development of autoimmune diseases such as myocarditis (Abel et al., 2006),

or using an alternate model of auto-immune arthritis (Jacobs et al., 2004).

1.5.5 Cancer

Osteopontin was originally identified as a secreted transformation associated

phosphoprotein whose expression was upregulated in transformed cells (Senger et al.,

1980). The protein was later identified as 2ar/osteopontin and was shown to be

upregulated by treatment with the tumor promoter TPA or in ras-transformed fibroblasts

(Craig et al., 1988; Craig et al., 1989; Chambers et al., 1992). OPN expression has since

been established in a wide variety of cancers, and is associated with metastatic potential

and poor prognosis. Expression of OPN antisense RNA in *ras*-transformed NIH3T3

fibroblasts reduced the tumorigenicity of the cells *in vivo* (Behrend et al., 1994). Another

study using a rat mammary epithelial line transfected with the human OPN gene

increased the ability of these cells to metastasize (Oates et al., 1996). Osteopontin

expression has been correlated with disease progression and decreased survival in lung

(Chambers et al., 1996), breast (Rudland et al., 2002), gastric (Ue et al., 1998), prostate

(Thalmann et al., 1999), ovarian (Bao et al., 2007) and uterine cervical cancer (Sakaguchi

et al., 2007) as well as head and neck squamous cell carcinomas (Le et al., 2003; Petrik et al., 2006).

The source of OPN serum levels in cancer patients remains controversial and may depend on the type and stage of cancer. Studies using *in situ* hybridization determined that for a large number of different tumors, OPN mRNA expression was localized to infiltrating macrophages (Brown et al., 1994). OPN protein in these samples however was associated with both the macrophages and tumor cells, suggesting that the macrophage OPN is bound by tumor cell surface receptors. In contrast, many tumor cell lines express high levels of OPN *in vitro,* and in another study benign breast lesions expressed low levels of osteopontin, however invasive breast carcinoma samples expressed high levels of OPN which was associated with microcalcifications (Bellahcene and Castronovo, 1995). Similarly OPN expression in melanoma was shown to be acquired along with invasiveness (Zhou et al., 2005).

Studies conducted *in vivo* have shed little light on this issue. When B16 melanoma cells, which express very little OPN, were injected into OPN-KO and wild-type mice by two different routes, the numbers of resulting tumors were significantly less in the OPN-KO mice (Nemoto et al., 2001). This result suggests that host OPN plays an important role in metastasis. However, studies in which cells expressing low levels of OPN were injected into mice have shown that tumor formation is enhanced in mice that received the cells expressing high levels of OPN (El-Tanani et al., 2001; Allan et al., 2006). Also, when both wildtype and OPN-KO *ras*-transformed cells were injected into nude mice, tumor formation occurred much more slowly in mice injected with the OPN-

KO cells, indicating that OPN expression is required for full expression of the transformed phenotype by the ras oncogene (Wu et al., 2000).

Regardless of the timing and source of expression, many of OPN's previously mentioned functions contribute to its role in cancer progression. OPN's ability to stimulate migration clearly contributes to the metastatic ability of tumor cells. OPN decreases nitric oxide expression and cytotoxicity of macrophages towards tumor cells (Feng et al., 1995; Rollo et al., 1996), and has also been shown to increase survival in a number of systems (Malyankar et al., 2000; Khan et al., 2002; Lee et al., 2007). The association of OPN with serum protein factor H has been shown to protect tumor cells from complement-mediated attack *in vitro* (Fedarko et al., 2000b; Jain et al., 2002). Osteopontin, along with two other SIBLING family members, has been demonstrated to bind and activate specific matrix metalloproteinases (MMPs) *in vitro* (Fedarko et al., 2004). This ability of OPN to bind and activate pro-and active MMP-3 as well as increase expression of pro-MMP-2 (Philip and Kundu, 2003) suggests a role in promoting extracellular matrix degradation facilitating tumor cell metastasis. Recently a splice variant of OPN has been shown to induce anchorage independence in human breast cancer cells (He et al., 2006)

1.5.6 Other

Osteopontin expression on the endosteal bone surface in bone marrow has recently been associated with the proliferation of hematopoietic stem cells (HSCs) and establishment of the niche to which HSCs migrate to (Nilsson et al., 2005). Using OPN-KO mice, it was shown that OPN is a negative regulatory element of the stem cell niche that limits the size of the stem cell pool (Stier et al., 2005).

Studies in multiple animal models have revealed that OPN is a major constituent of the uterine-placental microenvironment that is required for adhesion and signal transduction at the uterine-placental interface throughout pregnancy (Johnson et al., 2003). Phosphorylation of OPN has been implicated in this role. Dephosphorylation of OPN with TRAP reduced the migration of three human choriocarcinoma lines compared to native, fully phosphorylated OPN (Al-Shami et al., 2005). Because both OPN and TRAP are expressed in the uterus during early pregnancy, the authors suggest that extracellular phosphatases such as TRAP may modify OPN charge state and thus modulate cell migration.

1.6 Conclusion

Osteopontin plays a role in a variety of cellular processes from bone and extracellular matrix remodeling to immune cell activation. One of the best examples of the diverse functions of OPN is a recent paper examining fracture healing in wild-type and OPN-KO mice. OPN deficiency altered the functionality of multiple cell types, resulting in delayed early vascularization, altered collagen fiber and matrix organization, and late remodeling due to the osteoclast defect, resulting in reduced biomechanical properties (Duvall et al., 2007). A persistent theme throughout the characterization of OPN's role in these processes has been the importance of post-translational modifications, particularly phosphorylation.

In this study, OPN produced by two different murine cell lines was immuno-affinity purified and the degree of post-translational modification of the proteins was analyzed using trypsin digestion and MALDI-TOF mass spectrometry. The two forms of OPN differed substantially in the amount of phosphate modifications; OPN produced by

differentiating osteoblasts was significantly more phosphorylated than OPN produced by

ras-transformed fibroblasts. The difference in the amount of phosphate modification of

the OPN forms translated into a functional difference in the ability of these forms to

support cell adhesion. This cell adhesion was determined to be RGD-dependent,

suggesting that phosphate modification of OPN is able to affect integrin interaction with

the RGD motif of OPN.

In a related study, anti-OPN monoclonal antibodies were used to inhibit the

adhesion of a human breast cancer (MDA-MB-435) cell line to recombinant human OPN.

These antibodies recognized an epitope in the C-terminal region of OPN. It was

determined that cells are able to bind peptides corresponding to this region, but the

interaction is not an adhesive one. Cell adhesion studies using blocking antibodies

determined that the cells adhere to OPN via RGD-dependent adhesion by the $\alpha_v\beta_3$

integrin, suggesting that the C-terminal region is able to affect this interaction.

Chapter 2

Materials & Methods

2.1 Cells and Cell Culture:

MC3T3-E1 subclone 4 murine pre-osteoblast cells (Wang et al., 1999)(kind gift from Dr. R. Franceschi, University of Michigan) were maintained in α-MEM (Invitrogen Corp., Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT), 5 ug/ml penicillin, 5 U/ml streptomycin and 2 mM glutamine. For differentiation, cells were grown until confluent then switched to growth medium above containing 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO) for an additional 10-12 days before generating conditioned medium. The murine embryonic fibroblast lines (275 & 275-3-2) (Wu et al., 2000)(kind gift from Dr. Susan Rittling, Forsyth Institute, Boston, MA) and the MDA-MB-435 human breast cancer line (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's Medium (DMEM - Mediatech Inc., Herndon, VA) with 10% FBS (Hyclone), 5 μg/ml penicillin, 5 U/ml streptomycin and 2 mM glutamine. Conditioned media were generated from these cell lines by incubating them with serum-free medium overnight after washing twice with PBS.

2.2 Antibodies Used:

The following monoclonal anti-osteopontin antibodies were created and characterized in the Denhardt laboratory by Dr. Aaron Kowalski (2005): mAK2A1 (2A1), mAK3D9 (3D9), mAK7B4 (7B4), mAK1H3 (1H3), mAK2C5 (2C5), mAK10F6 (10F6). Polyclonal anti-OPN antibody LF124 was a kind gift from Dr. Larry Fisher. Anti-mouse CD44 monoclonal antibody (clone KM114) was purchased from BD Biosciences (San Diego,

CA). Anti-human CD44v6 antibody (clone 2F10) was purchased from R&D Systems (Minneapolis, MN). Anti-human CD44 monoclonal antibody clone A3 was a kind gift from Dr. Man-Sun Sy.

2.3 Peptides Used:

Human and mouse osteopontin peptides were initially designed for antibody binding studies using the human (hOPN) or mouse (mOPN) OPN amino acid sequences. All peptides contained an N-terminal biotin tag used to attach the peptides to streptavidin-coated microwell plates. Peptide amino acid sequences are listed in table 1. The integrin-blocking GRDGSP or GRADNP (control) peptides were purchased from BIOMOL International (Plymouth Meeting, PA).

2.4 Hybridoma Culture and Monoclonal Antibody Purification:

Hybridomas were maintained in DMEM with 10% FBS, 5 μg/ml penicillin, 5 U/ml streptomycin and 2 mM glutamine in 10cm tissue culture dishes. Antibody-containing conditioned medium was generated by seeding $75cm^2$ tissue culture flasks with cells from one 80% confluent 10cm dish. After 24hr, approximately 200ml serum-free DMEM was added and the cells were incubated until few live cells remained. The resulting antibody-containing medium was filtered through a 0.2μm bottle-top filter (Millipore) and stored at 4°C. Antibodies were purified from the conditioned medium with protein G-agarose columns (Pierce Biotech, Rockford, IL) following the manufacturer's protocol. Briefly, 2ml disposable columns were used to pack 0.5ml of protein G-agarose beads and the column was equilibrated with 100mM sodium acetate pH 5.0 (binding buffer). Antibody-containing conditioned medium was mixed 1:1 with binding buffer and pumped over the columns. The columns were then washed with

binding buffer and antibody was eluted with 100 mM glycine pH 2.5 and immediately
neutralized. Positive fractions were determined by OD_{280} and pooled. The purified
antibodies were dialyzed against PBS and stored at 4°C with 0.02% sodium azide. In
some cases, particularly the 2A1 antibody used for affinity purification below, antibody
was generated in ascites fluid and purified by Dr. Nomi Ron.

Table 1

Osteopontin Peptides Used in this Study

hOPN4	N- PQNAVSSEETNDFKQETLGG-Biotin
hOPN10	N- PTVDTYDGRGDSVVYGLRGG-Biotin
hOPN11	N- SVVYGLRSKSKKFRRPDIGG-Biotin
hOPN12	N- DIQYPDATDEDITSHMESGG-Biotin
hOPN19	N- KSKEEDKHLKFRISHELDGG-Biotin
hOPN20	N- HLKFRISHELDSASSEVNGG-Biotin
mOPN26	N- DRYKFRISHELESSSSEVNGG-Biotin
mOPN26P (phosphorylated)	N- DRYKFRIpSHELEpSSpSSEVNGG-Biotin

2.5 Antibody-Protein G coupling:

OPN was purified from media conditioned by MC3T3-E1 (ObOPN) and 275-3-2
murine *ras*-transformed fibroblasts (FbOPN). Cell lines were grown as described above
and conditioned medium was generated by incubating the cells in serum-free medium
overnight. OPN was affinity-purified using 2A1 monoclonal antibody coupled to protein
G-agarose beads. Typically 1mg of 2A1 antibody was bound to 0.5 ml protein G-agarose
beads by incubating overnight in PBS at 4°C with end-over-end rotation. The beads were

then washed twice with 0.2M triethanolamine pH 8.2 then incubated in triethanolamine containing 10mM dimethyl pimelimidate for 30 min at room temperature with end-over-end rotation. The reaction was stopped by washing the beads once then incubating the beads with 0.1M ethanolamine pH 8.2 for 2 hours with end-over-end rotation. Coupling was verified by SDS-PAGE and coomassie blue staining as described in Harlow and Lane (1999).

2.6 Immuno-Affinity Purification of Osteopontin:

Approximately 50 ml of conditioned medium was incubated with 1ml of antibody-coupled beads at 4 °C overnight with end-over-end rotation. The beads were gently pelleted, washed multiple times with cold phosphate-buffered saline and packed into 2 ml disposable columns (Pierce). OPN was eluted with 100 mM glycine, 500 mM NaCl pH2.5 and immediately neutralized. Fractions were analyzed by SDS-PAGE, and proteins visualized by non-ammoniacal silver staining or by western blotting. Positive fractions were pooled, desalted on PD-10 columns (GE Healthcare Bio-Sciences, Piscataway, NJ), quantitated by ELISA and lyophilized.

2.7 Polyacrylamide Gel Electrophoresis:

Freshly collected conditioned medium was used for western blotting of OPN produced by cell lines. Typically 10-20 μl/lane of conditioned medium was fractionated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide separating gels and a Tris-glycine buffer system. For purified proteins, equal amounts (typically 50 ng) in each lane were used. Gels were immediately transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) for western blotting or analyzed by non-ammoniacal silver staining as below.

2.8 Western Blotting:

Following transfer, PVDF membranes were allowed to dry at least 10 min before wetting in methanol and blocking in Tris-Tween buffered saline (TTBS) containing 1% nonfat milk for 1 hr at room temperature with gentle rocking. Primary antibody was diluted in the blocking solution and incubated for 1 hr at room temperature with gentle rocking. Membranes were then washed 5 times with TTBS and incubated with secondary antibody (typically goat anti-mouse IgG (H+L) HRP conjugate from BioRad) diluted in TTBS for 1 hr as above. In some cases the membranes were cut into strips after blocking and individual strips blotted with 1 µg/ml of purified monoclonal antibodies as above. Membranes were again washed 5 times with TTBS and detected using an enhanced chemiluminescence kit (New England Nuclear Western Lightning Western Blot Chemiluminescence Reagent).

2.9 Non-Ammoniacal Silver Staining:

Silver staining of polyacrylamide gels was conducted following the non-ammoniacal staining procedure described by Ausubel et al. (1987). Gels were fixed by incubating in 100ml of fixing solution for 30min at room temperature with gentle rocking, then incubated in destaining solution for 30min with gentle rocking. Following destaining, the gels were incubated with 50ml of 10% glutaraldehyde with rocking in a fume hood. The gels were then thoroughly rinsed under gently running water for 2 hr. The gels were then incubated with 100ml of 5µg/ml dithiothrietol for 30 min followed by 50 ml of 1% silver nitrate for 30 min with gentle rocking. The gels were then rinsed once with a small amount of water, followed by two quick rinses with a small amount of carbonate developing solution. The gels were then soaked in 100ml of carbonate

developing solution until the desired level of staining was achieved and the reaction

stopped by the addition of 5ml of 2.3 M citric acid for 10 min. The gels were then soaked

in water, and sealed into plastic pouches, scanned and stored at 4°C.

2.10 Peptide Affinity Immuno-Assay:

Biotin-labeled osteopontin peptides (kind gift of Dr. Larry Steinman) were added

to Neutra-Avidin-coated 96-well plates in varying concentrations and detected following

the manufacturer's protocol (Pierce Biotech.). Anti-OPN monoclonal antibodies were

then added at 1 μg/ml and detected with a fluorescently-conjugated anti-mouse IgG

(Alexa Fluor 594, Invitrogen) at 1 μg/ml. Plates were read using a Fluoroscan-Ascent

fluorometer (Thermo Labsystems, MA) using an excitation/emission wavelengths of

584/612nm. Verification that equal amounts of peptide remained bound to the wells was

conducted by examining the inhibition of binding of FITC-conjugated biotin (Invitrogen).

FITC-conjugated biotin was added to the wells and incubated for 30 min with shaking at

450rpm. After 3 washes, the wells were read at 485(Ex)/527(Em) nm using the

Fluoroscan-Ascent fluorometer.

2.11 Cell Adhesion Assays:

2.11.1 Native OPN: Flat-bottom 96-well tissue culture-treated polystyrene

microtiter plates (Corning, NY) were coated with 100 μl purified native OPN (10μg/ml)

or fibronectin (2.5μg/ml) in phosphate buffered saline at 4°C overnight and then blocked

with 1% BSA. MDA-MB-435 and 275-3-2 ras-transformed fibroblast cells were

trypsinized, then washed twice and re-suspended in DMEM containing 1mg/ml BSA.

Cells ($5x10^4$) were added to coated wells and allowed to adhere for 1 hr (275-3-2) or 18

hr as described (Xuan et al., 1995) (MDA-MB-435) at 37ºC in a humidified atmosphere

with 5% CO_2. Non-adhered cells were removed as described by Goodwin and Pauli (1995) with slight modifications. Cells were washed twice by pipetting 75µl Percoll wash solution (73% Percoll, 0.9% NaCl) slowly down the sides of the wells and adherent cells were fixed by adding 50µl fixative (10% glutaraldehyde in Percoll) in the same manner. The wash and fixative solutions were then washed from the wells with 2-3 washes of 100µl PBS. Fixed cells were stained with 100µl 0.1% crystal violet (25 min), washed with tap water and solubilized in 50µl 0.5% Triton X-100 at least 1 hour before reading at 570nm in a MRX revelation Reader (Thermo Labsystems, MA). In some experiments, cells were pre-incubated with 100µM GRDGSP or GRADNP peptides (BIOMOL International, PA) for 30 min at 37ºC prior to adding the cells to the 96-well plates.

2.11.2 Recombinant OPN: Flat-bottom 96-well tissue culture-treated polystyrene microtiter plates (Corning, NY) were coated with 100µl recombinant his-tagged human OPN (5µg/ml) or fibronectin (2.5 µg/ml) in phosphate-buffered saline at 4 °C overnight and then blocked with 1% BSA. MDA-MB-435 cells were trypsinized, then washed twice and re-suspended in Dulbecco's modified Eagle's Medium containing 1mg/ml BSA. Cells (5×10^4) were added to coated wells and allowed to adhere for 1.5 hr. Non-adhered cells were removed, fixed and stained as above. In some experiments, anti-OPN monoclonal antibodies were incubated in OPN-containing wells for 2 hr at room temperature, then washed twice with PBS prior to adding cells. In other experiments, cells were pre-incubated at 37°C with peptides (30 min), receptor-blocking antibodies (1 hr), or hyaluronon (15 min) prior to being added to the wells.

2.11.3 Biotinylated Peptides: Neutra-avidin coated pre-blocked plates were purchased from Pierce Biotech and used to assess cell adhesion to biotinylated peptides.

Biotinylated peptides (100μl of biotinylated peptide solution at 10μg/ml) were coated onto the wells as described by the manufacturer. The wells were then washed twice with PBS. MDA-MB-435 cells were trypsinized and washed as above. Cells ($5x10^4$) were added to the wells and allowed to adhere for 2 hr at 37 ºC in a humidified atmosphere with 5% CO_2. Non-adherent cells were removed by washing twice with 75μl Percoll wash solution and adherent cells were fixed with 50μl fixative (10% glutaraldehyde in Percoll). Fixed cells were stained with 100μl 0.1% crystal violet and solubilized in 50μl 0.5% Triton X-100 before reading at 570 nm in a MRX revelation Reader (Thermo Labsystems, MA).

2.12 Flow Cytometry Peptide Binding Assay

MDA-MB-435 cells were trypsinized, then washed twice and re-suspended in DMEM containing 1 mg/ml BSA. Cells ($5x10^5$) were added to clear polystyrene tubes. Biotinylated peptides were then added to the cells at a final concentration of 50μM. The tubes were then incubated at 37°C in a humidified atmosphere with 5% CO_2 for 30 min. The cells were washed twice by centrifugation in a GPKR swinging-bucket centrifuge (Beckman) at 1500rpm for 5 min and resuspended in PBS containing 1% BSA. The cells were then fixed by resuspension in 500μl cold 1% paraformaldehyde in PBS for 15 min at room temperature. The cells were again washed twice with PBS/1%BSA. Phycoerythrin-conjugated streptavidin (eBioscience) was then added at 0.5μg/ml in 500μl and the cells were incubated at room temperature with shaking at 250rpm for 1 hr. After washing twice with PBS/1%BSA, the cells were resuspended in 1% paraformaldehyde in PBS and analyzed using a FACS Calibur flow cytometer (BD Biosciences).

Chapter 3

Results

3.1 Project I: Variations in Post-Translational Modification of Osteopontin

3.1.1 Historical Background

As described in Chapter 1, OPN protein is highly post-translationally modified, and numerous studies have highlighted the importance of these PTMs in mediating osteopontin's varied functions. In addition, reports characterizing the degree of modification of OPN isolated from various sources have demonstrated that OPN PTMs can vary substantially depending on the source of the cells, tissue or fluid. A prime example of this variation has been described by Dr. J. Sodek and colleagues using diffentiating primary rat osteoblasts (Sodek et al., 1995). These cells, when in culture, go through distinct stages of differentiation towards mature, mineral-producing osteoblasts. They progress through a period of proliferation, followed by a period of extracellular matrix production, and finally mature into osteoblasts capable of mineralizing the resulting extracellular matrix. During these stages OPN possessing various PTMs is produced. However, the predominant form changes during this process. Initially, during the proliferation stage, an OPN that migrates at ~55kDa on SDS-PAGE gels is the major isoform produced. When the cells reach the mineralizing stage of differentiation, a 44kDa OPN molecule is the major species. This 44kDa OPN has been shown to have higher amounts of both phosphate and sulfate modifications when compared to the 55kDa OPN produced by the growing cells. Similar changes in OPN PTMs have been shown in transformed vs. non-transformed rat kidney cells (Nemir et al., 1989).

Although many studies have linked OPN's PTMs to many of its functions, these studies have primarily addressed this through *in vitro* modification of recombinant protein. Typically unmodified bacterially produced OPN is phosphorylated by various commercial enzymes prior to being used in cell-based assays. This approach has the following problems: 1) what is being compared are 'all-or-nothing' conditions of phosphorylation. However, the unphosphorylated state is most likely not physiologically relevant. In addition, the degree and variation in the enzymatically phosphorylated OPN is not known and may be relevant to the assay. 2) Enzymes typically used to phosphorylate OPN *in vitro* may not be the primary enzyme that phosphorylates OPN *in vivo*. The enzymes used most often to modify OPN *in vitro* are the casein kinases 1 & 2. However, the enzyme that is believed to be primarily responsible for phosphorylating OPN - mammary gland casein kinase or Golgi kinase with the primary phosphorylation motif: S-X-E/D/pS (Lasa et al., 1997), has not been highly purified and is not commercially available. One study has circumvented some of these issues by using purified osteoclast tartrate-resistant acid phosphatase (TRAP) to dephosphorylate purified native OPN *in vitro* (Al-Shami et al., 2005). This was a desireable approach because TRAP is postulated to dephosphorylate OPN in bone *in vivo* (Andersson et al., 2003). The authors demonstrated that the degree of phosphorylation of the OPN molecule affected the ability of human choriocarcinoma cells to migrate (Al-Shami et al., 2005). The authors were not able to determine whether the overall degree of phosphorylation or the phosphorylation state of specific amino acids were responsible for the observed effect.

3.1.2 Hypothesis

The primary hypothesis for this project is that differences in the modifications of native OPN from different sources (e.g. proliferating and differentiated osteoblasts) will have an effect on OPN's function by altering the cellular response.

3.1.3 Strategy

The strategy chosen for this project focuses on the availability of essentially unlimited amounts of highly specific anti-OPN monoclonal antibodies that can be used to affinity purify large amounts of OPN from various sources. These monoclonal antibodies were developed in the Denhardt lab by a former graduate student, Dr. Aaron Kowalski (2005). The resulting highly purified native OPN from multiple sources can then be characterized with respect to the degree of PTMs and be used for various cell-based assays. The system initially chosen for purification of different OPN isoforms was the osteoblast system described in section 3.1.1 above. The highly mineralizing pre-osteoblast murine cell line MC3T3-E1 (subclone 4) was chosen due to the ability to differentiate the cells into osteoblasts in culture, the ability of the cells to produce large amounts of OPN when in the differentiated mineralizing state, and ease of use of an established cell line compared to primary cells.

3.1.4 Characterization of the System

The MC3T3-E1 subclone 4 (MC3T3) osteoblast system chosen needed to be examined for OPN production both by undifferentiated proliferating cells and by differentiated mineralizing osteoblastic cells. In addition, Dr. Kowalski had developed two monoclonal antibodies that exhibited strong binding to native murine fibroblast OPN. A determination of which antibody would yield better affinity purification results was

needed. A 6-well plate was seeded with 1×10^5 MC3T3 cells and grown until confluent.

Once confluent, duplicate wells were incubated in DMEM/10%FBS containing 100μg/ml

ascorbic acid and 10mM β-glycerophosphate, 100 μg/ml ascorbic acid only, or medium

without additions (uninduced) with medium changes every 2 days. After 10 days the

wells were switched to serum-free medium overnight to generate conditioned medium

that was then assayed for OPN via SDS-PAGE and western blotting. The potential

antibody candidates 2A1 and 3D9 were assessed. As shown in figure 3 the growth

conditions can be ranked in the following manner with regard to the amount of OPN

produced: ascorbic acid and β-glycerophosphate > ascorbic acid only > uninduced.

Proliferating cells were independently assessed and yielded less OPN than the uninduced

wells above (data not shown). Interestingly, although both 2A1 and 3D9 showed strong

reactivity towards the recombinant mouse GST-OPN control, the 3D9 antibody did not

react with the OPN produced by the MC3T3 cells cultured under any of the conditions

tested.
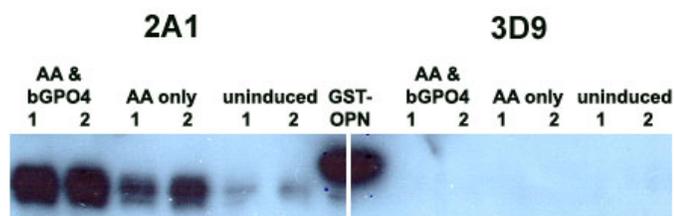
Figure 3: Characterization of the Osteoblast/Antibody System. MC3T3-E1 subclone 4 cells were grown in DMEM/10% FBS containing 100μg/ml ascorbic acid and 10mM β-glycerophosphate (AA & βGPO4), ascorbic acid (AA only), or medium control (uninduced). Ten μl of overnight conditioned media harvested after 10 days growth were assayed via SDS-PAGE and western blotting with anti-OPN monoclonal antibodies 2A1 and 3D9.

3.1.5 Identification of two OPNs Differing in Antibody Reactivity

As mentioned above, Dr. Kowalski had demonstrated that both 2A1 and 3D9 monoclonal antibodies showed very strong reactivity to OPN in medium conditioned by the murine *ras*-transformed fibroblast line (275-3-2). Since the antibodies were generated by immunizing OPN knock-out mice with unmodified recombinant OPN, the above result suggested that the OPN produced by MC3T3 cells may differ in the degree or location of post-translational modifications. The ability of the monoclonal antibodies to bind OPN produced in the conditioned medium generated by these cell lines is shown in figure 4. The MC3T3, 275-3-2 *ras*-transformed fibroblast, and 275 parental non-transformed fibroblast lines were cultured and conditioned media generated as described in chapter 2. Equal amounts of conditioned medium for each cell line was separated via SDS-PAGE and transferred to PVDF membranes. The membranes were then cut into strips which were blotted with the individual antibodies or a polyclonal control.

The OPN produced by the murine *ras*-transformed fibroblast 275-3-2 cell line was recognized by all antibodies assayed. However, only the 2A1 monoclonal antibody was able to bind to OPN produced by the 275 fibroblast line or by differentiating MC3T3 osteoblastic cells. This suggested that there may be substantial differences in post-translational modifications of the OPNs produced by the 275-3-2 and MC3T3 cell lines. These two lines were chosen for immuno-affinity purification of OPN and more detailed characterization.
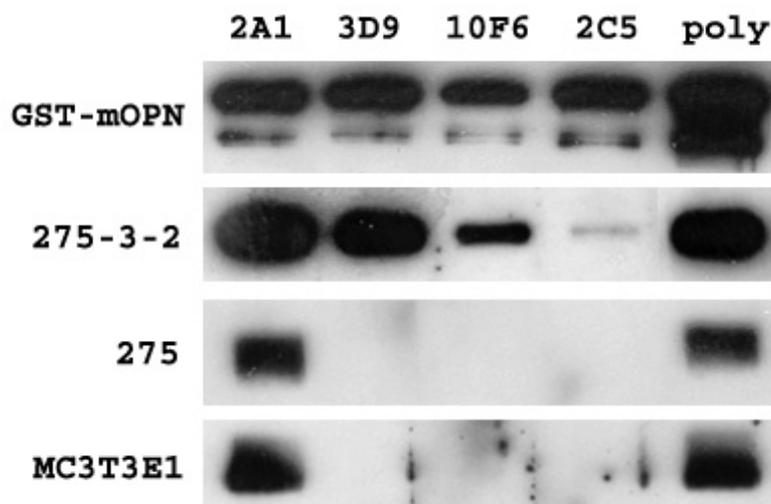
Figure 4: Monoclonal antibody recognition of OPN in conditioned medium. Western blotting results showing antibody recognition of OPN. Conditioned media (10μl/lane) from various cell lines or 50 ng of recombinant murine OPN (GST-mOPN) were separated on 12% tris-glycine polyacrylamide gels and transferred to PVDF membranes. The membranes were then cut into strips which were blotted with monoclonal antibodies at 1μg/ml or polyclonal control at 1:3000 (shown above each lane). 275-3-2: ras-tranformed murine fibroblast cell line. 275: non-transformed murine fibroblast cell line. MC3T3E1: pre-osteoblast cell line induced to differentiate for 12 days as described in materials & methods.

3.1.6 Immuno-Affinity Purification of Osteopontin

Native osteopontin was purified from media conditioned by *ras*-transformed fibroblasts and differentiating MC3T3 osteoblastic cells as using 2A1-coupled protein G-agarose beads as described in chapter 2. Typically 8 10cm plates were used to generate approximately 50ml of conditioned medium for purification. All native OPN preparations were analyzed via western blotting and silver staining. Positive fractions were pooled, desalted on a PD-10 column and quantitated by ELISA. Figure 5 shows silver stain and western blotting results of a typical purification of MC3T3 OPN. The single-step purification procedure yields extremely pure OPN preparations. Typically a smaller ~26kDa C-terminal OPN fragment is also purified because 2A1 binds on the C-terminal side of the thrombin cleavage site. The yield from approximately 50ml of conditioned

medium is typically between 50-100 µg for the MC3T3 cells and 200-300 µg for the 275-

3-2 *ras*-transformed fibroblasts.
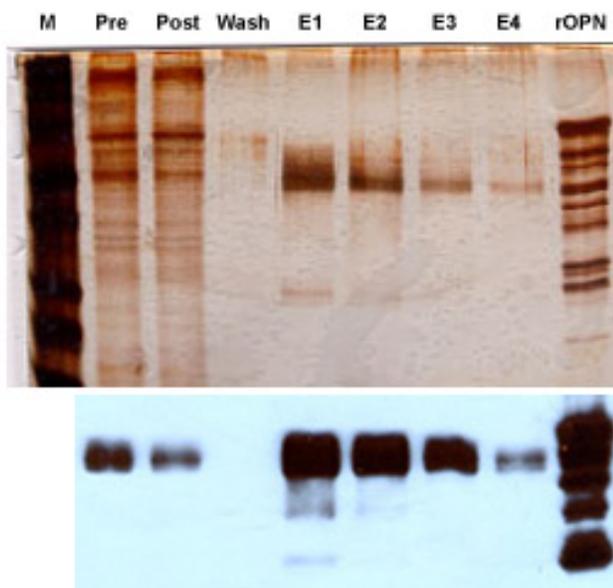
Figure 5: Immuno-affinity purification of native OPN from conditioned medium. Silver stain (top) and western blot results (bottom) of a typical affinity-purification of native OPN from medium conditioned by differentiating MC3T3 osteoblast cells. The MC3T3 cells were grown and differentiated as described in chapter 2. The medium was then changed to serum-free medium overnight. The resulting conditioned medium was incubated overnight with 2A1-coupled protein G-agarose beads. The beads were then washed extensively in PBS and packed into columns for elution. The purified native OPN was eluted with 0.1M glycine, 0.5M NaCl and immediately neutralized. Ten µl of each fraction was then separated in 12% tris-glycine polyacrylamide gels and assayed via non-ammoniacal silver staining and western blot with 2A1 antibody.

While working with OPN produced by the MC3T3 and 275-3-2 cells I noticed that there

was a slight difference in the migration of these two native OPN proteins in 12%

polyacrylamide gels. OPN was purified from both cell lines and compared via silver

staining (figure 6). The 275-3-2 ras-transformed fibroblast produced OPN (FbOPN)

shows an apparent molecular weight of approximately 55 kDa, whereas the MC3T3

produced OPN (ObOPN) migrates at a much smaller apparent molecular weight of 44

kDa. Both preparations contained a smaller molecular weight species believed to be a C-terminal thrombin cleavage fragment of OPN. Interestingly, this fragment also displayed a difference in apparent molecular weight with the FbOPN fragment migrating at approximately 28 kDa and the ObOPN fragment migrating at ~23 kDa.
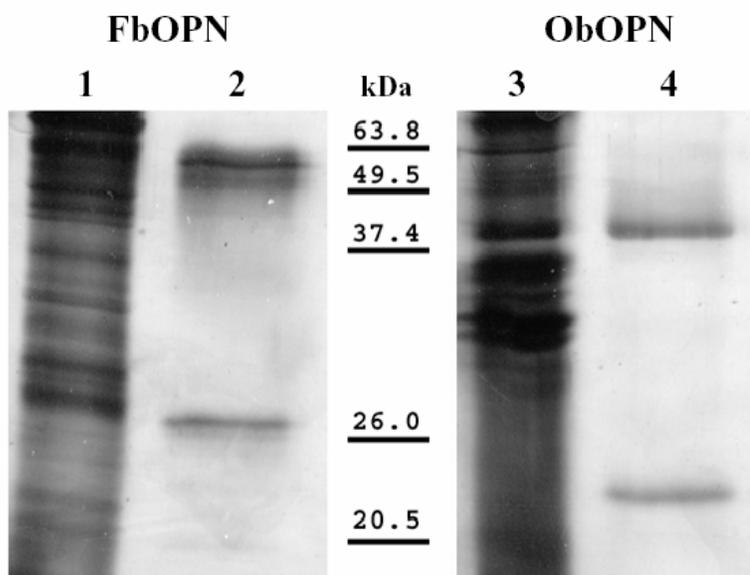
Figure 6: SDS-PAGE of affinity-purified FbOPN and ObOPN. Samples were separated on a 12% tris-glycine gel by SDS-PAGE, and silver stained using the non-ammoniacal procedure. Lane 1: Conditioned medium from 275-3-2 ras-transformed fibroblasts. Lane 2: First elution of FbOPN from 2A1-coupled protein G-agarose column. Lane 3: Conditioned medium from differentiating MC3T3-E1 subclone 4 osteoblasts. Lane 4: First elution of ObOPN from the 2A1-coupled protein G-agarose column. The bands at approximately 21-26 kDa in lane 2 and 4 are believed to represent C-terminal fragments of OPN.

3.1.7. Identification of Cell-Type Specific Differences in Post-Translational Modifications of Native OPN – a collaborative study

The observed difference in apparent molecular weight of the two purified OPNs, as well as the observed differences in monoclonal antibody reactivity described above led us to hypothesize that there were substantial differences in degree and/or type of post-translational modifications of these OPNs. In collaboration with Dr. Esben Sørensen (University of Aarhus, Denmark), we attempted to determine the type and number of

phosphate and glycosyl modifications on each of the two OPNs. Approximately 500 µg of each of the OPNs was generated by performing multiple purifications using the immuno-affinity purification methods described above and used for detailed analysis of the modifications.

In order to determine the molecular weights and amount of phosphate modification of the OPNs in a more precise manner, MALDI-TOF mass spectrometric analysis of the two purified OPNs before and after alkaline phosphatase (ALP) treatment was performed by Brian Christensen, a graduate student in Dr. Sørensen's laboratory. The mass of the fibroblast-produced FbOPN was determined to be 34.9 kDa and the mass of the osteoblast-produced ObOPN was determined to be 35.9 kDa (Figure 7). Also, the broadness of the MS peaks suggests that the modifications of both OPNs are heterogeneous. After ALP treatment the masses of the proteins were determined to be 34.6 for FbOPN and 34.2 kDa for ObOPN respectively (Figure 7). The difference in molecular weight before and after ALP treatment corresponds to a loss of 4 phosphates for FbOPN and approximately 21 phosphates for ObOPN.

The locations of phosphate and glycosylation modifications on the two OPNs were also determined in the Sørensen lab. The OPN proteins were digested with trypsin and separated via reverse-phase high performance liquid chromatography (RP-HPLC). One large fragment not susceptible to trypsin digestion (Gln^{35}/Gln^{54}-Asp^{128}) was separated out by gel filtration and digested with thermolysin. The fragments resulting from this digestion were also separated by RP-HPLC. All fragments from the trypsin and thermolysin digestions were analyzed by MALDI-TOF mass spectrometry and some were additionally analyzed by amino acid sequence analysis. In this manner, many of the

phosphorylation sites could be identified. However, several peptide fragments contained multiple serines and/or threonines such that the phosphorylation sites could not be unambiguously determined. In these cases, the sites of phosphorylations were assigned to residues matching the recognition motif for mammary gland casein kinase, taking into consideration phosphorylation sites previously identified in other species of OPN (Sørensen et al., 1995; Christensen et al., 2005).
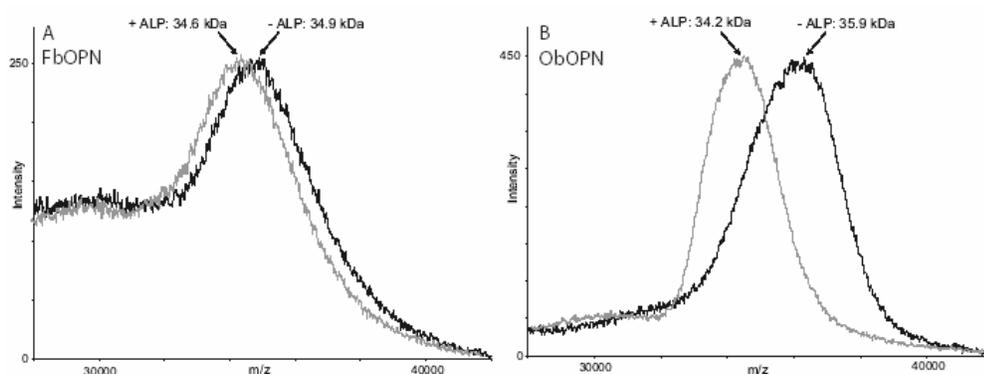
Figure 7: MALDI-TOF mass spectrometric analysis of intact FbOPN and ObOPN.
(A) The average mass peaks at approximately 34.9 kDa (black) and 34.6 kDa (grey) represent FbOPN before and after treatment with ALP, respectively. (B) The average mass peaks at approximately 35.9 kDa (black) and 34.2 kDa (grey) represent ObOPN before and after treatment ALP, respectively.

The amino acid sequence of murine OPN showing the identified modifications on both FbOPN and ObOPN is shown in figure 8. There were 17 sites of phosphorylation identified on the FbOPN and 27 sites of phosphorylation identified on the ObOPN. Analysis of the fragments showed heterogeneity in the phosphorylation of both OPNs. However, the ObOPN is considered to be significantly more phosphorylated *on average* because typically individual fragments from the ObOPN were phosphorylated, whereas the most abundant species of a typical fragment of the FbOPN was predominantly

unphosphorylated or singly phosphorylated (B. Christensen, data not shown). The
residues that differ in phosphorylation state between the OPNs are spread throughout the
molecule, with approximately equal number on both the N-terminal and C-terminal sides
of the thrombin cleavage site. These data represent the sum of all the observed sites of
phosphorylation of each OPN form. Combining these data with the MALDI-TOF mass
spectrometry data from figure 7 we conclude that the FbOPN contains an approximate
average of 4 phosphate modifications spread over the 17 potential sites, and the ObOPN
contains approximately 21 phosphates spread over the 27 potential phosphorylation sites.

Glycosylation of the two OPNs was also analyzed in the Sørensen lab. Five
residues were O-glycosylated in both OPNs: Thr^{107}, Ser^{109}, Thr^{110}, Thr^{116} and Thr^{121}. One
minor difference was that Thr^{107} in ObOPN was only partially glycosylated, it was
observed both with and without the modification (data not shown).

3.1.8 Different Adhesive Properties of OPN Forms

It has been established above that OPN produced by *ras*-transformed fibroblasts
and differentiating osteoblasts differ significantly in the number of phosphate
modifications. However, these data are only mildly interesting unless a functional
consequence of the observed differences can be established. Thus, I set out to determine
if the FbOPN and ObOPN produce different results in various functional assays. One of
OPN's most basic functions is as a cell adhesion molecule, supporting adhesion via its
RGD motif, the cryptic SVVYGLR sequence exposed after cleavage of the molecule by
thrombin, as well as unidentified CD44 adhesion sites.

```
         P PP                                              P P
Ob   LPVKVTDSGSSEEKLYSLHPDPIATWLVPDPSQKQNLLAPQNAVSSEEKD        50
          P PP                                             P P
Fb   LPVKVTDSGSSEEKLYSLHPDPIATWLVPDPSQKQNLLAPQNAVSSEEKD

              P P  P                        P   P   P   P   P   P
Ob   DFKQETLPSNSNESHDHMDDDDDDDDDDGDHAESEDSVDSDESDESHHSD        100
                P P                        P   P
Fb   DFKQETLPSNSNESHDHMDDDDDDDDDDGDHAESEDSVDSDESDESHHSD

          P     ◇ ♦♦       ♦      ♦                          P
Ob   ESDETVTASTQADTFTPIVPTVDVPNGRGDSLAYGLRSKSRSFQVSDEQY        150
          P     ♦ ♦♦       ♦      ♦                          P
Fb   ESDETVTASTQADTFTPIVPTVDVPNGRGDSLAYGLRSKSRSFQVSDEQY

           P            P       P
Ob   PDATDEDLTSHMKSGESKESLDVIPVAQLLSMPSDQDNNGKGSHESSQLD        200
                        P   P
Fb   PDATDEDLTSHMKSGESKESLDVIPVAQLLSMPSDQDNNGKGSHESSQLD

           P          P   P   P                   P
Ob   EPSLETHRLEHSKESQESADQSDVIDSQASSKASLEHQSHKFHSHKDKLV        250
                      P   P   P
Fb   EPSLETHRLEHSKESQESADQSDVIDSQASSKASLEHQSHKFHSHKDKLV

                      P       P P
Ob   LDPKSKEDDRYLKFRISHELESSSSEVN                             278
                      P
Fb   LDPKSKEDDRYLKFRISHELESSSSEVN
```

Figure 8. **Localization of PTMs in FbOPN and ObOPN**. Localization of posttranslational modifications in OPN produced by *ras*-transformed fibroblasts (FbOPN) and differentiating osteoblasts (ObOPN). The murine OPN amino acid sequence is displayed. Phosphorylations are indicated with P's. Glycosylations are indicated with filled diamonds. The half filled diamond represents a variable glycosylated site in ObOPN. Figure provided by Brian Christensen. Data generated in collaboration with Dr. Esben Sørensen.

The ability of the FbOPN and ObOPN to support adhesion of various cell lines was determined using 96-well cell adhesion assays. Adhesion to OPN was compared to adhesion to fibronectin (a positive control), which was set at 100%. The human breast cancer cell line MDA-MB-435 showed significant adhesion to FbOPN (78%), however much less adhesion was observed to the more highly phosphorylated ObOPN (21.5%) (Figure 9). In contrast, the murine *ras*-transformed fibroblast line 275-3-2 showed a significant increase (~36%) in adhesion to the ObOPN compared to the FbOPN (Figure 9). Adhesion to a recombinant human OPN was the same for both cell lines, although the MDA-MB-435 cell line showed better adhesion to the recombinant protein than to either of the native OPNs.
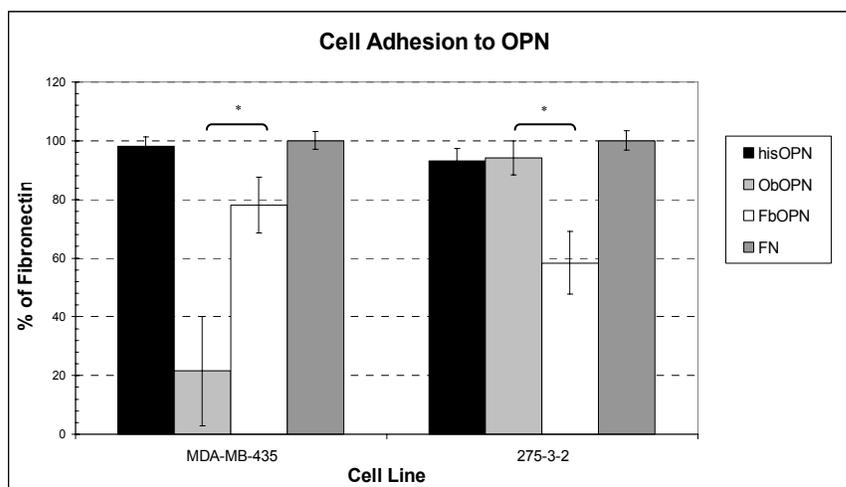
Figure 9: Cell Adhesion to OPN from various sources. OPN purified from various sources was coated onto 96-well plates at 10µg/ml and blocked with 1% BSA. Cells (5x10^4) MDA-MB-435 human breast cancer cells or 275-3-2 *ras*-transformed fibroblasts were added to the wells and allowed to adhere for 16 hr and 3.5 hr respectively. Nonadherent cells were washed away and the adherent cells fixed and stained with crystal violet as described in materials and methods. Data are expressed as % relative to fibronectin positive control, which was set to 100% after adhesion to BSA (negative control) was subtracted out. The chart represents the combined data from 5 independent experiments with n=5/exp (2 experiments for hisOPN). hisOPN – recombinant human his-tagged OPN, ObOPN – native murine OPN purified from differentiating MC3T3 osteoblasts, FbOPN – native murine OPN purified from 275-3-2 *ras*-transformed fibroblasts, FN – fibronectin. *, p < 0.001, Students *t* test.
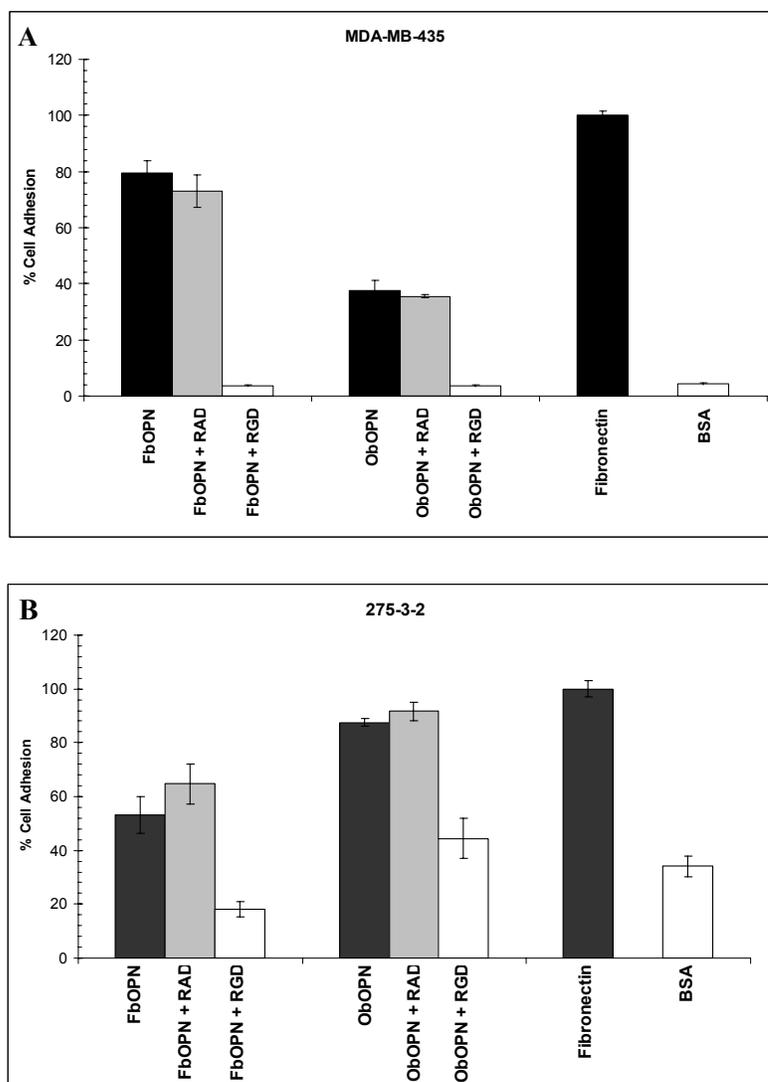
Figure 10: Peptide inhibition of adhesion of cells to OPN. Adhesion of MDA-MB-435 (A) and 275-3-2 (B) cells to surfaces coated with the specified proteins. OPN isoforms were coated at 10μg/ml. Cells were pre-incubated in the presence or absence of 100μM GRGDNP (RGD) or GRADSP (RAD) peptides. Cell adhesion to positive control fibronectin was set to 100% and 1% BSA was used as negative control. Numbers of attached cells were measured as described in Experimental Procedures. Bars show mean values for four wells per protein ± S.D. Data shown are representative of three independent experiments for each cell line (n=4/exp).

3.1.9. Inhibition of Adhesion to OPN by RGD Peptides

Adhesion to OPN is mediated by a number of integrins, as well as certain variants

of the CD44 receptor. Since the cell lines tested each displayed greater adhesion to a

different OPN, the observed differences in adhesion of the cell lines examined to the OPNs may be partially due to the receptor repertoire expressed by the cells. In order to determine the receptors that may be involved in the adhesion to OPN, adhesion assays were conducted using synthetic peptides to inhibit integrin binding. Cells were preincubated with GRDGNP (RGD) or GRADSP (RAD) control peptide for 30 min. prior to adding to the wells coated with OPN. As shown in figure 10A, the adhesion of the MDA-MB-435 cells was completely inhibited by the addition of the RGD peptides, but not by the control RAD peptide. This indicated that the adhesion to OPN by this cell line is predominantly through integrins binding the RGD motif of OPN. The 275-3-2 cell line (figure 10B) was also inhibited by the RGD peptide. Adhesion of 275-3-2 cells to OPN in the presence of RGD peptide was decreased by approximately 85-90%.

3.2 Project II: Characterization of Anti-OPN Monoclonal Antibodies and Inhibition of Cell Adhesion

3.2.1 Historical Background

It has been shown above that the anti-OPN monoclonal antibodies generated by Dr. Kowalski exhibit differences in their ability to bind murine OPN from different sources (figure 4). These antibodies were shown by Dr. Kowalski to also recognize human recombinant OPN and native OPN from a cell line engineered to express high levels of OPN via western blotting. However, few of the antibodies were able to recognize purified human milk OPN, which is very highly phosphorylated (Christensen et al., 2005). These results suggest that modification of OPN (primarily phosphorylation) may inhibit the binding of some of the monoclonal antibodies.

Dr. Kowalski also showed in a preliminary experiment that some of the antibodies were able to inhibit cell adhesion to OPN (Kowalski, 2005). The antibody showing the greatest inhibition of adhesion, 1H3, was not well characterized with respect to its recognition site on OPN. Other antibodies (2C5, 3D9, & 7B4) were observed to slightly inhibit adhesion, and their binding sites were well-characterized. The binding sites of two of these antibodies (3D9 and 7B4) were located in same region of OPN – the C-terminal region. This was extremely interesting because this region is located far from the RGDSVVYGLR integrin binding area on OPN, and also because the C-terminal half of the OPN molecule has been associated with CD44 binding (Katagiri et al., 1996; Weber et al., 1996). However the binding region of CD44 to the C-terminal half of OPN was not localized. All the antibodies mentioned above, 1H3, 3D9, and 7B4, bind in the C-terminal half of OPN and may be useful to determine the CD44-binding region.

3.2.2 Hypotheses

The first hypothesis of this project is that modification of OPN is blocking the binding of certain anti-OPN monoclonal antibodies to OPN. The second hypothesis is that the C-terminal binding antibodies may be inhibiting the adhesion of cells to OPN by blocking CD44 binding.

3.2.3 Strategy

The goals of this project were to further characterize the monoclonal antibodies with respect to their recognition sites and binding characteristics, and in the case of certain antibodies, to prove that phosphorylation blocks antibody binding to OPN. A second goal of this project was to examine in further detail the preliminary result that

certain antibodies were able to inhibit adhesion of cells to OPN and possibly determine the receptors or mechanisms involved.

3.2.4 Antibody Recognition of Human OPN

The observation that the ability of specific monoclonal antibodies to bind to murine OPN in a western blot varied depending on the source of OPN prompted us to determine whether this same phenomenon occurred when human OPN was used. All antibodies had previously been shown to bind well to recombinant human his-tagged OPN (hisOPN). However, only 2A1 was able to bind to purified highly modified human milk OPN (generously supplied by Dr. Esben Sørensen) (data not shown). Another easily attainable source of human OPN is from urine. Figure 11 shows western blot results using the monoclonal antibodies to detect OPN in the equivalent of 50µl of urine in each lane. The antibody showing the most intense binding to all four bands of OPN typically observed was 1H3. 10F6 was also able to recognize all 4 bands of OPN, as was the two different polyclonal antibodies tested. Interestingly, 3D9 was only able to recognize the top two bands of OPN, whereas 2A1 and 2C5 were not able to bind urine OPN at all. It is not surprising that 2C5 does not bind this OPN, as it displays very weak binding to all native OPNs examined to date and its binding site is located in a region that has many glycosylation sites. 3D9 binds the C-terminal region of OPN, and has been shown to in figure 4 to differ in its binding to murine OPN from different sources which are differentially phosphorylated in this region. The lower 2 bands not recognized by 3D9 may be truncated fragments of OPN missing the C-terminal region, or may be phosphorylated in the 3D9 binding region which could block binding. The most intriguing result is that 2A1 is not able to bind the urine OPN. This is somewhat

unexplainable as the region of OPN where 2A1 binds is relatively devoid of post-

translational modifications. The approximately 30kDa protein is a non-specific band later

determined to be cross-reacting with the goat anti-mouse secondary antibody used. This

is why this band does not appear in the LF124 lane, which requires a goat anti-rat

secondary antibody.
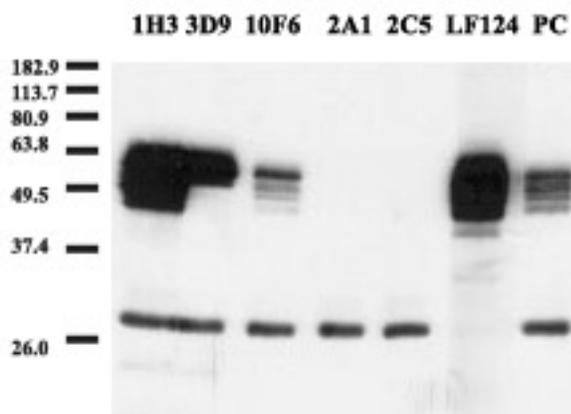
Figure 11: Western blot of human urine with anti-OPN monoclonal antibodies. Urine was collected and dialyzed extensively against 0.1M NaCl before approximately 10-fold concentration with Centriprep spin columns. Five μl of the concentrated, dialyzed urine was assayed via SDS-PAGE and western blotting with monoclonal antibodies 1H3, 3D9, 10F6, 2A1, and 2C5 at 1μg/ml or polyclonal antibodies LF124 and anti-OPN mouse serum (PC) at 1:750 and 1:3000 respectively.

3.2.5 Phosphorylation of OPN Blocks 3D9 Monoclonal Antibody Binding

The results in figure 4 showing that anti-OPN antibody 3D9 is able to recognize

the weakly modified FbOPN, but not the highly modified ObOPN strongly suggest that

phosphorylation of OPN in the 3D9 epitope can block its binding. This needed to be

definitively proven. Phosphorylated and unphosphorylated biotinylated peptides

corresponding to region where the 3D9 epitope on OPN was mapped (figure 12A) were

kindly synthesized by Dr. L. Steinman (Stanford University). The 3D9 antibody was used

to detect these peptides coated onto Neutra-avidin-coated plates. At all concentrations of

peptide tested, the signals generated in wells containing the unphosphorylated peptide (mOPN26) were significantly greater than from wells containing the phosphorylated peptide (mOPN26P) (figure 12B). The only difference between these two peptides is the three phosphorylated serines determined to be potential sites of phosphorylation of ObOPN (figure 12A). It was previously determined that 3D9 was able to bind the human peptide hOPN19, which was used as a positive control in this assay.

(A)

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mOPN26:      D-R-Y-K-F-R-I-S-H-E-L-E-S-S-S-S-E-V-N-G-G-(CH₂)-Biotin
mOPN26P:     D-R-Y-K-F-R-IpS-H-E-L-EpS-SpS-S-E-V-N-G-G-(CH₂)-Biotin
```

(B)
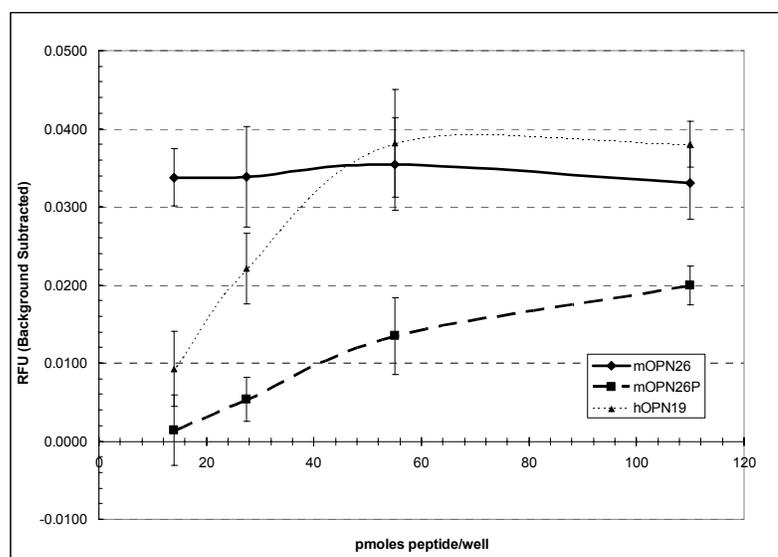
Figure 12: Phosphorylation blocks antibody 3D9 binding. (A) Amino acid sequences of the peptides used for the antibody-peptide binding assay kindly provided by Dr. Larry Steinman. (B) Binding of antibody AK3D9 to phosphorylated (mOPN26P) and non-phosphorylated (mOPN26) peptides at various concentrations. The human peptide sequence (hOPN19) was used as a positive control. The biotinylated peptides were coated onto Neutra-avidin plates at the indicated concentrations and detected with 1ug/ml 3D9 monoclonal antibody following the manufacturer's instructions (Pierce Biotech). The secondary antibody used was Alexafluor 594 and fluorescence was detected using excitation/emission wavelengths of 584/612nm. Data shown are combined from two independent experiments (n=4/exp).

3.2.6 Antibodies 3D9 and 7B4 Inhibit Cell Adhesion to OPN

Previous results by Dr. Kowalski had shown that some of the monoclonal

antibodies could inhibit adhesion of cells to OPN. This result was reexamined using the

MDA-MB-435 and 275-3-2 cell lines commonly used in this study and conducting

adhesion assays as previously described. Antibodies were added to wells pre-coated with

human recombinant his-tagged OPN (hisOPN) and allowed to bind OPN. The wells were

then washed prior to adding the cells. The ability of the antibodies to block adhesion of

the cells to hisOPN was assessed by comparing the adhesion to that in wells blocked with

non-specific mouse IgG (figure 13). The results show that for both cell lines examined,

the 3D9 and 7B4 antibodies are able to inhibit cell adhesion by approximately 40-50%.

Since the OPN used to coat the plate was human recombinant OPN, the human breast

cancer cell line MDA-MB-435 cell line was chosen for further study to avoid the

possibility of species specific differences affecting our conclusions.

These results are slightly different than those described by Dr. Kowalski, as his

results showed antibodies 1H3 and 2C5 to be the most effective, with only a slight

inhibition by antibodies 3D9 or 7B4. In the course of completing these experiments, it

was discovered that the characteristics of antibody 1H3 had changed. Verification of the

of the 1H3 epitope mapped by Dr. Kowalski using human OPN peptides revealed that the

hybridoma line was secreting antibodies able to recognize two distinct areas on OPN

(data not shown). The 1H3 hybridoma line was subsequently dilution cloned and

individual clones were epitope mapped using the synthetic OPN peptides. One clone –

1H3F7 displayed a binding site closer to the RGDSVVYGLR integrin binding area of

OPN and was able to inhibit adhesion of MDA-MB-435 cells approximately 50% (data

not shown). His data also used a different cell line which might also account for some of

the observed differences.
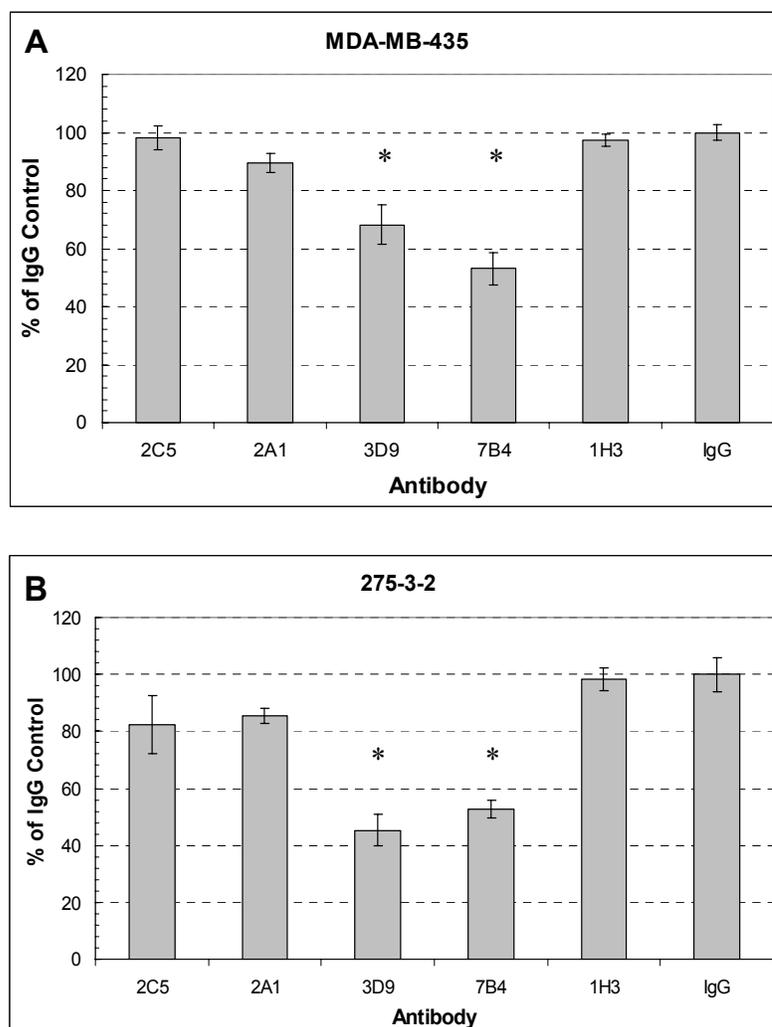
Figure 13: Antibody inhibition of cell adhesion to recombinant human OPN. Tissue culture treated 96-well plates were coated with 150μM human recombinant his-tagged OPN, then blocked with 1% BSA. Antibodies were then added at 125 μM and allowed to bind OPN for 2 hr. The wells were then washed and 5×10^4 MDA-MB-435 (A) or 275-3-2 (B) cells were added and allowed to adhere for 3 or 3.5 hr respectively. Non-adherent cells were removed by washing and adherent cells were quantitated by staining with crystal violet as described in materials and methods. Data are representative of 4 independent experiments for the MDA-MB-435 cell line and 2 independent experiments for the 275-3-2 cell line (n=4). *, p<0.001 Student's *t* test

In order to further characterize the inhibition of cell adhesion by antibody 3D9, a

dose response experiment was performed. Wells were coated with 5μg/ml (150 nM)

hisOPN, blocked with BSA, and the OPN blocked with varying amounts of 3D9 antibody or IgG control. Figure 14 shows that the maximum inhibition achievable in this experiment was approximately 40% and this occurred between 31 and 62 nM antibody. Similar results were obtained with the 275-3-2 *ras*-transformed fibroblast line (data not shown). The concentration of antibody required for maximum inhibition is significantly less than the amount of OPN originally applied to the wells, however this OPN concentration is most likely saturating and the amount of OPN actually bound to the wells is unknown.
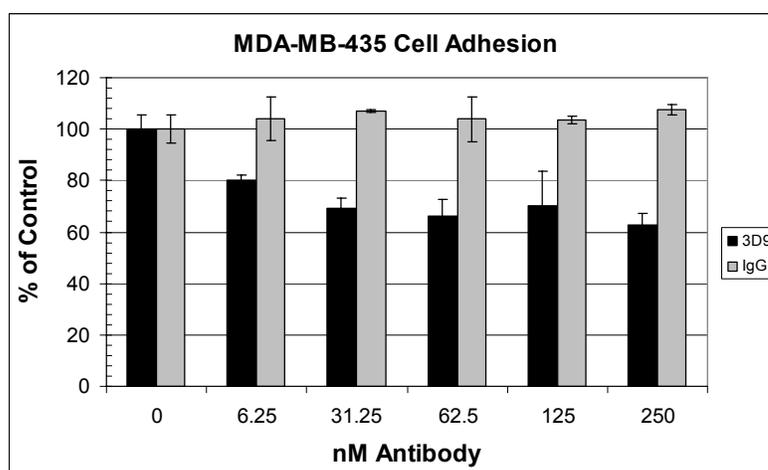
Figure 14: Dose response of 3D9 inhibition of cell adhesion. Tissue culture-treated 96-well plates were coated with 150 nM human recombinant his-tagged OPN, then blocked with 1% BSA. 3D9 or control IgG antibodies were then added at the indicated concentrations and allowed to bind OPN for 1 hr. The wells were then washed and 5×10^4 MDA-MB-435 cells were added and allowed to adhere. Non-adherent cells were removed by washing and adherent cells were quantitated by staining with crystal violet as described in materials and methods. Data are representative of 2 independent experiments (n=3).

3.2.7 Cell Adhesion to Synthetic OPN Peptides

The antibodies 3D9 and 7B4 both recognize the extreme C-terminal region of OPN, an area that has not been directly shown to have a role in mediating adhesion to

OPN. Adhesion to the entire C-terminal thrombin fragment of OPN has been

demonstrated, but the binding site was not further localized (Weber et al., 1996). My

results here indicate that the adhesion observed by others may be occurring at the extreme

C-terminal region where antibodies 3D9 and 7B4 bind OPN. I examined cell adhesion to

synthetic biotinylated human OPN peptides. The biotinylated C-terminal peptides

hOPN20 and hOPN19, which is shifted 7 amino acids towards the N-terminus, were used

to coat wells of 96-well Neutra-avidin plates. Positive control peptides hOPN10,

containing both the RGD and SVVYGLR integrin binding sites and hOPN11, containing

only the SVVYGLR integrin site were also used, as well as two other OPN peptides

randomly chosen as negative controls (hOPN4 and hOPN12).
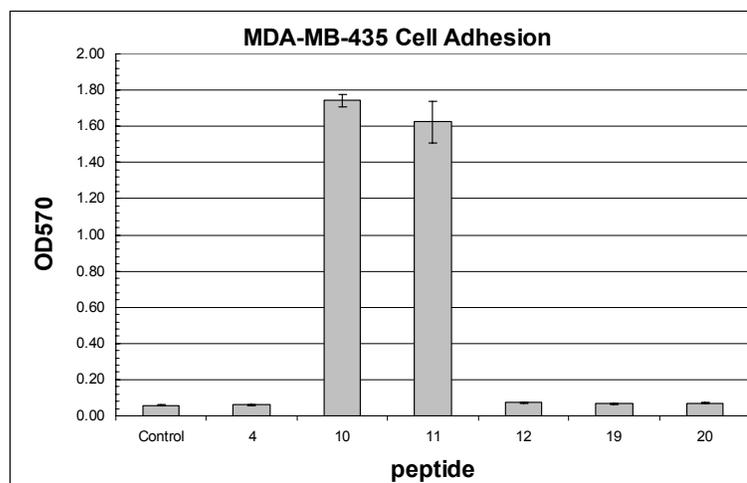
Figure 15: Cell adhesion to synthetic human OPN peptides. Biotinylated human peptides were coated onto Neutra-avidin plates at 10 μg/ml. Cells ($5x10^4$) MDA-MB-435 cells were added to each well and allowed to adhere for 2 hr. Non-adherent cells were removed by washing and adherent cells were fixed and quantitated by staining with crystal violet as described in materials and methods. Data are representative of two independent experiments.

The MDA-MB-435 cells were only able to adhere to the two peptides containing

integrin binding sites (hOPN10 & 11) confirming that adhesion can occur to peptides

attached to the plate via a C-terminal biotin-avidin interaction. No adhesion was observed to either of the C-terminal peptides hOPN19 or 20. This may be due to the design of the peptides, that the full binding region is not contained within the 18 amino acids of the peptides, or that the binding region is not exactly where the 3D9 and 7B4 antibodies bind.

3.2.8 Flow Cytometric Analysis of Peptide Binding

In order to determine whether a direct interaction was occurring in the region implicated by our inhibition results using the 3D9 and 7B4 antibodies, we attempted to assay cellular binding of the synthetic human peptides via flow cyometry as described in the materials and methods section. Trypsinized and washed MDA-MB-435 cells were incubated in the presence of 50 μM peptides for 30 min at 37°C in round bottom tubes, washed twice with PBS, then fixed with cold 3% paraformaldehyde in PBS. The ability of the cells to bind the peptides was assessed by using phycoerythrin-conjugated streptavidin (SA-PE) to detect bound biotinylated peptide, followed by flow cytometry. Initial experiments compared fixing the cells after the initial peptide incubation and washes versus fixing the cells after the addition of the SA-PE and subsequent washing. Decreased binding of all peptides was observed when the fixing was done at the end of the procedure (data not shown).

Very strong binding as indicated by a rightward shift of the peak in figure 16A was observed to positive control peptide hOPN11, verifying that this procedure is able to detect cell binding of peptides. Weaker, yet significant shifts were observed for both C-terminal peptides examined – hOPN19 and hOPN20, with 61% and 64% of cells respectively, located within the positive gate indicated by "M1" in figure 16A. Negative control hOPN4 did display some weak interaction with 10% positive cells and hOPN12

showed no binding. Interestingly, peptide hOPN10, which contains both the RGD and

SVVYGLR binding sites, did not show any binding in this assay (data not shown). These

data strongly suggest that a direct interaction is occurring between the C-terminal region

of OPN bound by antibodies 3D9 and 7B4 and an unknown receptor on the MDA-MB-

435 cells. The direct peptide adhesion study above (figure 15), as well as the loss of

binding if the peptides and cells are not fixed immediately after washing the cells to

remove unbound peptide, suggest that this interaction is a transient signaling one rather

than an adhesive interaction. The region of amino acid overlap (HLKFRISHELD)

between the two C-terminal peptides is very likely to be where this interaction is

occurring.

3.2.9 Peptide Inhibition of Cell Adhesion

My next experiment was to assess how occupying cell receptors with the C-

terminal peptides would affect the ability of the cells to adhere to OPN in the established

adhesion assay. MDA-MB-435 cells were preincubated with 50 μM peptides prior to

being added to hisOPN coated 96-well plates in a similar manner to figure 10 above

using RGD peptides. Cell adhesion assays were then carried out as described above. The

two peptides containing integrin binding motifs (hOPN10 & 11) were able to inhibit cell

adhesion to OPN by approximately 40% (Figure 17). The two C-terminal peptides

hOPN19 and 20 only showed slight 16% and 9% decreases in adhesion, respectively. The

decrease observed for hOPN19 or 20 is not considered significant since the pre-treatment
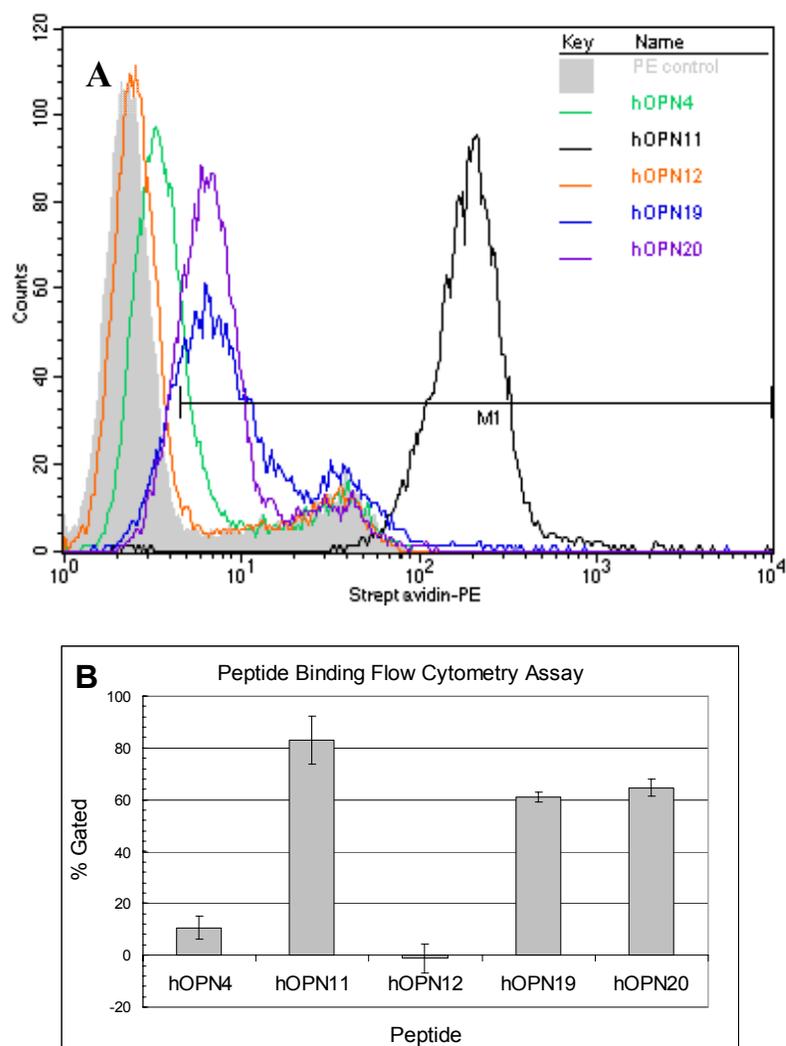
Figure 16: Flow cytometric analysis of peptide binding. (A) Flow Cytometry results of peptide binding assay. Biotinylated peptides were incubated with MDA-MB-435 cells for 30 min at 37°C, washed and fixed with paraformaldehyde. The cells were then incubated with phycoerythrin-conjugated streptavidin and analyzed using a FACSCalibur flow cytometer. (B) Percentage of cells within the gate (considered positive) designated with an M1 in (A) after subtracting out the percentage of positive cells from the unstained sample. Data presented are the mean ± S.D. of two independent experiments.

of cells with negative control peptides hOPN4 & 12 resulted in similar decreases in cell adhesion. The results in this assay were highly variable, with some experiments resulting in a statistically significant difference for hOPN19, while other experiments did not reproduce the observation. The seemly transient nature of the peptide-receptor interaction

measured in the flow cytometry assay above may limit the effectiveness of the pretreatment of cells with peptides in this assay. Also, it is unknown whether peptide binding would block the receptor without subsequent activation of signaling pathways normally activated by engagement of OPN.

3.2.10 Receptor Blocking Cell Adhesion Assays

Numerous attempts to block the CD44 receptor on the cell surface of MDA-MB-435 cells prior to conducting cell adhesion assays were also carried out. Pre-incubation of the cells with hyaluronan at 500μg/ml was able to inhibit cell adhesion by approximately 35%, but this concentration was extremely high and required the addition of a large volume of solution to the cells (data not shown). In addition, CD44-blocking antibodies were used with both the MDA-MB-435 and 275-3-2 cell lines. The CD44-blocking antibody A3 has been shown to inhibit hyaluronan binding by CD44 (Dr. M-S. Sy, personal communication), but had no effect on the adhesion of MDA-MB-435 cells. Neither did the anti-CD44v6 antibody (clone 2F10) which was shown to block an OPN-CD44 interaction (Lee et al., 2007)(Figure 18) . The CD44-blocking antibody KM114 was also unable to block the adhesion of 275-3-2 cells to hisOPN (data not shown).

The RDG-containing peptide was also tested with the MDA-MB-435 cells and was able to inhibit approximately 80% of cell adhesion (data not shown).The $\alpha_v\beta_3$-blocking antibody LM609 blocked all adhesion, indicating that the $\alpha_v\beta_3$ integrins is the primary receptor mediating binding to the recombinant human OPN used in these assays.
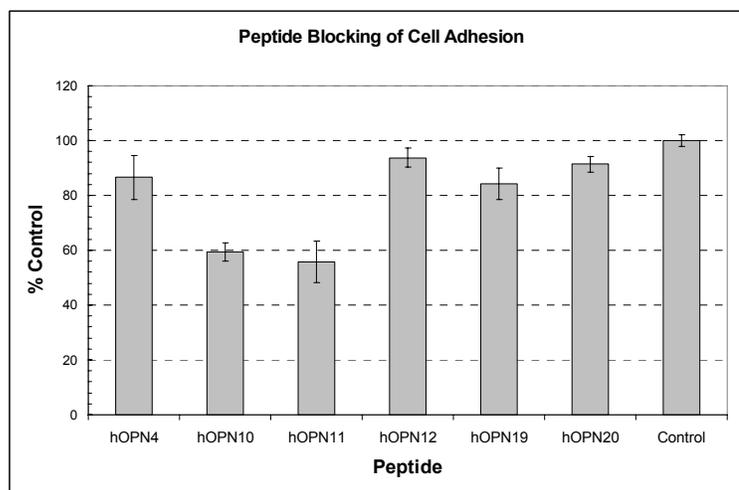
Figure 17: Peptide blocking of cell adhesion. The indicated peptides (50μM) were pre-incubated with MDA-MB-435 cells for 30 min at 37°C prior to the addition of $5x10^4$ cells to 96-well plate wells coated with 5μg/ml recombinant human OPN. The cells were allowed to adhere for 1.5 hr, and non-adherent cells were removed by washing. The remaining adherent cells were fixed and stained with crystal violet as described in materials and methods. Data shown are combined from three independent experiments (n=12).
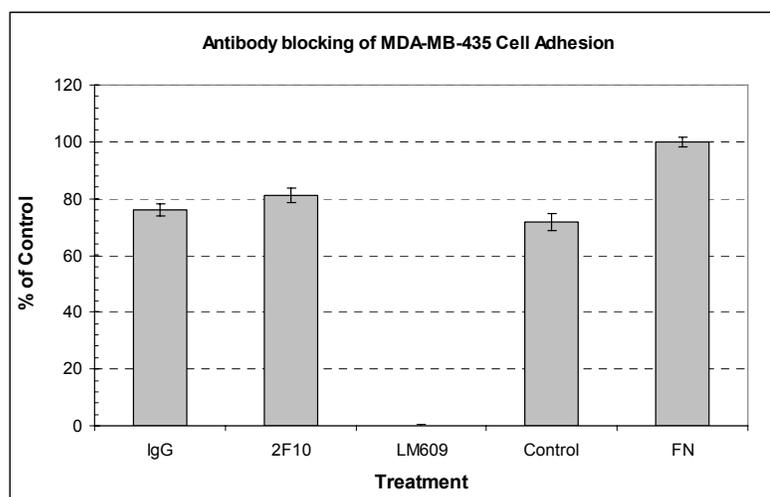
Figure 18: Antibody blocking of cell adhesion. MDA-MB-435 cells were pre-incubated with control IgG, anti-CD44v6 antibody (2F10), or anti-$\alpha_v\beta_3$antibody (LM609) for 1 hr at 37°C prior to the addition of $5x10^4$ cells to 96-well plate wells coated with 5μg/ml recombinant human OPN. The cells were allowed to adhere for 1.5 hr, and non-adherent cells were removed by washing. The remaining adherent cells were fixed and stained with crystal violet as described in materials and methods. Data shown is representative of two independent experiments (n=4/exp).

Chapter 4

Discussion

4.1 Project 1

4.1.1 Rationale

Osteopontin is expressed by a large, diverse group of cell types and has been associated with a wide variety of functions. Researchers are continuing to expand OPN's involvement in many processes and disease states, particularly those involving injury, inflammation and auto-immune diseases. OPN is also highly post-translationally modified, and these modifications are often required for OPN to carry out many of its functions. Many of these studies have used OPN that has been enzymatically altered *in vitro* to add phosphate groups to recombinant OPN or to remove phosphates from native OPN.

Post-translational modifications of OPN purified from various species have been characterized, although they have been from only two sources – milk and bone, due to the abundance of OPN in this fluid and tissue. As a result, OPN from these sources is typically used experimentally as native OPN. As a result, little is known of the differences in PTMs of OPN expressed by other cell types and whether potential cell-type specific differences can alter OPN's functions. My hypothesis was that OPN expressed by different cell types could differ in PTM status and that these differences could in turn modulate OPNs function or the cellular response to OPN. This hypothesis stemmed from the observations that OPN's PTM state was different in proliferating vs. mineralizing osteoblasts as well as normal and transformed kidney cells (Nemir et al., 1989; Sodek et

al., 1995), that vitamin D3 treatment altered the apparent pI of OPN (Safran et al., 1998), presumably by altering it's PTMs, and most importantly that tumor-produced OPN and macrophage OPN seemed to have different functions in an *in vivo* study (Crawford et al., 1998). The latter observation is a prime example of a situation where OPN from multiple sources can coexist. In tumors, OPN can be expressed by the tumor cells themselves, the surrounding host tissue, and infiltrating macrophages and lymphocytes. OPN's effects on these cells differ and can be contradictory – does OPN increase metastasis or help attract macrophages? Does OPN increase the activation of infiltrating immune cells or decrease their nitric oxide production and cytotoxicity? My hypothesis suggests that differences in the PTM state of the OPN produced by these various cell types, in addition to cell receptor repertoire, play a role in determining the effects of OPN signaling, modulating function or the degree of response in a given cell type. According to the example above, tumor-derived OPN would decrease the production of the cytotoxic molecule nitric oxide by macrophages, whereas macrophage OPN would serve as a more effective chemoattractant and activate T-lymphocytes to attack the tumor cells. The goal of my research was to purify OPN expressed by proliferating, undifferentiated osteoblasts, and mineralizing, differentiated osteoblasts and identify post-translational differences between the two OPN proteins. Cellular responses to these OPNs would then be examined.

The Denhardt laboratory was well-equipped to begin to test my hypothesis. Previous work by Dr. Kowalski had generated highly specific anti-OPN monoclonal antibody-producing hybridoma cell lines which could be used to generate large quantities of antibodies for subsequent immuno-affinity purification OPN. These antibodies were

raised against the recombinant OPN molecule utilizing the OPN-KO mouse. As a result, some of these antibodies were determined to be sensitive to post-translational modifications of the amino acids in the antibody-binding (epitope) region. For example the antibody 3D9 bound well to recombinant bovine OPN, but did not recognize the highly-modified bovine milk OPN. These antibodies could also be used as an initial screen to determine if differences in PTMs at specific sites exist.

4.1.2 Identification of Two Forms of OPN

Initial characterization of the chosen system did not yield the same differences in OPN mobility described by Dr. Sodek and colleagues. His group determined that proliferating osteoblasts expressed a 55-kDa form of OPN and mineralizing osteoblasts produced a 44-kDa form (Sodek et al., 1995). Proliferating or undifferentiated confluent cells of the MC3T3-E1 subclone 4 cell line did not produce large amounts of OPN. Also, the little OPN that was produced did not vary in apparent mobility in 12% SDS-PAGE gels as a function of differentiation state (figure 3). These differences are most likely due to differences in the cells used (MC3T3 vs primary rat calvarial cells) and the fact that the Sodek group used a radioactive label which can be more sensitive, especially in detecting the small amount of OPN produced by proliferating cells.

These initial experiments did determine that the antibody binding of the MC3T3 OPN differed from another murine cell line typically used by Dr. Kowalski – the *ras*-transformed cell line 275-3-2 (figure 3). This strongly suggested that there were differences in the post-translational modifications of OPN expressed by the cell lines tested. Interestingly, similar differences were observed when the *ras*-transformed 275-3-2 cell line was compared to the parent non-transformed fibroblast line (275) (figure 4). This

cell line's antibody reactivity was very similar to that of the MC3T3 line, suggesting that the observed decrease in PTMs could be a result of *ras*-transformation. This is not a novel idea as differences in OPN isoform expression between normal and transformed rat kidney cells have been described (Nemir et al., 1989). Additionally, OPN produced by other non-transformed cell lines such as the myogenic C2C12 cell line and NIH3T3 fibroblasts was undetectable when assayed using the 3D9 antibody. However, the PAP2 cell line, a *ras*-transformed subline of the NIH3T3 fibroblast cell line did show 3D9 reactivity (personal observation, data not shown), further supporting the hypothesis that *ras*-transformation causes a reduction in phosphorylation of OPN.

4.1.3 Cell-Type Specific Differences in Post-translational Modifications of Native OPN

In collaboration with Dr. Esben Sørensen (University of Aarhus, Denmark), the sites of PTMs of OPN expressed by MC3T3 (ObOPN) and 275-3-2 (FbOPN) cell lines were identified. This marks the first detailed characterization of post-translational modifications of murine-produced OPN, as well as OPN produced by a transformed "tumor-like" cell line. In addition, this is the first extensive characterization of differences in phosphorylation of OPN produced by two different cell lines of the same species. There was a dramatic difference in the degree of phosphorylation of OPN between the two cell lines. Analysis of proteolytic fragments of OPN identified 27 sites of phosphorylation for the ObOPN, but only 17 for the FbOPN. These numbers represent the total number of phosphorylation sites observed in a heterogeneous population of each OPN isoform. Any given OPN molecule will have a subset of these sites phosphorylated. The actual difference in phosphorylation is likely to be even greater since alkaline

phosphatase (ALP) treatment of the OPNs removed ~21 phosphates from the ObOPN and only ~4 from the FbOPN (Figure 7). It should be noted that ALP likely does not remove all phosphates from the molecules, so these numbers are only partially representative of the actual number of phosphates per molecule. However this greater difference is further supported by the fact that ObOPN peptides were most often identified to be fully phosphorylated, whereas for the FbOPN, the most abundant peptide encountered was the unphosphorylated one (data not shown). This may also explain the observation that the antibodies bound the OPNs in an 'all or none' manner – either signal was observed, or it was not. One would have predicted that due to the hetergeneity of the modifications, only a subset of the OPN produced would contain phosphates at the specific amino acids necessary to block OPN binding, decreasing the amount of singal in western blots, but not eliminating it.

The degree of modification of the ObOPN agrees well with earlier characterizations of PTMs of OPN from rat bone and from bovine and human milk with 27 potential sites of phosphorylation compared to 29, 28, and 36, respectively (Sørensen et al., 1995; Christensen et al., 2005; Keykhosravani et al., 2005). The milk OPN was determined in both cases to be almost completely modified, but the rat bone OPN was determined to contain an average of 10-11 phosphates (Keykhosravani et al., 2005). As described in the earlier characterizations, modifications of OPN are heterogeneous and these data are no exception. Both OPNs displayed heterogeneity in the phosphorylation, with many more potential sites of phosphorylation identified compared to phosphates removed by ALP.

The mass spectrometry data also confirmed that the immuno-affinity purified proteins were highly pure and devoid of any appreciable coprecipitation of molecules reported to be associated with OPN, such as osteocalcin or matrix metalloproteinases.

4.1.4 OPN Forms Have Different Adhesive Properties

Figure 9 shows that the observed differences in phosphorylation of the two characterized OPNs translate into a functional difference in the degree of cell adhesion they support. Two different cell lines were used to examine adhesion: MDA-MB-435, a human breast cancer cell line, and 275-3-2 the *ras*-transformed fibroblast cell line which was the source of one of the two OPNs. Surprisingly, the cells' ability to adhere to the two OPN forms was different for each cell line. The MDA-MB-435 cells showed a much greater adhesion to the less phosphorylated FbOPN than to the more phosphorylated ObOPN, which supported only slight cell adhesion. The 275-3-2 cell line however displayed greater binding to the ObOPN compared to the FbOPN. It is worth noting that the FbOPN was purified from this same cell line. The fact that the cell lines showed the opposite difference does alleviate some concern that the difference observed is due to a difference in the ability of the OPNs to adhere to the plastic of the 96-well plates used for the assays. During the course of these experiments, I observed that Costar microwell plates allowed the greatest cell adhesion, while overall adhesion and the differences between the OPNs were not as great when Falcon tissue culture plates were used for the adhesion assays. I believe this issue does not effect my conclusions. Also, it is curious that both cell lines adhered very well to the recombinant hisOPN, eliminating the possibility of a relationship between degree of phosphorylation and cell adhesion.

However, the two OPNs may adopt a different structure on the different plastics, changing the receptor interactions.

The method used for the cell adhesion assays was a modification of that described by Goodwin and Pauli (1995). It was observed that this method, which incorporates Percoll into the wash and fixing solutions to alter their density, thus allowing washing without removing the media, was much more gentle and effective than more common methods. Comparisons of this method with more common methods employing simple PBS washing determined that fewer adherent cells were removed from the wells when the Percoll solutions were used, increasing the accuracy of the results and also the sensitivity of the assay.

One explanation for the observed differences in adhesion of the two cell lines to the OPN forms is a difference in receptor expression by the cell lines. Both cell lines expressed high levels of CD44s, and the MDA-MB-435 cell line also expresses at least one of the specific variants (v6) known to bind to OPN (personal observation, data not shown). The 275-3-2 line was not assayed for CD44v6-7 expression. In order to determine if there was a difference in integrin vs. CD44 binding between the cell lines, the adhesion assays were repeated with integrin-blocking RGD peptides. Many other labs have demonstrated RGD-independent CD44v binding of OPN by observing adhesion in the presence of RGD peptides. This residual RGD-independent adhesion could then be eliminated by the addition of CD44-blocking antibodies along with the RGD peptides. As shown in figure 10, both cell lines were almost completely inhibited by the addition of RGD peptides, indicating that the majority of adhesion is integrin- and RGD-mediated. The 275-3-2 cell did show some cell adhesion (~10%) in the presence of the RGD

peptides but this was not consistent. The 275-3-2 cells tended to clump together during this assay, making them much more difficult to wash from the wells and resulting in higher BSA background values compared to the experiment shown in figure 9. I believe this is due to the extra 30 min preincubation with the peptides. However, when observed microscopically prior to washing the wells, there was no adhesion to the BSA, and only slight adhesion in the presence of RGD peptide. These data also eliminate any contribution from the small amount of C-terminal thrombin fragment of OPN that is co-purified by the 2A1 antibody columns, which has been shown to support CD44 variant binding (Katagiri et al., 1999).

Explanations for differences in RGD-mediated adhesion to the two OPNs include the fact that there is a species difference between the two cell lines with the 435 cells being of human origin and the 275-3-2 cells of murine origin. However, the observation that each cell line is able to display a difference in RGD-dependent adhesion to OPNs differing mainly in their phosphorylation state and that the cell lines showed the opposite effect are major findings of this project.

There are few sites of phosphorylation in proximity to the RGD site, and the closest observed site of differential phosphorylation between the two forms of OPN in this work is 24 amino acids away. Some data exist suggesting that phosphorylation is able to affect RGD-dependent adhesion. Katayama et al. (1998) showed that phosphorylation of OPN by casein kinase II increased the adhesion of osteoclasts but not osteoblasts to recombinant rat OPN. This attachment was also RGD-dependent and was completely abolished by 1mM RGD peptide. Also, an antibody to the integrin β_3 subunit but not a CD44-blocking antibody blocked the increase in adhesion to phosphorylated

OPN. Ashkar et al. (2000) also showed that an integrin interaction was phosphate dependent. Native, but not recombinant, OPN treatment of mouse peritoneal macrophages increased IL-12 production in an RGD-dependent manner. Also, Weber et al. (2002) showed that phosphorylation of OPN was required for RGD-dependent cell spreading of a murine monocytic cell line. These studies support my observations and conclusion that differences in phosphorylation are regulating RGD-dependent integrin attachment to OPN.

The reports by Ashkar and Weber above both describe a 10-kDa fragment of osteopontin isolated by protease digestion that retained cell attachment ability. Dephosphorylation of this fragment reduced its ability to support cell attachment. Further analysis determined that the N-terminal sequence was QETLPSN and the fragment was predicted to end at the thrombin cleavage site. This sequence contains 10 sites of potential phosphorylation in ObOPN and 5 sites in FbOPN. Differences in this region may explain the observed differences in cell adhesion supported by the OPN forms. OPN may contain one of more 'synergy' sites that may help regulate integrin binding to the RGD site, similar to those found in fibronectin (Clark et al., 2003).

A recent exciting publication offers an alternative explanation that could explain the regulation of RGD-binding integrins by specific phosphate groups. Lee et al. (2007) demonstrated that an OPN-CD44v interaction is able to increase integrin adhesion by activation of integrins via outside-in signaling. OPN treatment of AZ521 gastric cancer cells increased the activation of β_1 integrins and attachment to fibronectin. The activation of β_1 integrins was blocked by antiCD44v6 antibody pretreatment, and was unaffected by treatment with an RGE-mutant OPN. The activation was dependent on src-kinase

signaling, as the src-kinase family inhibitor PP2 eliminated the effect. It was determined that the observed OPN/CD44v-mediated anti-apoptotic effect on cancer cells occurred through activation of integrin receptors. The region of OPN where CD44v receptors bind is still controversial and no binding region has been unequivocally localized. It is possible that the OPN-CD44v interaction could be regulated by phosphorylation of OPN. Thus, phosphorylation of OPN could indirectly affect RGD binding by regulating integrin activation through outside-in signaling initiated by engagement of the CD44v receptors with OPN. A feedback loop may also exist as an interaction with OPN and α_v integrins was shown to increase CD44 expression and MMP-2 activity (Samanna et al., 2006). This was also due to outside-in signaling through the integrin and required the pp(60c-Src) kinase.

How do these data relate to an *in vivo* scenario? Tumor OPN has been hypothesized to be a soluble protein, not associated with matrix, possibly a result of reduced phosphorylation. This is supported by early experiments by Nemir et al. (1989) using normal rat kidney cells. These cells produced both a phosphorylated (pp69) and nonphosphorylated form (np69) of OPN, however when treated with vanadyl sulfate, which causes transformation, the expression of np69 was significantly increased and pp69 decreased. Tumor OPN and OPN expressed by *ras*-transformed fibroblasts was shown in one study to be soluble, and not associated with extracellular matrix (Rittling et al., 2002). In contrast, milk and bone OPN are in areas containing high levels of calcium and/or calcium-containing crystals. The additional phosphates observed in the OPN found in these areas increase the protein's negative charge and most likely allow an increase in the association of OPN with calcium and hydroxyapatite, allowing OPN to

control or prevent crystal growth. Gericke et al. (2005) showed that partial

dephosphorylation of bovine OPN with alkaline phosphatase removed its inhibitory effect

on hydroxyapatite growth, and reduced its ability to bind calcium. Mineralizing rat

osteoblasts were also shown to produce a more highly phosphorylated form of OPN

(Sodek et al., 1995).

I hereby suggest that tumor-produced OPN differs in PTMs from OPN made by

normal cells, possibly accounting for its increased solubility, and that cells are able to

distinguish the 'tumor-form' from the form produced by normal cells. I also suggest the

tumor form is more effective at promoting cancer progression, either by increasing

anchorage-independence and metastasis, or protecting the cells from the immune

response and apoptosis. The distinguishing factor is proposed to be differences in

phosphorylation which, as described above, may affect receptor interaction and signaling

in a direct or indirect manner - through an interaction with CD44. The latter seems much

more likely as there is a large body of data connecting CD44, OPN, and tumor

development. CD44 variant expression has been shown in many tumor types and in some

cases correlates with aggressiveness (Weber et al., 1997). Certain CD44 variants have

been associated with metastasis formation (Gunthert et al., 1991). Interestingly these

molecules contained the same variants (v6 & v7) shown to be required for OPN binding.

Many recent studies have linked CD44 and OPN expression in tumor cells and associated

the two with increased migration. OPN increases the expression of CD44v6 in liver

carcinoma cells and a human breast cancer cell line which also exhibited increased

CD44-dependent migration. In addition *ras*-transformation of mouse fibroblasts was

shown to upregulate both molecules in an autocrine manner and increased invasion in an *in vitro* assay (Teramoto et al., 2005).

This observed difference in binding to the OPN isoforms used in this study only partially supports this model. Since tumor-produced OPN may be soluble and not matrix-associated, one might expect this OPN to be less effective in supporting cell adhesion. One would also expect both cell lines to behave in a similar manner since one is a tumor line and the other is 'tumor-like', both adhering better to the more highly modified ObOPN. This was only true for the 275-3-2 cell line. As mentioned above, species differences may contribute to the lack of binding of the MDA-MB-435 cells to the ObOPN. For example, human CD44 receptors may not be able to bind mouse OPN, eliminating the possibility of integrin activation through CD44 described above.

4.2 Project 2: Anti-OPN antibodies and inhibition of cell adhesion

4.2.1 Rationale

The results in project 1 above strongly suggested that the 3D9 antibody was sensitive to post-translational modification of its epitope, but this needed to be definitively proven. In addition, this antibody and others were previously shown to partially inhibit adhesion of a cell line to recombinant OPN. Since the majority of these antibodies were determined to bind epitopes in the C-terminal half of OPN, this was especially interesting because CD44v is the only receptor yet identified that binds this region of OPN and its binding site has not been localized (Weber et al., 1997). I hypothesized that some of the antibodies that were preliminarily demonstrated to inhibit cell adhesion (2C5, 1H3 and to a lesser extent 3D9 & 7B4) may be doing so by inhibiting CD44 binding.

4.2.2 Antibody Sensitivity to Post-Translational Modifications

Western blotting of human urine for OPN showed the typical 4 closely migrating bands (figure 11). Interestingly, the antibody reactivity for urine OPN was quite different from that of the murine conditioned medium assayed in figure 4. Antibody 1H3 bound strongly to all four forms, whereas 3D9 only bound 2 and antibodies 2C5 and 2A1 did not bind at all. The 2A1 result was somewhat surprising as 2A1 binds well to OPN from many sources, including the highly modified human milk OPN, and has not been previously observed to be sensitive to the PTM state of OPN. Its minimal epitope (PVA) is quite small, and is located in an area with few PTMs. Binding of 3D9 to only the two higher molecular weight species of the urine OPN suggest that there are differently modified species of OPN in urine, reflecting the heterogeneity of modification of OPN in general, although the possibility exists that the unrecognized lower apparent molecular weight forms do not contain the extreme C-terminus due to splicing or protease cleavage.

Sensitivity of antibody 3D9 to phosphate modification of its epitope is proven in figure 12B. Similar results have been obtained with the other C-terminal binding antibody 7B4. These two antibodies (as well as the others) can be used to screen for the existence of phosphorylation (or splicing/cleavage) in the C-terminal half of the molecule. Such screening may become important if it can be related to the overall level of phosphorylation of the molecule or to a specific function. The data presented in this work suggest a receptor interaction in the extreme C-terminal region of OPN where 3D9 binds. Although the receptor and the role of phosphorylation in this area have yet to be determined, 3D9 may become a valuable tool for screening of OPN to determine whether this interaction can occur.

4.2.3 Cell Adhesion and the Extreme C-Terminus of OPN

Figure 13 shows that the extreme C-terminal-binding antibodies 3D9 and 7B4 are able to inhibit adhesion of both MDA-MB-435 and 275-3-2 cells to recombinant human OPN. A dose response experiment using 3D9 shows that the maximum inhibition achievable is ~35% and this can be achieved with an antibody concentration of approximately 60nM. This is less that the 150nM hisOPN originally applied to the wells, however this concentration is presumed to be saturating, resulting in less OPN bound per well (Katagiri et al., 1999).

From these results I hypothesized that the antibodies were inhibiting binding of a receptor to this area of OPN – possibly the CD44 receptor. Using synthetic peptides in adhesion and flow-cytometry assays to determine if there was a direct interaction of a cell-surface receptor and the C-terminal region of OPN indicated that there is likely an interaction occurring, but it is not an adhesive one. Figure 15 examined whether two overlapping peptides corresponding to the extreme C-terminus of OPN could support cell adhesion, and the results show no adhesion to these peptides. However, the same C-terminal region peptides were able to bind to approximately 60% of cells in the flow cytometry assay (Figure 16). As mentioned in section 3.2.8, the fixation step needed to be done after the washing of the initial incubation of peptides and cells. If the fixing was delayed until the end of the procedure (as is normal in flow cytometry procedures), the observed binding was significantly decreased. This suggests that the binding of the peptides to the receptor is a transient one more indicative of a signaling interaction than an adhesive one and supports the lack of adhesion observed in figure 15. Yet the possibility exists that the C-terminal biotin tag used to attach the peptides to the Neutra-

avidin plates sequesters an important part of the peptide making it unavailable to receptors. It is also possible that these antibodies are sterically interfering with binding of a receptor in the vicinity of the C-terminal region or even the integrin-binding region.

Analysis of the C-terminal region shows an extremely high degree of amino acid sequence similarity (figure 19), with approximately 26 of the last 40 amino acids of the protein have 80% or greater similarity among mammalian species. Considering that all other important function motifs of OPN are highly conserved among species (see figure 1), this is suggestive of an important role for this region of the protein.

Experiments using CD44 blocking antibodies had no effect on the adhesion of either cell line (data not shown & figure 18). The majority of these antibodies are termed "CD44 blocking antibodies" but this is in reference to the ability of CD44 to bind to hyaluronan. With the exception of one antibody (2F10 – anti-CD44v6) which was demonstrated to inhibit an interaction with OPN, it is not known whether these CD44 'blocking' antibodies are able to block the CD44v-OPN interaction. Hyaluronan was also used and only slightly inhibited binding at the extremely high concentration of 500 μg/ml. The addition of an RGD-containing peptide inhibited an average of 85% of cell adhesion, and the $\alpha_v\beta_3$ blocking antibody LM609 blocked 100% of adhesion, indicating that the adhesion in this system is predominantly $\alpha_v\beta_3$ –mediated (data not shown & figure 18).

The results of preincubating the C-terminal region peptides with cells prior to adhesion in attempts to block this presumed interaction and resultant signaling were inconclusive. As shown in figure 17, these peptides only showed mild inhibition of adhesion that did not significantly differ from OPN control peptides. During the course of conducting these

experiments, the inhibition observed by these peptides has been extremely variable, with

the greatest inhibition by peptide hOPN19 being as high as 27%. The seemingly transient

nature of the peptide-receptor interaction may be such that significant inhibition cannot

be observed. Receptor binding to the C-terminal region of OPN coated on the plate may

be of higher affinity than peptide binding, effectively eliminating any inhibitory effect. It

is also possible that peptide binding activates the signaling mechanism as OPN-binding

would. Clearly the interaction between the C-terminal region of OPN and cell surface-

receptors needs further study.

Figure 19: Analysis of the C-terminal regions of osteopontin from mammalian species. Amino acid sequences of bovine, pig, rabbit, human, mouse, and rat OPN were aligned using the ClustalW program (Chenna et al., 2003). The alignment was then viewed with the Jalview alignment viewer (Clamp et al., 2004). Amino acids highlighted in blue represent 80% of greater conservation among the sequences shown.

Another possibility is that receptor binding of the C-terminal region is not an

adhesive interaction, but rather a signaling interaction that activates integrins. The work

of Lee et al. (2007) described above showing that an OPN-CD44v interaction is able to

increase RGD-mediated adhesion by activating integrins via inside-out signaling is a

likely explanation, especially given the results of figure 16 which are suggestive of a

direct interaction at the C-terminal region of OPN. The differential phosphorylation

between the FbOPN and ObOPN observed in this area could regulate binding in this

region. As a result, outside-in signaling would be regulated, explaining the differences in

adhesion to the two OPNs observed in project 1.

The findings in this study can be extended to other members of the SIBLING family, which all have multiple phosphate modications. In some cases, such as bone sialoprotein and dentin matrix protein, the function of phosphate modification is unknown (Qin et al., 2004). Phosphorylation is a well-known modification in intracellular signalling cascades, where it is intimately involved in regulation of protein-protein interactions. The results described in this study suggest that despite the high number and heterogeneity of phosphate modifications of OPN, they may also regulate protein-protein interactions in the extracellular compartment.

4.3 Future Directions

4.3.1 Project I

The difference in cell adhesion between the two OPN forms characterized in this study agrees with many other studies using OPNs comparing phosphorylated and non-phosphorylated OPN. As noted above, some studies have used enzymes to alter the phosphorylation state of OPN. The fact that the OPNs used in this study are purified from two cell lines of the same species increases the relevance of the results. In addition to cell adhesion, many other functions of OPN are affected by its phosphorylation state. It would be beneficial to know whether the degree of phosphorylation plays a role in other processes besides cell adhesion. Both OPN forms used in this study are implicated in the migration of cells. OPN produced by *ras*-transformed fibroblasts has been shown to increase invasion and osteoblast-produced OPN is implicated in migration and homing of hematopoietic stem cells (HSCs) (Nilsson et al., 2005). An assessment of the ability of the described forms of OPN to support cell migration of cells such as HSCs or

macrophages, as well as the migration and invasion of tumor cells, would help determine the role of phosphorylation of native OPNs in these processes.

Phosphorylation of OPN differs according to the stage of osteoblast differentiation, and has been shown to be important in OPN's ability to modulate crystal growth. The two OPNs purified in this study seem to resemble the OPNs produced by proliferating and differentiating rat osteoblasts (Sodek et al., 1995). The two OPNs can therefore be used to examine differences in signaling in the osteoblast system, including gene expression and effects on mineral production. It would also be interesting to examine the differences of the two forms of OPN to bind and modulate the growth of hydroxyapatite crystals.

Ashkar et al. (2000) showed that a phosphorylated OPN-dependent integrin interaction increased IL-12 production, while a phosphorylation-independent OPN-CD44 interaction decreased IL-10 production in murine macrophages. Recombinant OPN has also been shown to decrease nitric oxide production in macrophages and kidney epithelial cells (Hwang et al., 1994; Rollo et al., 1996). Similar studies comparing the two forms of OPN may reveal differences in the ability of the forms to alter macrophage production of these molecules and shed light on this issue of whether OPN from different sources (e.g. tumor-produced OPN and host-produced OPN) has similar or divergent effects on macrophages.

The suggestion that phosphorylation of OPN affects RGD-dependent adhesion is a significant finding and needs further study. The effect may be direct, for example by altering local structure affecting RGD availability, or indirect by affecting the outside-in signaling through a CD44 interaction. The latter model could be examined using

antibodies directed against CD44 or signaling inhibitors such as a src-kinase inhibitor in attempts to block the outside-in signaling. The former model is much more difficult to examine. Due to the heterogeneous nature of phosphorylation of OPN it would be extremely difficult to isolate specific phospho-serines involved. The integrin(s) involved should be determined and a cellular system chosen that assays the binding of a single integrin type to the OPN forms. OPN fragments from the two forms can be used to narrow down the area in which differential phosphorylation is important. A particular fragment of interest is the 10kDa Lys-C fragment described above that has been shown by others to support cell adhesion depending on its phosphorylation state (ashkar and weber).

Another interesting aspect of this project was the observed difference in the recognition of OPN produced by various murine cell lines by our monoclonal antibodies (figure 4), specifically the difference between the *ras*-transformed fibroblast line and its non-transformed parent line. A similar binding differences were observed for another matched pair of non- and *ras*-transformed fibroblast lines (NIH3T3 & PAP2, respectively – data not shown) strongly suggesting that *ras*-transformation downregulates the phosphorylation of OPN. Since ras mutations are a common mutation found in cancers and OPN is highly expressed by many types of cancer, it can be speculated that tumor-produced OPN might also have significantly less phosphate modifications. It would be interesting to examine the antibody binding (indirectly measuring phosphorylation) in a set of tumor and host tissue samples, or cell lines differing in metastatic potential. Many studies have detected serum osteopontin levels by ELISA and have correlated them with poor prognosis or detection of cancer (Fedarko et al., 2001; Bramwell et al., 2006; Zhang

et al., 2006). Our antibodies may be useful in such an application, especially if they are able to specifically identify tumor-produced OPN, or 'metastasis-associated' OPN from host tissue-produced OPN. The immuno-affinity purification method described in this work yields highly pure native OPN in a single purification step. This procedure could be easily applied to OPN from other sources. The subsequent purified OPNs could then be used in the functional assays described above. An attractive example would be to purify OPN from tumor and host cells, or normal and transformed cell lines, for use functional assay comparison.

4.3.2 Project II.

This work has uncovered a novel region of osteopontin that has a role in cellular adhesion - the C-terminal region. The studies described in this work indicate that receptor(s) may be interacting with the C-terminal region and subsequently affecting cell adhesion. However, the interaction does not seem to be an adhesive one, although switching the biotin tag from the C-terminal end to the N-terminus would eliminate a caveat to this conclusion. The use of C-terminal peptides to block the potential interaction has not been reliably effective, primarily because so little is known about this interaction. As mentioned above, the peptides themselves may cause activation of signaling pathways. More definitive proof of an interaction and identification of the receptor(s) involved are required. Experiments are ongoing which will cross-link the hOPN19 or 20 peptides to cells and attempt to affinity-purify the peptide-receptor complex. This procedure utilizes the biotin tag on the peptides for precipitation using avidin-coupled agarose beads. If successful, blots can then be probed for specific receptors such as

CD44, or the resulting proteins can be further purified and identified by MALDI-TOF analysis.

The creation of a series of truncated mutants of OPN would be quite informative in determining the role of the C-terminal region in adhesion and other functional assays. In addition, the C-terminal thrombin fragment has been shown to support adhesion and migration of lymphocyes through CD44 binding. A comparison of binding of the C-terminal thrombin fragment vs. truncated C-terminal mutant thrombin fragments in such a system may reveal the CD44 binding region(s).

The result showing that the anti-OPN antibodies 3D9 and 7B4 are able to inhibit cell adhesion to OPN opens the door for testing of these antibodies in other OPN functional assays. Indeed, preliminary data by undergraduate student Bhumika Desai indicated that the 3D9 antibody may also block the ability of OPN to increase IL-12 production by mouse peritoneal macrophages.

REFERENCES

Abel, B., Kurrer, M., Shamshiev, A., Marty, R.R., Eriksson, U., Gunthert, U., and Kopf, M. (2006). The osteopontin - CD44 pathway is superfluous for the development of autoimmune myocarditis. Eur J Immunol *36*, 494-499.

Agnihotri, R., Crawford, H.C., Haro, H., Matrisian, L.M., Havrda, M.C., and Liaw, L. (2001). Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). J Biol Chem *276*, 28261-28267.

Al-Shami, R., Sorensen, E.S., Ek-Rylander, B., Andersson, G., Carson, D.D., and Farach-Carson, M.C. (2005). Phosphorylated osteopontin promotes migration of human choriocarcinoma cells via a p70 S6 kinase-dependent pathway. J Cell Biochem *94*, 1218-1233.

Allan, A.L., George, R., Vantyghem, S.A., Lee, M.W., Hodgson, N.C., Engel, C.J., Holliday, R.L., Girvan, D.P., Scott, L.A., Postenka, C.O.*, et al.* (2006). Role of the integrin-binding protein osteopontin in lymphatic metastasis of breast cancer. Am J Pathol *169*, 233-246.

Andersson, G., Ek-Rylander, B., Hollberg, K., Ljusberg-Sjolander, J., Lang, P., Norgard, M., Wang, Y., and Zhang, S.J. (2003). TRACP as an osteopontin phosphatase. J Bone Miner Res *18*, 1912-1915.

Ashkar, S., Weber, G.F., Panoutsakopoulou, V., Sanchirico, M.E., Jansson, M., Zawaideh, S., Rittling, S.R., Denhardt, D.T., Glimcher, M.J., and Cantor, H. (2000). Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science *287*, 860-864.

Asou, Y., Rittling, S.R., Yoshitake, H., Tsuji, K., Shinomiya, K., Nifuji, A., Denhardt, D.T., and Noda, M. (2001). Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone. Endocrinology *142*, 1325-1332.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds. (1987). Current Protocols in Molecular Biology (John Wiley & Sons, Inc.).

Bao, L.H., Sakaguchi, H., Fujimoto, J., and Tamaya, T. (2007). Osteopontin in metastatic lesions as a prognostic marker in ovarian cancers. J Biomed Sci *[epub ahead of print]*.

Barry, S.T., Ludbrook, S.B., Murrison, E., and Horgan, C.M. (2000a). Analysis of the alpha4beta1 integrin-osteopontin interaction. Exp Cell Res *258*, 342-351.

Barry, S.T., Ludbrook, S.B., Murrison, E., and Horgan, C.M. (2000b). A regulated interaction between alpha5beta1 integrin and osteopontin. Biochem Biophys Res Commun *267*, 764-769.

Behrend, E.I., Craig, A.M., Wilson, S.M., Denhardt, D.T., and Chambers, A.F. (1994). Reduced malignancy of ras-transformed NIH 3T3 cells expressing antisense osteopontin RNA. Cancer Res *54*, 832-837.

Bellahcene, A., and Castronovo, V. (1995). Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. Am J Pathol *146*, 95-100.

Beninati, S., Senger, D.R., Cordella-Miele, E., Mukherjee, A.B., Chackalaparampil, I., Shanmugam, V., Singh, K., and Mukherjee, B.B. (1994). Osteopontin: its transglutaminase-catalyzed posttranslational modifications and cross-linking to fibronectin. J Biochem (Tokyo) *115*, 675-682.

Beshensky, A.M., Wesson, J.A., Worcester, E.M., Sorokina, E.J., Snyder, C.J., and Kleinman, J.G. (2001). Effects of urinary macromolecules on hydroxyapatite crystal formation. J Am Soc Nephrol *12*, 2108-2116.

Boskey, A.L., Maresca, M., Ullrich, W., Doty, S.B., Butler, W.T., and Prince, C.W. (1993). Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite formation and growth in a gelatin-gel. Bone Miner *22*, 147-159.

Boskey, A.L., Spevak, L., Paschalis, E., Doty, S.B., and McKee, M.D. (2002). Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcif Tissue Int *71*, 145-154.

Bramwell, V.H., Doig, G.S., Tuck, A.B., Wilson, S.M., Tonkin, K.S., Tomiak, A., Perera, F., Vandenberg, T.A., and Chambers, A.F. (2006). Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. Clin Cancer Res *12*, 3337-3343.

Brown, L.F., Papadopoulos-Sergiou, A., Berse, B., Manseau, E.J., Tognazzi, K., Perruzzi, C.A., Dvorak, H.F., and Senger, D.R. (1994). Osteopontin expression and distribution in human carcinomas. Am J Pathol *145*, 610-623.

Chabas, D., Baranzini, S.E., Mitchell, D., Bernard, C.C., Rittling, S.R., Denhardt, D.T., Sobel, R.A., Lock, C., Karpuj, M., Pedotti, R.*, et al.* (2001). The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science *294*, 1731-1735.

Chackalaparampil, I., Peri, A., Nemir, M., McKee, M.D., Lin, P.H., Mukherjee, B.B., and Mukherjee, A.B. (1996). Cells in vivo and in vitro from osteopetrotic mice homozygous for c-src disruption show suppression of synthesis of osteopontin, a multifunctional extracellular matrix protein. Oncogene *12*, 1457-1467.

Chambers, A.F., Behrend, E.I., Wilson, S.M., and Denhardt, D.T. (1992). Induction of expression of osteopontin (OPN; secreted phosphoprotein) in metastatic, ras-transformed NIH 3T3 cells. Anticancer Res *12*, 43-47.

Chambers, A.F., Wilson, S.M., Kerkvliet, N., O'Malley, F.P., Harris, J.F., and Casson, A.G. (1996). Osteopontin expression in lung cancer. Lung Cancer *15*, 311-323.

Chang, P.L., and Prince, C.W. (1991). 1 alpha,25-dihydroxyvitamin D3 stimulates synthesis and secretion of nonphosphorylated osteopontin (secreted phosphoprotein 1) in mouse JB6 epidermal cells. Cancer Res *51*, 2144-2150.

Chang, P.L., and Prince, C.W. (1993). 1 alpha,25-Dihydroxyvitamin D3 enhances 12-O-tetradecanoylphorbol-13-acetate- induced tumorigenic transformation and osteopontin expression in mouse JB6 epidermal cells. Cancer Res *53*, 2217-2220.

Chellaiah, M.A., and Hruska, K.A. (2003). The integrin alpha(v)beta(3) and CD44 regulate the actions of osteopontin on osteoclast motility. Calcif Tissue Int *72*, 197-205.

Chellaiah, M.A., Kizer, N., Biswas, R., Alvarez, U., Strauss-Schoenberger, J., Rifas, L., Rittling, S.R., Denhardt, D.T., and Hruska, K.A. (2003). Osteopontin Deficiency Produces Osteoclast Dysfunction Due to Reduced CD44 Surface Expression. Mol Biol Cell *14*, 173-189.

Chen, Y., Bal, B.S., and Gorski, J.P. (1992). Calcium and collagen binding properties of osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 from bone. J Biol Chem *267*, 24871-24878.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., and Thompson, J.D. (2003). Multiple Sequence Alignment with the Clustal Series of Programs. Nucleic Acids Res *31*, 3497-3500.

Chiba, S., Okamoto, H., Kon, S., Kimura, C., Murakami, M., Inobe, M., Matsui, Y., Sugawara, T., Shimizu, T., Uede, T., *et al.* (2002). Development of atherosclerosis in osteopontin transgenic mice. Heart Vessels *16*, 111-117.

Christensen, B., Nielsen, M.S., Haselmann, K.F., Petersen, T.E., and Sørensen, E.S. (2005). Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. Biochem J *390*, 285-292.

Clamp, M., Cuff, J., Searle, S.M., and Barton, G.J. (2004). The Jalview Java Alignment Editor. Bioinformatics *20*, 426-427.

Clark, R.A.F., An, J.-Q., Greiling, D., Khan, A., and Schwarzbauer, J.E. (2003). Fibroblast migration on fibronectin requires three distinct functional domains. J Invest Dermatol *121*, 695-705.

Craig, A.M., and Denhardt, D.T. (1991). The murine gene encoding secreted phosphoprotein 1 (osteopontin): promoter structure, activity, and induction in vivo by estrogen and progesterone. Gene *100*, 163-171.

Craig, A.M., Nemir, M., Mukherjee, B.B., Chambers, A.F., and Denhardt, D.T. (1988). Identification of the major phosphoprotein secreted by many rodent cell lines as 2ar/osteopontin: enhanced expression in H-ras-transformed 3T3 cells. Biochem Biophys Res Commun *157*, 166-173.

Craig, A.M., Smith, J.H., and Denhardt, D.T. (1989). Osteopontin, a transformation-associated cell adhesion phosphoprotein, is induced by 12-O-tetradecanoylphorbol 13-acetate in mouse epidermis. J Biol Chem *264*, 9682-9689.

Crawford, H.C., Matrisian, L.M., and Liaw, L. (1998). Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo. Cancer Res *58*, 5206-5215.

Denda, S., Reichardt, L.F., and Muller, U. (1998). Identification of osteopontin as a novel ligand for the integrin alpha8 beta1 and potential roles for this integrin-ligand interaction in kidney morphogenesis. Mol Biol Cell *9*, 1425-1435.

Denhardt, D.T., and Noda, M. (1998). Osteopontin expression and function: role in bone remodeling. J Cell Biochem Suppl *30-31*, 92-102.

Denhardt, D.T., Noda, M., O'Regan, A.W., Pavlin, D., and Berman, J.S. (2001). Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest *107*, 1055-1061.

Duvall, C.L., Taylor, W.R., Weiss, D., Wojtowicz, A.M., and Guldberg, R.E. (2007). Impaired angiogenesis, early callus formation, and late stage remodeling in fracture healing of osteopontin-deficient mice. J Bone Miner Res *22*, 286-297.

Ek-Rylander, B., Flores, M., Wendel, M., Heinegard, D., and Andersson, G. (1994). Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. J Biol Chem *269*, 14853-14856.

El-Tanani, M., Barraclough, R., Wilkinson, M.C., and Rudland, P.S. (2001). Metastasis-inducing dna regulates the expression of the osteopontin gene by binding the transcription factor Tcf-4. Cancer Res *61*, 5619-5629.

Faccio, R., Grano, M., Colucci, S., Zallone, A.Z., Quaranta, V., and Pelletier, A.J. (1998). Activation of alphav beta3 integrin on human osteoclast-like cells stimulates adhesion and migration in response to osteopontin. Biochem Biophys Res Commun *249*, 522-525.

Fedarko, N.S., Fohr, B., and Fisher, L.W. (2000a). DMP1 inactivates complement by bridging Factor H to cell surface receptors. Journal of Dental Research *79*, 339.

Fedarko, N.S., Fohr, B., Robey, P.G., Young, M.F., and Fisher, L.W. (2000b). Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. J Biol Chem *275*, 16666-16672.

Fedarko, N.S., Jain, A., Karadag, A., and Fisher, L.W. (2004). Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. Faseb J *18*, 734-736.

Fedarko, N.S., Jain, A., Karadag, A., Van Eman, M.R., and Fisher, L.W. (2001). Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res *7*, 4060-4066.

Feng, B., Rollo, E.E., and Denhardt, D.T. (1995). Osteopontin (OPN) may facilitate metastasis by protecting cells from macrophage NO-mediated cytotoxicity: evidence from cell lines down-regulated for OPN expression by a targeted ribozyme. Clin Exp Metastasis *13*, 453-462.

Fet, V., Dickinson, M.E., and Hogan, B.L. (1989). Localization of the mouse gene for secreted phosphoprotein 1 (Spp-1) (2ar, osteopontin, bone sialoprotein 1, 44-kDa bone phosphoprotein, tumor-secreted phosphoprotein) to chromosome 5, closely linked to Ric (Rickettsia resistance). Genomics *5*, 375-377.

Fisher, L.W., and Fedarko, N.S. (2003). Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. Connect Tissue Res *44 Suppl 1*, 33-40.

Fisher, L.W., Torchia, D.A., Fohr, B., Young, M.F., and Fedarko, N.S. (2001). Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun *280*, 460-465.

Gadeau, A.P., Campan, M., Millet, D., Candresse, T., and Desgranges, C. (1993). Osteopontin overexpression is associated with arterial smooth muscle cell proliferation in vitro. Arterioscler Thromb *13*, 120-125.

Gao, C., Guo, H., Downey, L., Marroquin, C., Wei, J., and Kuo, P.C. (2003). Osteopontin-dependent CD44v6 expression and cell adhesion in HepG2 cells. Carcinogenesis *24*, 1871-1878.

Gericke, A., Qin, C., Spevak, L., Fujimoto, Y., Butler, W.T., Sorensen, E.S., and Boskey, A.L. (2005). Importance of Phosphorylation for Osteopontin Regulation of Biomineralization. Calcif Tissue Int *77*, 45-54.

Giachelli, C.M., Bae, N., Almeida, M., Denhardt, D.T., Alpers, C.E., and Schwartz, S.M. (1993). Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest *92*, 1686-1696.

Giachelli, C.M., Lombardi, D., Johnson, R.J., Murry, C.E., and Almeida, M. (1998). Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. Am J Pathol *152*, 353-358.

Goodwin, A.E., and Pauli, B.U. (1995). A new adhesion assay using bouyancy to remove non-adherent cells. Journal of Immunological Methods *187*, 213-219.

Gorski, J.P., Kremer, E., Ruiz-Perez, J., Wise, G.E., and Artigues, A. (1995). Conformational analyses on soluble and surface bound osteopontin. Ann N Y Acad Sci *760*, 12-23.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H., and Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell *65*, 13-24.

Hamada, Y., Nokihara, K., Okazaki, M., Fujitani, W., Matsumoto, T., Matsuo, M., Umakoshi, Y., Takahashi, J., and Matsuura, N. (2003). Angiogenic activity of osteopontin-derived peptide SVVYGLR. Biochem Biophys Res Commun *310*, 153-157.

Harlow, E., and Lane, D. (1999). Using Antibodies: A laloratory manual (Cold Spring Harbor, Cold Spring Harbor Laboratory Press).

He, B., Mirza, M., and Weber, G.F. (2006). An osteopontin splice variant induces anchorage independence in human breast cancer cells. Oncogene *25*, 2192-2202.

Hijiya, N., Setoguchi, M., Matsuura, K., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1994). Cloning and characterization of the human osteopontin gene and its promoter. Biochem J *303 (Pt 1)*, 255-262.

Hirama, M., Takahashi, F., Takahashi, K., Akutagawa, S., Shimizu, K., Soma, S., Shimanuki, Y., Nishio, K., and Fukuchi, Y. (2003). Osteopontin overproduced by tumor cells acts as a potent angiogenic factor contributing to tumor growth. Cancer Lett *198*, 107-117.

Hirota, S., Imakita, M., Kohri, K., Ito, A., Morii, E., Adachi, S., Kim, H.M., Kitamura, Y., Yutani, C., and Nomura, S. (1993). Expression of osteopontin messenger RNA by macrophages in atherosclerotic plaques. A possible association with calcification. Am J Pathol *143*, 1003-1008.

Hoyer, J.R., Asplin, J.R., and Otvos, L. (2001). Phosphorylated osteopontin peptides suppress crystallization by inhibiting the growth of calcium oxalate crystals. Kidney Int *60*, 77-82.

Hruska, K.A., Rolnick, F., Huskey, M., Alvarez, U., and Cheresh, D. (1995). Engagement of the osteoclast integrin alpha v beta 3 by osteopontin stimulates phosphatidylinositol 3-hydroxyl kinase activity. Endocrinology *136*, 2984-2992.

Hu, D.D., Hoyer, J.R., and Smith, J.W. (1995a). Ca2+ suppresses cell adhesion to osteopontin by attenuating binding affinity for integrin alpha v beta 3. J Biol Chem *270*, 9917-9925.

Hu, D.D., Lin, E.C., Kovach, N.L., Hoyer, J.R., and Smith, J.W. (1995b). A biochemical characterization of the binding of osteopontin to integrins alpha v beta 1 and alpha v beta 5. J Biol Chem *270*, 26232-26238.

Huang, W., Carlsen, B., Rudkin, G., Berry, M., Ishida, K., Yamaguchi, D.T., and Miller, T.A. (2004). Osteopontin is a negative regulator of proliferation and differentiation in MC3T3-E1 pre-osteoblastic cells. Bone *34*, 799-808.

Hunter, G.K., Hauschka, P.V., Poole, A.R., Rosenberg, L.C., and Goldberg, H.A. (1996). Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. Biochem J *317 (Pt 1)*, 59-64.

Hunter, G.K., Kyle, C.L., and Goldberg, H.A. (1994). Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J *300 (Pt 3)*, 723-728.

Hur, E.M., Youssef, S., Haws, M.E., Zhang, S.Y., Sobel, R.A., and Steinman, L. (2007). Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. Nat Immunol *8*, 74-83.

Hwang, S.M., Lopez, C.A., Heck, D.E., Gardner, C.R., Laskin, D.L., Laskin, J.D., and Denhardt, D.T. (1994). Osteopontin inhibits induction of nitric oxide synthase gene expression by inflammatory mediators in mouse kidney epithelial cells. J Biol Chem *269*, 711-715.

Ihara, H., Denhardt, D.T., Furuya, K., Yamashita, T., Muguruma, Y., Tsuji, K., Hruska, K.A., Higashio, K., Enomoto, S., Nifuji, A.*, et al.* (2001). Parathyroid hormone-induced bone resorption does not occur in the absence of osteopontin. J Biol Chem *276*, 13065-13071.

Ishijima, M., Rittling, S.R., Yamashita, T., Tsuji, K., Kurosawa, H., Nifuji, A., Denhardt, D.T., and Noda, M. (2001). Enhancement of osteoclastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do not occur in the absence of osteopontin. J Exp Med *193*, 399-404.

Ishijima, M., Tsuji, K., Rittling, S.R., Yamashita, T., Kurosawa, H., Denhardt, D.T., Nifuji, A., and Noda, M. (2002). Resistance to unloading-induced three-dimensional bone loss in osteopontin-deficient mice. J Bone Miner Res *17*, 661-667.

Ito, S., Saito, T., and Amano, K. (2004). In vitro apatite induction by osteopontin: interfacial energy for hydroxyapatite nucleation on osteopontin. J Biomed Mater Res A *69*, 11-16.

Jacobs, J.P., Pettit, A.R., Shinohara, M.L., Jansson, M., Cantor, H., Gravallese, E.M., Mathis, D., and Benoist, C. (2004). Lack of requirement of osteopontin for inflammation, bone erosion, and cartilage damage in the K/BxN model of autoantibody-mediated arthritis. Arthritis Rheum *50*, 2685-2694.

Jain, A., Karadag, A., Fohr, B., Fisher, L.W., and Fedarko, N.S. (2002). Three SIBLINGs (small integrin-binding ligand, N-linked glycoproteins) enhance factor H's cofactor activity enabling MCP-like cellular evasion of complement-mediated attack. J Biol Chem *277*, 13700-13708.

Jang, J.H., and Kim, J.H. (2005). Improved cellular response of osteoblast cells using recombinant human osteopontin protein produced by Escherichia coli. Biotechnol Lett *27*, 1767-1770.

Johnson, G.A., Burghardt, R.C., Bazer, F.W., and Spencer, T.E. (2003). Osteopontin: roles in implantation and placentation. Biol Reprod *69*, 1458-1471.

Jono, S., Peinado, C., and Giachelli, C.M. (2000). Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. J Biol Chem *275*, 20197-20203.

Kaartinen, M.T., Murshed, M., Karsenty, G., and McKee, M.D. (2006). Osteopontin Upregulation and Polymerization by Transglutaminase 2 in Calcified Arteries of Matrix Gla Protein-deficient Mice. J Histochem Cytochem *55*, 375-386.

Kaartinen, M.T., Pirhonen, A., Linnala-Kankkunen, A., and Maenpaa, P.H. (1997). Transglutaminase-catalyzed cross-linking of osteopontin is inhibited by osteocalcin. J Biol Chem *272*, 22736-22741.

Kaartinen, M.T., Pirhonen, A., Linnala-Kankkunen, A., and Maenpaa, P.H. (1999). Cross-linking of osteopontin by tissue transglutaminase increases its collagen binding properties. J Biol Chem *274*, 1729-1735.

Katagiri, Y.U., Murakami, M., Mori, K., Iizuka, J., Hara, T., Tanaka, K., Jia, W.Y., Chambers, A.F., and Uede, T. (1996). Non-RGD domains of osteopontin promote cell adhesion without involving alpha v integrins. J Cell Biochem *62*, 123-131.

Katagiri, Y.U., Sleeman, J., Fujii, H., Herrlich, P., Hotta, H., Tanaka, K., Chikuma, S., Yagita, H., Okumura, K., Murakami, M*., et al.* (1999). CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res *59*, 219-226.

Katayama, Y., House, C.M., Udagawa, N., Kazama, J.J., McFarland, R.J., Martin, T.J., and Findlay, D.M. (1998). Casein kinase 2 phosphorylation of recombinant rat osteopontin enhances adhesion of osteoclasts but not osteoblasts. J Cell Physiol *176*, 179-187.

Kavukcuoglu, N.B., Denhardt, D.T., Guzelsu, N., and Mann, A.B. (2007). Effects of Osteopontin Defficiency and Aging on Nanomechanics of Mouse Bone. Journal of Biomedical Materials Research *[epub ahead of print]*.

Kerr, J.M., Fisher, L.W., Termine, J.D., and Young, M.F. (1991). The cDNA cloning and RNA distribution of bovine osteopontin. Gene *108*, 237-243.

Keykhosravani, M., Doherty-Kirby, A., Zhang, C., Brewer, D., Goldberg, H.A., Hunter, G.K., and Lajoie, G. (2005). Comprehensive identification of post-translational modifications of rat bone osteopontin by mass spectrometry. Biochemistry *44*, 6990-7003.

Khan, S.A., Cook, A.C., Kappil, M., Gunthert, U., Chambers, A.F., Tuck, A.B., and Denhardt, D.T. (2005). Enhanced cell surface CD44 variant (v6, v9) expression by osteopontin in breast cancer epithelial cells facilitates tumor cell migration: novel post-transcriptional, post-translational regulation. Clin Exp Metastasis *22*, 663-673.

Khan, S.A., Lopez-Chua, C.A., Zhang, J., Fisher, L.W., Sorensen, E.S., and Denhardt, D.T. (2002). Soluble osteopontin inhibits apoptosis of adherent endothelial cells deprived of growth factors. J Cell Biochem *85*, 728-736.

Kiefer, M.C., Bauer, D.M., and Barr, P.J. (1989). The cDNA and derived amino acid sequence for human osteopontin. Nucleic Acids Res *17*, 3306.

Kitahara, K., Ishijima, M., Rittling, S.R., Tsuji, K., Kurosawa, H., Nifuji, A., Denhardt, D.T., and Noda, M. (2003). Osteopontin deficiency induces parathyroid hormone enhancement of cortical bone formation. Endocrinology *144*, 2132-2140.

Kohri, K., Suzuki, Y., Yoshida, K., Yamamoto, K., Amasaki, N., Yamate, T., Umekawa, T., Iguchi, M., Sinohara, H., and Kurita, T. (1992). Molecular cloning and sequencing of cDNA encoding urinary stone protein, which is identical to osteopontin. Biochem Biophys Res Commun *184*, 859-864.

Konya, E., Umekawa, T., Iguchi, M., and Kurita, T. (2003). The role of osteopontin on calcium oxalate crystal formation. Eur Urol *43*, 564-571.

Kowalski, A. (2005). Creation, Characterization, and Application of Novel Anti-Osteopontin Monoclonal Antibodies. Dissertation In Department of Microbiology and Molecular Genetics (Piscataway, Rutgers University, the State University of New Jersey).

Koyama, Y., Rittling, S.R., Tsuji, K., Hino, K., Salincarnboriboon, R., Yano, T., Taketani, Y., Nifuji, A., Denhardt, D.T., and Noda, M. (2006). Osteopontin deficiency suppresses high phosphate load-induced bone loss via specific modulation of osteoclasts. Endocrinology *147*, 3040-3049.

Lampe, M.A., Patarca, R., Iregui, M.V., and Cantor, H. (1991). Polyclonal B cell activation by the Eta-1 cytokine and the development of systemic autoimmune disease. J Immunol *147*, 2902-2906.

Lasa, M., Chang, P.L., Prince, C.W., and Pinna, L.A. (1997). Phosphorylation of osteopontin by Golgi apparatus casein kinase. Biochem Biophys Res Commun *240*, 602-605.

Le, Q.T., Sutphin, P.D., Raychaudhuri, S., Yu, S.C., Terris, D.J., Lin, H.S., Lum, B., Pinto, H.A., Koong, A.C., and Giaccia, A.J. (2003). Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. Clin Cancer Res *9*, 59-67.

Leali, D., Dell'Era, P., Stabile, H., Sennino, B., Chambers, A.F., Naldini, A., Sozzani, S., Nico, B., Ribatti, D., and Presta, M. (2003). Osteopontin (Eta-1) and fibroblast growth factor-2 cross-talk in angiogenesis. J Immunol *171*, 1085-1093.

Lee, J.L., Wang, M.J., Sudhir, P.R., Chen, G.D., Chi, C.W., and Chen, J.Y. (2007). Osteopontin Promotes Integrin Activation through Outside-In and Inside-Out Mechanisms: OPN-CD44V Interaction Enhances Survival in Gastrointestinal Cancer Cells. Cancer Res *67*, 2089-2097.

Liaw, L., Almeida, M., Hart, C.E., Schwartz, S.M., and Giachelli, C.M. (1994). Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. Circ Res *74*, 214-224.

Liaw, L., Birk, D.E., Ballas, C.B., Whitsitt, J.S., Davidson, J.M., and Hogan, B.L. (1998). Altered wound healing in mice lacking a functional osteopontin gene (spp1). J Clin Invest *101*, 1468-1478.

Liaw, L., Skinner, M.P., Raines, E.W., Ross, R., Cheresh, D.A., Schwartz, S.M., and Giachelli, C.M. (1995). The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest *95*, 713-724.

Lieske, J.C., Hammes, M.S., Hoyer, J.R., and Toback, F.G. (1997). Renal cell osteopontin production is stimulated by calcium oxalate monohydrate crystals. Kidney Int *51*, 679-686.

Malyankar, U.M., Scatena, M., Suchland, K.L., Yun, T.J., Clark, E.A., and Giachelli, C.M. (2000). Osteoprotegerin is an alpha vbeta 3-induced, NF-kappa B-dependent survival factor for endothelial cells. J Biol Chem *275*, 20959-20962.

Marroquin, C.E., Downey, L., Guo, H., and Kuo, P.C. (2004). Osteopontin increases CD44 expression and cell adhesion in RAW 264.7 murine leukemia cells. Immunology letters *95*, 109-112.

Masuda, K., Takahashi, N., Tsukamoto, Y., Honma, H., and Kohri, K. (2000). N-Glycan structures of an osteopontin from human bone. Biochem Biophys Res Commun *268*, 814-817.

McKee, M.D., and Nanci, A. (1995). Osteopontin and the bone remodeling sequence. Colloidal-gold immunocytochemistry of an interfacial extracellular matrix protein. Ann N Y Acad Sci *760*, 177-189.

McKee, M.D., and Nanci, A. (1996). Osteopontin at mineralized tissue interfaces in bone, teeth, and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover, and repair. Microsc Res Tech *33*, 141-164.

McKee, M.D., Nanci, A., and Khan, S.R. (1995). Ultrastructural immunodetection of osteopontin and osteocalcin as major matrix components of renal calculi. J Bone Miner Res *10*, 1913-1929.

Miyauchi, A., Alvarez, J., Greenfield, E.M., Teti, A., Grano, M., Colucci, S., Zambonin-Zallone, A., Ross, F.P., Teitelbaum, S.L., Cheresh, D*., et al.* (1991). Recognition of osteopontin and related peptides by an alpha v beta 3 integrin stimulates immediate cell signals in osteoclasts. J Biol Chem *266*, 20369-20374.

Miyazaki, Y., Setoguchi, M., Yoshida, S., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1990). The mouse osteopontin gene. Expression in monocytic lineages and complete nucleotide sequence. J Biol Chem *265*, 14432-14438.

Mukherjee, B.B., Nemir, M., Beninati, S., Cordella-Miele, E., Singh, K., Chackalaparampil, I., Shanmugam, V., DeVouge, M.W., and Mukherjee, A.B. (1995). Interaction of osteopontin with fibronectin and other extracellular matrix molecules. Ann N Y Acad Sci *760*, 201-212.

Nasu, K., Ishida, T., Setoguchi, M., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1995). Expression of wild-type and mutated rabbit osteopontin in Escherichia coli, and their effects on adhesion and migration of P388D1 cells. Biochem J *307 (Pt 1)*, 257-265.

Neame, P.J., and Butler, W.T. (1996). Posttranslational modification in rat bone osteopontin. Connect Tissue Res *35*, 145-150.

Nemir, M., DeVouge, M.W., and Mukherjee, B.B. (1989). Normal rat kidney cells secrete both phosphorylated and nonphosphorylated forms of osteopontin showing different physiological properties. J Biol Chem *264*, 18202-18208.

Nemoto, H., Rittling, S.R., Yoshitake, H., Furuya, K., Amagasa, T., Tsuji, K., Nifuji, A., Denhardt, D.T., and Noda, M. (2001). Osteopontin deficiency reduces experimental tumor cell metastasis to bone and soft tissues. J Bone Miner Res *16*, 652-659.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J., and Haylock, D.N. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood *106*, 1232-1239.

Nystrom, T., Duner, P., and Hultgardh-Nilsson, A. (2007). A constitutive endogenous osteopontin production is important for macrophage function and differentiation. Exp Cell Res *313*, 1149-1160.

O'Regan, A., and Berman, J.S. (2000). Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. Int J Exp Pathol *81*, 373-390.

O'Regan, A.W., Hayden, J.M., and Berman, J.S. (2000). Osteopontin augments CD3-mediated interferon-gamma and CD40 ligand expression by T cells, which results in IL-12 production from peripheral blood mononuclear cells. J Leukoc Biol *68*, 495-502.

Oates, A.J., Barraclough, R., and Rudland, P.S. (1996). The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. Oncogene *13*, 97-104.

Ohri, R., Tung, E., Rajachar, R., and Giachelli, C.M. (2005). Mitigation of ectopic calcification in osteopontin-deficient mice by exogenous osteopontin. Calcif Tissue Int *76*, 307-315.

Oldberg, A., Franzen, A., and Heinegard, D. (1986). Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc Natl Acad Sci U S A *83*, 8819-8823.

Owen, T.A., Aronow, M., Shalhoub, V., Barone, L.M., Wilming, L., Tassinari, M.S., Kennedy, M.B., Pockwinse, S., Lian, J.B., and Stein, G.S. (1990). Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol *143*, 420-430.

Pampena, D.A., Robertson, K.A., Litvinova, O., Lajoie, G., Goldberg, H.A., and Hunter, G.K. (2004). Inhibition of hydroxyapatite formation by osteopontin phosphopeptides. Biochem J *378*, 1083.

Patarca, R., Freeman, G.J., Singh, R.P., Wei, F.Y., Durfee, T., Blattner, F., Regnier, D.C., Kozak, C.A., Mock, B.A., Morse, H.C., 3rd*, et al.* (1989). Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. J Exp Med *170*, 145-161.

Petrik, D., Lavori, P.W., Cao, H., Zhu, Y., Wong, P., Christofferson, E., Kaplan, M.J., Pinto, H.A., Sutphin, P., Koong, A.C.*, et al.* (2006). Plasma osteopontin is an independent prognostic marker for head and neck cancers. J Clin Oncol *24*, 5291-5297.

Philip, S., and Kundu, G.C. (2003). Osteopontin induces nuclear factor kappa B-mediated promatrix metalloproteinase-2 activation through I kappa B alpha /IKK signaling pathways, and curcumin (diferulolylmethane) down-regulates these pathways. J Biol Chem *278*, 14487-14497.

Pollack, S.B., Linnemeyer, P.A., and Gill, S. (1994). Induction of osteopontin mRNA expression during activation of murine NK cells. J Leukoc Biol *55*, 398-400.

Prince, C.W. (1989). Secondary structure predictions for rat osteopontin. Connect Tissue Res *21*, 15-20.

Prince, C.W., and Butler, W.T. (1987). 1,25-Dihydroxyvitamin D3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in clonal osteoblast-like osteosarcoma cells. Coll Relat Res *7*, 305-313.

Prince, C.W., Dickie, D., and Krumdieck, C.L. (1991). Osteopontin, a substrate for transglutaminase and factor XIII activity. Biochem Biophys Res Commun *177*, 1205-1210.

Prince, C.W., Oosawa, T., Butler, W.T., Tomana, M., Bhown, A.S., Bhown, M., and Schrohenloher, R.E. (1987). Isolation, characterization, and biosynthesis of a phosphorylated glycoprotein from rat bone. J Biol Chem *262*, 2900-2907.

Qin, C., Baba, O., and Butler, W.T. (2004). Post-Translational Modifications of SIBLING Proteins and Their Roles in Osteogenesis and Dentinogenesis. Crit Rev Oral Biol Med *15*, 126-136`.

Razzouk, S., Brunn, J.C., Qin, C., Tye, C.E., Goldberg, H.A., and Butler, W.T. (2002). Osteopontin posttranslational modifications, possibly phosphorylation, are required for in vitro bone resorption but not osteoclast adhesion. Bone *30*, 40-47.

Reinholt, F.P., Hultenby, K., Oldberg, A., and Heinegard, D. (1990). Osteopontin--a possible anchor of osteoclasts to bone. Proc Natl Acad Sci U S A *87*, 4473-4475.

Ritter, N.M., Farach-Carson, M.C., and Butler, W.T. (1992). Evidence for the formation of a complex between osteopontin and osteocalcin. J Bone Miner Res *7*, 877-885.

Rittling, S.R., Chen, Y., Feng, F., and Wu, Y. (2002). Tumor-derived osteopontin is soluble, not matrix associated. J Biol Chem *277*, 9175-9182.

Rittling, S.R., Matsumoto, H.N., McKee, M.D., Nanci, A., An, X.R., Novick, K.E., Kowalski, A.J., Noda, M., and Denhardt, D.T. (1998). Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. J Bone Miner Res *13*, 1101-1111.

Rollo, E.E., Laskin, D.L., and Denhardt, D.T. (1996). Osteopontin inhibits nitric oxide production and cytotoxicity by activated RAW264.7 macrophages. J Leukoc Biol *60*, 397-404.

Rosenthal, A.K., Gohr, C.M., Uzuki, M., and Masuda, I. (2007). Osteopontin promotes pathologic mineralization in articular cartilage. Matrix Biol *26*, 96-105.

Ross, F.P., Chappel, J., Alvarez, J.I., Sander, D., Butler, W.T., Farach-Carson, M.C., Mintz, K.A., Robey, P.G., Teitelbaum, S.L., and Cheresh, D.A. (1993). Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. J Biol Chem *268*, 9901-9907.

Rudland, P.S., Platt-Higgins, A., El-Tanani, M., De Silva Rudland, S., Barraclough, R., Winstanley, J.H., Howitt, R., and West, C.R. (2002). Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer. Cancer Res *62*, 3417-3427.

Safran, J.B., Butler, W.T., and Farach-Carson, M.C. (1998). Modulation of osteopontin post-translational state by 1, 25-(OH)2-vitamin D3. Dependence on Ca2+ influx. J Biol Chem *273*, 29935-29941.

Sakaguchi, H., Fujimoto, J., Hong, B.L., and Tamaya, T. (2007). Clinical implications of osteopontin in metastatic lesions of uterine cervical cancers. Cancer Lett *247*, 98-102.

Samanna, V., Wei, H., Ego-Osuala, D., and Chellaiah, M.A. (2006). Alpha-V-dependent outside-in signaling is required for the regulation of CD44 surface expression, MMP-2 secretion, and cell migration by osteopontin in human melanoma cells. Exp Cell Res *312*, 2214-2230.

Scatena, M., Almeida, M., Chaisson, M.L., Fausto, N., Nicosia, R.F., and Giachelli, C.M. (1998). NF-kappaB mediates alphavbeta3 integrin-induced endothelial cell survival. J Cell Biol *141*, 1083-1093.

Senger, D.R., Ledbetter, S.R., Claffey, K.P., Papadopoulos-Sergiou, A., Peruzzi, C.A., and Detmar, M. (1996). Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am J Pathol *149*, 293-305.

Senger, D.R., Wirth, D.F., and Hynes, R.O. (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell *16*, 885-893.

Senger, D.R., Wirth, D.F., and Hynes, R.O. (1980). Transformation-specific secreted phosophoproteins. Nature *286*, 619-621.

Shanmugam, V., Chackalaparampil, I., Kundu, G.C., Mukherjee, A.B., and Mukherjee, B.B. (1997). Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells. Biochemistry *36*, 5729-5738.

Shinohara, M.L., Lu, L., Bu, J., Werneck, M.B., Kobayashi, K.S., Glimcher, L.H., and Cantor, H. (2006). Osteopontin expression is essential for interferon-alpha production by plasmacytoid dendritic cells. Nat Immunol *7*, 498-506.

Shiraga, H., Min, W., VanDusen, W.J., Clayman, M.D., Miner, D., Terrell, C.H., Sherbotie, J.R., Foreman, J.W., Przysiecki, C., Neilson, E.G.*, et al.* (1992). Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci U S A *89*, 426-430.

Singh, K., DeVouge, M.W., and Mukherjee, B.B. (1990a). Physiological properties and differential glycosylation of phosphorylated and nonphosphorylated forms of osteopontin secreted by normal rat kidney cells. J Biol Chem *265*, 18696-18701.

Singh, K., Mukherjee, A.B., De Vouge, M.W., and Mukherjee, B.B. (1992). Differential processing of osteopontin transcripts in rat kidney- and osteoblast-derived cell lines. J Biol Chem *267*, 23847-23851.

Singh, R.P., Patarca, R., Schwartz, J., Singh, P., and Cantor, H. (1990b). Definition of a specific interaction between the early T lymphocyte activation 1 (Eta-1) protein and murine macrophages in vitro and its effect upon macrophages in vivo. J Exp Med *171*, 1931-1942.

Smith, J.H., and Denhardt, D.T. (1987). Molecular cloning of a tumor promoter-inducible mRNA found in JB6 mouse epidermal cells: induction is stable at high, but not at low, cell densities. J Cell Biochem *34*, 13-22.

Smith, J.H., and Denhardt, D.T. (1989). Evidence for two pathways of protein kinase C induction of 2ar expression: correlation with mitogenesis. J Cell Physiol *139*, 189-195.

Smith, L.L., Cheung, H.K., Ling, L.E., Chen, J., Sheppard, D., Pytela, R., and Giachelli, C.M. (1996). Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by alpha9beta1 integrin. J Biol Chem *271*, 28485-28491.

Smith, L.L., and Giachelli, C.M. (1998). Structural requirements for alpha 9 beta 1-mediated adhesion and migration to thrombin-cleaved osteopontin. Exp Cell Res *242*, 351-360.

Sodek, J., Batista Da Silva, A.P., and Zohar, R. (2006). Osteopontin and mucosal protection. J Dent Res *85*, 404-415.

Sodek, J., Chen, J., Nagata, T., Kasugai, S., Todescan, R., Jr., Li, I.W., and Kim, R.H. (1995). Regulation of osteopontin expression in osteoblasts. Ann N Y Acad Sci *760*, 223-241.

Sodek, J., Ganss, B., and McKee, M.D. (2000). Osteopontin. Crit Rev Oral Biol Med *11*, 279-303.

Sørensen, E.S., Hojrup, P., and Petersen, T.E. (1995). Posttranslational modifications of bovine osteopontin: identification of twenty-eight phosphorylation and three O-glycosylation sites. Protein Sci *4*, 2040-2049.

Sørensen, E.S., and Petersen, T.E. (1994). Identification of two phosphorylation motifs in bovine osteopontin. Biochem Biophys Res Commun *198*, 200-205.

Speer, M.Y., Chien, Y.C., Quan, M., Yang, H.Y., Vali, H., McKee, M.D., and Giachelli, C.M. (2005). Smooth muscle cells deficient in osteopontin have enhanced susceptibility to calcification in vitro. Cardiovasc Res *66*, 324-333.

Steitz, S.A., Speer, M.Y., McKee, M.D., Liaw, L., Almeida, M., Yang, H., and Giachelli, C.M. (2002). Osteopontin inhibits mineral deposition and promotes regression of ectopic calcification. Am J Pathol *161*, 2035-2046.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., Cheng, T., Dombkowski, D., Calvi, L.M., Rittling, S.R.*, et al.* (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med *201*, 1781-1791.

Suzuki, K., Zhu, B., Rittling, S.R., Denhardt, D.T., Goldberg, H.A., McCulloch, C.A., and Sodek, J. (2002). Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. J Bone Miner Res *17*, 1486-1497.

Takahashi, F., Akutagawa, S., Fukumoto, H., Tsukiyama, S., Ohe, Y., Takahashi, K., Fukuchi, Y., Saijo, N., and Nishio, K. (2002). Osteopontin induces angiogenesis of murine neuroblastoma cells in mice. Int J Cancer *98*, 707-712.

Teramoto, H., Castellone, M.D., Malek, R.L., Letwin, N., Frank, B., Gutkind, J.S., and Lee, N.H. (2005). Autocrine activation of an osteopontin-CD44-Rac pathway enhances invasion and transformation by H-RasV12. Oncogene *24*, 489-501.

Tezuka, K., Denhardt, D.T., Rodan, G.A., and Harada, S. (1996). Stimulation of mouse osteopontin promoter by v-Src is mediated by a CCAAT box-binding factor. J Biol Chem *271*, 22713-22717.

Tezuka, K., Sato, T., Kamioka, H., Nijweide, P.J., Tanaka, K., Matsuo, T., Ohta, M., Kurihara, N., Hakeda, Y., and Kumegawa, M. (1992). Identification of osteopontin in isolated rabbit osteoclasts. Biochem Biophys Res Commun *186*, 911-917.

Thalmann, G.N., Sikes, R.A., Devoll, R.E., Kiefer, J.A., Markwalder, R., Klima, I., Farach-Carson, C.M., Studer, U.E., and Chung, L.W. (1999). Osteopontin: possible role in prostate cancer progression. Clin Cancer Res *5*, 2271-2277.

Ue, T., Yokozaki, H., Kitadai, Y., Yamamoto, S., Yasui, W., Ishikawa, T., and Tahara, E. (1998). Co-expression of osteopontin and CD44v9 in gastric cancer. Int J Cancer *79*, 127-132.

Umekawa, T., Iguchi, M., and Kurita, T. (2001). The effect of osteopontin immobilized collagen granules in the seed crystal method. Urol Res *29*, 282-286.

Umekawa, T., Kohri, K., Kurita, T., Hirota, S., Nomura, S., and Kitamura, Y. (1995). Expression of osteopontin messenger RNA in the rat kidney on experimental model of renal stone. Biochem Mol Biol Int *35*, 223-230.

Vernon, H.J., Osborne, C., Tzortzaki, E.G., Yang, M., Chen, J., Rittling, S.R., Denhardt, D.T., Buyske, S., Bledsoe, S.B., Evan, A.P.*, et al.* (2005). Aprt/Opn double knockout mice: Osteopontin is a modifier of kidney stone disease severity. Kidney Int *68*, 938-947.

Wada, T., McKee, M.D., Steitz, S., and Giachelli, C.M. (1999). Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. Circ Res *84*, 166-178.

Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P.H., and Franceschi, R.T. (1999). Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res *14*, 893-903.

Weber, G.F., Ashkar, S., and Cantor, H. (1997). Interaction between CD44 and osteopontin as a potential basis for metastasis formation. Proc Assoc Am Physicians *109*, 1-9.

Weber, G.F., Ashkar, S., Glimcher, M.J., and Cantor, H. (1996). Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science *271*, 509-512.

Weber, G.F., Zawaideh, S., Hikita, S., Kumar, V.A., Cantor, H., and Ashkar, S. (2002). Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. J Leukoc Biol *72*, 752-761.

Wesson, J.A., Johnson, R.J., Mazzali, M., Beshensky, A.M., Stietz, S., Giachelli, C., Liaw, L., Alpers, C.E., Couser, W.G., Kleinman, J.G.*, et al.* (2003). Osteopontin is a critical inhibitor of calcium oxalate crystal formation and retention in renal tubules. J Am Soc Nephrol *14*, 139-147.

Worcester, E.M., Blumenthal, S.S., Beshensky, A.M., and Lewand, D.L. (1992). The calcium oxalate crystal growth inhibitor protein produced by mouse kidney cortical cells in culture is osteopontin. J Bone Miner Res *7*, 1029-1036.

Wrana, J.L., Zhang, Q., and Sodek, J. (1989). Full length cDNA sequence of porcine secreted phosphoprotein-I (SPP-I, osteopontin). Nucleic Acids Res *17*, 10119.

Wu, Y., Denhardt, D.T., and Rittling, S.R. (2000). Osteopontin is required for full expression of the transformed phenotype by the ras oncogene. Br J Cancer *83*, 156-163.

Xuan, J.W., Hota, C., Shigeyama, Y., D'Errico, J.A., Somerman, M.J., and Chambers, A.F. (1995). Site-directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. J Cell Biochem *57*, 680-690.

Yasui, T., Fujita, K., Asai, K., and Kohri, K. (2002). Osteopontin regulates adhesion of calcium oxalate crystals to renal epithelial cells. Int J Urol *9*, 100-108.

Yokosaki, Y., Matsuura, N., Sasaki, T., Murakami, I., Schneider, H., Higashiyama, S., Saitoh, Y., Yamakido, M., Taooka, Y., and Sheppard, D. (1999). The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. J Biol Chem *274*, 36328-36334.

Yokosaki, Y., Tanaka, K., Higashikawa, F., Yamashita, K., and Eboshida, A. (2005). Distinct structural requirements for binding of the integrins alphavbeta6, alphavbeta3, alphavbeta5, alpha5beta1 and alpha9beta1 to osteopontin. Matrix Biol *24*, 418-427.

Yoon, K., Buenaga, R., and Rodan, G.A. (1987). Tissue specificity and developmental expression of rat osteopontin. Biochem Biophys Res Commun *148*, 1129-1136.

Yoshitake, H., Rittling, S.R., Denhardt, D.T., and Noda, M. (1999). Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. Proc Natl Acad Sci U S A *96*, 8156-8160.

Young, M.F., Kerr, J.M., Termine, J.D., Wewer, U.M., Wang, M.G., McBride, O.W., and Fisher, L.W. (1990). cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics *7*, 491-502.

Yue, T.L., McKenna, P.J., Ohlstein, E.H., Farach-Carson, M.C., Butler, W.T., Johanson, K., McDevitt, P., Feuerstein, G.Z., and Stadel, J.M. (1994). Osteopontin-stimulated vascular smooth muscle cell migration is mediated by beta 3 integrin. Exp Cell Res *214*, 459-464.

Yumoto, K., Ishijima, M., Rittling, S.R., Tsuji, K., Tsuchiya, Y., Kon, S., Nifuji, A., Uede, T., Denhardt, D.T., and Noda, M. (2002). Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice. Proc Natl Acad Sci U S A *99*, 4556-4561.

Zhang, H., Ye, Q.H., Ren, N., Zhao, L., Wang, Y.F., Wu, X., Sun, H.C., Wang, L., Zhang, B.H., Liu, Y.K*., et al.* (2006). The prognostic significance of preoperative plasma levels of osteopontin in patients with hepatocellular carcinoma. J Cancer Res Clin Oncol *132*, 709-717.

Zhou, Y., Dai, D.L., Martinka, M., Su, M., Zhang, Y., Campos, E.I., Dorocicz, I., Tang, L., Huntsman, D., Nelson, C., *et al.* (2005). Osteopontin expression correlates with melanoma invasion. J Invest Dermatol *124*, 1044-1052.

Zohar, R., Cheifetz, S., McCulloch, C.A., and Sodek, J. (1998). Analysis of intracellular osteopontin as a marker of osteoblastic cell differentiation and mesenchymal cell migration. Eur J Oral Sci *106 Suppl 1*, 401-407.

Zohar, R., Lee, W., Arora, P., Cheifetz, S., McCulloch, C., and Sodek, J. (1997). Single cell analysis of intracellular osteopontin in osteogenic cultures of fetal rat calvarial cells. J Cell Physiol *170*, 88-100.

Zohar, R., Suzuki, N., Suzuki, K., Arora, P., Glogauer, M., McCulloch, C.A., and Sodek, J. (2000). Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J Cell Physiol *184*, 118-130.

CURRICULUM VITA

CHRISTIAN CHARLES KAZANECKI

Education:

2000 – Present Rutgers University   Piscataway, NJ
Doctor of Philosophy, Microbiology and Molecular Genetics

1998 - 2000   Rutgers University   Piscataway, NJ
Masters Program, Microbiology and Molecular Genetics

1989 - 1993   Lehigh University   Bethlehem, PA
Bachelor of Science, Molecular Biology

Experience:

1995 - 1998   Roche Molecular Systems, Inc. Branchburg, NJ
Associate Scientist

1994 - 1995   Lab Support, Inc.   Piscataway, NJ
Assistant Scientist

Publications:

Denhardt, D. T., Burger, E. H., Kazanecki, C.C., Krishna, S., Semeins, C. M., and Klein-Nulend, J. (2001). Osteopontin-deficient bone cells are defective in their ability to produce NO in response to pulsatile fluid flow. *Biochem Biophys Res Commun 288*, 448-453.

Christensen, B.*, Kazanecki, C.C.*, Petersen, T.E., Rittling, S.R., Denhardt, D.T., Sorensen, E.S. (2007). Cell-type specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. *J Biol Chem*, submitted.
* - these authors contributed equally to this study

Kazanecki C.C.*, Kowalski A.J.*, Ding T., Rittling S. R., and Denhardt, D.T. (2007). Characterization of anti-Osteopontin Monoclonal Antibodies: Sensitivity to Post-Translational Modification. In preparation. * - these authors contributed equally to this study.

Kazanecki, C.C., Denhardt, D.T. (2007). Establishment and Characterization of Pre-Osteoblast Cell Lines from the Long Bones of the Osteopontin Knock-out Mouse. In preparation

Haders, D.*, Kazanecki, C.C.*, Burukhin, A., Denhardt, D.T., Riman, R. (2007). Hydrothermally Synthesized Hydroxyapatite Film – an *in-vitro* Study of Bioactivity. In preparation. * - these authors contributed equally to this study.

Honors and Awards:

2004 National Science Foundation Integrative Graduate Education & Research Traineeship (IGERT) Fellowship.

2003 James B. Leatham scholarship.