(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 December 2007 (27.12.2007)

PCT

(10) International Publication Number
WO 2007/149948 A2

(51) International Patent Classification:
G01N 33/50 (2006.01)

(21) International Application Number:
PCT/US2007/071712

(22) International Filing Date: 20 June 2007 (20.06.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/805,298 20 June 2006 (20.06.2006) US

(71) Applicant *(for all designated States except US)*: **THE GOV. OF THE USA AS REPRESENTED BY THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; National Institutes Of Health, Office Of Technology Transfer, 6011 Executive Boulevard, Suite 325, Rockville, MD 20852-3804 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **TAKAFUJI, Vivian** [US/US]; 6373 Beryl Road, #201, Alexandria, VA 22312 (US). **WANG, Xin-Wei** [US/US]; 11409 Crownwood Lane, Rockville, MD 20850-3573 (US). **UNSWORTH, Edward, J.** [US/US]; 4414 Glenridge Street, Kensington, MD 20895 (US). **GOLDSMITH, Paul, K.** [US/US]; 202 Lynn Manor Drive, Rockville, MD 20850-4431 (US).

(74) Agent: **DEGRANDIS, Paula**; Klarquist Sparkman, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** COMPOSITIONS AND METHODS FOR DIAGNOSIS AND TREATMENT OF TUMORS

(57) **Abstract:** Based on the observation of the cooperation of osteopontin (OPN) and matrixmetalloproteinase-9 (MMP-9) in the promotion of the metastatic phenotype, therapies and diagnostic assays are disclosed for the treatment of a tumor that overexpresses OPN, such as hepatocellular carcinoma (HCC), for example metastatic HCC. In one example, methods of treating a tumor include administration of an agent that reduces cellular invasion resulting from the interaction between a fragment of OPN (OPN-5kD) generated by MMP-9 cleavage and CD44 receptor. Examples of such agents include fragments of OPN-5kD and antibodies specific for OPN-5kD. Therapeutic compositions are also provided that include such agents. Also provided are methods of diagnosing or prognosing a tumor, for example by detecting expression of OPN-5kD peptide or OPN-c mRNA in a biological sample obtained from the subject. Also provided are antibodies that specifically bind OPN-5kD.

WO 2007/149948 A2

COMPOSITIONS AND METHODS FOR DIAGNOSIS AND TREATMENT OF TUMORS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from US provisional application number 60/805,298, filed June 20, 2006, which is herein incorporated by reference.

FIELD

This application relates to methods of diagnosing and treating a tumor that has increased expression of osteopontin (OPN), such as metastatic hepatocellular carcinoma (HCC), as well as compositions and kits that can be used for such methods. Also provided are antibodies that specifically bind to a fragment of OPN (OPN-5kD) generated by metalloproteinase-9 (MMP-9) cleavage.

BACKGROUND

Elucidating the molecular events that promote tumor cell invasion continues to be a challenge for the treatment and prevention of hepatocellular carcinoma (HCC). HCC is a highly aggressive carcinoma of the liver which has been reported to occur world-wide in increasing numbers. Intra-hepatic metastatic recurrences via the portal vein are the main cause of death in HCC patients who have undergone partial hepatectomy or liver transplantation (Korn, *World J. Gastroenterol.*, 7:777-8, 2001). Identifying the main 'players' and how they contribute to the tumor cell metastatic cascade, for example at the early stages of cellular invasion, can present opportunities for lessening the severity of HCC through new therapeutic interventions.

Osteopontin (OPN, SPP1) is a secreted multi-functional glycoprotein expressed at high levels in tumors and the surrounding stroma of numerous cancers, including those of the liver, and is alternatively spliced into at least 3 isoforms (OPN-a, OPN-b, OPN-c) (Butler, *Ann. N.Y. Acad. Sci.*, 760:6-11, 1995; Coppola *et al.*, *Clin. Cancer Res.*, 10:184-90, 2004). Increased serum and plasma OPN levels (~ 4-10 fold) are associated with advanced stage lung, hepatic, breast, colon, and prostate carcinomas (Oates *et al.*, *Invasion Metastasis*, 17:1-15, 1997; Fedarko *et*

al., *Clin. Cancer Res.,* 7:4060-6, 2001; Singhal *et al.*, *Clin. Cancer Res.,* 3:605-11, 1997.). OPN expression can predict high grade, late stage and early recurrence HCC (Pan *et al.*, *Cancer*, 98:119-27, 2003) and is highly correlated with tumor recurrence and decreased patient survival following orthotopic liver transplantation (Wang, *Hepatology*, 42(4), Suppl. 1:391A, 2005). Prominent OPN levels have been detected in metastatic HCC tumor cells at the leading edge of pseudopodia and filopodia (Suzuki *et al.*, *J. Bone Miner. Res.*, 17:1486-97, 2002) and in macrophages at the tumor-stroma interface (Senger *et al.*, *Ann. N.Y. Acad. Sci.*, 760: 83-100, 1995). A correlation between OPN mRNA expression and primary HCC tumor metastasis has been shown (Ye *et al.*, *Nat. Med.*, 9: 416-423, 2003). Cytoplasmic OPN was detected in vascularized regions of primary HCC tumors but not in normal liver. In addition, a neutralizing antibody to OPN decreased pulmonary secondary lesions in nude mice and inhibited tumor cell invasion.

Local and extra-hepatic HCC tumor cell invasion is associated with extensive matrix remodeling, angiogenesis, and hepatocyte injury (McKenna *et al.*, *Am. J. Surg.*, 183: 588-94, 2002). The members of the zinc-dependent endopeptidase family of matrix metalloproteinases (MMPs) catabolize extracellular matrix components (Theret *et al.*, *Hepatology*, 34:82-88, 2001; Liaw and Crawford, *Braz. J. Med. Biol. Res.*, 32:805-812, 1999). Each MMP contains a catalytic and pro-peptide regulatory domain and a variable number of carboxy-terminal hemopoexin-like structural domains and are broadly divided into subclasses based on substrate activity. The two gelatinases (MMP-2 and MMP-9) play roles in tumor invasion and angiogenesis and participate in cancer progression in several neoplasias (Turpeenniemi-Hujanen, *Biochimie*, 87:287-97, 2005; Hanemaaijer *et al.*, *Int. J. Cancer*, 86:204-7, 2000; Scorilas *et al.*, *Br. J. Cancer,* 84:1488-96, 2001). Active MMP-9 enzymatically cleaves proteins of the basement membrane (such as collagens type IV, V, VII, X, and XIV) and can be detected at the invasive front of HCC (Kaneyoshi *et al.*, *Clin. Cancer Res.,* 7:4027-32, 2001). A substantial increase in MMP-9 mRNA levels in HCC primary metastatic tumors has been observed (Ye *et al.*, *Nat. Med.* 9:416-23, 2003), which is consistent with indications of HCC tumor malignancy and MMP-9 abundance (Ashida *et al.*, *Am. J. Pathol.*, 149:1803-11, 1996.; Wei *et al.*, *Hunan. Yi. Ke. Da. Xue. Xue. Bao.*, 28:212-6, 2003).

Stromelysin-1 (MMP-3) and matrilysin (MMP-7) are reported to cleave OPN at residues 166 and 210 (Agnihotri *et al.*, *J. Biol. Chem.*, 276:28261-7, 2001). MMP-3/-7 digested OPN fragments increase AsPC-1 and HeLa tumor cell adhesion via cell surface integrin receptors and MMP-3 cleaved OPN can increase mouse peritoneal macrophage cell migration. The thrombin coagulation factor also cleaves OPN at residue 168, resulting in two fragments of similar molecular weight (~28-30kD) can be detected in the serum and plasma of patients with cancer (Senger *et al.*, Cancer Res., 48:5770-4, 1988). Thrombin-cleaved OPN can mediate increased tumor and macrophage cell adhesion and migration via exposure of the amino-terminal reactive RGD sequence and binding to cell surface integrins, namely the vitronectin receptor αVβ3, although interactions with αVβ1, αVβ5, α4β1, and α9β1 have been described (Sodek *et al.*, *Crit. Rev. Oral Biol. Med.*, 11:279-303, 2000; Wai and Kuo, *J. Surg. Res.*, 121:228-41, 2004; Weber, *Biochim. Biophys. Acta*, 1552:61-85, 2001). Conversely, the COOH-terminal thrombin-cleaved fragment has been proposed to induce macrophage migration primarily through CD44 receptors (Weber *et al.*, *J. Leukoc. Biol.*, 72:752-61, 2002).

SUMMARY

Methods of treating a tumor that overexpresses osteopontin (OPN), such as a tumor that overexpresses OPN and MMP-9, as well as diagnosing and prognosing such tumors, are provided. Examples of such tumors include cancers of the liver, breast, colon, and prostate. The disclosed treatment, diagnostic, and prognostic methods can be used in combination or individually.

The inventors have determined that MMP-9 mediates OPN proteolytic cleavage into several fragments including OPN-5kD (such as SEQ ID NO: 4), which is a ligand for CD44 receptor. The interaction of OPN-5kD and the CD44 receptor increases HCC tumor cell invasion. The inventors have identified several fragments of OPN-5kD that significantly reduce HCC tumor cell invasion, which may be achieved by disrupting the interaction between OPN-5kD and CD44 receptor. Based on this observation, new methods of treating such tumors are disclosed, for example by using short invasive-blocking peptides targeted to the effects of OPN-5kD. The inventors have also identified monoclonal antibodies that bind to OPN-

5kD which can be used to significantly reduce HCC tumor cell invasion by disrupting the interaction between OPN-5kD and the CD44 receptor. Therefore, new methods of treating such tumors are disclosed, for example by administering therapeutically effective amounts of one or more peptides or antibodies that reduce or inhibit the effects of OPN-5kD.

Methods of treating a tumor in a subject are provided, such as a tumor that overexpresses OPN, MMP-9, or both. A particular example of such a tumor is HCC. In one example, the method includes administering to the subject a therapeutically effective amount of an agent that decreases cellular invasion of a tumor cell effected by the interaction of OPN-5kD and the CD44 receptor, such as a peptide that includes at least 5 contiguous amino acids of OPN-5kD, or an antibody that binds to OPN-5kD, thereby treating the tumor. A particular example of OPN-5kD sequence is provided in SEQ ID NO: 4. However, one skilled in the art will appreciate that variants of this sequence (such as polymorphisms) can retain OPN-5kD activity (such as the ability to bind to CD44 receptor with high affinity). For example, a variant OPN-5kD sequence may include at least one amino acid deletion, substitution, addition, or combinations thereof, such as 1-5 conservative amino acid substitutions, while retaining the ability to stimulate cellular invasion upon interaction with the CD44 receptor.

Exemplary therapeutic peptides can include at least one peptide sequence, or a plurality of peptide sequences, wherein each peptide sequence includes at least 5 contiguous amino acids of OPN-5kD. For example, the peptide can be a composition that includes one or more peptides consisting of the amino acid sequence shown in SEQ ID NO: 5, 6, 7, or 8. Exemplary therapeutic antibodies can include a single monoclonal antibody or an equivalent specific binding agent the binds to one epitope or multiple antibodies that bind to more than one epitope in OPN-5kD. If desired, therapeutically effective amounts of one or more additional therapeutic agents, such as an anti-neoplastic chemotherapeutic agent, can be administered to the subject. In particular examples, treatment includes reducing cellular invasion by a tumor, such as reducing or preventing metastasis of a tumor. In some examples, treating the tumor prolongs survival time of the subject (for example by at least 2 months, at least 6 months, or even at least 12 months).

Also provided by the present disclosure are therapeutic compositions that can be used to treat a tumor, such as a tumor that overexpresses OPN (such as overexpresses OPN and MMP-9), for example HCC. Such compositions can be used in the therapeutic methods provided herein. In one example, the composition includes one or more peptides, each including at least 5 contiguous amino acids of OPN-5kD (such as SEQ ID NO: 4). In a specific example, the composition includes one or more peptides consisting of the amino acid sequence of SEQ ID NO: 5, 6, 7, or 8. In another example the therapeutic compositions include antibodies that specifically bind to OPN-5kD. The disclosed compositions can include one or more pharmaceutically acceptable carriers, as well as additional therapeutic agents (such as other anti-neoplastic agents). Such therapeutic compositions can also be part of a kit, such as a kit that includes one more other anti-neoplastic agents (such as IL-2, IL-12, GM-CSF, a chemotherapeutic agent, or combinations thereof).

Methods of diagnosing and prognosing a tumor (such as a tumor that overexpresses OPN, MMP-9, or both) are provided. In some examples, such methods are performed prior to the treatment methods described herein. However, such methods can also be used independently of the disclosed treatment methods. In particular examples, the method includes contacting a sample from the subject that includes peptides (such as serum) with an agent that specifically binds to OPN-5kD (such as an antibody that specifically binds to SEQ ID NO: 4), then determining whether the agent specifically bound to proteins in the sample. If specific binding of the agent to proteins in the sample is detected, this indicates that the subject has a tumor (such as HCC), has a poor prognosis (for example because this indicates that the tumor has metastasized), or both. Such a method provides a non-invasive means for diagnosis and prognosis of a tumor, such as metastatic HCC. In another example, the method includes contacting a sample from the subject that includes nucleic acid molecules (such as a tumor sample) with an agent that permits detection of OPN-c nucleic acid molecules (such as cDNA or mRNA), then determining a relative amount of OPN-c nucleic acid molecules in the sample. For example, if increased OPN-c mRNA expression is detected in non-cancerous tissue adjacent to the tumor or in the tumor itself (or both), this indicates that the subject's tumor has

increased metastatic potential. Similar methods can be used to measure OPN-c protein expression, as an alternative to OPN-c nucleic acid molecule expression.

Also provided are antibodies that specifically bind OPN-5kD, such as an antibody that binds to an epitope sequence within SEQ ID NO: 4, or a fragment thereof such as any of SEQ ID NOS: 5-8. Such antibodies are useful for detection and treatment of tumors and can be part of a kit. Diagnostic kits can include other agents to permit detection of the antibody, such as a labeled secondary antibody.

The foregoing and other objects and features of the disclosure will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A are two plots showing that mRNA expression of OPN (left) and MMP-9 (right) correlate with HCC tumor metastasis. NM=non-metastatic (n=11), M=metastatic (n=14), MM=metastatic intra-hepatic lesion (n=10). All data were normalized to mRNA levels detected in the normal tissue immediately adjacent to the tumor samples (T/N). Statistical analysis was performed using Mann Whitney non-parametric t-tests (α=0.05).

FIG. 1B is a graph showing OPN and MMP-9 expression level correlation as determined using linear regression analysis after plotting values within each patient case.

FIG. 2A is a digital image of a Western blot showing a time-dependent MMP-9-cleavage of recombinant human OPN (~65 kD) (lanes 2-6). MMP inhibitors were added to separate reactions and analyzed after a 1-hour digestion at 37°C: EDTA (10 mM), MMP-9 inhibitor I (2 μM) and TIMP-1 (0.1 μM) (lanes 8, 10, 11 respectively). Undigested OPN (lane 1), OPN exposed to thrombin cleavage (0.05 U) (lane 7) and TIMP-1 alone lanes (9) were included as controls.

FIG. 2B is a graph showing the kinetics of the MMP-9 specific OPN cleavage quantified by densitometric analysis of the three bands marked by arrows in FIG. 2A and presented as a fraction of starting values at Time=0.

FIG. 3A is a schematic drawing showing the expression vectors generated to determine the activity level of OPN fragments.

FIG. 3B is a digital image of a Western blot showing expression of the four constructs in FIG. 3A in HEK 293 cells.

FIG. 4A is a digital image of a Western blot (left) and a bar graph showing densitometric analysis of OPN-5kD expression in FIG. 4A (right), demonstrating that endogenous OPN protein levels correlate with HCC cell line metastatic potential. Data were adjusted relative to Hep3B levels (=1) following normalization to b-actin levels.

FIG. 4B is a digital image of a protein gel showing gelatinase activity relative to pro-MMP-9, active MMP-9, and active MMP-2 standards.

FIG. 4C is a bar graph showing the percent cell invasion for three HCC cell lines. Data are presented as the mean percent cell invasion and standard deviation following normalization to fluorescence readings corresponding to cells migrating through uncoated control membranes.

FIG. 5A is a bar graph showing that MMP-9-cleaved OPN increased HCC cell adhesion relative to uncleaved OPN. Student un-paired t-tests were used to compare cleaved versus un-cleaved OPN adhesion (α=0.05).

FIG. 5B is a bar graph showing adhesion of HCC cells in response to four OPN constructs. Student un-paired t-tests were used to compare media versus OPN adhesion at each time point (α=0.05).

FIG. 5C is a graph showing the effect of different length OPN molecules on migration of SMMC-7721. Data are presented relative to vector-transfected cell controls at each of the time points. * denotes significance $p<0.017$.

FIG. 6A is a bar graph showing that the OPN-5kD fragment induced reproducible increases in cellular invasion. Data are presented as the mean percent cell invasion and standard deviation following normalization to fluorescence readings corresponding to cells migrating through uncoated control membranes. Un-paired student's t-tests were used to compare mean and standard deviation values (α=0.05).

FIG. 6B is a bar graph showing the effect of the presence of blocking antibodies to the integrin $\alpha V\beta 3$ and CD44 receptors on the invasion of HCC cells transiently expressing OPN full-length or OPN-5kD. Un-paired student's t-tests were used to compare mean and standard deviation values (α=0.05).

FIG. 6C is a bar graph showing the effect of varying concentrations of the OPN-5kD peptide on the invasion of HCC cells. Un-paired student's t-tests were used to compare mean and standard deviation values of media versus treated cells (α=0.05).

FIGS. 7A and B are bar graphs showing that some 10-mer peptides can inhibit OPN-5kD induced HCC cellular invasion. Data were normalized to uncoated membrane chamber values and adjusted by media control values (=1). (A) Un-paired student's t-tests were used to compare mean and standard deviation values of OPN-5kD peptide versus each of the 10-mer peptides (α=0.05). (B) Significance of the small peptide inhibition of the OPN-5kD peptide increased cellular invasion was tested using un-paired student's t-tests (α=0.05). * denotes significance (A) $p<0.037$ and (B) $p<0.002$.

FIGS. 8A and 8B are plots showing the OPN splice variants expressed in (A) tumor or (B) non-cancerous tissue. Data are shown following normalization to 18S rRNA (Applied Biosystems, CA) and relative to a normal liver tissue reference pool (n=8).

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and one letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NO: 1 is a cDNA sequence for a human osteopontin-a.

SEQ ID NO: 2 is the protein encoded by SEQ ID NO: 1.

SEQ ID NO: 3 is a cDNA sequence for a human osteopontin-c.

SEQ ID NO: 4 is an amino acid sequence of OPN-5kD.

SEQ ID NO: 5 is the p3 fragment of OPN-5kD.

SEQ ID NO: 6 is the p6 fragment of OPN-5kD.

SEQ ID NO: 7 is the p7 fragment of OPN-5kD.

SEQ ID NO: 8 is a fragment of OPN-5kD.

SEQ ID NO: 9 is the protein encoded by SEQ ID NO: 3.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Abbreviations and Terms

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. The singular forms "a," "an," and "the" refer to one or more than one, unless the context clearly dictates otherwise. For example, the term "comprising a peptide" includes single or plural peptides and is considered equivalent to the phrase "comprising at least one peptide." The term "or" refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. As used herein, "comprises" means "includes." Thus, "comprising A or B," means "including A, B, or A and B," without excluding additional elements.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs.

anti-CTLA-4: cytotoxic T-lymphocyte antigen-4

HCC: hepatocellular carcinoma

IL-2: interleukin-2

MMP-9: matrix metalloproteinase-9

OPN: osteopontin

Administration: To provide or give a subject an agent, such as a composition that includes a peptide fragment of OPN-5kD, such as one or more of SEQ ID NOS: 5-8, or OPN-5kD specific antibodies, by any effective route. Exemplary routes of administration include, but are not limited to, oral, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), sublingual, rectal, transdermal (e.g. topical), intranasal, vaginal and inhalation routes.

Antibody: Immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, that is, molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen (such as OPN-5kD).

A naturally occurring antibody (such as IgG, IgM, IgD) includes four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. As used herein, the term antibody also includes recombinant antibodies produced by expression of a nucleic acid that encodes one or more antibody chains in a cell (for example see U.S. Patent No. 4,745,055; U.S. Patent No. 4,444,487; WO 88/03565; EP 256,654; EP 120,694; EP 125,023; Faoulkner *et al.*, *Nature* 298:286, 1982; Morrison, *J. Immunol.* 123:793, 1979; Morrison *et al.*, *Ann Rev. Immunol* 2:239, 1984).

The term antibody also includes an antigen binding fragment of a naturally occurring or recombinant antibody. Specific, non-limiting examples of binding fragments encompassed within the term antibody include Fab, $(Fab')_2$, Fv, and single-chain Fv (scFv). Fab is the fragment that contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain or equivalently by genetic engineering. Fab' is the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule. $(Fab')_2$ is the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction or equivalently by genetic engineering. $F(Ab')_2$ is a dimer of two FAb' fragments held together by disulfide bonds. Fv is a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains. Single chain antibody ("SCA") is a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Methods of making these fragments are routine in the art.

Binding affinity: Affinity of an antibody for an antigen, such as the affinity of an antibody for an OPN-5kD peptide. In one example, affinity is calculated by a modification of the Scatchard method described by Frankel *et al.*, *Mol. Immunol.*,

16:101-106, 1979. In another example, binding affinity is measured by an antigen/antibody dissociation rate. In yet another example, a high binding affinity is measured by a competition radioimmunoassay. In several examples, a high binding affinity is at least about 1 x 10^{-8} M. In other example, a high binding affinity is at least about 1.5 x 10^{-8}, at least about 2.0 x 10^{-8}, at least about 2.5 x 10^{-8}, at least about 3.0 x 10^{-8}, at least about 3.5 x 10^{-8}, at least about 4.0 x 10^{-8}, at least about 4.5 x 10^{-8}, or at least about 5.0 x 10^{-8} M.

In a particular example, a functional variant of an OPN-5kD peptide retains a similar binding affinity for an antibody than the binding affinity of the native OPN-5kD peptide (such as SEQ ID NO: 4) for the same antibody.

Cancer: Malignant neoplasm that has undergone characteristic anaplasia with loss of differentiation, increased rate of growth, invasion of surrounding tissue, and is capable of metastasis.

CD44 receptors: A family of cell-surface adhesion molecules found on both normal and malignant cell types that can mediate cell-matrix and cell-cell interactions. CD44 receptors have been associated with increased inflammation and metastasis. Based on the results shown herein, it is proposed that OPN-5kD is a ligand for CD44 receptors, and this interaction enhances cellular invasion, for example of HCC cells.

Chemotherapeutic agent: In cancer treatment, refers to the administration of one or a combination of compounds to kill or slow the reproduction of rapidly multiplying cells. Chemotherapeutic agents include anti-neoplastics known by those skilled in the art, including, but not limited to: 5-fluorouracil (5-FU), azathioprine, cyclophosphamide, antimetabolites (such as Fludarabine), antineoplastics (such as Etoposide, Doxorubicin, methotrexate, and Vincristine), carboplatin, cis-platinum and the taxanes, such as taxol, monoclonal antibodies such as Avastin or Herceptin, and growth pathway inhibitors such as Gleevac. In particular examples, such chemotherapeutic agents are administered in combination with a therapy that reduces cellular invasion (for example before, during or after administration of a therapeutic amount of one or more peptides that include at least 5 contiguous amino acids of OPN-5kD, such as SEQ ID NOS: 5-8, or OPN-5kD specific antibodies).

Chimeric antibodies: Antibodies whose light and heavy chain genes have been constructed, for example by genetic engineering, from immunoglobulin variable and constant region genes belonging to different species. For example, the variable segments of the genes from a mouse monoclonal antibody can be joined to human constant segments, such as kappa and gamma 1 or gamma 3. In one example, a chimeric antibody is a hybrid protein composed of the variable or antigen-binding domain from a mouse antibody and the constant or effector domain from a human antibody (such as an antibody that recognizes OPN-5kD), although other mammalian species can be used, or the variable region can be produced by molecular techniques. Methods of making chimeric antibodies are well known in the art, for example, see U.S. Patent No. 5,807,715.

Conservative substitution: One or more amino acid substitutions for amino acid residues having similar biochemical properties. Typically, conservative substitutions have little to no impact on the activity of a resulting polypeptide. For example, a conservative substitution in an OPN-5kD fragment (such as one or more conservative substitutions in any of SEQ ID NOS: 5-8) ideally does not substantially affect the ability of the peptide to reduce invasion of an HCC cell. Methods that can be used to determine the amount of invasion are disclosed herein (for example, see Examples 4-8). In another particular example, a conservative substitution in OPN-5kD (such as SEQ ID NO: 4) ideally does not significantly decrease the ability of OPN-5kD to specifically bind to CD44, and in some examples may not affect the ability of such an interaction to stimulate cellular invasion. The interaction between an OPN-5kD variant containing one or more conservative amino acid substitutions and CD44 can be measured using methods known in the art, such as incubating a labeled OPN-5kD antibody with CD44 receptor-expressing cells in the presence of OPN-5kD, wherein the presence of detectable label indicates the presence of bound OPN-5kD to the cells.

For example, an alanine scan can be used to identify which amino acid residues in SEQ ID NOS: 4-8 can tolerate an amino acid substitution. In one example, one or more conservative substitutions in SEQ ID NOS: 5-8 ideally do not decrease the observed reduction of invasion by more than 25%, for example not more than 20%, for example not more than 10%, when an alanine, or other

conservative amino acid (such as those listed below), is substituted for one or more native amino acids. In one example, one or more conservative substitutions in SEQ ID NO: 4 ideally does not reduce the observed interaction of SEQ ID NO: 4 with CD44 receptor by more than 25%, for example not more than 20%, for example not more than 10%, when an alanine, or other conservative amino acid (such as those listed below), is substituted for one or more native amino acids.

In one example, one conservative substitution is included in the peptide, such as a single conservative amino acid substitution in any of SEQ ID NOS: 4-8. In another example, two conservative substitutions are included in the peptide (such as any of SEQ ID NOS: 4-8). In a further example, three conservative substitutions are included in the peptide (such as any of SEQ ID NOS: 4-8). A peptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR. Alternatively, a peptide can be produced to contain one or more conservative substitutions by using standard peptide synthesis methods.

Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Ser for Cys; Asn for Gln; Asp for Glu; Pro for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln for Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val.

Further information about conservative substitutions can be found in, among other locations in, Ben-Bassat *et al.*, (*J. Bacteriol.* 169:751-7, 1987), O'Regan *et al.*, (*Gene* 77:237-51, 1989), Sahin-Toth *et al.*, (*Protein Sci.* 3:240-7, 1994), Hochuli *et al.*, (*Bio/Technology* 6:1321-5, 1988) and in standard textbooks of genetics and molecular biology.

Decrease: To reduce the quality, amount, or strength of something.

In one example, a therapy decreases a tumor (such as the size of a tumor, the number of tumors, the metastasis of a tumor, or combinations thereof), or one or

more symptoms associated with a tumor, for example as compared to the response in the absence of the therapy. In a particular example, a therapy decreases the size of a tumor, the number of tumors, the metastasis of a tumor, or combinations thereof, subsequent to the therapy, such as a decrease of at least 10%, at least 20%, at least 50%, or even at least 90%. Such decreases can be measured using the methods disclosed herein.

Deletion: The removal of a one or more nucleotides from a nucleic acid molecule or the removal of one or more amino acids from a protein, the regions on either side being joined together.

Epitope: An antigenic determinant. An epitope is the particular structure formed by chemical groups or peptide sequences in a molecule that is antigenic, meaning that elicits a specific immune response. An antibody specifically binds a particular antigenic epitope, for example on an OPN-5kD peptide. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5, 6, 7, 8, 9 or 10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, for example, "Epitope Mapping Protocols" in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed (1996).

Hepatocellular carcinoma (HCC): A malignant collection of abnormal and uncontrollably growing cells that derive from hepatocytes, the epithelial cells of the liver. Risk factors for development of liver cancer include chronic infection with the hepatitis B or C virus and cirrhosis (scarring of the liver). HCC can be a primary tumor, an HCC tumor that has metastasized to another part of the body, or can result from a metastasis to the liver from another part of the body (such as from colon, stomach, skin or ovarian cancer).

Humanized antibodies: An immunoglobulin including a human framework region and one or more CDRs from a non-human (such as a mouse, rat, or synthetic)

immunoglobulin. A humanized antibody binds to the same antigen as the donor antibody that provides the CDRs.

The non-human immunoglobulin providing the CDRs is termed a "donor" and the human immunoglobulin providing the framework is termed an "acceptor." In one example, all the CDRs are from the donor immunoglobulin in a humanized immunoglobulin. Constant regions need not be present, but if they are, they are substantially identical to human immunoglobulin constant regions, for instance, at least about 85-90%, such as about 95% or more identical.

The donor CDRs of a humanized antibody can have a limited number of substitutions using amino acids from the acceptor CDR. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. The acceptor framework of a humanized immunoglobulin or antibody can have a limited number of substitutions by amino acids taken from the donor framework. Humanized or other monoclonal antibodies can have additional amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. Exemplary conservative substitutions are described above (see also U.S. Patent No. 5,585,089). Humanized immunoglobulins can be constructed by means of genetic engineering, for example, see U.S. Patent Nos. 5,225,539 and 5,585,089.

Isolated: An "isolated" biological component (such as a nucleic acid molecule, protein, or cell) has been substantially separated or purified away from other components, such as other components in the cell of the organism, or the organism itself, in which the component occurs, such as other chromosomal and extra-chromosomal DNA and RNA, proteins and cells. Nucleic acid molecules and proteins that have been "isolated" include nucleic acid molecules (such as DNA or RNA) and proteins purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acid molecules and proteins. For example, an isolated antibody is one that is substantially separated from the animal or cells in which it was generated, or from cell culture medium.

Label: A detectable compound or composition that is conjugated directly or indirectly to another molecule, such as an antibody or a protein, to facilitate

detection of that molecule. For example, the label can be capable of detection by ELISA, spectrophotometry, flow cytometry, or microscopy. Specific, non-limiting examples of labels include fluorophores, chemiluminescent agents, enzymatic linkages, and radioactive isotopes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed for example in Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989) and Ausubel *et al.* (In Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1998).

Malignant: Cells which have the properties of anaplasia invasion and metastasis.

Matrix metalloproteinase-9 (MMP-9): A member of the metalloproteinase family, also referred to as gelatinase B. It is called a gelatinase because it has a high affinity for digestion of denatured collagen I. MMP-9 can also cleave a variety of proteins including many components of the extracellular matrix such as collagens I, III, IV, and V, entactin, and elastin. MMP-9 is frequently up-regulated in cancer cells and also in the adjacent host tissues, and its expression by tumor cells contributes to metastasis.

MMP-9 has a signal peptide that leads to its secretion, but the secreted molecule is not itself enzymatically active. MMP-9 has a proregion adjacent to the signal peptide. The proregion contains a signature sequence including an unpaired cysteine that interacts with the Zn^{2+} that is complexed with three histidines to form the active site. This interaction allows the proregion to act as a competitive inhibitor of MMP-9. Hence, the proregion is cleaved and dissociated to allow enzymatic activity

MMP-9 has been cloned from a variety of organisms, and nucleic acid and protein sequences are publicly available, for example from GenBank and EMBL. Human MMP-9 protein is Mr 92,000 although the murine form is Mr 105,000 because of an insert of an additional 24 amino acids.

Neoplasm: Abnormal growth of cells.

Normal cells: Non-tumor, non-malignant cells.

Osteopontin (OPN, SPP1): A secreted multi-functional phosphorylated glycoprotein expressed at high levels in tumors and the surrounding stroma of

numerous cancers, including those of the liver. OPN proteins contain a functional Gly-Arg-Gly-Asp-Ser (GRGDS; amino acids 131-135 of SEQ ID NO: 4) cell-binding sequence.

Several splice variants of OPN have been identified, including OPN-a (native sequence) and OPN-c (truncated sequence). OPN-c lacks exon 4 (27 amino acids) in the NH_2-terminal region of the mature sequence. OPN-c includes a transglutaminase reactive domain (Gly-X-Gly) which can mediate covalent homodimer cross-linking as well as heterodimer formation to other matrix components (such as fibronectin).

The term osteopontin includes any osteopontin gene, cDNA, mRNA, or protein from any organism that retains OPN biological activity. OPN sequences are publicly available. For example, GenBank Accession Nos: D28759 (nucleic acid) and BAA05949 (protein) disclose human OPN-a sequences, and GenBank Accession Nos: D2876 (nucleic acid) and BAA05951 (protein) disclose human OPN-c sequences.

In one example, an OPN sequence includes a full-length wild-type (or native) sequence, as well as OPN allelic variants, variants, fragments, homologs or fusion sequences that retain OPN biological function. In certain examples, OPN has at least 80% sequence identity, for example at least 85%, at least 90%, at least 95%, or at least 98% sequence identity to a native OPN. In other examples, an OPN nucleic acid sequence has a sequence that hybridizes under very high stringency conditions to a sequence set forth in GenBank Accession No. D28759 or D2876 and retains OPN activity.

OPN-5kD: One of three osteopontin peptide fragments generated when exposed to MMP-9 (when run on a SDS gel this peptide can migrate at approximately 10kD, however, its predicted size based upon amino acid sequence is 5kD). This peptide binds to the CD44 receptor, thereby enhancing cellular invasion of HCC cells. A particular example of an OPN-5kD sequence is provided in SEQ ID NO: 4. However, variants of SEQ ID NO: 4 (such as a truncated sequence, fusion sequence, a sequence that includes one or more substitutions, deletions, or insertions, or combinations thereof) can also be an OPN-5kD peptide, as long as such peptide retains the ability to bind CD44 receptor an stimulate cellular invasion

of HCC cells (for example using the methods described in Examples 4-7). In a particular example, an OPN-5kD sequence has at least 80% sequence identity to SEQ ID NO: 4, such as at least 90% or at least 95% sequence identity to SEQ ID NO: 4. In one example, an OPN-5kD peptide sequence includes 1-8 conservative amino acid sequences in SEQ ID NO: 4, such as 1, 2, 3, 4, 5, 6, 7 or 8 conservative amino acid sequences in SEQ ID NO: 4.

Peptide: A chain of amino acids of which is at least 4 amino acids in length, regardless of post-translational modification (such as glycosylation or phosphorylation). In one example, a peptide is at least 6 amino acids in length, such as at least 8, at least 9, at least 10, at least 11, or at least 12 amino acids in length. In particular examples, a peptide is 4 to 30 amino acids in length, for example 5 to 25 amino acids in length, 5 to 20 amino acids in length, 9 to 15 amino acids in length, or 9 to 10 amino acids in length.

In one example, a peptide is an OPN peptide or a fragment thereof, such as OPN-5kD (for example SEQ ID NO: 4) or a fragment thereof (such as SEQ ID NOS: 5-8) (or variants thereof). In particular examples a fragment of an OPN-5kD peptide consists of at least 5 contiguous amino acids of SEQ ID NO: 4, such as at least 6, at least 7, at least 8, at least 9, or at least 10 contiguous amino acids of SEQ ID NO: 4, for example 5-25, 5-20, 8-10, 9-10, 9, or 10 contiguous amino acids of SEQ ID NO: 4.

Pharmaceutically Acceptable Carriers: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic agents, such as one or more compositions that include a peptide that includes at least 5 contiguous amino acids of OPN-5kD (such as at least 9 contiguous amino acids of SEQ ID NO: 4) or an OPN-5kD specific antibody.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations can include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose,

glycerol or the like as a vehicle. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate, sodium lactate, potassium chloride, calcium chloride, and triethanolamine oleate.

Purified: The term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide is one in which the peptide is more pure than the peptide in its natural environment, such as within a cell.

In particular examples, purified OPN peptides or fragments thereof (such as SEQ ID NOS: 4-8) refers to peptides that are at least 75% pure, at least 80% pure, at least 90% pure, at least 95% pure, at least 97% pure, at least 98% pure, or at least 99% pure. The purity of a peptide can be measured using methods known in the art, such as by a Western blot.

Radiological agent: In cancer treatment, refers to the administration of one or a combination of radioactive compounds to damage the DNA of cells, thereby killing or slowing the reproduction of rapidly multiplying cells. Exemplary methods of administering radiological agents to a subject include external beam radiotherapy (XBRT) or teletherapy, brachytherapy or sealed source radiotherapy, and unsealed source radiotherapy. The radiological agents that can be administered to a subject in combination with the disclosed therapies that include a peptide that includes at least 5 contiguous amino acids of OPN-5kD (such as at least 9 contiguous amino acids of SEQ ID NO: 4) or OPN-5kD antibodies. Exemplary radiological agents include those known by those skilled in the art, such as ionizing radiation (for example x-rays and gamma rays).

Sample: Includes biological samples that contain cells, genomic DNA, RNA, or proteins (or combinations thereof) obtained from a subject, such as those present in peripheral blood (or a fraction thereof such as plasma or serum), urine, saliva, tissue biopsy, surgical specimen, fine needle aspirate, and autopsy material. In a particular example, a sample is obtained from a subject having or suspected of having a metastatic HCC.

Sequence identity: The identity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al. Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options can be set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (such as C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (such as C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (such as C:\output.txt); -q is set to –1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison

between two sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastn –o c:\output.txt –q –1 –r 2.

To compare two amino acid sequences, the options of Bl2seq can be set as follows: -i is set to a file containing the first amino acid sequence to be compared (such as C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (such as C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (such as C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastp –o c:\output.txt. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1154 nucleotides is 75.0 percent identical to the test sequence (1166÷1554*100=75.0). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (that is, 15÷20*100=75).

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). Homologs are typically characterized by possession of at least 70% sequence

identity counted over the full-length alignment with an amino acid sequence using the NCBI Basic Blast 2.0, gapped blastp with databases such as the nr or swissprot database. Queries searched with the blastn program are filtered with DUST (Hancock and Armstrong, 1994, *Comput. Appl. Biosci.* 10:67-70). Other programs use SEG. In addition, a manual alignment can be performed. Proteins with even greater similarity will show increasing percentage identities when assessed by this method, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity. For example, a peptide having substantial sequence identity to an OPN-5kD sequence can share at least 80% sequence identity to SEQ ID NO: 4, such as at least 90% or at least 95% sequence identity to SEQ ID NO: 4.

When aligning short peptides (fewer than around 30 amino acids), the alignment can be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). For example, when less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and can possess sequence identities of at least 85%, at least 90%, at least 95% or at least 98% depending on their identity to the reference sequence. For example, a peptide having substantial sequence identity to an OPN-5kD fragment can share at least 80% sequence identity to any of SEQ ID NOS: 5-8, such as at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to SEQ ID NOS: 5-8. Methods for determining sequence identity over such short windows are described at the NCBI web site.

One indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions, as described above. Nucleic acid sequences that do not show a high degree of identity may nevertheless encode identical or similar (conserved) amino acid sequences, due to the degeneracy of the genetic code. Changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein. Such homologous nucleic acid sequences can, for example, possess at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity determined by this method. For example, a nucleic acid encoding an OPN-5kD sequence can have substantial sequence identity to a native

sequence if it shares at least 80% sequence identity to nucleotides 566-697 of SEQ ID NO: 1, such as at least 90% or at least 95% sequence identity to nucleotides 566-697 of SEQ ID NO: 1. An alternative (and not necessarily cumulative) indication that two nucleic acid sequences are substantially identical is that the peptide which the first nucleic acid sequence encodes is immunologically cross reactive with the peptide encoded by the second nucleic acid sequence.

One of skill in the art will appreciate that the particular sequence identity ranges are provided for guidance only; it is possible that strongly significant homologs could be obtained that fall outside the ranges provided.

Specific binding agent: An agent that binds substantially only to a defined target. Thus an OPN-5kD specific binding agent is an agent that binds substantially to a OPN-5kD peptide. In one example, the specific binding agent is an antibody that specifically binds the OPN-5kD peptide.

The term "**specifically binds**" refers, with respect to an antigen such as OPN-5kD, to the preferential association of an antibody or other specific binding agent, in whole or part, to the antigen and not to other antigens. A certain degree of non-specific interaction can occur between a specific binding agent and a non-target antigen. Nevertheless, specific binding can be distinguished as mediated through specific recognition of the antigen. Specific binding results in a significant association between the antibody (or other specific binding agent) and the antigen than between the antibody and a non-antigen. Specific binding typically results in greater than 2-fold, such as greater than 5-fold, greater than 10-fold, or greater than 100-fold increase in amount of bound antibody or other specific binding agent to the antigen as compared to binding to a non-specific antigen.

The determination that a particular agent binds substantially only to OPN-5kD can be made using or adapting routine procedures. For example, western blotting can be used to determine that a specific binding agent, such as an antibody, binds substantially only to the protein (such as substantially only binds OPN-5kD but not to other proteins in an HCC cell) (for example see Harlow and Lane, *Antibodies: A Laboratory Manual*. 1988). A variety of immunoassay formats are appropriate for selecting antibodies or other specific binding agent specifically immunoreactive with a particular protein (such as OPN-5kD). For example, solid-phase ELISA

immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein.

Subject: Living multi-cellular vertebrate organisms, a category that includes human and non-human mammals (such as laboratory or veterinary subjects).

Therapeutically effective amount: An amount of a therapeutic agent (such as an OPN-5kD specific antibody, or a composition that includes a peptide that includes at least 5 contiguous amino acids of OPN-5kD, for example at least 9 contiguous amino acids of SEQ ID NO: 4), that alone, or together with one or more additional therapeutic agents, induces the desired response, such as treatment of a tumor that overexpresses OPN, such as a metastatic HCC tumor. In one example, it is an amount of an OPN-5kD specific antibody, or one or more peptides that include at least 5 contiguous amino acids of OPN-5kD needed to prevent or delay the development of a tumor, prevent or delay the metastasis of a tumor, cause regression of an existing tumor, or treat one or more signs or symptoms associated with a tumor, in a subject, such as a subject having HCC. Ideally, a therapeutically effective amount provides a therapeutic effect without causing a substantial cytotoxic effect in the subject. The preparations disclosed herein are administered in therapeutically effective amounts.

In one example, a desired response is to decrease the size, volume, or number (such as metastases) of a tumor that overexpresses OPN, such as HCC. For example, the therapeutic compositions can decrease the size, volume, or number of tumors (such as HCC) by a desired amount, for example by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, or even at least 90%, as compared to a response in the absence of the composition.

The effective amount of a composition that includes an OPN-5kD specific antibody, or one or more peptides that include at least 5 contiguous amino acids of OPN-5kD, that is administered to a human or veterinary subject will vary depending upon a number of factors associated with that subject, for example the overall health of the subject. An effective amount of a composition can be determined by varying the dosage of the product and measuring the resulting therapeutic response, such as the regression of a tumor. Effective amounts also can be determined through

various *in vitro*, *in vivo* or *in situ* immunoassays. The disclosed therapeutic agents can be administered in a single dose, or in several doses, as needed to obtain the desired response. However, the effective amount of can be dependent on the source applied, the subject being treated, the severity and type of the condition being treated, and the manner of administration.

In particular examples, a therapeutically effective dose of one or more peptides that include at least 5 contiguous amino acids of OPN-5kD or an OPN-5kD antibody includes at least 1 μg daily (such as 1 - 100 μg or 5 – 50 μg) if administered via injection, or at least 1 mg daily if administered topically (such as 1 - 100 mg or 5 – 50 mg) of one or more peptides that include at least 5 contiguous amino acids of OPN-5kD or an OPN-5kD antibody. In particular examples, such daily dosages are administered in one or more divided doses (such as 2, 3, or 4 doses) or in a single formulation.

The disclosed compositions that include one or more peptides that include at least 5 contiguous amino acids of OPN-5kD or an OPN-5kD antibody can be administered alone, in the presence of a pharmaceutically acceptable carrier, in the presence of other therapeutic agents (such as other anti-neoplastic agents), or both.

Transgene: An exogenous gene introduced into a cell.

Treating or treatment: Refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition related to a disease (such as a tumor, for example HCC). Treatment can also induce remission or cure of a condition, such as a tumor. In particular examples, treatment includes preventing a tumor, for example by inhibiting the full development of a tumor, such as preventing development of a metastasis or the development of a primary tumor. Prevention does not require a total absence of a tumor.

Reducing a sign or symptom associated with a tumor (such as a tumor that overexpresses OPN, for example HCC) can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject (such as a subject having HCC which has not yet metastasized), a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease (for example by prolonging the life of a subject having tumor), a reduction in the number of relapses of the disease, an improvement in the overall health or well-being of the

subject, or by other parameters well known in the art that are specific to the particular tumor.

Tumor: A neoplasm.

Under conditions sufficient for: A phrase that is used to describe any environment that permits the desired activity.

In one example, includes administering a therapeutically effective amount of a composition that includes an OPN-5kD specific antibody, or one or more peptides that include at least 5 contiguous amino acids of OPN-5kD, sufficient to allow the desired activity. In particular examples the desired activity is treatment of a tumor, such as a tumor that expresses OPN, for example HCC.

Unit dose: A physically discrete unit containing a predetermined quantity of an active material calculated to individually or collectively produce a desired effect, such as a therapeutic effect. A single unit dose or a plurality of unit doses can be used to provide the desired effect, such as treatment of a tumor, for example a metastatic HCC. In one example, a unit dose includes a desired amount of an agent that decreases cellular invasion induced by OPN-5kD binding to CD44.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector can include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. A vector may also include one or more therapeutic genes and/or selectable marker genes and other genetic elements known in the art. A vector can transduce, transform or infect a cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. In a particular example, a vector includes a nucleic acid molecule encoding one or more of SEQ ID NOS: 4-8.

Methods of Treating a Tumor

It is shown herein that MMP-9 liberates an OPN-5kD fragment, and that OPN-induced cellular invasion proceeds via binding of the OPN-5kD ligand to CD44 cell surface receptors. Without being bound to a particular theory, it is proposed that exposure of a non-RGD cryptic binding site region of OPN by

extracellular proteolytic cleavage increases binding to cell surface CD44 receptors and mediates an invasive response, which is distinct and independent from integrin αVβ3 function. It is also demonstrated herein that small peptide fragments of OPN-5kD (for example any of SEQ ID NOS: 5-8) effectively decrease or inhibit cellular invasion induced by OPN-5kD. Without being bound to a particular theory, it is proposed that these peptides structurally compete with OPN-5kD for available cell-surface CD44 docking sites, thereby decreasing cellular invasion. This observation also supports the use of antibodies directed to epitopes within the OPN-5kD peptide as therapeutics. Based on these observations, methods of treating a tumor that overexpresses OPN, MMP-9, or both, are disclosed. These methods include administering peptides, antibodies, or combinations thereof, that effectively decrease or inhibit cellular invasion.

Methods are disclosed herein for treating tumors, such as those that overexpress OPN, for example in combination with overexpression of MMP-9. In one example, increased expression of OPN or MMP-9 can be detected in serum or plasma obtained from a subject having such a tumor. For example, detection of OPN-5kD in the serum of a subject (for example at a level of at least twice that found in a subject not having a tumor), detection of increased levels of OPN-c in the tumor (for example relative to expression of OPN-c in adjacent non-tumor cells, or relative to expression of OPN-a in the tumor), or both, indicates that the subject can benefit from the disclosed therapies. In some examples, subjects are initially screened to determine if they have increased levels of OPN-5kD in their serum, whether they have a tumor that has increased expression of OPN-c (for example relative to adjacent non-tumor cells, or relative to expression of OPN-a), or combinations thereof. For example, the diagnostic methods provided herein can be used to screen subjects to determine if they are candidates for the disclosed therapies.

In some examples, the tumor is treated *in vivo*, for example in a mammalian subject, such as a human subject. A tumor is an abnormal growth of tissue that results from excessive cell division. A particular example of a tumor is cancer. For example, the current application is useful for the treatment (such as the prevention or reduction of metastasis) of tumors (such as cancers). Exemplary tumors that can be

treated using the disclosed methods include, but are not limited to: cancers of the liver, breast, colon, and prostate, including metastases of such tumors. For example, the tumor can be a metastatic HCC, such as an intra-hepatic metastasis or an extra-hepatic metastasis.

Treatment of a tumor, such as HCC, can include preventing or delaying the development of the tumor in a subject (such as preventing metastasis of a tumor), and also includes reducing signs or symptoms associated with the presence of such a tumor (for example by reducing the size or volume of the tumor or a metastasis thereof). In a specific example, treatment includes reducing the growth of cells of the tumor, or even killing the tumor cells (for example by causing the cells to undergo apoptosis). Such reduced growth can in some examples decrease or slow metastasis of the tumor, or reduce the size or volume of the tumor. In one example, treatment of a tumor includes reducing the invasive activity of the tumor in the subject, for example by reducing the ability of the tumor to metastasize. In some examples, treatment using the methods disclosed herein prolongs the time of survival of the subject.

In particular examples, the method includes administering to the subject a therapeutically effective amount of one or more agents that reduce cellular invasion resulting from the interaction between OPN-5kD and CD44 receptor, thereby treating the tumor. In particular examples, cellular invasion is reduced by at least 10% (such as at least 20%, at least 50%, or at least 90%), for example as compared to an amount of cellular invasion in the absence of the therapeutic agent. Examples of such agents include small peptide fragments of OPN-5kD or antibodies specific to OPN-5kD that reduce the interaction between OPN-5kD and CD44 receptor and reduce cellular invasion. Specific examples of small peptides include peptide sequences having at least 5 contiguous amino acids of an OPN-5kD sequence, such as the OPN-5kD sequence shown in SEQ ID NO: 4.

Particular examples of agents that can be used in the methods disclosed herein include therapeutic amounts of peptides that include or consist of at least 5 contiguous amino acids of SEQ ID NO: 4, such as at least 6, at least 7, at least 8, at least 8, or at least 9 contiguous amino acids of SEQ ID NO: 4, for example 5-20, 10-15, or 8-10 contiguous amino acids of SEQ ID NO: 4. For example, the therapeutic

peptide can include or consist of 5, 6, 7, 8, 9, 10, 12, 15, 20, or 30 contiguous amino acids of SEQ ID NO: 4. In one example, the peptide include or consists of the amino acid sequence of SEQ ID NO: 5, 6, 7, or 8. One skilled in the art will appreciate that more than one peptide can be used, such as one or more peptides that include at least 5 contiguous amino acids of SEQ ID NO: 4, such as one or more peptides consisting of SEQ ID NO: 5, 6, 7, or 8. In some examples the therapeutic peptide is a fusion peptide, such as a fusion peptide including at least 5 contiguous amino acids of SEQ ID NO: 4, for example a fusion peptide that includes any of SEQ ID NOS: 5-8.

Screening subjects

Subjects can be screened prior to initiating the disclosed therapies, for example to determine whether the subject has a tumor that overexpresses OPN, MMP-9, or both. The presence of a tumor that overexpresses OPN, MMP-9, or both indicates that the tumor can be treated using the methods provided herein.

In one example, the tumor (or a portion thereof, such as a fine needle aspirate or other biopsy sample) is analyzed using immunodetection methods. For example, the biological sample can be incubated with an antibody that specifically binds to OPN or to MMP-9, or both antibodies. The primary antibody can include a detectable label. For example, the primary antibody can be directly labeled, or the sample can be subsequently incubated with a secondary antibody that is labeled (for example with a fluorescent label). The label can then be detected, for example by microscopy, ELISA, flow cytometery, or spectrophotometry. In another example, the biological sample is analyzed by Western blotting for the presence of OPN or to MMP-9. In one example, a subject is screened by determining whether that have increased levels of OPN-5kD in their serum (for example relative to a level present in a serum sample from a subject not having a tumor), for example using an antibody that specifically binds OPN-5kD (such as those described in Example 8).

As an alternative to analyzing the sample for the presence of proteins, the presence of nucleic acids can be determined. For example, the biological sample can be incubated with primers that permit the amplification of OPN or to MMP-9, under conditions sufficient to permit amplification of OPN or to MMP-9.

Exemplary methods include PCR and RT-PCR. In another example, the biological sample is incubated with probes that can bind to OPN or to MMP-9 nucleic acid (such as cDNA, genomic DNA, or RNA (such as mRNA)) under high stringency conditions. The resulting hybridization can then be detected using methods known in the art. In one example, a subject is screened by determining whether that have increased levels of OPN-c in their serum (for example relative to a level present in adjacent non-tumor cells from the same subject, or relative to a level of OPN-a in the same tumor sample), for example detecting OPN-c mRNA expression.

The presence of increased expression of OPN or MMP-9 indicates that the tumor overexpresses OPN or MMP-9 or both, and that the tumor is one that can be treated using the disclosed therapies. In one example, the presence of increased OPN-5kD in the serum, the presence of increased OPN-c in the tumor, or both, indicates that the subject has a tumor that can be treated using the disclosed therapies.

Administration

Methods of administration of the disclosed therapeutic agents are routine, and can be determined by a skilled clinician. For example, the disclosed therapies (such as those that include a peptide having at least 5 contiguous amino acids of OPN-5kD, for example as at least 9 contiguous amino acids of SEQ ID NO: 4 and antibodies directed to OPN-5kD) can be administered via injection (for example via embolization), orally, topically, transdermally, parenterally, or via inhalation or spray. In a particular example, a peptide having at least 5 contiguous amino acids of OPN-5kD is administered intravenously to a mammalian subject, such as a human.

The therapeutic compositions, such as those that include one or more peptides having at least 5 contiguous amino acids of OPN-5kD, for example as at least 9 contiguous amino acids of SEQ ID NO: 4, or antibodies specific for OPN-5kD, can further include one or more biologically active or inactive compounds (or both), such as anti-neoplastic agents and conventional non-toxic pharmaceutically acceptable carriers, respectively.

In a particular example, a therapeutic composition that includes a therapeutically effective amount of one or more agents that reduce cellular invasion

due to the interaction of OPN-5kD with CD44 (such as peptides having at least 5 contiguous amino acids of OPN-5kD) further includes one or more biologically inactive compounds. Examples of such biologically inactive compounds include, but are not limited to: carriers, thickeners, diluents, buffers, preservatives, and carriers. The pharmaceutically acceptable carriers useful for these formulations are conventional (see Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition (1995)). In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations can include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can include minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

In a particular example, a therapeutic composition that includes a therapeutically effective amount of one or more agents that reduce cellular invasion due to the interaction of OPN-5kD with CD44 (such as peptides having at least 5 contiguous amino acids of OPN-5kD) further includes therapeutically effective amounts of one or more other biologically active compounds. Examples of biologically active compounds include, but are not limited to: anti-neoplastic agents (such as chemotherapeutics), antibiotics, alkylating agents, antioxidants, adjuvants, and so forth (such as those listed below under "additional treatments"). However, one skilled in the art will appreciate that peptides having at least 5 contiguous amino acids of OPN-5kD and the other biologically active compounds can also be administered separately (instead of in a single composition).

The therapeutically effective amount of the agents administered can vary depending upon the desired effects and the subject to be treated. In one example, the method includes daily administration of at least 1 μg of one or more peptides having

at least 5 contiguous amino acids of OPN-5kD to the subject (such as a human subject). For example, a human can be administered at least 1 μg daily or at least 1 mg daily of one or more of SEQ ID NOS: 5-8, such as 10 μg to 100 μg daily, 100 μg to 1000 μg daily, for example 10 μg daily, 100 μg daily, or 1000 μg daily. In one example, the subject is administered at least 1 μg (such as 1-100 μg or 5-50 μg) intravenously of each of one or more of SEQ ID NOS: 5-8. In one example, the subject is administered at least 1 mg intramuscularly (for example in an extremity) or topically of each of one or more of SEQ ID NOS: 5-8. The dosage can be administered in divided doses (such as 2, 3, or 4 divided doses per day), or in a single dosage daily.

Therapeutic amounts of OPN-5kD specific antibodies can also be administered. In some examples, therapeutic amounts are amounts which eliminate or reduce the patient's tumor burden, or which prevent or reduce the proliferation of metastatic cells. The dosage will depend on many parameters, including the nature of the tumor, patient history, patient condition, the possible co-use of other oncolytic agents, and methods of administration. Methods of administration include injection (e.g., parenteral, subcutaneous, intravenous, intraperitoneal, etc.) for which the antibodies are provided in a nontoxic pharmaceutically acceptable carrier such as water, saline, Ringer's solution, dextrose solution, 5% human serum albumin, fixed oils, ethyl oleate, or liposomes. Typical dosages may range from about 0.01 to about 20 mg/kg, such as from about 0.1 to about 10 mg/kg. Other methods of administration include oral and transdermal (such as at least 1 mg, for example 1-1000 mg). Acceptable carriers for oral ingestion include pharmaceutically acceptable liquid carriers or pharmaceutically acceptable solid carriers in the form of tablets, capsules, caplets, or gel-seals. Other effective methods of administration and dosages may be determined by routine experimentation and are within the scope of this invention.

Therapeutic methods employing OPN-5kD specific antibodies or peptides having at least 5 contiguous amino acids of OPN-5kD can be combined with chemotherapy, surgery, and radiation therapy, depending on type of the tumor, patient condition, other health issues, and a variety of factors. The methods can also include immunoconjugates for targeted immunotoxin-mediated therapy, wherein

OPN-5kD antibodies are covalently or non-covalently conjugated to various cytotoxic agents, further enhancing toxicity to targeted cells. See, for example, U.S. Pat. No. 5,872,223. Such agents, including various bacterial toxins (e.g., *Pseudomonas* exotoxin), ricin A-chain, daunorubicin, methotrexate, and ribosome inhibitors (e.g., trichosantin). Also, OPN-5kD antibodies can be labeled with alpha, beta, or Auger electron emitters, resulting in immunoconjugates for targeted radiotherapy.

Thus, OPN-5kD specific antibodies can be used in a variety of methods and compositions for detecting and treating metastatic disease.

In particular examples, the subject is administered the therapeutic composition that includes OPN-5kD specific antibodies, or one or more peptides having at least 5 contiguous amino acids of OPN-5kD, on a multiple daily dosing schedule, such as at least two consecutive days, 10 consecutive days, and so forth, for example for a period of weeks, months, or years. In one example, the subject is administered the therapeutic composition for a period of at least 30 days, such as at least 2 months, at least 4 months, at least 6 months, at least 12 months, at least 24 months, or at least 36 months.

Additional treatments

In particular examples, prior to, during, or following administration of a therapeutic amount of an agent that reduces cellular invasion due to the interaction of OPN-5kD with CD44 (such as one or more peptides having at least 5 contiguous amino acids of OPN-5kD or OPN-5kD antibodies), the subject can receive one or more other therapies. In one example, the subject receives one or more treatments to remove or reduce the tumor prior to administration of a therapeutic amount of one or more agents that reduce cellular invasion due to the interaction of OPN-5kD with CD44.

Examples of such therapies include, but are not limited to, surgical treatment for removal or reduction of the tumor (such as surgical resection, cryotherapy, or chemoembolization), as well as anti-tumor pharmaceutical treatments which can include radiotherapeutic agents, anti-neoplastic chemotherapeutic agents, antibiotics, alkylating agents and antioxidants, kinase inhibitors, and other agents. Particular

examples of additional therapeutic agents can that can be used include microtubule binding agents, DNA intercalators or cross-linkers, DNA synthesis inhibitors, DNA and/or RNA transcription inhibitors, antibodies, enzymes, enzyme inhibitors, gene regulators, and angiogenesis inhibitors. These agents (which are administered at a therapeutically effective amount) and treatments can be used alone or in combination. Methods and therapeutic dosages of such agents are known to those skilled in the art, and can be determined by a skilled clinician.

"Microtubule binding agent" refers to an agent that interacts with tubulin to stabilize or destabilize microtubule formation thereby inhibiting cell division. Examples of microtubule binding agents that can be used in conjunction with the disclosed therapy include, without limitation, paclitaxel, docetaxel, vinblastine, vindesine, vinorelbine (navelbine), the epothilones, colchicine, dolastatin 15, nocodazole, podophyllotoxin and rhizoxin. Analogs and derivatives of such compounds also can be used and are known to those of ordinary skill in the art. For example, suitable epothilones and epothilone analogs are described in International Publication No. WO 2004/018478. Taxoids, such as paclitaxel and docetaxel, as well as the analogs of paclitaxel taught by U.S. Patent Nos. 6,610,860; 5,530,020; and 5,912,264 can be used.

Suitable DNA and/or RNA transcription regulators, including, without limitation, actinomycin D, daunorubicin, doxorubicin and derivatives and analogs thereof also are suitable for use in combination with the disclosed therapies.

DNA intercalators and cross-linking agents that can be administered to a subject include, without limitation, cisplatin, carboplatin, oxaliplatin, mitomycins, such as mitomycin C, bleomycin, chlorambucil, cyclophosphamide and derivatives and analogs thereof.

DNA synthesis inhibitors suitable for use as therapeutic agents include, without limitation, methotrexate, 5-fluoro-5'-deoxyuridine, 5-fluorouracil and analogs thereof.

Examples of suitable enzyme inhibitors include, without limitation, camptothecin, etoposide, formestane, trichostatin and derivatives and analogs thereof.

Suitable compounds that affect gene regulation include agents that result in increased or decreased expression of one or more genes, such as raloxifene, 5-azacytidine, 5-aza-2'-deoxycytidine, tamoxifen, 4-hydroxytamoxifen, mifepristone and derivatives and analogs thereof.

"Angiogenesis inhibitors" include molecules, such as proteins, enzymes, polysaccharides, oligonucleotides, DNA, RNA, and recombinant vectors, and small molecules that function to reduce or even inhibit blood vessel growth. Angiogenesis is implicated in most types of human solid tumors. Angiogenesis inhibitors are known in the art and examples of suitable angiogenesis inhibitors include, without limitation, angiostatin K1-3, staurosporine, genistein, fumagillin, medroxyprogesterone, suramin, interferon-alpha, metalloproteinase inhibitors, platelet factor 4, somatostatin, thromobospondin, endostatin, thalidomide, and derivatives and analogs thereof.

Kinase inhibitors include Gleevac, Iressa, and Tarceva that prevent phosphorylation and activation of growth factors.

Antibodies that can be used include Herceptin and Avastin that block growth factors and the angiogenic pathway.

Other therapeutic agents, for example anti-tumor agents, that may or may not fall under one or more of the classifications above, also are suitable for administration in combination with the disclosed therapies (such as those that include one or more peptides having at least 5 contiguous amino acids of OPN-5kD). By way of example, such agents include adriamycin, apigenin, rapamycin, zebularine, cimetidine, and derivatives and analogs thereof.

In one example, the therapeutic peptide composition (such as one or more peptides having at least 5 contiguous amino acids of OPN-5kD), or OPN-5kD is injected into the subject in the presence of an adjuvant, thus generating an immune response to OPN-5kD wherein such immune response decreases OPN-5kD binding to CD44. An adjuvant is an agent that when used in combination with an immunogenic agent augments or otherwise alters or modifies a resultant immune response. In some examples, an adjuvant increases the titer of antibodies induced in a subject by the immunogenic agent. In one example, the one or more peptides are administered to the subject as an emulsion with IFA and sterile water for injection

(for example an intravenous or intramuscular injection). Incomplete Freund's Adjuvant (Seppic, Inc.) can be used as the Freund's Incomplete Adjuvant (IFA) (Fairfield, NJ). In some examples, IFA is provided in 3 ml of a mineral oil solution based on mannide oleate (Montanide ISA-51). At the time of injection, the peptide(s) is mixed with the Montanide ISA.51 and then administered to the subject. Other adjuvants can be used, for example, Freund's complete adjuvant, B30-MDP, LA-15-PH, montanide, saponin, aluminum hydroxide, alum, lipids, keyhole lympet protein, hemocyanin, a mycobacterial antigen, and combinations thereof.

In some examples, the subject receiving a therapeutic composition (such as one or more peptides having at least 5 contiguous amino acids of OPN-5kD or OPN-5kD antibodies) is also administered interleukin-2 (IL-2), for example via intravenous administration. In particular examples, IL-2 (Chiron Corp., Emeryville, CA) is administered at a dose of at least 500,000 IU/kg as an intravenous bolus over a 15 minute period every eight hours beginning on the day after administration of the peptides and continuing for up to 5 days. Doses can be skipped depending on subject tolerance.

In some examples, the therapeutic compositions can be co-administered with a fully human antibody to cytotoxic T-lymphocyte antigen-4 (anti–CTLA-4). In some examples subjects receive at least 1 mg/kg anti-CTLA-4 (such as 3 mg/kg every 3 weeks or 3 mg/kg as the initial dose with subsequent doses reduced to 1 mg/kg every 3 weeks).

In one example, at least a portion of the tumor (such as a metastatic HCC) is surgically removed (for example via cryotherapy), irradiated, chemically treated (for example via chemoembolization) or combinations thereof, prior to administration of the disclosed therapies. For example, a subject having HCC can have all or part of the tumor surgically excised prior to administration of the disclosed therapies. In another particular example, the subject has HCC and is administered radiation therapy, chemoembolization therapy, or both, prior to administration of the disclosed therapies.

Methods of Diagnosing and Prognosing a Tumor

Metastasis is a major complication in the pathogenesis of tumors, such as HCC, and is typically indicative of poor prognosis. It is shown herein that OPN-5kD abundance in HCC cell lines correlates with degree of metastatic potential. Without wishing to be bound to a particular theory, it is proposed that the interaction of OPN and MMP-9 is related to enhanced HCC tumor cell metastasis. The abundance of the OPN-5kD fragment in HCC cells correlates to degree of metastatic potential. It is also shown herein that OPN-c mRNA expression correlates with metastatic HCC but not non-metastatic HCC in non-cancerous tissue adjacent to primary HCC lesions. Based on these observations, methods of diagnosing or prognosing a tumor that overexpresses OPN, MMP-9, or both, based on detecting OPN-5kD, OPN-c, or both, are disclosed. In some examples, such methods can be used to identify those subjects that will benefit from the disclosed treatment methods. For example, such diagnostic methods can be performed prior to the subject undergoing the treatments described herein.

In one example, detection of OPN-5kD (such as SEQ ID NO: 4 or a variant thereof, such as a polymorphism thereof) in a biological sample from the subject is used to diagnose or prognose a tumor that overexpresses OPN, MMP-9, or both (such as HCC). Methods of detecting a peptide in a sample, such as OPN-5kD, are known in the art and are routine. In some examples, the relative amount of OPN-5kD present is determined, for example by quantitating the amount of OPN-5kD present. For example, the relative or absolute quantity of OPN-5kD in a sample can be determined.

In another example, detection of OPN-c mRNA (such as an mRNA corresponding to SEQ ID NO: 3 or 9, or a variant thereof, such as a polymorphism thereof) in a biological sample from the subject is used to diagnose or prognose a tumor that overexpresses OPN, MMP-9, or both (such as metastatic HCC). Methods of detecting a nucleic acid molecule in a sample, such as OPN-c, are known in the art and are routine. In some examples, the relative amount of OPN-c mRNA present is determined, for example by quantitating the amount of OPN-c mRNA present. For example, the relative or absolute quantity of OPN-c mRNA in a sample can be

determined. However, one skilled in the art will recognize that OPN-c protein can be detected as an alternative to detecting OPN-c nucleic acid molecules.

In some examples, detection of both OPN-5kD protein and OPN-c mRNA in one or more biological samples obtained from the subject (such as serum or a tumor sample) are used to diagnose or prognose a tumor that overexpresses OPN, MMP-9, or both (such as metastatic HCC).

Biological samples

Methods of obtaining a biological sample from a subject are known in the art. For example, methods of obtaining a blood sample or a fraction thereof (such as serum) are routine. Similarly, a sample from a tumor that contains cellular material can be obtained by surgical excision of all or part of the tumor, by collecting a fine needle aspirate from the tumor, as well as other methods known in the art. If desired, the sample can be concentrated or purified before use. For example, proteins or nucleic acids can be isolated from the sample. Alternatively, the sample can be used directly. In particular examples, a serum sample obtained from the subject is analyzed to determine if it contains detectable levels of OPN-5kD. In particular examples, a tumor sample obtained from the subject is analyzed to determine if it contains detectable levels of OPN-c mRNA or protein.

Detection of OPN-5kD peptide

In particular examples, a sample obtained from the subject is analyzed to determine if it contains detectable levels of OPN-5kD, such as a serum sample.

Methods of detecting proteins are routine. In some examples, immunoassays are used to detect the presence of OPN-5kD protein in the sample. Generally, immunoassays include the use of one or more specific binding agents (such as antibodies) that can substantially only bind to OPN-5kD. Such binding agents can include a detectable label (such as a radiolabel, fluorophore or enzyme), that permits detection of the binding to the protein. Exemplary immunoassays that can be used include, but are not limited to: Western blotting, ELISA, fluorescence microscopy, and flow cytometry.

In one example, the specific binding agent is an antibody, such as a polyclonal or monoclonal antibody, or fragment thereof. In some examples, the antibody is a humanized antibody. In some examples, the antibody is a chimeric antibody. If desired, the antibody can include a detectable label to permit detection and in some cases quantitation of the OPN-5kD/antibody complex. In a particular example, the antibody used is the antibody described in Example 8.

The presence of detectable signal above background or control levels indicates that the presence of an OPN-5kD peptide in the sample. For example, the level of OPN-5kD detected can be compared to a control or reference value, such as a value that represents a level of OPN-5kD expected if a tumor that overexpresses OPN is present in the subject (such as metastatic HCC), a level of OPN-5kD expected if a tumor that overexpresses OPN is absent in the subject (such as HCC), a level of OPN-5kD expected if a tumor that overexpresses OPN is has metastasized in the subject (such as metastatic HCC), or combinations thereof.

In some examples, detection of OPN-5kD at a level of at least twice (such as at least three or at least five times) that observed in a subject not having a tumor, indicates that that subject has a tumor that overexpresses OPN, has a tumor with metastatic potential, has a tumor that has metastasized, has a poor prognosis, or combinations thereof. For example, if serum levels of OPN-5kD from a subject are twice that observed for a subject not having a tumor, such as an increase of at least 100%, at least 200%, or at least 500%, this indicates that the subject has a tumor that overexpresses OPN, has a tumor with metastatic potential, has a tumor that has metastasized, has a poor prognosis, or combinations thereof.

For example, if the level of OPN-5kD detected in the subject's sample is significantly less than the level of OPN-5kD expected if a tumor that overexpresses OPN is present in the subject, is similar to or less than the level of OPN-5kD expected if a tumor that overexpresses OPN is not present in the subject, or both, this indicates that the subject does not have a tumor that overexpresses OPN. In another example, if the level of OPN-5kD detected in the subject's sample is similar or greater than the level of OPN-5kD expected if a tumor that overexpresses OPN is present in the subject, is significantly greater than the level of OPN-5kD expected if a tumor that overexpresses OPN is not present in the subject, or both, this indicates

that the subject has a tumor that overexpresses OPN, such as HCC. In another example, if the level of OPN-5kD detected in the subject's sample is similar or greater than the level of OPN-5kD expected if a tumor that overexpresses OPN is present in the subject, is significantly greater than the level of OPN-5kD expected if a tumor that overexpresses OPN is not present in the subject, is similar to or greater than the level of a level of OPN-5kD expected if a tumor that overexpresses OPN is has metastasized in the subject, or combinations thereof, this indicates that the subject has a metastatic tumor that overexpresses OPN, such as metastatic HCC.

Detection of OPN-c

In particular examples, a sample obtained from the subject is analyzed to determine if it contains detectable levels of OPN-c, such as a tumor sample, a sample adjacent to the tumor, or both. In particular examples, OPN-c nucleic acid molecules (such as mRNA) are measured. However, one skilled in the art will appreciate that OPN-c protein could also be measured, for example using routine immunoassays known in the art (such as those described above).

Methods of detecting nucleic acid molecules are routine. In particular examples, a tumor sample and a sample adjacent to the tumor obtained from the subject are analyzed to determine if each contains detectable levels of OPN-c nucleic acid molecules, such as cDNA or mRNA. For example, assays that permit detection of nucleic acids can be used. Exemplary assays that can be used include, but are not limited to: Northern blotting, Southern blotting, PCR (such as RT-PCR), and DNA arrays. For example, OPN-c can be amplified from a sample using PCR, and the amplicons detected and in some examples quantitated, wherein the presence of detectable amplicons above background or control levels indicates that the tumor (or adjacent tissue) expresses OPN-c nucleic acid molecules. In one example, a nucleic acid probe that hybridizes to an OPN-c nucleic acid is contacted with the sample. For example, the probe can be incubated with the sample under high stringency conditions (such as when the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO_4 (pH 7.4), 5X SSC, 5X Denhart's solution, 50 μg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about $5x10^7$ cpm/μg), while the washes are

performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% sodium dodecyl sulfate), wherein the presence of detectable signal from the probe above background or control levels indicates that the tumor (or adjacent tissue) expresses OPN-c nucleic acid molecules.

The presence of detectable signal above background or control levels indicates that the presence of OPN-c nucleic acid molecules in the sample. For example, the level of OPN-c detected can be compared to a control or reference value, such as a value that represents a level of OPN-c expected if a tumor is or is not metastatic (such as metastatic HCC). In one example, the level of OPN-c detected in a tumor sample is compared to the level of OPN-c detected in an adjacent non-tumor sample.

In some examples, detection of at least twice (such as at least 3-times or at least 5- times) the relative amount of OPN-c mRNA observed in a tumor sample, as compared to the relative amount of OPN-c mRNA in an adjacent non-tumor sample from the same subject, indicates that that subject has a tumor with metastatic potential, has a tumor that has metastasized, has a poor prognosis, or combinations thereof. For example, if tumor levels of OPN-c mRNA are twice that observed for an adjacent non-tumor sample, such as an increase of at least 100%, at least 200%, or at least 500%, this indicates that the subject has a tumor with metastatic potential, has a tumor that has metastasized, has a poor prognosis, or combinations thereof.

In some examples, detection of statistically similar relative amounts of OPN-c mRNA observed in a tumor sample, as compared to the relative amount of OPN-c mRNA in an adjacent non-tumor sample from the same subject, indicates that that subject does not have a tumor with metastatic potential, does not have a tumor that has metastasized, has a good prognosis, or combinations thereof.

In some examples, the ratio of OPN-a to OPN-c in tumor versus non-tumor cells is determined. It is shown herein that the ratio for OPN-a versus OPN-c in metastatic tumors is slightly lower than for non-metastatic tumors, indicating higher levels of OPN-c in metastasis (for example a ratio of 2.27 versus 2.85).

Therapeutic Compositions

Another aspect of the disclosure includes pharmaceutical compositions prepared for administration to a subject and which include a therapeutically effective amount of one or more of the currently disclosed compounds. Such compositions can also be part of a kit. For example, compositions that include a therapeutic amount of an agent that decreases or inhibits cellular invasion that results from the interaction of OPN-5kD with CD44 receptor can be formulated for use in treating a tumor that overexpresses OPN, such as HCC. Particular examples of such agents include OPN-5kD specific antibodies and peptides having at least 5 contiguous amino acids of OPN-5kD (such as at least 9 contiguous amino acids of SEQ ID NO: 4), for example the peptide sequences shown in SEQ ID NOS: 5-8.

In one example, compositions include a therapeutic amount of one or more agents that decrease or inhibit tumor cellular invasion (for example a reduction of at least 10%, at least 20%, at least 50%, or even at least 90%) such as by decreasing the binding of OPN-5kD to the CD44 receptor. Such compositions can include one or more additional biologically active agents, one or more biologically inactive compounds, or combinations thereof. Examples of such biologically active agents are described above, and can include anti-neoplastic agents. Examples of biologically inactive agents are described above, and can include pharmaceutically acceptable carriers. In a specific example, the therapeutic composition includes an antibody to cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4).

Particular examples of agents that can be used in the disclosed compositions include peptide fragments of OPN-5kD that decrease tumor cellular invasion resulting from the interaction of OPN-5kD with CD44 receptor. One example of OPN-5kD peptide fragments includes peptides having at least 5 contiguous amino acids of OPN-5kD (such as at least 5 contiguous amino acids of SEQ ID NO: 4), such as at least 6, at least 7, at least 8, at least 9, at least 10, at least 12, at least 15, or even at least 20 contiguous amino acids of OPN-5kD, for example 5-30, 5-25, 6-20, 6-12, or 8-10 contiguous amino acids of OPN-5kD (such as this number of contiguous amino acids of SEQ ID NO: 4). Exemplary examples of such peptides include those shown in SEQ ID NOS: 5-8.

In a particular example, the composition includes at least two peptides, each including at least 5 contiguous amino acids of OPN-5kD (such as at least 5 contiguous amino acids of SEQ ID NO: 4). For example, the composition can include a mixture of at least 2 of the peptides shown in SEQ ID NOS: 5-8, such as a composition that includes 2, 3, or 4 of these peptides. Exemplary combinations include the peptides shown in SEQ ID NOS: 5 and 6, SEQ ID NOS: 5 and 7, SEQ ID NOS: 5 and 8, SEQ ID NOS: 6 and 7, SEQ ID NOS: 6 and 8, SEQ ID NOS: 7 and 8, SEQ ID NOS: 5, 6, and 7, SEQ ID NOS: 5, 6, and 8, SEQ ID NOS: 5, 7, and 8, SEQ ID NOS: 6, 7, and 8, or SEQ ID NOS: 5, 6, 7, and 8. In one example, the composition includes one or more peptides consisting of the amino acid sequence shown in SEQ ID NO: 5, 6, 7, or 8.

In particular examples, the composition includes 1-1000 μg of one or more peptide fragments of OPN-5kD that decrease tumor cellular invasion resulting from the interaction of OPN-5kD with CD44 receptor, such as 1-1000 μg of one or more of SEQ ID NOS: 5-8. In some examples, the composition includes 1-1000 mg of one or more peptide fragments of OPN-5kD that decrease tumor cellular invasion resulting from the interaction of OPN-5kD with CD44 receptor, such as 1-1000 mg of one or more of SEQ ID NOS: 5-8.

In another particular example, the composition includes one or more OPN-5kD specific antibodies. Such antibodies bind to OPN-5kD or fragments thereof and inhibit or decrease binding to CD44 receptors. In particular examples, the composition includes 1-1000 μg or 1-1000 mg of one or more OPN-5kD specific antibodies that decrease tumor cellular invasion resulting from the interaction of OPN-5kD with CD44 receptor.

Kits

Provided by this disclosure are kits that can be used to diagnose, prognose, or treat a tumor that overexpresses OPN (or combinations thereof). The disclosed kits can include instructional materials disclosing means of use of the compositions in the kit. The instructional materials can be written, in an electronic form (such as a computer diskette or compact disk) or can be visual (such as video files).

Kits are provided that can be used in the therapies and diagnostic assays disclosed herein. For example, kits can include one or more of the disclosed therapeutic compositions (such as a composition including one or more peptides having 5-20 contiguous amino acids of SEQ ID NO: 4 or OPN-5kD specific antibodies), one or more of the disclosed diagnostic compositions (such as an antibody that specifically binds OPN-5kD), or combinations thereof. One skilled in the art will appreciate that the kits can include other agents to facilitate the particular application for which the kit is designed.

In one example, a kit is provided for treating a tumor that overexpresses OPN, such as HCC. For example, such kits can include one or more of the disclosed therapeutic compositions (such as a composition including one or more peptides having 5-20 contiguous amino acids of SEQ ID NO: 4 or OPN-5kD specific antibodies). Such a diagnostic kit can additionally contain other therapeutic molecules, such as therapeutic doses of IL-2, anti-CTLA-4, an adjuvant, or combinations thereof.

In one example, a kit is provided for detecting OPN-5kD in a biological sample, such as serum. Kits for detecting OPN-5kD can include one or more antibodies that specifically bind OPN-5kD, such as any of the antibodies disclosed herein. In some examples, the antibody is labeled (for example, with a fluorescent, radioactive, or an enzymatic label). Such a diagnostic kit can additionally contain means of detecting a label (such as enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a secondary antibody, or the like), as well as buffers and other reagents routinely used for the practice of a particular diagnostic method.

OPN-5kD Antibodies

The present disclosure provides antibodies that specifically bind OPN-5kD (such as SEQ ID NO: 4). Such antibodies are useful for both therapeutic and diagnostic purposes. In some examples OPN-5kD specific antibodies have an equilibrium constant (K_d) of 1 nM or less. For example, antibodies are provided that bind human OPN-5kD with a binding affinity of 0.1×10^{-8} M, at least about 0.3×10^{-8} M, at least about 0.5×10^{-8} M, at least about 0.75×10^{-8} M, at least about $1.0 \times$

10^{-8} M, at least about 1.3 x 10^{-8} M at least about 1.5 x 10^{-8}M, or at least about 2.0 x 10^{-8} M. In additional examples, the antibody (such as a human monoclonal) binds an epitope of OPN-5kD with an equilibrium disassociation constant (K_d) of 1 nM or less. In particular examples, the antibody can be used to diagnose a tumor, or be used to predict the metastatic potential of the tumor. In other examples, the antibodies can be used in therapeutic compositions to treat a subject. The disclosed antibodies can be part of a kit, such as a kit used to diagnose a tumor, such as HCC (such as metastatic HCC).

In one example, the antibody is a monoclonal antibody. The monoclonal antibody can be of any isotype, such as an IgM or an IgG antibody, for example IgG_1or an IgG_2. The class of an antibody that specifically binds OPN-5kD can be switched with another. In one aspect, a nucleic acid molecule encoding V_L or V_H is isolated using methods well-known in the art, such that it does not include any nucleic acid sequences encoding the constant region of the light or heavy chain, respectively. The nucleic acid molecule encoding V_L or V_H is then operatively linked to a nucleic acid sequence encoding a C_L or C_H from a different class of immunoglobulin molecule. This can be achieved using a vector or nucleic acid molecule that includes a C_L or C_H chain, as known in the art. For example, an antibody that specifically binds OPN-5kD that was originally IgM can be class switched to an IgG. Class switching can be used to convert one IgG subclass to another, such as from IgG_1 to IgG_2.

Antibody fragments are encompassed by the present disclosure, such as Fab, $F(ab')_2$, and Fv which include a heavy chain and light chain variable region and are capable of binding the epitopic determinant on OPN-5kD. These antibody fragments retain the ability to selectively bind with the antigen. Methods of making these fragments are known in the art (see for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988). In a specific example, the antibody is an Fv antibody.

Antibody fragments can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by

enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see U.S. Patent No. 4,036,945 and U.S. Patent No. 4,331,647, and references contained therein; Nisonhoff *et al.*, *Arch. Biochem. Biophys.* 89:230, 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman *et al.*, *Methods in Enzymology*, Vol. 1, page 422, Academic Press, 1967; and Coligan *et al.* at sections 2.8.1-2.8.10 and 2.10.1-2.10.4). Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

One of skill will realize that conservative variants of the antibodies can be produced. Such conservative variants employed in antibody fragments, such as dsFv fragments or in scFv fragments, will retain critical amino acid residues necessary for correct folding and stabilizing between the V_H and the V_L regions, and will retain the charge characteristics of the residues in order to preserve the low pI and low toxicity of the molecules. Amino acid substitutions (such as at most one, at most two, at most three, at most four, or at most five amino acid substitutions) can be made in the V_H and the V_L regions to increase yield. Conservative amino acid substitution tables providing functionally similar amino acids are well known to one of ordinary skill in the art.

Effector molecules, such as therapeutic, diagnostic, or detection moieties can be linked to an antibody of interest, such as an antibody that specifically binds OPN-5kD using any number of means known to those of skill in the art. Both covalent and noncovalent attachment means can be used. The procedure for attaching an effector molecule to an antibody varies according to the chemical structure of the effector. Polypeptides typically contain a variety of functional groups; such as carboxylic acid (COOH), free amine ($-NH_2$) or sulfhydryl (-SH) groups, which are available for reaction with a suitable functional group on an antibody to result in the binding of the effector molecule. Alternatively, the antibody is derivatized to

expose or attach additional reactive functional groups. The derivatization can involve attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford, IL. The linker can be any molecule used to join the antibody to the effector molecule. The linker is capable of forming covalent bonds to both the antibody and to the effector molecule. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the antibody and the effector molecule are polypeptides, the linkers may be joined to the constituent amino acids through their side groups (such as through a disulfide linkage to cysteine) or to the alpha carbon amino and carboxyl groups of the terminal amino acids.

An antibody that specifically binds OPN-5kD can be labeled with a detectable moiety. Useful detection agents include fluorescent compounds, including fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-napthalenesulfonyl chloride, phycoerythrin, lanthanide phosphors, and the cyanine family of dyes (such as Cy-3 or Cy-5) and the like. Bioluminescent markers are also of use, such as luciferase, Green fluorescent protein (GFP), Yellow fluorescent protein (YFP). An antibody can also be labeled with enzymes that are useful for detection, such as horseradish peroxidase, β- galactosidase, luciferase, alkaline phosphatase, glucose oxidase and the like. When an antibody is labeled with a detectable enzyme, it can be detected by adding additional reagents that the enzyme uses to produce a reaction product that can be discerned. For example, when the agent horseradish peroxidase is present the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is visually detectable. An antibody can also be labeled with a radiolabeled amino acid, such as ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, or ^{131}I.

Nucleic acid molecules encoding antibodies

Nucleic acid molecules encoding the disclosed OPN-5kD antibodies can readily be produced by one of skill in the art. Upon generation of a monoclonal antibody, such as those that can be produced from the parental clones described in Example 9, the amino acid sequence can be determined and a nucleic acid sequence

encoding the amino acid sequence can be engineered. In addition, one of skill can readily construct a variety of clones containing functionally equivalent nucleic acid molecules, such as nucleic acid molecules which differ in sequence but which encode the same effect or molecule ("EM") or antibody sequence. Thus, nucleic acids encoding antibodies, conjugates and fusion proteins are provided herein.

Nucleic acid sequences encoding the antibodies that specifically bind OPN-5kD can be prepared by any suitable method including, for example, cloning of appropriate sequences or by direct chemical synthesis by methods such as the phosphotriester method of Narang *et al., Meth. Enzymol.* 68:90-99, 1979; the phosphodiester method of Brown *et al., Meth. Enzymol.* 68:109-151, 1979; the diethylphosphoramidite method of Beaucage *et al., Tetra. Lett.* 22:1859-1862, 1981; the solid phase phosphoramidite triester method described by Beaucage & Caruthers, *Tetra. Letts.* 22(20):1859-1862, 1981, for example, using an automated synthesizer as described in, for example, Needham-VanDevanter *et al., Nucl. Acids Res.* 12:6159-6168, 1984; and, the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This can be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is generally limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Exemplary nucleic acid molecules encoding sequences encoding an antibody that specifically binds OPN-5kD can be prepared by cloning techniques. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook *et al., supra*, Berger and Kimmel (eds.), *supra*, and Ausubel, *supra*. Product information from manufacturers of biological reagents and experimental equipment also provide useful information. Such manufacturers include the SIGMA Chemical Company (Saint Louis, MO), R&D Systems (Minneapolis, MN), Pharmacia Amersham (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika

Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen (San Diego, CA), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

Nucleic acid molecules can also be prepared by amplification methods. Amplification methods include polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR). A wide variety of cloning methods, host cells, and *in vitro* amplification methodologies are well known to persons of skill.

In one example, an antibody that specifically binds OPN-5kD is prepared by inserting the cDNA which encodes a variable region from an antibody into a vector which includes the cDNA encoding an EM, such as an enzyme or label. The insertion is made so that the variable region and the EM are read in frame so that one continuous polypeptide is produced. Thus, the encoded polypeptide contains a functional Fv region and a functional EM region. In one example, cDNA encoding an enzyme is ligated to a scFv so that the enzyme is located at the carboxyl terminus of the scFv. For example, a cDNA encoding horseradish peroxidase or alkaline phosphatase, or a polypeptide marker of interest can be ligated to a scFv so that the enzyme (or polypeptide marker) is located at the amino terminus of the scFv. In another example, the label is located at the amino terminus of the scFv. In a further example, cDNA encoding the protein or polypeptide marker is ligated to a heavy chain variable region of an antibody, so that the enzyme or polypeptide marker is located at the carboxyl terminus of the heavy chain variable region. The heavy chain-variable region can subsequently be ligated to a light chain variable region of the antibody using disulfide bonds. In a yet another example, cDNA encoding an enzyme or a polypeptide marker is ligated to a light chain variable region of an antibody, so that the enzyme or polypeptide marker is located at the carboxyl terminus of the light chain variable region. The light chain-variable region can subsequently be ligated to a heavy chain variable region of the antibody using disulfide bonds.

Once the nucleic acids encoding the antibody, labeled antibody, or fragment thereof are isolated and cloned, the protein can be expressed in a recombinantly

engineered cell such as bacteria, plant, yeast, insect and mammalian cells using a suitable expression vector. One or more DNA sequences encoding the antibody or fragment thereof can be expressed *in vitro* by DNA transfer into a suitable host cell. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art. Hybridomas expressing the antibodies of interest are also encompassed by this disclosure.

Polynucleotide sequences encoding the antibody, labeled antibody, or functional fragment thereof, can be operatively linked to expression control sequences. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. The expression control sequences include, but are not limited to appropriate promoters, enhancers, transcription terminators, a start codon (ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons.

The polynucleotide sequences encoding the antibody, labeled antibody, or functional fragment thereof can be inserted into an expression vector including, but not limited to a plasmid, virus or other vehicle that can be manipulated to allow insertion or incorporation of sequences and can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art.

Transformation of a host cell with recombinant DNA can be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art.

Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired, or by electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate coprecipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with polynucleotide sequences encoding the antibody, labeled antibody, or functional fragment thereof, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for example, *Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982). One of skill in the art can readily use an expression systems such as plasmids and vectors of use in producing proteins in cells including higher eukaryotic cells such as the COS, CHO, HeLa and myeloma cell lines.

Isolation and purification of recombinantly expressed polypeptide can be carried out by conventional means including preparative chromatography and immunological separations. Once expressed, the antibody, labeled antibody or functional fragment thereof can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, and the like (see, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y., 1982). Substantially pure compositions of at least about 90 to 95% homogeneity are disclosed herein, and 98 to 99% or more homogeneity can be used for pharmaceutical purposes. Once purified, partially or to homogeneity as desired, if to be used therapeutically, the polypeptides should be substantially free of endotoxin.

Methods for expression of single chain antibodies and/or refolding to an appropriate active form, including single chain antibodies, from bacteria such as *E. coli* have been described and are well-known and are applicable to the antibodies disclosed herein. See, Buchner *et al.*, *Anal. Biochem.* 205:263-270, 1992; Pluckthun, *Biotechnology* 9:545, 1991; Huse *et al.*, *Science* 246:1275, 1989 and Ward *et al.*, *Nature* 341:544, 1989.

Often, functional heterologous proteins from *E. coli* or other bacteria are isolated from inclusion bodies and require solubilization using strong denaturants, and subsequent refolding. During the solubilization step, as is well known in the art, a reducing agent must be present to separate disulfide bonds. An exemplary buffer with a reducing agent is: 0.1 M Tris pH 8, 6 M guanidine, 2 mM EDTA, 0.3 M DTE (dithioerythritol). Reoxidation of the disulfide bonds can occur in the presence of low molecular weight thiol reagents in reduced and oxidized form, as described in Saxena *et al.*, *Biochemistry* 9: 5015-5021, 1970, and especially as described by Buchner *et al.*, *supra.*

Renaturation is typically accomplished by dilution (for example, 100-fold) of the denatured and reduced protein into refolding buffer. An exemplary buffer is 0.1 M Tris, pH 8.0, 0.5 M L-arginine, 8 mM oxidized glutathione (GSSG), and 2 mM EDTA.

As a modification to the two chain antibody purification protocol, the heavy and light chain regions are separately solubilized and reduced and then combined in the refolding solution. An exemplary yield is obtained when these two proteins are mixed in a molar ratio such that a 5 fold molar excess of one protein over the other is not exceeded. Excess oxidized glutathione or other oxidizing low molecular weight compounds can be added to the refolding solution after the redox-shuffling is completed.

In addition to recombinant methods, the antibodies, labeled antibodies and functional fragments thereof that are disclosed herein can also be constructed in whole or in part using standard peptide synthesis. Solid phase synthesis of the polypeptides of less than about 50 amino acids in length can be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany & Merrifield, *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.* pp. 3-284; Merrifield *et al.*, *J. Am. Chem. Soc.* 85:2149-2156, 1963, and Stewart *et al.*, *Solid Phase Peptide Synthesis, 2nd ed.*, Pierce Chem. Co., Rockford, Ill., 1984. Proteins of greater length may be synthesized by condensation of the amino and carboxyl termini of shorter fragments. Methods of forming peptide

bonds by activation of a carboxyl terminal end (such as by the use of the coupling reagent N, N'-dicylohexylcarbodimide) are well known in the art.

Use of the antibodies

The disclosure provides a method for detecting OPN-5kD in a biological sample, wherein the method includes contacting a biological sample with a human antibody that binds OPN-5kD under conditions conductive to the formation of an immune complex, and detecting the immune complex, to detect the OPN-5kD in the biological sample. The disclosure also provides a method for treating a subject suspected of having an OPN expressing tumor with OPN-5kD specific antibodies, wherein the method includes contacting a biological sample with an antibody that binds OPN-5kD and inhibits or reduces the binding of OPN to CD44 receptors (such methods are described herein and more specifically in the section describing methods of treatment and therapeutic compositions).

In one example, the detection of OPN-5kD in the sample indicates that the subject has a malignancy. In another example, the detection of OPN-5kD in the sample indicates that a tumor in the subject is prone to metastasis.

In one example, the antibody that specifically binds OPN-5kD is directly labeled with a detectable label. In another example, the antibody that specifically binds OPN-5kD (the first antibody) is unlabeled and a second antibody or other molecule that can bind the human antibody that specifically binds OPN-5kD is labeled. As is well known to one of skill in the art, a second antibody is chosen that is able to specifically bind the specific species and class of the first antibody. For example, if the first antibody is a human IgG, then the secondary antibody can be an anti-human-lgG. Other molecules that can bind to antibodies include, without limitation, Protein A and Protein G, which are available commercially.

Suitable labels for the antibody or secondary antibody are described above, and include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, magnetic agents and radioactive materials. Non-limiting examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase. Non-limiting examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin. Non-limiting

examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin. A non-limiting exemplary luminescent material is luminol; a non-limiting exemplary a magnetic agent is gadolinium, and non-limiting exemplary radioactive labels include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

In an alternative example, OPN-5kD can be assayed in a biological sample by a competition immunoassay utilizing OPN-5kD standards labeled with a detectable substance and an unlabeled antibody that specifically binds OPN-5kD. In this assay, the biological sample (such as serum), the labeled OPN-5kD standards and the antibody that specifically binds OPN-5kD are combined and the amount of labeled OPN-5kD standard bound to the unlabeled antibody is determined. The amount of OPN-5kD in the biological sample is inversely proportional to the amount of labeled OPN-5kD standard bound to the antibody that specifically binds OPN-5kD.

The immunoassays and methods disclosed herein can be used for a number of purposes. In one example, the antibodies that specifically bind OPN-5kD are used to detect the OPN-5kD in serum obtained from a subject known to have or suspected of having a tumor, such as HCC. Increased expression of OPN-5kD is associated with a tumor that expresses OPN, such as HCC, as well as increased likelihood of metastasis of the tumor. Thus, the level of OPN-5kD can be used to diagnose, or determine the prognosis of, HCC in a subject.

Example 1

OPN and MMP-9 are co-upregulated in HCC

This example describes methods used to demonstrate that OPN and MMP-9 are co-upregulated in HCC.

Messenger RNA (mRNA) levels of OPN and MMP-9 were quantified in normal and HCC tissue (obtained from human subjects) by microarray analysis. As shown in FIG. 1A, expression of both OPN and MMP-9 was significantly increased in primary HCC tumors and their corresponding metastatic lesions as compared to non-metastatic tumors (Ye *et al.*, *Nat. Med.* 9:416-23, 2003). As shown in FIG. 1B,

up-regulated expression was significantly correlated within each patient case (p=0.0013).

Example 2

OPN is a Substrate for MMP-9 Activity

This example describes methods used to demonstrate that MMP-9 cleaves OPN into several fragments.

OPN cleavage assays were performed as follows. Human MMP-9 (Calbiochem, CA) was incubated at 37°C for 3 hours with recombinant human OPN (R&D systems, MN) at an optimized ratio (5:1 substrate:enzyme) in a buffered solution (200 mM NaCl, 5 mM CaCl, 50 mM Tris-Cl, pH 7.5). Digested OPN was analyzed by 16% tris-glycine SDS-PAGE followed by colloidal coomassie blue staining (SimplyBlueTM, Invitrogen, CA). Separate cleavage reactions (2 µg OPN) were transferred to PDVF membranes for amino-end terminal microsequencing.

For western blot analysis, MMP-9-digested OPN samples and whole cell lysates (50 µg) were transferred to 0.22 um PVDF membranes and probed with a polyclonal antibody generated to a 14-residue peptide just downstream of the residue 169 thrombin cleavage site (KSKKFRRPDIQYPD; amino acids 169-183 of SEQ ID NO: 2) (Abcam, MA). HEK-293 cell lysates were probed with an anti-FLAG antibody (Sigma, MO). Immuno-reactive proteins were detected by chemilluminescence (ECL, GE Healthcare Life Sciences, NJ).

Recombinant MMP-9 cleaved OPN at two predominant sites *in vitro*, residues 166 and 210, resulting in 4 identified fragments: OPN-34kD (residues 1-166), OPN-32kD (residues 167-314), OPN-5kD (residues 167-210), and OPN-24kD (residues 211-314). Residue numbering is based on the sequence shown in SEQ ID NO: 2. Two of the fragments, OPN-32kD and OPN-5kD, were detected using an antibody that recognizes residues 169-183 (FIG. 2A). The OPN-5kD fragment was distinct from the products of thrombin digestion and could not be detected in cleavage reactions partially inhibited by the concomitant addition of EDTA, a naturally-occurring inhibitor of MMP-9 (tissue inhibitor of matrix metalloproteinase-1, or TIMP-1), or the hydroxamate MMP-9 inhibitor I. Cleavage

was similarly inhibited by a monoclonal antibody to MMP-9. OPN-5kD formation appeared dependent on further cleavage of OPN-32kD over time (FIG. 2B).

Expression vectors were generated that included an OPN fragment with its C-termini fused to a FLAG-tag and its N-termini following the native OPN putative secretory leading 16 amino acid peptide. Complementary DNA sequences encoding the wild-type OPN protein (OPN-a) and three identified truncated protein sequences described above (OPN-34 kD, OPN-5kD, and OPN-24 kD) were cloned into a CMV-promoter mammalian expression vector with a C-terminal 1X FLAG tag (pDest-490) (Gateway cloning system, Invitrogen, CA). The 16-residue predicted signal peptide sequence (MRIAVICFCLLGITCA, amino acids 1-16 of SEQ ID NO: 2) was fused to the N-terminus of each construct following a Kozak translation initiation sequence to ensure similar secretion. Entry clones were verified by sequencing and gel electrophoresis. As shown in FIG. 3A, the native 16 amino acid signal sequence (indicated in black) was included at the N-terminus of each of the 4 sequences. The RGD domain was present in 2 of the 4 constructs (indicated in white).

Effective expression was demonstrated in HEK 293 cells by immunoblotting for the C-terminal FLAG tagged sequences as follows. Secreted levels were also detected in conditioned media samples. HEK 293 cells were transiently transfected (Lipofectamine 2000, Invitrogen, CA) with 5 μg plasmid DNA and harvested after 24 hours. Total protein cell lysates (50 μg) were run on a 4-20% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with an anti-FLAG polyclonal antibody. As shown in FIG. 3B, all of the constructs were expressed in HEK 293 cells.

Example 3

OPN-5kD and MMP-9 expression Correlate with Metastatic Activity

This example describes methods used to demonstrate that expression of the OPN-5kD fragment (as well as full-length OPN) and expression of MMP-9 correlated with metastatic potential.

Three human HCC cell lines (Hep3B, SMMC-7721, and MHCC-97) of established inherent differences in invasiveness were comparatively screened for OPN protein expression.

Single-cell clones of the low-metastatic HCC cell line SMMC-7721 were produced by a limiting dilution cloning technique (Morley *et al.*, *Exp. Hematol.*, 11:418-24, 1983). Briefly, early passage parental cells were plated in 96-well plates (0.5 cell/well) in supplemented media (20% conditioned media) for two weeks with bi-weekly media changes. Cells were propagated by sequential splitting to generate large cell cultures for frozen storage. The subclone most closely resembling the parental cell line by morphology, growth, and a preliminary evaluation of adhesion (see assay methods below), SMMC-7721-SC2, was used for subsequent assays at early passage numbers (<10).

Total protein cell lysates (50 μg) were run by 16% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a polyclonal OPN antibody (pAb8448) and a monoclonal actin antibody (mAb1501). Recombinant human OPN and OPN-5kD peptide samples were run as controls (50 ng). As shown in FIG. 4A, endogenous OPN expression corresponds to degree of metastatic potential, with the highly-invasive cell line MHCC-97 expressing the most prominent levels. Similarly, relative levels of the OPN-5kD cleavage fragment correlated with metastatic potential (FIG. 4A, left panel and FIG. 4C). These results were reproduced using the OPN-5kD specific polyclonal antibody described in Example 8.

To determine the level of MMP-9 activity in the three human HCC cell lines (Hep3B, SMMC-7721, and MHCC-97), gelatinase activities in the cell lines were analyzed by zymography of conditioned media samples. Gelatin zymography was performed as follows. Cells were cultured for 48 hours in serum-free media. Cell number-adjusted serum-free conditioned media harvested from the HCC cell lines cultured to near-confluence were run on 10% tris-glycine gels containing 0.1% gelatin (NOVEX, Invitrogen, CA). The separated proteins were allowed to renature at 25°C for 1 hour (2.5% Triton X-100) and develop at 37°C for 16 hours (50 mM Tris, 0.2 M NaCl, 5 mM CaCl2, 0.02% Brij 35) followed by staining (0.1% amido

black). Pro-MMP-9, active MMP-9, and active MMP-2 standards were included as controls (0.5 ug).

As shown in FIG. 4B, the highest levels of gelatinases (pro-MMP-9 and pro-MMP-2) were observed in MHCC-97 cell media. Conversely, the two low-metastatic cell lines, Hep3B and SMCC-7721, secreted nearly undetectable MMP-9 levels. These combined findings implicate a correlated MMP-9/OPN abundance which may confer differential levels of OPN-5kD.

To measure the invasion of HCC cell lines, the following methods were used. An equal number of cells (5×10^4) was seeded into the upper chambers of Matrigel-coated 8-um pore membranes (HTSTM Fluoroblok tumor invasion system, BD Biosciences). After 36 hours, the invaded cells were labeled with calcein (37°C, 1 hour) and fluorescence was measured (EX485, EM530). As shown in FIG. 4C, the highest levels of invasion were observed in MHCC-97 cells, while the two low-metastatic cell lines, Hep3B and SMCC-7721, had lower levels of invasion.

Example 4

OPN Regions Differentially Modulate Cellular Adhesion and Migration

This example describes methods used to demonstrate the effects of OPN fragments on cellular adhesion and migration of HCC cells.

Cellular adhesion was measured using the following methods. SMMC-7721-SC2 cells were seeded into the wells of a 96-well fluorescent-read plate in serum-free media ($6x10^4$ cells/well) with human OPN (0-250 nM) with or without prior digestion by MMP-9. Media and MMP-9 alone treated cells were included as controls. A GRGDS blocking peptide (400 μM) was added to separate media control, intact OPN, and MMP-9 cleaved OPN (250 nM) conditions. Non-adherent cells were removed with a PBS wash (100 μl) after one hour and adherent cells were quantified by CyQuant GR dye incorporation (Molecular Probes, OR) and detection at EX485, EM530.

As shown in FIG. 5A, increased SMMC-7721-SC2 adhesion was observed in the presence of an exogenous MMP-9 cleaved OPN pool versus intact OPN at > 50 nM concentrations; addition of an integrin-binding GRGDS peptide blocked both intact and cleaved OPN-mediated adhesion.

To determine the affect of OPN fragments on adhesion, the following methods were used. SMMC-7721-SC2 cells (1 x 10^6) were trypsinized (E-PET, Biosource, CA) and transfected with the four OPN expression constructs shown in FIG. 3A and empty vector DNA (5 μg) (50-60% efficiency) using optimized conditions (Nucleofection, AMAXA, MD) and plated to 6-well plates. After a 24-hour recovery period, the cells were trypsinized and seeded by equal numbers (6 x 10^4) into fluorescent-read 96-well plates in serum-free media. Non-adherent cells were removed with a PBS wash after 2-60 minutes and fluorescence was quantified at EX485, EM530.

As shown in FIG. 5B, increased adhesion was observed in response to all four of the OPN constructs at early time points following transient over-expression (2-10 minutes). However, at later time points (30-60 minutes), the OPN fragments containing the RGD integrin binding motif (OPN-full-length and OPN-34kD) increased adhesion, whereas the non-RGD containing regions (OPN-5kD and OPN-24kD) decreased adhesion relative to controls (FIG. 5B).

Migration and invasion assays were performed as follows. Transfected SMMC-7721-SC2 cells (transfected with 5 μg plasmid DNA) were allowed to recover in 5% serum containing media and were re-counted prior to dilution in serum-free media. Cells were seeded (5x10^4) into the upper chambers of Matrigel coated 8 um pore membranes (HTSTM Fluoroblok tumor invasion system, BD Biosciences, MA) or uncoated 0.8 um pore membranes (HTSTM Fluoroblok insert system, BD Biosciences, MA). Media containing serum (5%) was added to the lower chambers as a chemotactic agent. Blocking antibodies to either the integrin αVβ3 receptor (clone LM609, Chemicon, CA) or a common determinant of the CD44 receptors (clone A020, Calbiochem, CA) were added to the upper chambers (4 μg/ml) at the time of cell seeding. Migrated cells were labeled with calcein AM (Molecular Probes, OR) at 37°C for 1 hour, and fluorescence was measured at 24, 36, and 48 hours (EX 490nm, EM 530nm).

As shown in FIG. 5C, full-length OPN and OPN-34kD induced SMMC-7721-SC2 migration whereas OPN-5kD and OPN-24kD expression decreased migration relative to controls.

Example 5

OPN-5kD induces cellular invasion via CD44, but not integrin αVβ3, receptors

This example describes methods used to determine the effect of OPN fragments on invasion of HCC cells.

Cell invasion was measured as described in Example 4. Briefly, each of the OPN vectors shown in FIG. 3A (5 μg) were separately transfected into SMMC-7721 cells (1 x 10^6) using optimized conditions (Nucleofection, AMAXA). An equal number of cells (5 x 10^4) was seeded into the upper chambers of Matrigel-coated 8 um pore membranes (HTSTM Fluoroblok tumor invasion system, BD Biosciences). After 36 hours, the invaded cells were labeled with calcein at 37°C for 1 hour and fluorescence was measured (EX485, EM530).

As shown in FIG. 6A, only the internal OPN-5kD region of OPN induced reproducible > 2-fold increases in cellular invasion in SMMC-7721-SC2 cells (p=0.001). Full-length OPN slightly increased invasion, but not to the extent observed by OPN-5kD. Conversely, over-expression of the RGD-containing OPN-34kD fragment slightly decreased invasiveness compared to vector transfected cells at 36 hours which was more noticeable at a longer 48 hour time point (p=0.005). No significant difference was observed with OPN-34kD or OPN-24kD.

To determine the effect of blocking antibodies to the integrin αVβ3 and CD44 receptors on the affect of invasion of HCC cells expression OPN full-length or OPN-5kD, integrin αVβ3 (Chemicon, CA) or CD44 (Calbiochem, CA) blocking antibodies were added (4 μg/ml) to cells transiently expressing OPN full-length and OPN-5kD. Invading cells were measured at 36 hours following calcein incorporation as described above.

To determine the effect of varying concentrations of the OPN-5kD peptide on invasion of HCC cells, SMMC-7721 cells were exposed to increasing concentrations of the OPN-5kD peptide (0.4 μM, 1 μM and 2 μM) and invading cells were measured after 36 hours following calcein incorporation as described above. A 44 residue peptide corresponding to the OPN-5kD fragment: LRSKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQD (SEQ ID NO: 4), was synthesized and purified >97% by HPLC (EZBiolabs, IN). A GRADSP

peptide of no known biological function was tested in parallel as a control. Cells transiently transfected with the OPN-5kD plasmid were included for comparison.

As shown in FIG. 6B, addition of a blocking antibody that recognizes a common determinant of CD44 receptors significantly reduced OPN-5kD induced invasion ($p=0.0005$), whereas a blocking antibody to the integrin $\alpha V\beta 3$ receptor had no significant effect. Similar results were observed when a synthetic peptide corresponding to OPN-5kD was used in a concentration-dependent manner (FIG. 6C).

These results indicate the OPN-5kD peptide induced cellular invasion is contingent upon CD44 receptor interaction. Similar patterns in adhesive, migratory, and invasive responses were reproduced in the other low-metastatic HCC cell line Hep3B.

Example 6

Fragments of OPN-5kD reduce OPN-5kD-induced cellular invasion

This example describes methods used to identify a minimum region responsible for the observed OPN-5kD-mediated cellular invasion and the identification of fragments that reduce OPN induced cellular invasion.

A 44 residue peptide corresponding to the OPN-5kD fragment (SEQ ID NO: 4), was synthesized and purified >97% by HPLC (EZBiolabs, IN). A series of short peptides spanning the region of 167-210 residues were generated as follows. Eight small overlapping peptides spanning the OPN-5kD peptide were similarly synthesized to >75% purity (p1=LRSKSKKFRR, amino acids 167-176 of SEQ ID NO: 2; p2=KKFRRPDIQY, amino acids 172-181 of SEQ ID NO: 2; p3=PDIQYPDATD, amino acids 177-186 of SEQ ID NO: 2; p4= PDATDEDITS, amino acids 182-191 of SEQ ID NO: 2; p5=EDITSHMESE, amino acids 187-196 of SEQ ID NO: 2; p6=HMESEELNGA, amino acids 192-201 of SEQ ID NO: 2; p7=ELNGAYKAIP, amino acids 197-206 of SEQ ID NO: 2; and p8=YKAIPVAQD amino acids 202-210 of SEQ ID NO: 2) (EZBiolabs, IN). The lyophilized peptides were resuspended in PBS and applied with cells to the upper wells of Matrigel invasion chambers at equal molar concentrations (2 μM). Invading cells were

measured after 36 hours following calcein incorporation as described in the examples above.

Exogenous treatment of these short peptides to SMMC-7721 SC2 cells at equal molar concentrations did not increase cellular invasion as compared to OPN-5kD or a control peptide of no known ability to bind cell surface receptors (GRADSP). However, three peptides (p3=PDIQYPDATD, amino acids 177-186 of SEQ ID NO: 2; p6=HMESEELNGA, amino acids 192-201 of SEQ ID NO: 2; and p7=ELNGAYKAIP, amino acids 197-206 of SEQ ID NO: 2) had a suppressive effect relative to media alone treated cells ($p<0.05$) (FIG. 7A).

Upon separate addition of p3, p6, and p7 in conjunction with OPN-5kD (1:1 molar concentrations, 2 μM each), a partial reduction in invasion was observed ($p<0.002$) which contrasted the insignificant effect of another peptide, p2 (FIG. 7B). The combined addition of p3, p6, and p7 completely abolished the OPN-5kD-mediated invasion down to the levels of media controls ($p=2x10^{-5}$).

Example 7

OPN-c is highly expressed in metastatic HCC

This example describes methods used to identify the OPN splice variants expressed in HCC.

Quantitative RT-PCR was performed as follows. RNA was isolated from HCC primary metastatic tumors (n=17), primary non-metastatic tumors (n=15), and paired non-cancerous tissues collected >2 cm from the lesions, using Trizol (Invitrogen, CA). Normal liver tissue was used as a reference pool (n=8). Total RNA was quality assessed prior to reverse-transcription (cDNA archive kit, Applied Biosystems, CA). Primers were designed upstream and downstream from unique reporter sequences for OPN-a (TCTCCTAGCCCCACAGAATGCTGTG (25-mer, SEQ ID NO: 10), 66-bp final amplicon) and OPN-c (AGGAAAAGCAGAATGCTGTGTCCTC (25-mer, SEQ ID NO: 11), 92-bp final amplicon) (TaqMan gene expression assays, Applied Biosystems, CA). CT threshold values were normalized to 18S rRNA (Applied Biosystems, CA) and data were presented as fold changes relative to the pooled normal liver samples.

Increased mRNA levels corresponding to both the wild-type OPN-a and the variant OPN-c were detected in metastatic HCC tumor samples compared with non-metastatic HCC (FIG. 8A). This effect was notably greater for OPN-c ($p=0.00074$ vs. $p=0.019$ for OPN-a). Further, increased OPN-c levels were detected in non-cancerous tissue surrounding metastatic lesions but were not observed for OPN-a, indicating the involvement of the surrounding stroma in a differential expression of this OPN variant and conditions favoring metastasis (FIG. 8B). A similar difference in OPN-c transcript levels was reflected in the degree of metastatic potential in three highly metastatic HCC cell lines evaluated relative to two low-metastatic cell lines following normalization to primary hepatocytes.

These results indicate greater mRNA level expression of OPN-c than the wild-type OPN-a in metastatic HCC. The adjacent non-cancerous tissues of the metastatic tumor samples are also higher in OPN-c but not OPN-a levels. Histological analysis of this surrounding tissue indicates a predominance of Kupffer-like cells, likely indicating the contribution of macrophages in the tumor cell environment to this observed transcript level response. Thus, these results indicate a macrophage contribution to MMP-9 levels in the tumor cell microenvironment. Such tumor–stromal cell interactions and cross-talk via soluble cytokines and other factors appear to play a role in malignant cell invasion in a multi-cellular tumor microenvironment.

Example 8

Antibody generation

This example describes methods used to generate antibodies to the OPN-5kD peptide.

Un-conjugated OPN-5kD synthetic peptide (synthesized as described in Example 6) was injected into 2 rabbits (3 x 500ug doses), followed by a boost injection (100 μg) at 84 days following initial immunization (Animal Pharm Services, CA). Post-boost bleedings were conducted every 2 weeks for 6 months and immuno-specific antibodies were affinity purified using an OPN-5kD peptide column (Affigel 15, Biorad, CA). The resulting IgG antibody was characterized using standard immunoblotting and immunoprecipitation methods.

Methods of generating monoclonal antibodies are known in the art (for example see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988). For example any of SEQ ID NOS: 4-8, or nucleic acids encoding these peptides can be injected into mice, followed by boost injections, to produce monoclonal antibodies. In addition, protocols for producing humanized forms of monoclonal antibodies and fragments of monoclonal antibodies are known in the art.

In one example, monoclonal antibody to epitopes of OPN-5kD protein can be prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495, 1975) or derivative methods thereof. Briefly, a mouse (such as Balb/c) is repetitively inoculated with a few micrograms of the selected protein (such as any of SEQ ID NOS: 4-8) over a period of a few weeks. The mouse is then sacrificed, and the antibody-producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall (*Enzymol.* 70:419, 1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use.

In another example, monoclonal antibody to epitopes of OPN-5kD protein can be prepared by repetitively inoculating a mouse (such as Balb/c) with one or more plasmids encoding the desired epitope (such as any of SEQ ID NOS: 4-8). For example, pcDNA3 (Invitrogen, Carlsbad, CA) or a vector derived there from, can be manipulated using standard molecular biology methods to include a coding sequence for an OPN-5kD epitope (such as any of SEQ ID NOS: 4-8). For example, BALB/c mice (6–8 weeks old) are immunized three times with the appropriate plasmid (20 μg in phosphate-buffered saline), and one boost can be given with cells before fusion. Mice can be injected three times intradermally into the base of the tail on days 0, 10, and 20 using an insulin syringe with a 28–gauge

needle attached. Serum can be drawn on days 30 and 45 for evaluation of the anti-serum titer. To boost the immunized mice, cells expressing the desired plasmid are injected (for example on day at least 50). These injections can be intravenous and intraperitoneal. Spleens are harvested about 80–90 hours after the last cell boost for cell fusion.

Cell fusions of the splenocytes can be performed according to the protocol of Oi and Herzenberg (*Selected Methods in Cellular Immunology*, Freeman Press, San Fransisco, 1980). Splenocytes and SP2/0 cells are mixed, for example at a 4:1 ratio. The mixed cells are centrifuged and the cell pellet resuspended in polyethylene glycol (such as 40% -50% (w/v) polyethylene glycol) and appropriate medium. The resulting suspension is centrifuged and the cell pellet resuspended in HAT medium, and seeded in 96-well plates at 100 µl/ well ($2.5x10^5$ cells/well) and cultured in a CO_2 incubator. On the day after fusion, 100 µl of fresh HAT medium containing 500 µg/ml geneticin (Invitrogen) is added. On days 4 and 7, half of the spent medium is replaced by fresh HAT medium containing 250 µg/ml geneticin. On day 8, the growth of the hybridoma in each well is checked under a microscope. mAb production in culture supernatants can be assayed on day 10 by enzyme-linked immunosorbent assay (ELISA) or days 9 and 10 by fluorescence-activated cell sorter (FACS). Positive clones can be expanded and the specific hybridomas cloned by a limiting dilution method. The isotype of established MAbs can be determined by a mouse MAb isotyping kit (Zymed, South San Francisco, CA). Immunoglobulin concentrations in the culture supernatants can be determined by a sandwich ELISA as described previously (Nagata *et al.*, *Hybridoma*, 10(3):369-78, 1991).

Example 9

OPN-5kD specific antibodies reduce OPN-5kD-induced cellular invasion

Parental clones have been generated using standard monoclonal antibody generation techniques, such as those described in Example 8. The antigen used to generate these parental clones was the OPN-5kD. 5 parental clones were identified using ELISA and western blotting. These 5 parental clones showed OPN binding activity.

Monoclonal antibodies can be identified from the 5 parental lines by selecting for monoclonal antibodies that bind to SEQ ID NOS. 5-8.

Antibodies, such as those generated from the 5 parental clones described above, are then tested to determine their ability to inhibit cellular invasion.

Such antibodies are suspended in PBS and applied with cells to the upper wells of Matrigel invasion chambers at equal molar concentrations (2 μM). Invading cells are measured after 36 hours following calcein incorporation as described in the examples above.

Therapeutic antibodies are identified as those that when added at equal molar amounts with OPN-5kD do not increase cellular invasion as compared to OPN-5kD (alone) or a control peptide of no known ability to bind cell surface receptors (GRADSP). Therapeutic antibodies are also identified as those that decrease migration as compared to the controls.

These monoclonal antibodies will then be used for generation of ELISA assays to measure the levels of OPN-5kD for diagnosis of disease and monitoring of therapeutic success.

Example 10

Treatment of Metastatic HCC in an Animal Model

This example describes methods than can be used to demonstrate that a therapeutic composition that includes one or more peptides having at least 5 contiguous amino acids of SEQ ID NO: 4, can be used to treat HCC in an animal model.

Mice recognized in the art as a model of HCC can be used. For example, the nude mice model LCI-D20 (see *Tang et al., J. Cancer Res. Clin. Oncol.* 130:187-96, 2004) can be used. Mice are administered at least 1 μg of one or more of SEQ ID NOS: 5-8 intravenously or at least 100 μg (such as 1 mg) intramuscularly. These peptides can be formulated with an inert diluent or with a pharmaceutically acceptable carrier. If desired, other therapeutic molecules can also be administered, such as one or more anti-neoplastic agents. The therapeutic compositions can be administered in a single dose delivery, via continuous delivery over an extended time period, in a repeated administration protocol (for example, by a, daily, weekly,

or monthly repeated administration protocol). In one example, mice receive at least weekly doses of the peptide over at least 6 months. Control mice can receive no peptide (such as an injection that only includes the pharmaceutical carrier).

Following the administration of one or more OPN-5kD peptide fragments, mice are monitored for tumor treatment, such as regression or reduction in metastatic lesions. In particular examples, mice are analyzed one or more times, starting 7 days following treatment. Mice can be monitored using any method known in the art. For example, diagnostic imaging can be used (such as x-rays, CT scans, MRIs, ultrasound,), as well as analysis of biological samples (for example analysis of blood, tissue biopsy, or other biological samples), such as analysis of the type of cells present, or analysis for a particular tumor marker.

A reduction in the number or size of tumors, or a prolonged survival time, in the experimental mice, as compared to the control mice, indicates that the one or more OPN-5kD peptide fragments have an anti-neoplastic therapeutic effect.

Example 11

Treatment of Metastatic HCC in an Animal Model

This example describes methods than can be used to demonstrate that a therapeutic composition that includes one or more OPN-5kD specific antibodies, can be used to treat HCC in an animal model.

Monoclonal OPN-5kD specific antibodies, particularly those that can be produced from one of the 5 parental clones described in Example 9, will be effective in blocking the growth and invasiveness of HCC cancer cells *in vivo*. The effectiveness of such antibodies in blocking the growth of HCC tumors is tested using subcutaneous implantation of HCC tumor cells in mice as a model. The primary advantages of subcutaneous models are the ease of implantation and subsequent monitoring of tumor size. 5×10^5 HCC cells are inoculated subcutaneously into the right craniolateral thorax (axilla) using aseptic technique. Tumors are measured every 34 days using vernier calipers until they reach a volume of 0.2-0.3 cm^3. At that time, the mice are divided into four groups (8-10 animals each): Group 1 (vehicle control), Groups 2-4 are treated with 0.1, 1.0, or 10 mg/kg a particular test antibody, administered intraperitoneally, twice a week. The mice are

then monitored every 3 days to measure tumor volume (with vernier calipers), body weight, and life span. After no greater than 60 days past implantation, the animals are sacrificed and postmortem evaluations of tumorigenesis, including measurement and weight of implanted tumors and proximal lymph nodes, macroscopic evaluation of soft tissues for tumors (lymph nodes and lung), and formalin fixation of the primary tumor and tissues, are performed. The tissues are evaluated by immunohistochemistry using OPN specific antibodies to determine the level of OPN expression in the tumors. In particular, tumor cells that escape treatment are studied to determine whether they have low levels of OPN expression.

While subcutaneous implantation is a popular and valuable method for modeling tumor cell growth, differences between the microenvironment of the skin and liver can cause rather dramatic differences in cell behavior. For example, HCC may not metastasize when implanted subcutaneously whereas intrahepatic implantation (orthotopic) may facilitate metastasis. Thus, HCC cells are implanted in the liver by exposing the liver via laparotomy and inoculating tumor cells into liver using a surgical microscope. After seven days, the mice begin receiving treatment with antibody, as described above, and the animals are sacrificed no later than 60 days after implantation (or if the animals become moribund). The tumors are palpated at 3-5 day intervals, at which time data on tumor size, animal weight, and survival are collected. Post-mortem evaluations are also performed as described above, with emphasis upon the effect of antibody upon metastatic potential (to lungs and regional lymph nodes). The monoclonal antibodies are believed to block the primary tumor and metastatic potential of HCC cells in a dose-dependent manner.

To minimize identification of strain or clonal-specific effects, identical analyses using other model systems can be employed.

Example 12

Treatment of Metastatic HCC in Humans

This example describes a particular method that can be used to treat a primary or metastatic HCC tumor in humans by administration of one or more peptides having at least 5 contiguous amino acids of SEQ ID NO: 4. Although particular methods, dosages, and modes of administrations are provided, one skilled

in the art will appreciate that variations can be made without substantially affecting the treatment.

Therefore human patients are treated intravenously with at least 1 μg (such as 1-100 μg) of one or more peptides that include or consist of the sequence shown in any of SEQ ID NOS: 5-8, for example for a period of at least 6 months, at least one year, at least 2 years, or at least five years. Administration of the peptides can be used in conjunction with normal cancer therapy (for example rather than replacing the therapy). Thus the therapeutic peptides can be added to the usual and customary chemotherapy, surgery and/or radiation treatments conventionally used for the particular tumor type, such as HCC. Administration of the therapeutic peptides can be continued after chemotherapy and radiation therapy was stopped and can be taken long term (for example over a period of months or years).

Briefly, the method can include screening subjects to determine if they have HCC, such as primary or metastatic HCC. Subjects having HCC are selected. In one example, subject having increased levels of OPN-5kD in their serum are selected. In a clinical trial, half of the subjects would follow the established protocol for treatment of HCC (such as a normal chemotherapy/radiotherapy/surgery regimen). The other half would follow the established protocol for treatment of the tumor (such as a normal chemotherapy/radiotherapy/surgery regimen) in combination with administration of the therapeutic peptides described above. In some examples, the tumor is surgically excised (in whole or part) prior to treatment with the therapeutic peptides.

Screening subjects

In particular examples, the subject is first screened to determine if they have HCC. Examples of methods that can be used to screening for HCC include a combination of ultrasound, tissue biopsy, and examination of alpha-feta protein (AFP) levels, wherein serum AFP levels greater than 20 ng/ml or greater than 400 ng/ml and a positive imaging result indicate that the subject has HCC.

In some examples, the tumor is analyzed to determine if it overexpresses OPN, MMP-9, or both, wherein the presence of such overexpression indicates that the tumor can be treated with the disclosed therapies.

However, such pre-screening is not required prior to administration of the therapeutic compositions disclosed herein (such as those that include a peptide having at least 5 contiguous amino acids of SEQ ID NO: 4).

Pre-treatment of subjects

In particular examples, the subject is treated prior to administration of a therapeutic composition that includes one or more peptides having at least 5 contiguous amino acids of SEQ ID NO: 4. However, such pre-treatment is not always required, and can be determined by a skilled clinician. For example, the tumor can be surgically excised (in total or in part) prior to administration of one or more peptides having at least 5 contiguous amino acids of SEQ ID NO: 4. In addition, the subject can be treated with an established protocol for treatment of the particular tumor present (such as a normal chemotherapy/radiotherapy regimen).

Administration of therapeutic compositions

Administration can be achieved by any method known in the art, such as oral administration, inhalation, or inoculation (such as intramuscular, ip, or subcutaneous). In some examples, the therapeutic composition includes one or more peptides having at least 5 contiguous amino acids of SEQ ID NO: 4.

The amount of one or more peptides having at least 5 contiguous amino acids of SEQ ID NO: 4 administered is sufficient to treat a subject having HCC. An effective amount can being readily determined by one skilled in the art, for example using routine trials establishing dose response curves. In addition, particular exemplary dosages are provided above. The therapeutic compositions can be administered in a single dose delivery, via continuous delivery over an extended time period, in a repeated administration protocol (for example, by a, daily, weekly, or monthly repeated administration protocol).

In one example, therapeutic compositions that include one or more peptides having at least 5 contiguous amino acids of SEQ ID NO: 4 are administered iv to a human. As such, these compositions may be formulated with an inert diluent or with an pharmaceutically acceptable carrier.

Assessment

Following the administration of one or more therapies, subjects having a tumor (for example HCC) can be monitored for tumor treatment, such as regression or reduction in metastatic lesions. In particular examples, subjects are analyzed one or more times, starting 7 days following treatment

Subjects can be monitored using any method known in the art. For example, diagnostic imaging can be used (such as x-rays, CT scans, MRIs, ultrasound, fiberoptic examination, and laparoscopic examination), as well as analysis of biological samples from the subject (for example analysis of blood, tissue biopsy, or other biological samples), such as analysis of the type of cells present, or analysis for a particular tumor marker. In one example, if the subject has a metastatic HCC, assessment can be made using ultrasound, MRI, or CAT scans, analysis of the type of cells contained in a tissue biopsy, and AFP levels.

One of ordinary skill in the art will appreciate that substantially similar methods can be used to treat human subjects with OPN-5kD specific antibodies.

Example 13

Evaluation Following Treatment

During or following therapeutic treatment, subjects can be monitored for the response of their tumor(s) to the therapy.

Subjects can receive a complete physical evaluation, CBC, acute care, hepatic and mineral panels and appropriate evaluations of all evaluable lesions (for example by x-ray, MRI, CT scan, ultrasound) are obtained every 6-12 weeks during the first six months of therapy and if stable, every 3-6 months thereafter. Other evaluations can be performed as indicated by symptoms or physical findings. For example, surveillance CT of the chest, abdomen and pelvis can be obtained with at least every other assessment and as indicated by symptoms or physical findings.

Example 14

Additional Treatment

In particular examples, if subjects are stable or have a minor, mixed or partial response to treatment, they can be re-treated after re-evaluation with the same

schedule and preparation of peptide that they previously received for up to a year of total therapy.

A mixed response is the shrinkage of some lesions but an increase in others. Subjects with mixed responses may only receive treatment for an additional 2-3 months without showing true disease stability or a bona fide minor or major response (i.e. no further progression). Two re-treatment cycles can be given following a complete response.

Example 15

Diagnosis of Metastatic HCC in Humans

This example describes particular methods that can be used to diagnose or prognose HCC in a human subject. However, one skilled in the art will appreciate that similar methods can be used. In some examples, such diagnosis is performed before treating the subject (for example as described in Example 10).

Biological samples are obtained from the subject. If blood or a fraction thereof (such as serum) is used 1-100 µl of blood is collected. Serum can either be used directly or fractionated using filter cut-offs to remove high molecular weight proteins. If desired, the serum can be frozen and thawed before use. If a tissue biopsy sample is used, 1-100 µg of tissue is obtained, for example using a fine needle aspirate RNA is isolated from the tissue using routine methods (for example using a commercial kit).

In one example, OPN-5kD protein levels are determined in a serum sample obtained from the subject. The serum sample described above is incubated with the antibody described in Example 8 for a time sufficient for the antibody to bind to OPN-5kD in the serum. The OPN-5kD/antibody complexes are detected, for example using an ELISA. Alternatively, the serum sample is subjected to 16% SDS-PAGE, and transferred to a membrane (such as nitrocellulose), which is probed with the antibody described in Example 8. The antibody/OPN-5kD complexes can be detected with a secondary labeled antibody, or by observing the appropriated sized protein on the gel. The relative amount of OPN-5kD/antibody complexes in the serum sample from the subject can be compared to a reference value, such as a relative amount of OPN-5kD/antibody complexes present in a serum sample from a

subject not having a tumor, wherein the presence of significantly more OPN-5kD/antibody complexes in the test sample as compared to the reference sample (such as an increase of at least 2-fold, at least 3-fold, or at least 5-fold) indicates that the subject has HCC, has metastatic HCC, has a poor prognosis, or combinations thereof.

In one example, OPN-c mRNA expression levels are determined in a tumor sample obtained from the subject and in a tissue sample adjacent to (but not including) the tumor. cDNA is generated from the RNA isolated from the tissue samples described above (for example using a commercial reverse transcription kit). OPN-c cDNA is amplified using appropriate primers (for example using primers having a detectable label), and the resulting OPN-c amplicons detected. The relative amount of OPN-c amplicons in the tumor tissue sample can be compared to a reference value, such as a relative amount of OPN-c amplicons present in the adjacent non-tumor sample from the subject, wherein the presence of significantly more OPN-c amplicons in the tumor sample as compared to the non-tumor sample (such as an increase of at least 2-fold, at least 3-fold, or at least 5-fold) indicates that the subject has metastatic HCC, has an increased likelihood of a primary HCC metastasizing, has a poor prognosis, or combinations thereof.

In some examples, relative amount of OPN-5kD protein and OPN-c mRNA expression are determined in the same subject using the methods described above.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A method of diagnosing an osteopontin (OPN)-expressing tumor in a subject, comprising:

contacting a sample comprising peptides from the subject with an antibody that specifically binds to SEQ ID NO: 4; and

determining whether the antibody specifically bound to proteins in the sample, wherein detection of a significant amount of binding as compared to a control value indicates that the subject has detectable OPN-5kD and has an OPN-expressing tumor.

2. The method of claim 1, wherein the sample comprises blood or a fraction thereof.

3. The method of claim 2, wherein the blood fraction comprises serum.

4. The method of claim 1, wherein the antibody is a polyclonal or monoclonal antibody, or fragment thereof.

5. The method of claim 1, wherein the antibody comprises a detectable label.

6. A method of diagnosing an OPN-expressing tumor in a subject, comprising:

determining an amount of OPN-c expression in a tumor sample obtained from the subject;

determining an amount of OPN-a expression in a tumor sample obtained from the subject; and

comparing the amount of OPN-c expression in the tumor sample to the amount of OPN-a expression in the tumor sample, wherein a statistically significant increase in OPN-c expression in the tumor sample compared to the amount of OPN-a expression in the tumor sample indicates that the subject has an OPN-expressing tumor.

7. The method of claim 6, further comprising determining whether the tumor expresses a greater level of OPN-c mRNA than adjacent non-tumor tissue, wherein a statistically significant increase in OPN-c mRNA expression in the tumor sample compared to the adjacent non-tumor tissue indicates that the subject has a tumor with increased metastatic potential.

8. A method of determining the metastatic potential of an OPN-expressing tumor in a subject, comprising:

determining an amount of OPN-c expression in a tumor sample obtained from the subject;

determining an amount of OPN-c expression in a sample obtained adjacent to the tumor from the subject; and

comparing the amount of OPN-c expression in the tumor sample to the adjacent sample, wherein a statistically significant increase in OPN-c expression in the tumor sample compared to the adjacent sample indicates that the subject has an OPN-expressing tumor with increased metastatic potential.

9. The method of claim 6 or 8, wherein OPN-a and OPN-c expression is determined by measuring an amount of mRNA expression or protein expression.

10. A method of treating an OPN-expressing tumor in a subject, comprising:

administering to the subject a therapeutically effective amount of an OPN-5kD specific antibody or a peptide comprising at least 5 contiguous amino acids of SEQ ID NO: 4, thereby treating the tumor.

11. The method of claim 1, 6, 8, or 10, wherein the OPN-expressing tumor comprises hepatocellular carcinoma (HCC).

12. The method of claim 11, wherein the HCC is a metastasis.

13. The method of claim 12, wherein the metastasis is an intra-hepatic metastasis.

14. The method of claim 12, wherein the metastasis is an extra-hepatic metastasis.

15. The method of claim 10, wherein the wherein the subject has detectable OPN-5kD in its serum, wherein the tumor expresses a greater amount of OPN-c mRNA than OPN-a mRNA, or both.

16. The method of claim 10, wherein the peptide comprising at least 5 contiguous amino acids of SEQ ID NO: 4 comprises 5-20 contiguous amino acids of SEQ ID NO: 4.

17. The method of claim 10, wherein the peptide comprising at least 5 contiguous amino acids of SEQ ID NO: 4 comprises 10-15 contiguous amino acids of SEQ ID NO: 4.

18. The method of claim 10, wherein the peptide comprising at least 5 contiguous amino acids of SEQ ID NO: 4 comprises one or more peptides consisting of the amino acid sequence of SEQ ID NO: 5, 6, 7, or 8.

19. The method of claim 10, wherein administration of the peptide is via injection.

20. The method of claim 19, wherein the therapeutically effective amount of the peptide comprises 1 – 1000 μg daily to the subject.

21. The method of claim 10, wherein the subject is a mammalian subject.

22. The method of claim 21, wherein the mammalian subject is a human subject.

23. The method of claim 10, wherein the method further comprises administering one or more additional therapeutic agents at a therapeutically effective amount to the subject.

24. The method of claim 23, wherein the one or more additional therapeutic agents comprise one or more anti-neoplastic agents.

25. The method of claim 23, wherein the one or more additional therapeutic agents comprise radiological agents.

26. The method of claim 25, further comprising exposing to the subject a therapeutically effective amount of radiation therapy.

27. The method of claim 10, wherein the method further comprises surgical excision of at least part the tumor prior to administering the therapeutically effective amount of the antibody or peptide.

28. The method of claim 10, wherein treating the tumor comprises reducing invasive activity of the tumor in the subject.

29. The method of claim 10, wherein treating the tumor prolongs survival time of the subject.

30. The method of claim 10, further comprising determining whether the subject has significantly increased levels of OPN-5kD in its serum compared to a subject not having a tumor, wherein the presence of significantly increased levels OPN-5kD in the serum indicates that the subject has an OPN-expressing tumor.

31. A composition comprising one or more peptides, wherein each peptide comprises 5-20 contiguous amino acids of SEQ ID NO: 4.

32. The composition of claim 31, wherein the composition comprises one or more peptides consisting of the amino acid sequence of SEQ ID NO: 5, 6, 7, or 8.

33. A method of treating a subject having an OPN-expressing tumor, comprising administering a therapeutically effective amount of the composition of any one of claims 31-32, thereby treating the tumor.

34. Use of the composition of any of claims 31-32 to treat a human having a metastatic HCC tumor.

35. Use of one or more peptides consisting of the amino acid sequence of SEQ ID NO: 5, 6, 7, or 8 for the manufacture of a medicament for the treatment of HCC.

36. A kit for treating an OPN-expressing tumor, comprising:
the composition of claim 31 or 32; and
at least one anti-neoplastic agent.

37. An isolated antibody that specifically binds to SEQ ID NO: 4 with an equilibrium dissociation constant (K_d) of 1 nM or less.

38. The isolated antibody of claim 37, wherein the antibody is a polyclonal antibody, monoclonal antibody, or fragment thereof.

39. The isolated antibody of claim 38, wherein the fragment comprises a Fab' fragment, a $F(ab)'_2$ fragment, a single chain Fv protein ("scFv"), or a disulfide stabilized Fv protein ("dsFv").

40. The isolated antibody of claim 37, wherein the antibody binds to any of SEQ ID NOS: 5-8.

41. The isolated antibody of claim 39, wherein the antibody comprises a detectable label.

42. A composition comprising the antibody of any one of claims 37-41 in a pharmaceutically acceptable carrier.

43. A method of diagnosing an OPN-expressing tumor in a subject, comprising

contacting a sample from the subject with the isolated antibody of any of claims 37-41; and

detecting binding of the antibody to the sample, wherein an increase in the binding of the antibody to the sample as compared to a control indicates that the subject has an OPN-expressing tumor.

44. The method of claim 43, further comprising

contacting a second antibody that specifically binds the isolated antibody with the sample, and

detecting the binding of the second antibody, wherein an increase in the binding of the second antibody as compared to a control indicates that the subject has an OPN-expressing tumor.

45. An isolated nucleic acid molecule encoding the antibody of claim 37.

46. The isolated nucleic acid molecule of claim 45, operably linked to a promoter.

47. An expression vector comprising the isolated nucleic acid molecule of claim 45.

48. An isolated host cell transformed with the nucleic acid molecule of claim 45.

49. A kit for diagnosing or prognosing an OPN-expressing tumor, comprising:

the antibody of claim 37; and

one or more agents that permit detection of binding of the antibody to OPN-5kD in a biological sample.

50. The kit of claim 49, wherein one or more of the agents that permit detection of binding of the antibody to OPN-5kD comprise a labeled secondary antibody that can specifically bind to the antibody.

51. A kit for treating an OPN-expressing tumor, comprising:
the isolated antibody of any of claims 37-41; and
at least one anti-neoplastic agent.

FIG. 1A

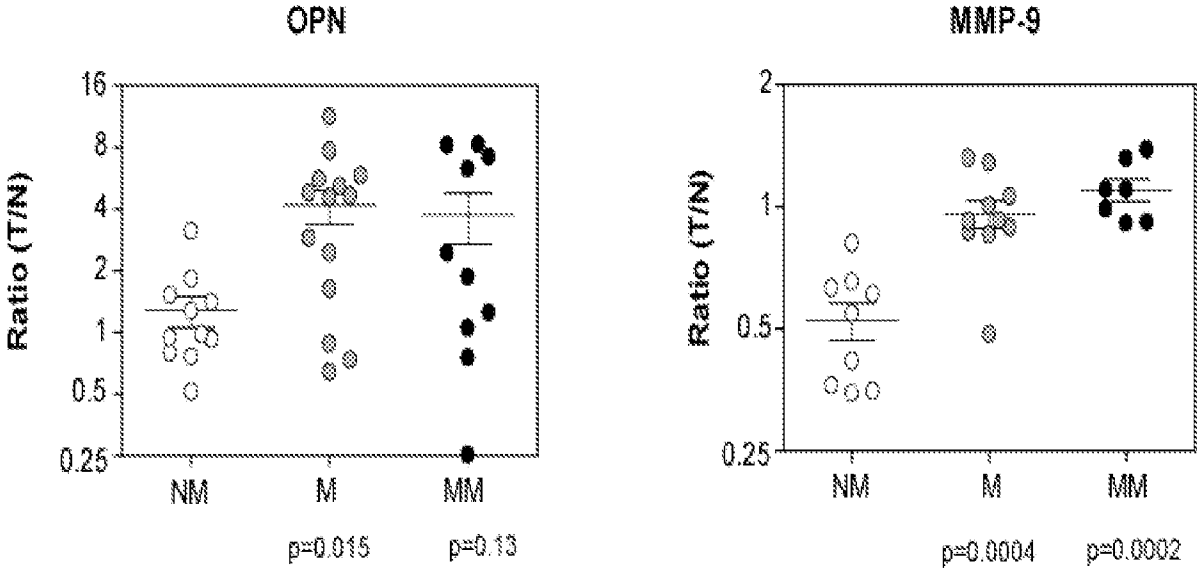

OPN
Ratio (T/N)
16
8
4
2
1
0.5
0.25
NM
M
MM
p=0.015
p=0.13
MMP-9
Ratio (T/N)
2
1
0.5
0.25
NM
M
MM
p=0.0004
p=0.0002

FIG. 1B

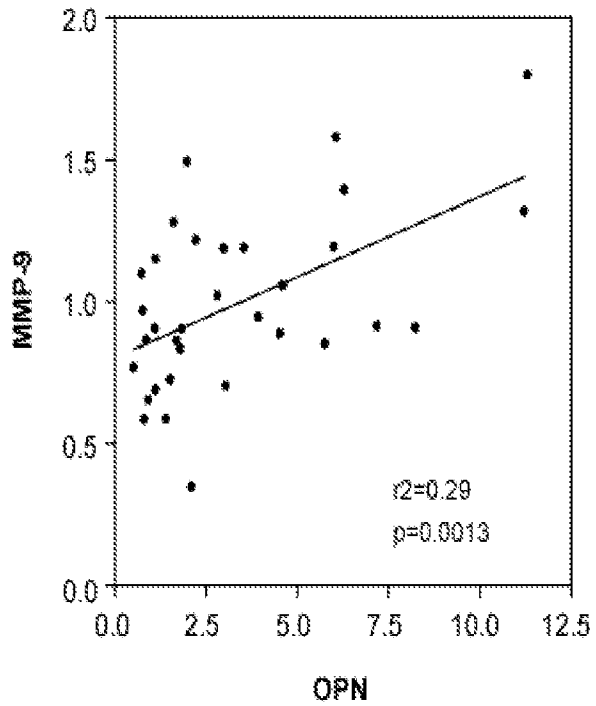

MMP-9
2.0
1.5
1.0
0.5
0.0
r2=0.29
p=0.0013
0.0
2.5
5.0
7.5
10.0
12.5
OPN

FIG. 2A

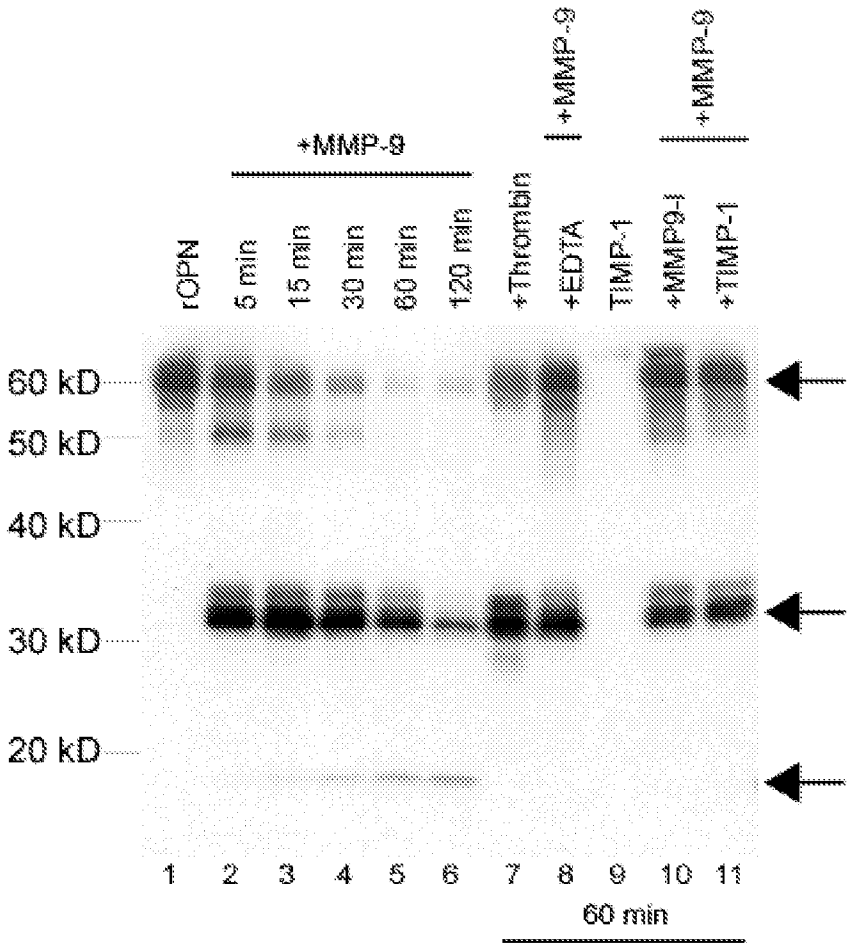

+MMP-9
+MMP-9
+MMP-9
rOPN
5 min
15 min
30 min
60 min
120 min
+Thrombin
+EDTA
TIMP-1
+MMP9-i
+TIMP-1
60 kD
50 kD
40 kD
30 kD
20 kD
1 2 3 4 5 6 7 8 9 10 11
60 min

FIG. 2B

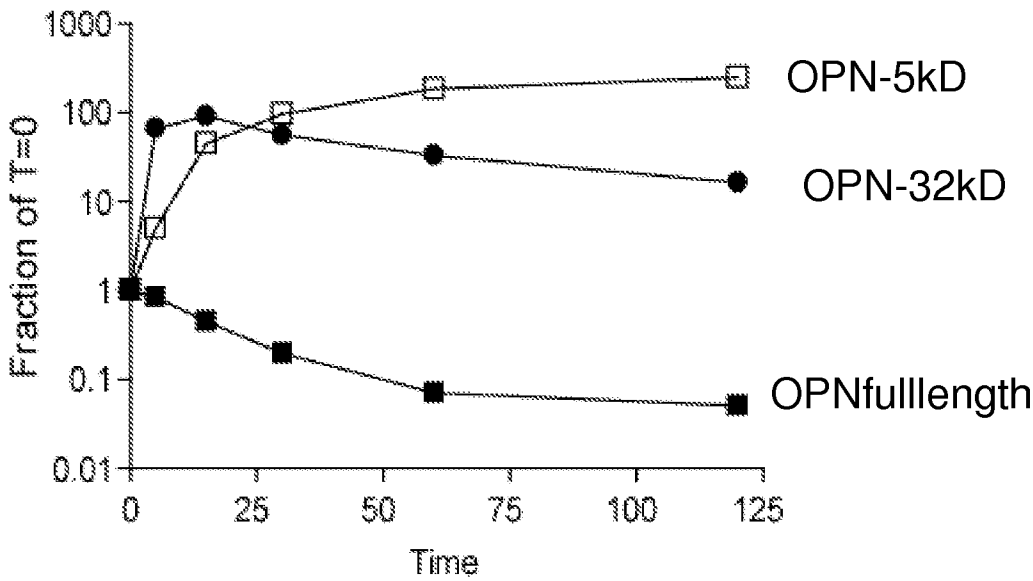

1000
100
10
1
0.1
0.01
Fraction of T=0
0 25 50 75 100 125
Time
OPN-5kD
OPN-32kD
OPNfulllength

FIG. 3A

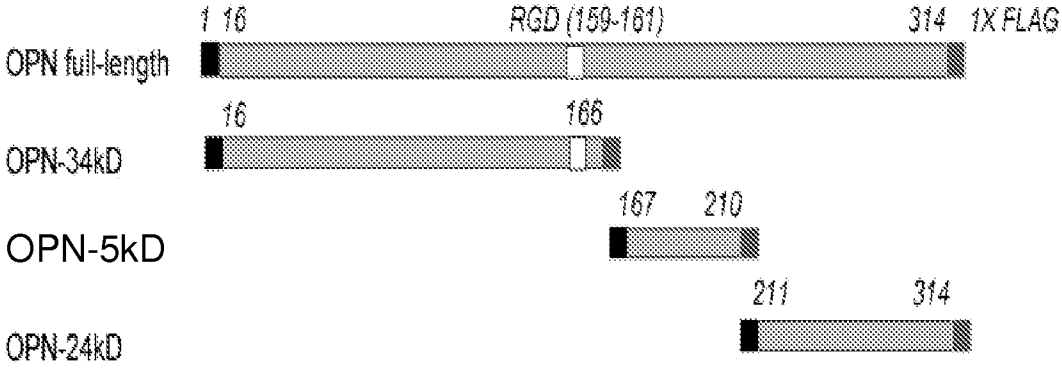

1 16
RGD (159-161)
314 1X FLAG
OPN full-length
16
166
OPN-34kD
167 210
OPN-5kD
211 314
OPN-24kD

FIG. 3B

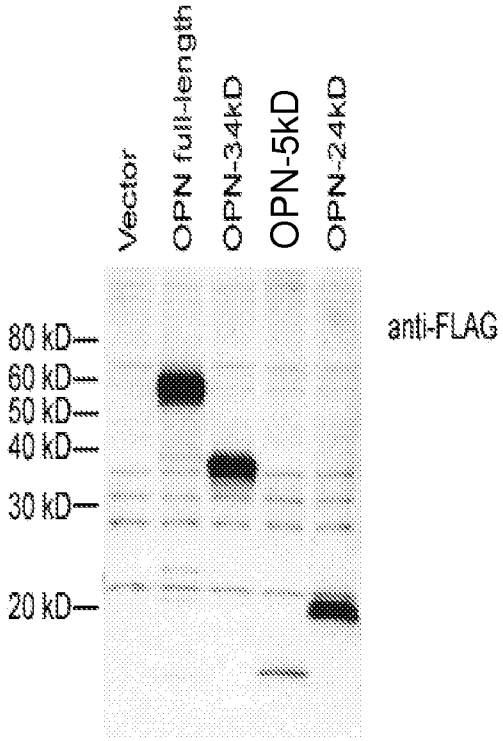

Vector
OPN full-length
OPN-34kD
OPN-5kD
OPN-24kD
anti-FLAG
80 kD
60 kD
50 kD
40 kD
30 kD
20 kD

FIG. 4A

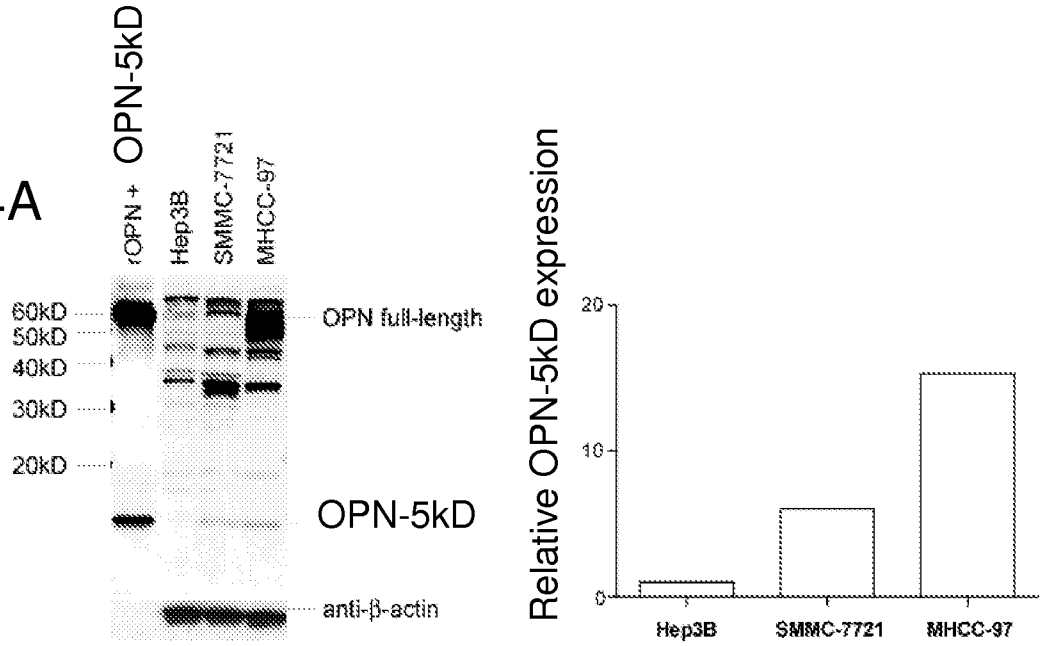


FIG. 4B

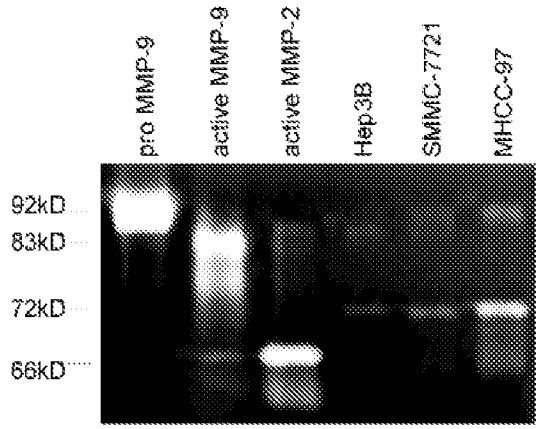


FIG. 4C

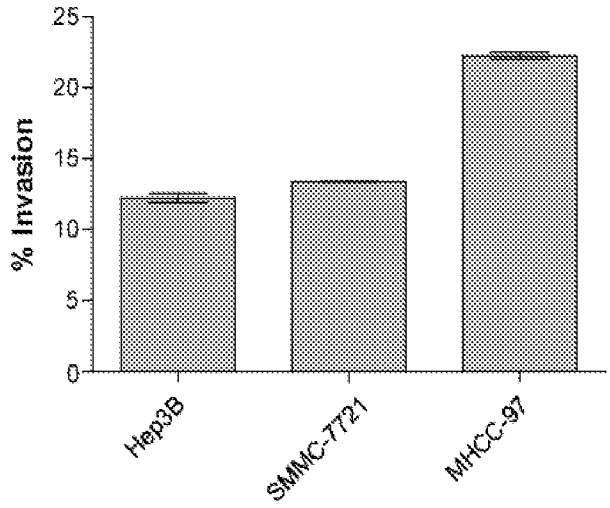


FIG. 5A

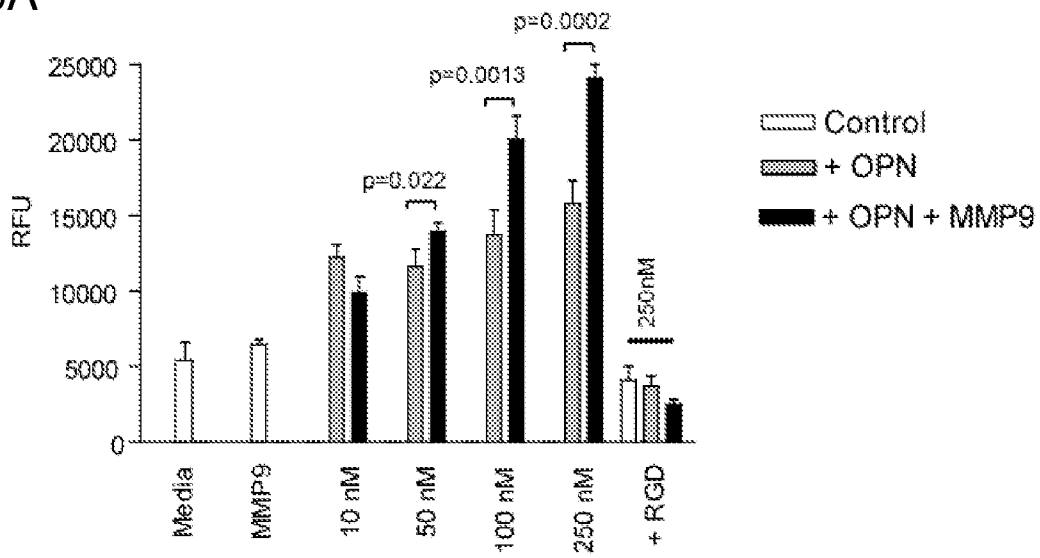


FIG. 5B

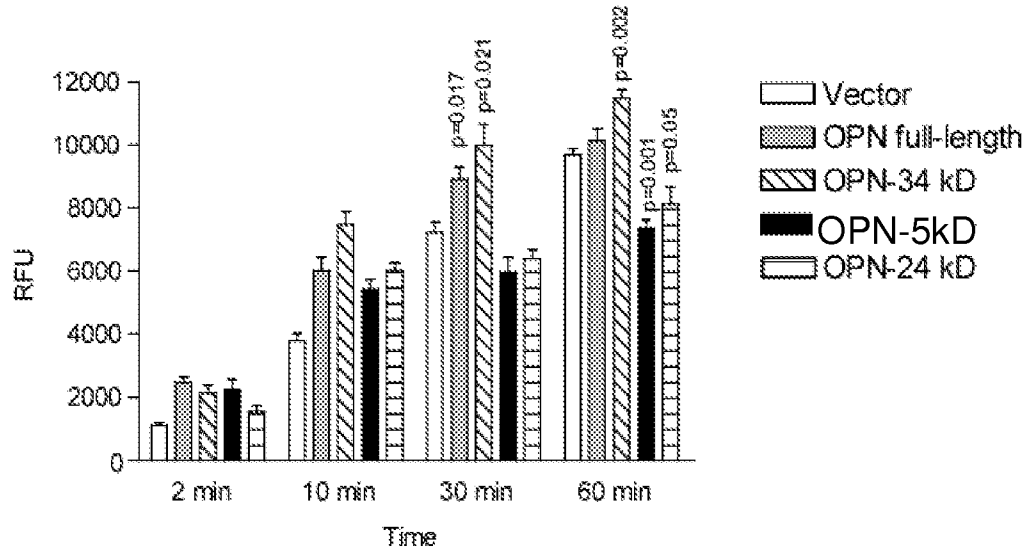


FIG. 5C

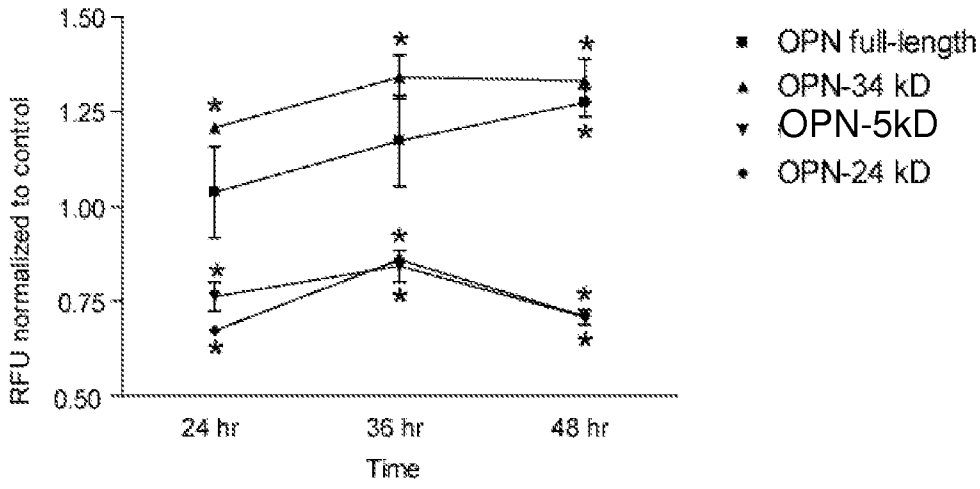


FIG. 6A

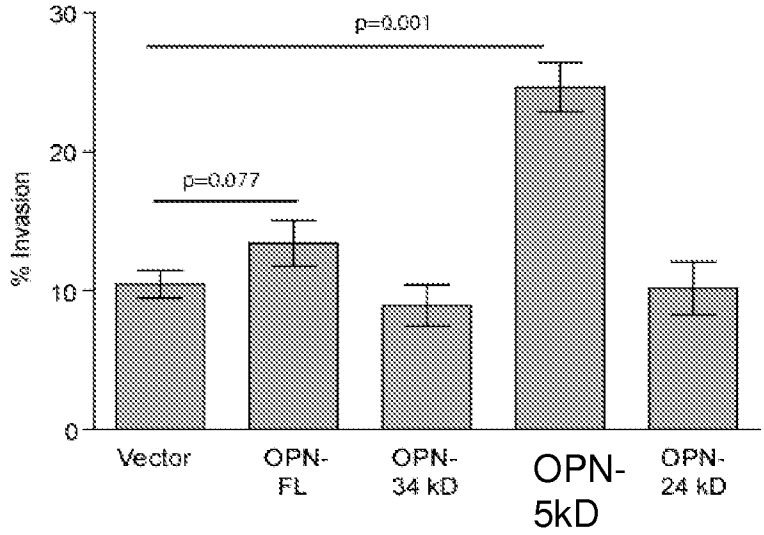


FIG. 6B

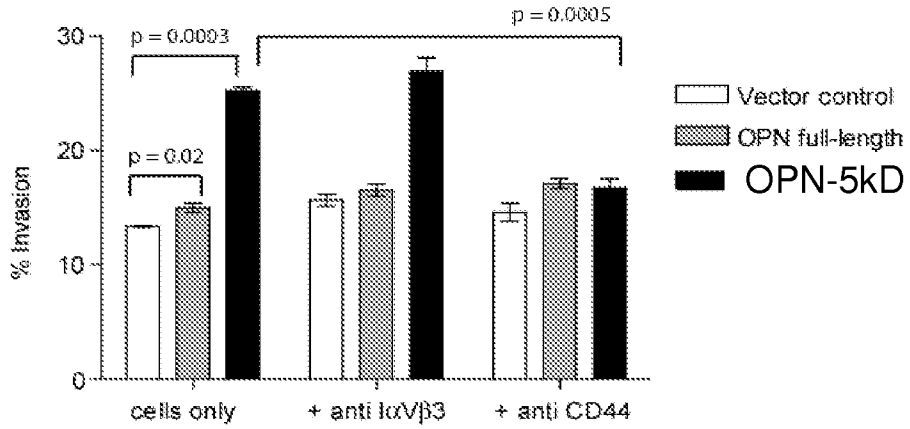


FIG. 6C

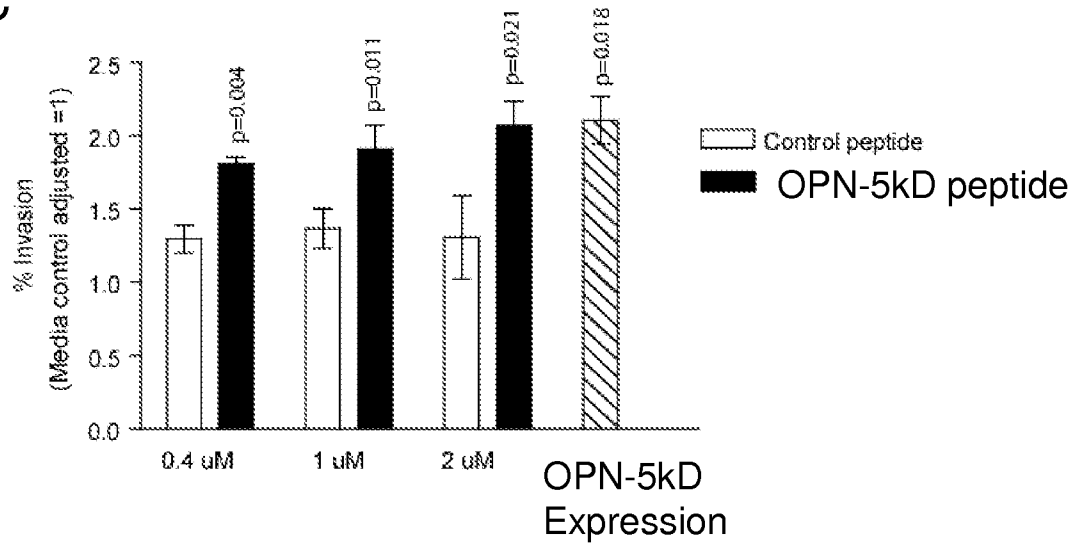


FIG. 7A

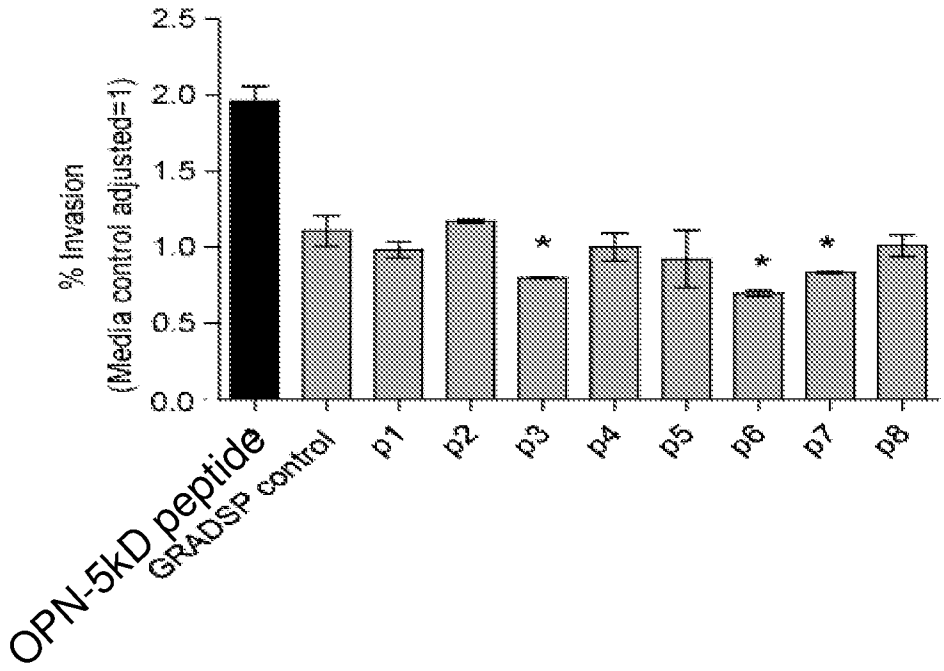

% Invasion
(Media control adjusted=1)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
OPN-5kD peptide
GRADSP control
p1
p2
p3
p4
p5
p6
p7
p8

FIG. 7B

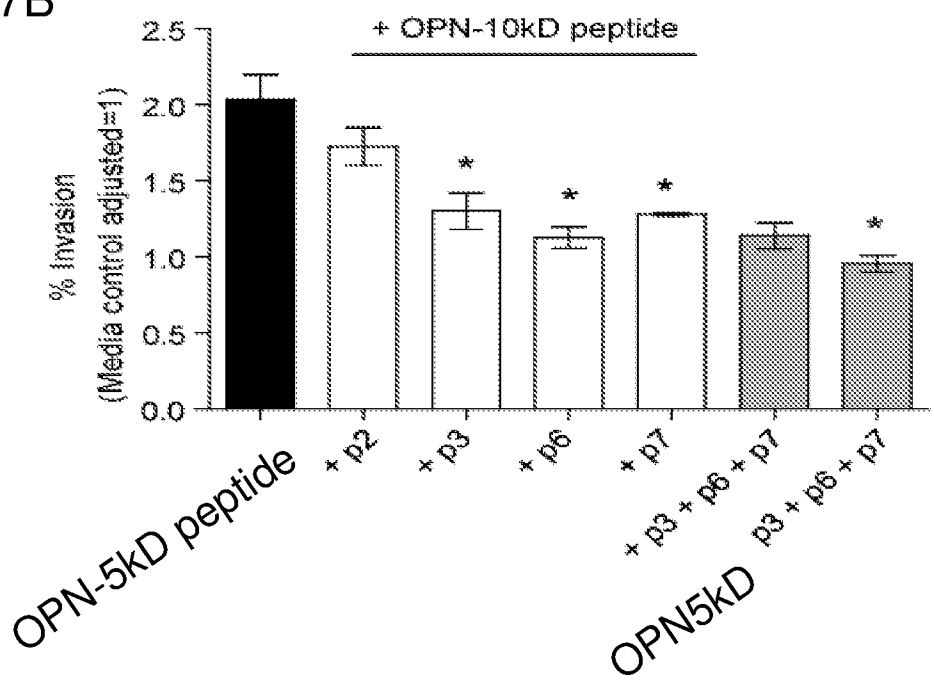

+ OPN-10kD peptide
% Invasion
(Media control adjusted=1)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
OPN-5kD peptide
+ p2
+ p3
+ p6
+ p7
+ p3 + p6 + p7
p3 + p6 + p7
OPN5kD

FIG. 8A

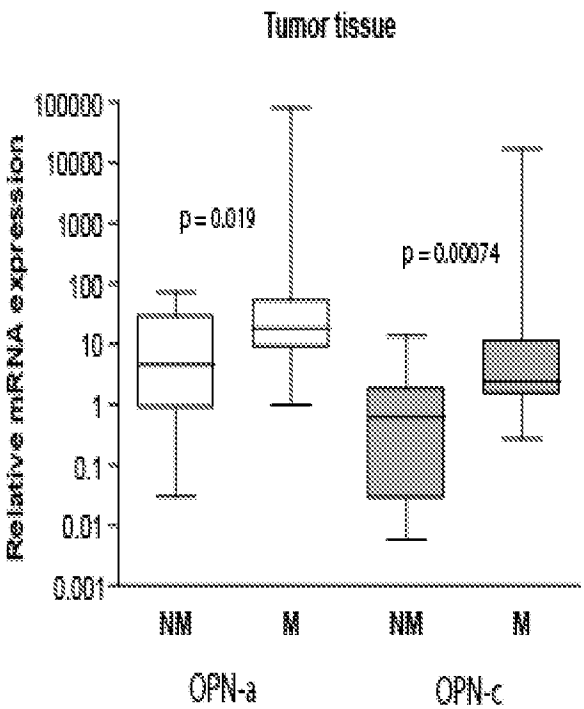
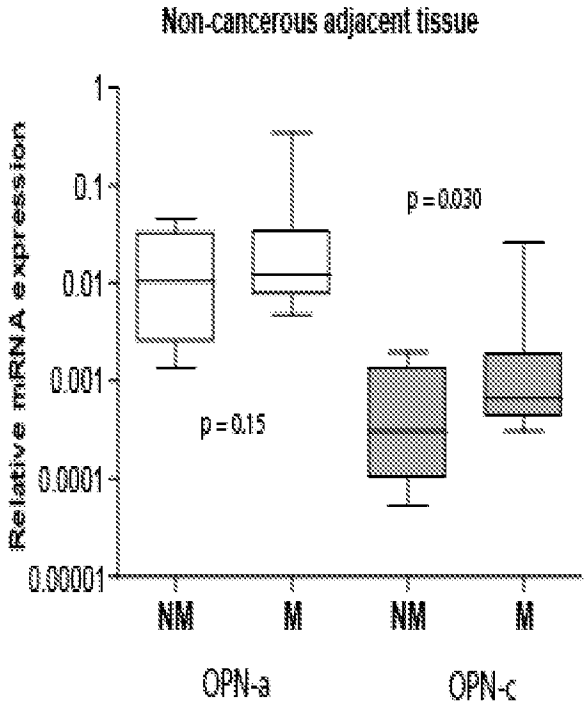

Tumor tissue
Relative mRNA expression
100000
10000
1000
100
10
1
0.1
0.01
0.001
p = 0.019
p = 0.00074
NM
M
NM
M
OPN-a
OPN-c

FIG. 8B


Non-cancerous adjacent tissue
Relative mRNA expression
1
0.1
0.01
0.001
0.0001
0.00001
p = 0.030
p = 0.15
NM
M
NM
M
OPN-a
OPN-c