

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 August 2008 (14.08.2008)

PCT

(10) International Publication Number  
**WO 2008/097466 A2**

(51) International Patent Classification:  
*G01N 33/574* (2006.01)

(74) Agents: MILLER, Alan, D. et al.; Amster, Rothstein & Ebenstein LLP, 90 Park Avenue, New York, NY 10016 (US).

(21) International Application Number:  
PCT/US2008/001343

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, 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.

(22) International Filing Date: 31 January 2008 (31.01.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/899,303 2 February 2007 (02.02.2007) US

(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, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicants (for all designated States except US): ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY [US/US]; 1300 Morris Park Avenue, Bronx, NY 10461 (US). MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US). IFO-REGINA ELENA CANCER INSTITUTE [IT/IT]; Via Elio Chianesi 53, I-00144 Rome (IT).

(72) Inventors; and

(75) Inventors/Applicants (for US only): CONDEELIS, John, S. [US/US]; 122 Horton Street, Bronx, NY 10464 (US). GOSWAMI, Sumanta [IN/US]; 611A Larchmont Acres East, Larchmont, NY 10538 (US). GERTLER, Frank [US/US]; 237 W. Newton Street, #5, Boston, MA 02116 (US). NISTICO, Paola [IT/IT]; Vicolo Delle Palline No. 7, I-00193 Rome (IT).

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



WO 2008/097466 A2

(54) Title: METASTASIS SPECIFIC SPLICE VARIANTS OF MENA AND USES THEREOF IN DIAGNOSIS, PROGNOSIS AND TREATMENT OF TUMORS

(57) Abstract: Methods and kits for diagnosis, prognosis and treatment of metastatic tumors are provided where the metastatic tumor is characterized by overexpression of splice variants of Mena.

METASTASIS SPECIFIC SPLICE VARIANTS OF MENA AND USES THEREOF IN  
DIAGNOSIS, PROGNOSIS AND TREATMENT OF TUMORS

CROSS-REFERENCE TO RELATED APPLICATION

[1] This application claims the benefit of U.S. Provisional Application No. 60/899,303, filed February 2, 2007, the content of which is incorporated by reference.

STATEMENT OF GOVERNMENT SUPPORT

[2] The invention disclosed herein was made with U.S. Government support under grant number CA100324 from the National Cancer Institute, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[3] Various publications are referred to in parentheses throughout this application. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference in their entireties into the subject application to more fully describe the art to which the subject application pertains.

[4] One out of three cancers diagnosed among U.S. women is due to breast cancer; 212,920 new invasive breast cancer cases and an additional 61,980 *in situ* breast cancer cases are expected to be diagnosed in the U.S. in 2006. Around 40,970 women are expected to die from breast cancer in 2006 in the U.S. alone (American Cancer Society, Breast Cancer Facts and Figures 2006). The metastasis of 10-15% of patients with breast cancer is aggressive and can take between 3-10 years to be manifested after the initial diagnosis. Currently, the prognosis in 70% of patients cannot be accurately determined resulting in the unnecessary treatment of many patients who will not benefit and may be injured by radiation and chemotherapy. The availability of an antibody and associated polymerase chain reaction (PCR) primer pair that uniquely and specifically identifies metastatic disease will allow for accurate prediction of disease course and allow appropriate treatment.

[5] Invasion of tumor cells into surrounding tissue and intravasation into blood and lymphatic vessels is implicated in the progression of metastatic breast cancer. This multi-

-2-

step process involves a number of phenotypic changes which occur sequentially and give rise to a hyper-invasive cell (Condeelis et al., 2005). In an effort to identify these individual events and to understand the molecular events underlying these phenotypic changes, animal models have been developed as well as a chemotaxis assay that isolates the *in vivo* invasive cells from the average primary tumor cells (APTC) (Wyckoff et al., 2000). Chemotaxis based isolation of the invasive cells and subsequent gene expression analysis have resulted in the identification of an invasion specific gene expression signature in invasive cells (Wang et al., 2004). In these studies a number of genes have been identified which need to be co-ordinately up-regulated in the invasive cells in order for invasion to lead to metastasis (Wang et al., 2006).

[6] One of the key genes of the invasion signature is that coding for the cytoskeletal protein Mena. Mena is a member of the Ena/VASP family of proteins. These proteins are regulatory molecules which control cell movement, motility and shape in a number of cell types and organisms. They are proposed to function by preventing the actin filaments from being capped by capping proteins at their barbed ends (Barzik et al., 2005). The anti-capping activity of Mena has been proposed to amplify the barbed end output of the cofilin and Arp2/3 complex pathways, which is sufficient to increase metastatic potential in mammary tumors (Wang et al., 2006). Ena/VASP proteins are also constituents of the adherence junctions necessary to seal membranes in the epithelial sheet and control actin organization on cadherin adhesion contact (Scott et al., 2006). This process is frequently perturbed in cancer. Ena/VASP proteins contain specific domains including the N-terminal EVH1 domain, which plays an essential role in intracellular protein localization by interacting with proline-rich motifs found in proteins such like zyxin and vinculin (Prehoda et al., 1999). The proline-rich domain in the center is known to mediate interaction with proteins having the SH3 and WW domains and also with the actin monomer binding protein profilin (Gertler et al., 1996). The C-terminal domain of Mena contains an EVH2 domain that is involved in tetramerization of the protein and also binding to G- and F-actin (Kuhnel et al., 2004). The interaction of the EVH2 domain with the growing ends of the actin filaments is essential for targeting the Ena/VASP to lamellipodia and filopodia (Loureiro et al., 2002). Mena is upregulated in mouse and rat invasive breast cancer cells (Wang et al., 2004) and overexpressed in human breast cancer tissues (Di Modugno et al., 2004). Both mouse and human Mena homologs have been cloned and sequenced, and a number of splice variants have been identified

(Gertler et al., 1996; Urbanelli et al., 2006).

[7] Recently it has been shown that splice variants can work very efficiently as cancer biomarkers (Brinkman 2004; Venables 2006). However, there remains a need to identify splice variants that are upregulated specifically in metastatic cancer cells, such as metastatic breast cancer cells.

#### SUMMARY OF THE INVENTION

[8] The invention provides a method for determining whether a subject has a metastatic tumor comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein overexpression of the ++ and/or +++ variant of Mena indicates the presence of a metastatic tumor.

[9] The invention also provides a method for assessing the efficacy of therapy to treat a metastatic tumor in a subject who has undergone or is undergoing treatment for a metastatic tumor, the method comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein overexpression of the ++ and/or +++ variant of Mena is indicative of a need to continue therapy to treat the tumor.

[10] The invention further provides a method for assessing the prognosis of a subject who has a metastatic tumor, comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein the subject's prognosis improves with a decrease in expression of the ++ and/or +++ variant of Mena.

[11] The invention provides a method of inhibiting metastasis of a tumor in a subject, the method comprising reducing the presence or activity of the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena in the subject.

[12] The invention provides a method for screening for a candidate compound that inhibit metastasis of a tumor, the method comprising contacting the compound with a cell line or tissue culture that express the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena, wherein reduction in the expression of the ++ and/or +++ isoform of Mena is indicative that the compound is a candidate compound for inhibiting metastasis of a tumor.

[13] The invention provides a purified polypeptide, where the polypeptide is overexpressed in a metastatic tumor, and an isolated nucleic acid molecule encoding the polypeptide, where the polypeptide comprises the amino acid sequence of the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena.

[14] The invention provides kits for detecting the presence or absence of a metastatic tumor, where the kits comprise an antibody, a peptide or an aptamer that specifically binds to the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena and/or a probe or PCR primers that specifically hybridize to nucleic acid encoding the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[15] Figure 1A-1B. Quantification of Mena isoforms in MTLn3 rat allograft model (A) and PyMT mouse transgenic model (B). The pan Mena primer shows a four to five fold over-expression in the invasive cells. The ++ and +++ exons as seen by QPCR reactions specific for them indicate that they are both over-expressed in the invasive cells as well.

[16] Figure 2. Mena isoforms ++ and +++ are over-expressed in metastatic MTLn3 cells as determined by QRT-PCR. The pan Mena primer shows a four fold over-expression in the invasive cells. The ++ and +++ exons as seen by QPCR reactions specific for them indicate that they are both over-expressed in cancer cells collected from blood and from successful lung mets.

[17] Figure 3. Mena +++ splice variant is expressed in human breast cancer cell lines. MDA231 shown on left and T47D shown on right.

[18] Figure 4A-4B. Sequence alignment for ++ and +++ exons in invasive cells aligned with published mouse and human sequences. The ++ exon nucleotides (SEQ ID NO:1) and their inferred amino acid sequence (SEQ ID NO:2) are aligned in A and the +++ exon nucleotides (SEQ ID NO:3) and their inferred amino acid sequence (SEQ ID NO:4) are aligned in B.

[19] Figure 5. Strategy for primer design for each of the Mena exons and Smart RACE.

[20] Figure 6. Effect of magnetic bead separation process in the gene expression pattern of the invasive cells.

[21] Figure 7. Effect of needle containment in the gene expression pattern of the invasive cells.

[22] Figure 8. Enhancement of tumor cell migration when Mena 3+ is expressed. Area is a measure of cell migration. wt = wild type.

[23] Figure 9. Inhibition of metastatic tumor cell migration when Mena is inhibited with mito, a molecule that redirects Mena to the wrong place in the cell.

## DETAILED DESCRIPTION OF THE INVENTION

[24] The invention provides a method for determining whether a subject has a metastatic tumor comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein overexpression of the ++ and/or +++ variant of Mena indicates the presence of a metastatic tumor.

[25] The invention also provides a method for assessing the efficacy of therapy to treat a metastatic tumor in a subject who has undergone or is undergoing treatment for a metastatic tumor, the method comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein overexpression of the ++ and/or +++ variant of Mena is indicative of a need to continue therapy to treat the tumor.

[26] The invention further provides a method for assessing the prognosis of a subject who has a metastatic tumor, comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein the subject's prognosis improves with a decrease in expression of the ++ and/or +++ variant of Mena.

[27] The tumor can be, for example, a secretory epithelial tumor. The tumor can be, for example, a breast, pancreas, prostate, colon, brain or liver tumor.

[28] As used herein, overexpression of the ++ and/or +++ variants of Mena means overexpression relative to their levels in normal tissue or relative to their levels in *in situ* (non-metastatic) carcinomas. The expression of the variant that is overexpressed can be normalized relative to the expression of protein variants that are not overexpressed in a metastatic tumor. Examples of proteins that could be used as controls include those of the Ena/VASP family that are unchanged in their expression in metastatic cells, including the 140K and 80K isoforms of Mena, Men11a and VASP. Other examples of proteins or genes that could be used as controls include those listed as relatively unchanged in expression in Condeelis et al. (2005). Such controls include N-WASP, Rac1, Pak1, and PKCalpha and beta. Preferred controls include the Mena-11a, the 80K and 140K isoforms of Mena, and VASP.

[29] The expression of the ++ and/or +++ variants of Mena may be detected *in vitro* or *in vivo*. The expression may be detected at the level of the nucleic acid variant and/or at the level of the protein isoform. Where expression is detected *in vitro*, a sample of blood, tumor, tissue or cells from the subject may be removed using standard procedures, including biopsy and aspiration. Cells which are removed from the subject may be analyzed using

immunocytofluorometry (FACS analysis). The expression of the ++ and +++ variants of Mena may be detected by detection methods readily determined from the known art, including, without limitation, immunological techniques such as Western blotting, hybridization analysis, fluorescence imaging techniques, and/or radiation detection.

[30] The blood, tissue, cell or tumor sample can be assayed using an agent that specifically binds to the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena. The agent that specifically binds to the ++ or +++ isoform of Mena can be, for example, an antibody, a peptide or an aptamer. As used herein, the term "antibody" encompasses whole antibodies and fragments of whole antibodies wherein the fragments specifically bind to the ++ or +++ variant of Mena. Antibody fragments include, but are not limited to, F(ab')<sub>2</sub> and Fab' fragments and single chain antibodies. F(ab')<sub>2</sub> is an antigen binding fragment of an antibody molecule with deleted crystallizable fragment (Fc) region and preserved binding region. Fab' is 1/2 of the F(ab')<sub>2</sub> molecule possessing only 1/2 of the binding region. The term antibody is further meant to encompass polyclonal antibodies and monoclonal antibodies. Antibodies may be produced by techniques well known to those skilled in the art. Polyclonal antibody, for example, may be produced by immunizing a mouse, rabbit, or rat with purified polypeptides encoded by the ++ and/or +++ variants of Mena. Monoclonal antibody may then be produced by removing the spleen from the immunized mouse, and fusing the spleen cells with myeloma cells to form a hybridoma which, when grown in culture, will produce a monoclonal antibody. The antibody can be, e.g., any of an IgA, IgD, IgE, IgG, or IgM antibody. The IgA antibody can be, e.g., an IgA1 or an IgA2 antibody. The IgG antibody can be, e.g., an IgG1, IgG2, IgG2a, IgG2b, IgG3 or IgG4 antibody. A combination of any of these antibodies subtypes can also be used. One consideration in selecting the type of antibody to be used is the size of the antibody. For example, the size of IgG is smaller than that of IgM allowing for greater penetration of IgG into tissues. The antibody can be a human antibody or a non-human antibody such as a goat antibody or a mouse antibody. Antibodies can be "humanized" using standard recombinant DNA techniques.

[31] Aptamers are single stranded oligonucleotides or oligonucleotide analogs that bind to a particular target molecule, such as a protein. Thus, aptamers are the oligonucleotide analogy to antibodies. However, aptamers are smaller than antibodies. Their binding is highly dependent on the secondary structure formed by the aptamer oligonucleotide. Both RNA and single stranded DNA (or analog) aptamers can be used. Aptamers that bind to

virtually any particular target can be selected using an iterative process called SELEX, which stands for Systematic Evolution of Ligands by EXponential enrichment.

[32] The agent that specifically binds to the ++ or +++ isoform of Mena may be labeled with a detectable marker. Labeling may be accomplished using one of a variety of labeling techniques, including peroxidase, chemiluminescent, and/or radioactive labels known in the art. The detectable marker may be, for example, a nonradioactive or fluorescent marker, such as biotin, fluorescein (FITC), acridine, cholesterol, or carboxy-X-rhodamine, which can be detected using fluorescence and other imaging techniques readily known in the art. Alternatively, the detectable marker may be a radioactive marker, including, for example, a radioisotope. The radioisotope may be any isotope that emits detectable radiation, such as, for example, <sup>35</sup>S, <sup>32</sup>P, or <sup>3</sup>H. Radioactivity emitted by the radioisotope can be detected by techniques well known in the art. For example, gamma emission from the radioisotope may be detected using gamma imaging techniques, particularly scintigraphic imaging.

[33] The expression of the ++ and/or +++ variants of Mena in a subject may be detected through hybridization analysis of nucleic acid extracted from a blood, tumor, tissue or cell sample from the subject using one or more nucleic acid probes which specifically hybridize to nucleic acid encoding the ++ and/or +++ isoforms of Mena. The nucleic acid encoding the ++ isoform of Mena can have the nucleotide sequence set forth in SEQ ID NO:1. The nucleic acid encoding the +++ isoform of Mena can have the nucleotide sequence set forth in SEQ ID NO:3. The nucleic acid probes may be DNA or RNA, and may vary in length from about 8 nucleotides to the entire length of the ++ or +++ nucleic acid variant of Mena. Hybridization techniques are well known in the art, see e.g. Sambrook and Russell (2001). The probes may be prepared by a variety of techniques known to those skilled in the art, including, without limitation, restriction enzyme digestion of Mena nucleic acid; and automated synthesis of oligonucleotides whose sequence corresponds to selected portions of the nucleotide sequence of the Mena nucleic acid, using commercially-available oligonucleotide synthesizers, such as the Applied Biosystems Model 392 DNA/RNA synthesizer. Combinations of two or more nucleic acid probes, corresponding to different or overlapping regions of the ++ or +++ variant of Mena, may be used to assay a diagnostic sample for expression of the ++ or +++ variant of Mena.

[34] The nucleic acid probes may be labeled with one or more detectable markers. Labeling of the nucleic acid probes may be accomplished using a number of methods known

in the art (e.g., nick translation, end labeling, fill-in end labeling, polynucleotide kinase exchange reaction, random priming, or SP6 polymerase) with a variety of labels (e.g., radioactive labels, such as <sup>35</sup>S, <sup>32</sup>P, or <sup>3</sup>H, or nonradioactive labels, such as biotin, fluorescein (FITC), acridine, cholesterol, or carboxy-X-rhodamine (ROX)).

[35] The sample can be assayed using PCR primers that specifically hybridize to nucleic acid encoding the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena. The nucleic acid encoding the ++ isoform of Mena can have the nucleotide sequence set forth in SEQ ID NO:1. The nucleic acid encoding the +++ isoform of Mena can have the nucleotide sequence set forth in SEQ ID NO:3.

[36] The sample can be assayed for the ++ variant of Mena, for the +++ variant of Mena, or for both the ++ variant and the +++ variant of Mena.

[37] In addition, or alternatively, other splice variants of Mena may be overexpressed during metastasis of some tumors.

[38] Overexpression of the ++ and/or +++ variant of Mena can occur in combination with overexpression of one or more of, for example, Arp 2/3 complex subunit p21, Arp 2/3 complex subunit p16, actinin alpha 3, capping protein alpha 1, epidermal growth factor receptor (EGFR), WAVE 3, actin gamma, LIM-kinase 1, cofilin 1, Rock 1, RhoA or protein kinase Cz. The detection of the expression of these genes has been described (e.g., Kamai et al., 2003; Otsubo et al., 2004; Wang et al., 2004).

[39] The invention still further provides methods of inhibiting metastasis of a tumor in a subject, the method comprising reducing the presence or activity of the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena in the subject. The method can involve intervention at the level of DNA, RNA, and/or protein. For example, the presence or activity of the isoform can be reduced by addition of an antisense molecule, a ribozyme, or an RNA interference (RNAi) molecule to the tumor, where the antisense molecule, ribozyme or RNAi molecule specifically inhibits expression of the isoform. The antisense molecule, ribozyme, or RNAi molecule can be comprised of nucleic acid (e.g., DNA or RNA) or nucleic acid mimetics (e.g., phosphorothionate mimetics) as are known in the art. Methods for treating tissue with these compositions are also known in the art. The antisense molecule, ribozyme or RNAi molecule can be added directly to the cancerous tissue in a pharmaceutical composition that preferably comprises an excipient that enhances penetration of the antisense molecule, ribozyme or RNAi molecule into the cells of the tissue. The antisense molecule,

ribozyme or RNAi can be expressed from a vector that is transfected into the cancerous tissue. Such vectors are known in the art.

[40] The presence or activity of the isoform can be reduced by addition of an antibody or aptamer to the tissue, wherein the antibody or aptamer specifically binds to and reduces the activity of the isoform in the tissue. The antibody or aptamer can be added directly to the tissue, preferably in a pharmaceutical composition comprising an agent that enhances penetration of the antibody or aptamer into the tissue. The antibody or aptamer can be encoded on a vector that is used to transfect the cancerous tissue.

[41] The invention also provides methods for screening for a candidate compound that inhibit metastasis of a tumor, where the method comprises contacting the compound with a cell line or tissue culture that express the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena, wherein reduction in the expression of the ++ and/or +++ isoform is indicative that the compound is a candidate compound for inhibiting metastasis of a tumor.

[42] The invention provides a purified polypeptide, where the polypeptide is overexpressed in a metastatic tumor, the polypeptide comprising the amino acid sequence of the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena. The invention also provides isolated nucleic acids encoding these polypeptides. The isolated nucleic acid can be DNA or RNA. The nucleic acid can comprise the nucleotide sequence for ++ variant (SEQ ID NO:1) and/or +++ variant (SEQ ID NO:3) of Mena.

[43] Laboratory tests of patient biopsy tissue using standard protocols for detection of the expression of nucleic acid variants or protein isoforms can be performed in conventional pathology labs. The invention provides kits for these tests. Kits of the present invention for detecting the presence or absence of a metastatic tumor can contain an antibody, a peptide or an aptamer that specifically binds to the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena. Alternatively, or in addition, the kits can contain a probe or PCR primers that specifically hybridize to nucleic acid encoding the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena. The nucleic acid encoding the ++ isoform of Mena can have the nucleotide sequence set forth in SEQ ID NO:1. The nucleic acid encoding the +++ isoform of Mena can have the nucleotide sequence set forth in SEQ ID NO:3.

[44] The present invention is illustrated in the following Experimental Details section, which is set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims that follow thereafter.

## EXPERIMENTAL DETAILS

### Materials and Methods

[45] *Isolation of Invasive Tumor Cells by in vivo Invasion Assay and Fluorescence-Activated Cell Sorting of Primary Tumor Cells.* *MTLn3*-derived mammary tumors in rats (Wang et al., 2004), the PyMT driven mouse breast cancer transgenic model, and the *in vivo* invasion assay were used as described previously (Wang et al., 2004; Wyckoff et al., 2000) to study the gene expression pattern of invasive subpopulations of carcinoma cells within live primary tumors. The *in vivo* invasion assay uses microneedles filled with Matrigel and growth factors to collect invasive tumor cells from primary tumors. Microneedles are held in a clamping device and positioned in the primary tumor with a micromanipulator. One tenth of the volume from each needle was used to determine the number of cells collected. Collected cells were a mixture of carcinoma cells (75%) and macrophages (25%). From the remaining 9/10 volume from the microneedle, macrophages were removed by magnetic separation using CD11b beads (Mitenyl Biotech, USA), and RNA was extracted from purified carcinoma cells as described before (Wang et al., 2004). To isolate the average primary tumor cells (APTCs), a small piece of tumor was separated from the whole tumor, minced, and filtered twice through a nylon filter to obtain a single cell suspension. To isolate the tumor cells from blood, right auricular puncture was performed in anesthetized animals; red blood cells were lysed using ammonium chloride lysis buffer. To purify cancer cells from the lung metastasis, a portion of the lung was minced, and filtered twice through a nylon filter to obtain a single cell suspension. Fluorescence-activated cell sorting (FACS) was performed on the resulting single cell suspensions based on their green fluorescent protein (GFP) expression in tumor cells. GFP-positive tumor cells were collected into a tube and lysed directly for RNA extraction. All of the procedures were done on ice or at 4°C.

[46] *Controls for Invasion Specific Gene Expression Pattern.* To detect microneedle-sampling effects on gene expression, cell lines used to prepare tumors and tumor cells FACS sorted from primary tumors were subjected to microneedle collection, matrigel, and epidermal growth factor (EGF). The gene patterns resulting from these stimuli were not related to the invasion signature shown previously (Wang et al., 2004) as the genes regulated by EGF and matrigel were removed from the final analysis. The effect of needle containment of the invasive cells after they enter the microneedle was analyzed and the data is presented

in Figure 6. Finally, concerning the effect of using antibody beads directed against invasive cells to separate cell types, the expression of genes related to the invasion signature of tumor cells is unaffected as shown in Figure 7 and as discussed in the results and discussion section. Thus, only the environment within the primary tumor generates the pattern of gene expression of invasive cells.

[47] *Cell lines and Cell Culture.* MTLn3 rat adenocarcinoma, and human breast cancer cell lines MDA-231 and T47D were procured from the American Type Culture Collection (ATCC), Manassas, Virginia. The culture conditions for MTLn3 were alpha MEM with 5% FBS. MDA-231 and T47D were grown in Dulbecco/Vogt Modified Eagle's Minimal Essential Medium (DMEM) with 10% fetal bovine serum (FBS), insulin and Selenium.

[48] *RT-PCR and QRT-PCR:* Real time-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (QRT-PCR) were performed using primers mentioned in Table 1. QRT-PCR was performed using SyBr Green kit, ABI 9700 sequence detector, and data analysis was performed using ABI Prism 2.0 software (Applied Biosystems Foster City, CA). A strategy for the primer sequence design is given in Figure 5.

[49] *RACE, Cloning and Sequencing:* Both 3' and 5' RACE were performed using Invitrogen RACE ready cDNA kit (sequences given in Table 1 and the RACE primer design strategy is given in Figure 5) and cloned using Invitrogen TOPO TA cloning kit, following manufacturer's protocol. Briefly PCR was performed using two internal primers for the ++ and +++ sequences (Table 1) and an oligo dT primer for the 5' and poly G primer for the 3' ends. The PCR products were eluted from the gel and cloned into pCR-TOPO vector. The ligated vector was transformed into chemically competent cells; the selected clones were sequenced using M13 primers. Sequence alignment was performed using DNASTAR software.

Table 1. Primer Sequences

## Mena primer sequences

1. AGAGGATGCCAATGTCTTCG	(SEQ ID NO:5)
2. TGTCTAGGCAATGTTGGCC	(SEQ ID NO:6)
3. GATTCAAGACCATCAGGTTGTG	(SEQ ID NO:7)
4. CAATGTTGGCCCTAAATAGAA	(SEQ ID NO:8)
d4. TTCTATTTAGGGCCAACATTG	(SEQ ID NO:9)
5. TACATCGCAAATTAGTGCTGTC	(SEQ ID NO:10)
d5. GACAGCACTAATTTGCGATGT	(SEQ ID NO:11)
6. CCAACCAGAAAACCTTGGG	(SEQ ID NO:12)
7. TGCTTCAGCCTCTCATAGTCA	(SEQ ID NO:13)
8. GAGCGAGAGAGGCAGAG	(SEQ ID NO:14)
9. GCTCGGAAGCAGAGGAGTCT	(SEQ ID NO:15)

## Pan Mena Primer

Forward: CGGCAGTAAGTCACCTGTCA (SEQ ID NO:16)

Reverse: CTCAGCTTTGCCAGCTCTT (SEQ ID NO:17)

## Smart Primers

## SMART II™ A Oligonucleotide

AAGCAGTGGTATCAACGCAGAGTACGCGGG (SEQ ID NO:18)

## 3'-RACE CDS Primer A

AAGCAGTGGTATCAACGCAGAGTAC (T) 30V N (SEQ ID NO:19)

(N = A, C, G, or T; V = A, G, or C)

5'-RACE CDS Primer A (T) 25V N (SEQ ID NO:20)

(N = A, C, G, or T; V = A, G, or C)

## Long:

CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT  
(SEQ ID NO:21)

## Short:

CTAATACGACTCACTATAGGGC (SEQ ID NO:22)

## Results and Discussion

**[50]** Specific isoforms of Mena that are upregulated in invasive breast cancer cells have been identified in this study. Three models were utilized in this report, i.e., the MTLn3 rat adenocarcinoma allograft model, the PyMT mouse transgenic breast cancer model and a number of human breast cancer cell lines. Mena expression is upregulated 3-4 fold in invasive primary breast cancer cells (Wang et al., 2004). Controls done to determine the effects of manipulations used to collect invasive tumor cells from the primary mammary tumor demonstrate that the expression of the invasion isoform of Mena is not induced by cell collection. Only the tumour microenvironment induces the expression of these isoforms. In this study the stability of this overexpression was determined, i.e. the invasive cells collected

by *in vivo* invasion assay showed a 3-4 fold upregulation in Mena expression when compared to APTCs. Therefore, the cells were followed and separated from the blood and from the lung met. Using the MTLn3 and mouse PyMT transgenic models, the invasive cells were collected and separated by *in vivo* invasion assay, and the APTCs by FACS sorting. RT-PCR analysis of the invasive cells showed that amplicons specific for the ++ and +++ exons were upregulated in both models. An amplicon specific for + exon was also detected. However, it showed no change between the invasive cells and the APTCs. QRT-PCR studies confirmed the RT-PCR finding and showed an up-regulation of both ++ and +++ exons in the invasive cells for MTLn3 (Figure 1A) and for mouse PyMT model (Figure 1B). Figure 2 shows that the ++ and +++ splice variant of Mena message remains up-regulated in the cells that have intravasated into blood and the cells that have formed successful mets in the lung. This indicates that the change in expression level is due to a stable genetic change in the metastatic cells.

[51] The results of the RT-PCR and QRT-PCR showed that both the ++ and +++ exons are up-regulated in the invasive cells. However, it was unclear if the exons are present in a single transcript or on separate transcripts. To address this question, ++ and +++ bearing transcripts from the invasive PyMT mouse transgenic tumors were cloned and sequenced. RACE analysis was selected in order to identify transcripts that contained the ++ and +++ exons. The results provide a consensus sequence from at least 10 clones for each transcript. The results show a 100% match with the published mouse sequences and demonstrated that the ++ and +++ exons are in separate transcripts. The alignments for the ++ and +++ sequences are shown in Figure 4.

[52] Figures 6 and 7 show that both the 2+ and 3+ variants remain elevated at the mRNA level in tumor cells circulating in the blood, thus making possible a blood assay for these variants. PCR, nucleic acid probes and /or antibody staining can thus be used to diagnose metastatic disease using a blood sample.

[53] Based on the above sequence alignment data, a molecular probe, either nucleic acid or antibody or both, against either the ++ or +++ variant would provide an important diagnostic/prognostic tool. Since the up-regulation of expression of the ++ and +++ exons observed here is a stable change in invasive and metastatic mammary tumor cells, probes specifically directed at these exons would be powerful diagnostic markers for the presence of metastatic cells and therefore the potential of metastatic disease.

[54] Figure 8 shows enhancement of tumor cell migration when Mena 3+ is expressed. Importantly, as shown in Figure 9, inhibition of metastatic tumor cell migration occurs when Mena is inhibited, in this case using mito, a molecule that redirects Mena to the wrong place in the cell. This result establishes that inhibition of Mena function inhibits migration of metastatic cells and therefore is a good strategy for inhibiting metastasis.

[55] All human adenocarcinomas are derived from epithelial organs that may share a common morphogenetic strategy at the molecular level (Condeelis and Pollard, 2006; Wang et al., 2004, 2005). The invasion signature, of which Mena 2+ and 3+ are invasion isoforms, predicts that the same morphogenetic strategy is used for normal organ morphogenesis and tumor metastasis. This suggests that Mena 2+ and 3+ will be useful targets for the diagnosis and therapy of all common adenocarcinomas in adult humans. In particular, the invention described herein will be applicable to tumors such as breast, prostate, pancreas, colon, brain and liver tumors.

#### REFERENCES

Barzik M, Kotova TI, Higgs HN et al. Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins. *J Biol Chem.* 2005 Aug 5; 280(31):28653-62.

Brinkman BM. Splice variants as cancer biomarkers. *Clin Biochem.* 2004 Jul; 37(7):584-94.

Condeelis and Pollard (2006) Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124: 263-266.

Condeelis J, Singer RH, Segall JE. The great escape: when cancer cells hijack the genes for chemotaxis and motility. *Annu Rev Cell Dev Biol.* 2005;21: 695-718.

Di Modugno F, Bronzi G, Scanlan MJ et.al. Human Mena protein, a serex-defined antigen overexpressed in breast cancer eliciting both humoral and CD8+ T-cell immune response. *Int J Cancer.* 2004 May 10;109(6):909-18.

Gertler FB, Niebuhr K, Reinhard M et. al. Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics. *Cell.* 1996 Oct 18;87(2):227-39.

Kamai T, Tsujii T, Arai K, Takagi K, Asami H, Ito Y, Oshima H. Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. *Clin Cancer Res*. 2003 Jul;9(7):2632-41.

Kuhnel K, Jarchau T, Wolf E et. al. The VASP tetramerization domain is a right-handed coiled coil based on a 15-residue repeat. *Proc Natl Acad Sci U S A*. 2004 Dec 7; 101(49):17027-32.

Loureiro JJ, Rubinson DA, Bear JE et. al. Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration. *Mol Biol Cell*. 2002 Jul;13 (7):2533-46.

Otsubo T, Iwaya K, Mukai Y, Mizokami Y, Serizawa H, Matsuoka T, Mukai K. Involvement of Arp2/3 complex in the process of colorectal carcinogenesis. *Mod Pathol*. 2004 Apr;17(4):461-7.

Prehoda KE, Lee DJ, Lim WA. Structure of the enabled/VASP homology 1 domain-peptide complex: a key component in the spatial control of actin assembly. *Cell*. 1999 May 14;97(4):471-80.

Sambrook J and Russell DW. *Molecular Cloning. A Laboratory Manual*, third edition, Cold Spring Harbor Laboratory Press, 2001.

Scott JA, Shewan AM, den Elzen NR et. al. Ena/VASP proteins can regulate distinct modes of actin organization at cadherin-adhesive contacts. *Mol Biol Cell*. 2006 Mar; 17(3):1085-95.

Urbanelli L, Massini C, Emiliani C et.al. Characterization of human Enah gene. *Biochim Biophys Acta*. 2006 Jan-Feb; 1759(1-2):99-107.

Venables JP. Unbalanced alternative splicing and its significance in cancer. *Bioessays*. 2006 Apr; 28(4):378-86.

Wang W, Goswami S, Lapidus K, et. al. Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. *Cancer Res.* 2004 Dec 1; 64(23):8585-94.

Wang, Goswami, Sahai, Wyckoff, Segall, Condeelis (2005) Tumor Cells caught in the act of invading: their strategy for enhanced cell motility. *Trends Cell Biol.* 15:138-145.

Wang W, Mouneimne G, Sidani M et.al The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. *J Cell Biol.* 2006 May 8;173(3):395-404.

Wyckoff JB, Segall JE, Condeelis JS. The collection of the motile population of cells from a living tumor. *Cancer Res.* 2000 Oct 1; 60(19):5401-4.

What is claimed is:

1. A method for determining whether a subject has a metastatic tumor comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein overexpression of the ++ and/or +++ variant of Mena indicates the presence of a metastatic tumor.
2. A method for assessing the efficacy of therapy to treat a metastatic tumor in a subject who has undergone or is undergoing treatment for a metastatic tumor, the method comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein overexpression of the ++ and/or +++ variant of Mena is indicative of a need to continue therapy to treat the tumor.
3. A method for assessing the prognosis of a subject who has a metastatic tumor, comprising assaying a blood, tissue and/or tumor sample of the subject for expression of the ++ and/or +++ variant of Mena, wherein the subject's prognosis improves with a decrease in expression of the ++ and/or +++ variant of Mena.
4. The method of Claim 1, 2, or 3, wherein the tumor is a secretory epithelial tumor.
5. The method of Claim 1, 2, or 3, wherein the tumor is a breast, pancreas, prostate, colon, brain or liver tumor.
6. The method of Claim 1, 2, or 3, wherein the tumor is a breast tumor.
7. The method of Claim 1, 2 or 3, wherein the sample is assayed using an agent that specifically binds to the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena.
8. The method of Claim 7, wherein the agent is an antibody, a peptide or an aptamer.
9. The method of Claim 7, wherein the agent is labeled with a detectable marker.

10. The method of Claim 1, 2, or 3, wherein the sample is assayed using at least one nucleic acid probe that specifically hybridizes to nucleic acid encoding the ++ isoform of Mena or the +++ isoform of Mena, wherein the ++ isoform of Mena has the amino acid sequence set forth in SEQ ID NO:2 and the +++ isoform of Mena has the amino acid sequence set forth in SEQ ID NO:4.
11. The method of Claim 10, wherein the nucleic acid probe is DNA or RNA.
12. The method of Claim 10, wherein the nucleic acid probe is labeled with a detectable marker.
13. The method of Claim 1, 2, or 3, wherein the sample is assayed using PCR primers that specifically hybridize to nucleic acid encoding the ++ isoform of Mena or the +++ isoform of Mena, wherein the ++ isoform of Mena has the amino acid sequence set forth in SEQ ID NO:2 and the +++ isoform of Mena has the amino acid sequence set forth in SEQ ID NO:4.
14. The method of Claim 10 or 13, wherein the nucleic acid encoding the ++ isoform of Mena has the nucleotide sequence set forth in SEQ ID NO:1.
15. The method of Claim 10 or 13, wherein the nucleic acid encoding the +++ isoform of Mena has the nucleotide sequence set forth in SEQ ID NO:3.
16. The method of Claim 1, 2, or 3, wherein the sample is assayed for the ++ variant of Mena.
17. The method of Claim 1, 2, or 3, wherein the sample is assayed for the +++ variant of Mena.
18. The method of Claim 1, 2, or 3, wherein the sample is assayed for both the ++ variant and the +++ variant of Mena.

19. The method of Claim 1, 2, or 3, wherein overexpression of the ++ and/or +++ variant of Mena occurs in combination with overexpression of one or more of Arp 2/3 complex subunit p21, Arp 2/3 complex subunit p16, actinin alpha 3, capping protein alpha 1, epidermal growth factor receptor (EGFR), WAVE 3, actin gamma, LIM-kinase 1, cofilin 1, Rock 1, RhoA or protein kinase Cz.
20. A method of inhibiting metastasis of a tumor in a subject, the method comprising reducing the presence or activity of the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena in the subject.
21. The method of claim 20, wherein the presence or activity of the isoform is reduced by addition of an antisense molecule, a ribozyme, or an RNAi molecule to the tumor, where the antisense molecule, ribozyme or RNAi molecule specifically inhibits expression of the isoform.
22. The method of claim 20, wherein the presence or activity of the isoform is reduced by addition of an antibody or aptamer to the tumor, wherein the antibody or aptamer specifically binds to and reduces the activity of the isoform.
23. The method of Claim 20, wherein the tumor is a secretory epithelial tumor.
24. The method of Claim 20, wherein the tumor is a breast, pancreas, prostate, colon, brain or liver tumor.
25. The method of Claim 20, wherein the tumor is a breast tumor.
26. A method for screening for a candidate compound that inhibit metastasis of a tumor, the method comprising contacting the compound with a cell line or tissue culture that express the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena, wherein reduction in the expression of the ++ and/or +++ isoform of Mena is indicative that the compound is a candidate compound for inhibiting metastasis of a

tumor.

27. The method of Claim 26, wherein the tumor is a secretory epithelial tumor.
28. The method of Claim 26, wherein the tumor is a breast, pancreas, prostate, colon, brain or liver tumor.
29. The method of Claim 26, wherein the tumor is a breast tumor.
30. A purified polypeptide, where the polypeptide is overexpressed in a metastatic tumor, the purified polypeptide comprising the amino acid sequence of the ++ isoform (SEQ ID NO:2) and/or +++ isoform (SEQ ID NO:4) of Mena.
31. An isolated nucleic acid encoding the polypeptide of Claim 30.
32. The isolated nucleic acid of Claim 31, wherein the nucleic acid is DNA or RNA.
33. The isolated nucleic acid of Claim 31, wherein the nucleic acid comprises the nucleotide sequence for ++ variant (SEQ ID NO:1) and/or +++ variant (SEQ ID NO:3) of Mena.
34. A kit for detecting the presence or absence of a metastatic tumor, the kit comprising an antibody, a peptide or an aptamer that specifically binds to the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena.
35. A kit for detecting the presence or absence of a metastatic tumor, the kit comprising a probe or PCR primers that specifically hybridize to nucleic acid encoding the ++ isoform (SEQ ID NO:2) or +++ isoform (SEQ ID NO:4) of Mena.
36. The kit of Claim 35, wherein the nucleic acid encoding the ++ isoform of Mena has the nucleotide sequence set forth in SEQ ID NO:1.

-21-

37. The kit of Claim 35, wherein the nucleic acid encoding the +++ isoform of Mena has the nucleotide sequence set forth in SEQ ID NO:3.
  
38. The kit of Claim 35, wherein the nucleic acid probe is labeled with a detectable marker.

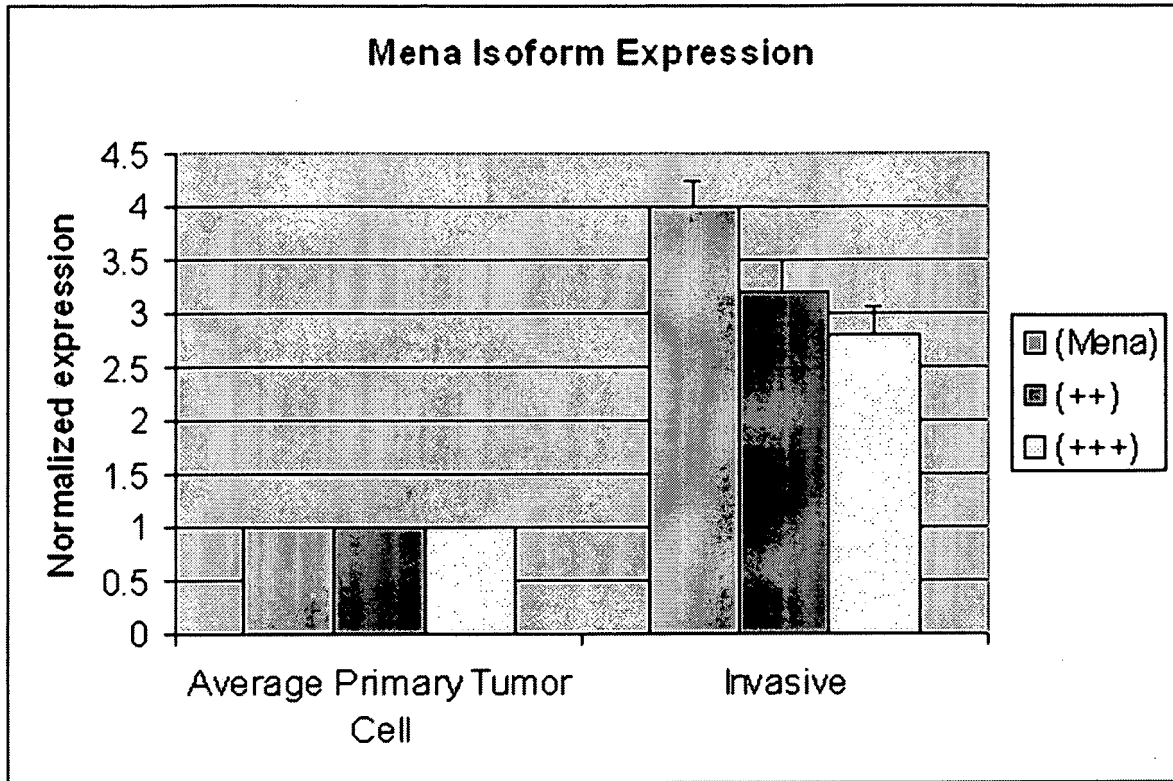


FIGURE 1A

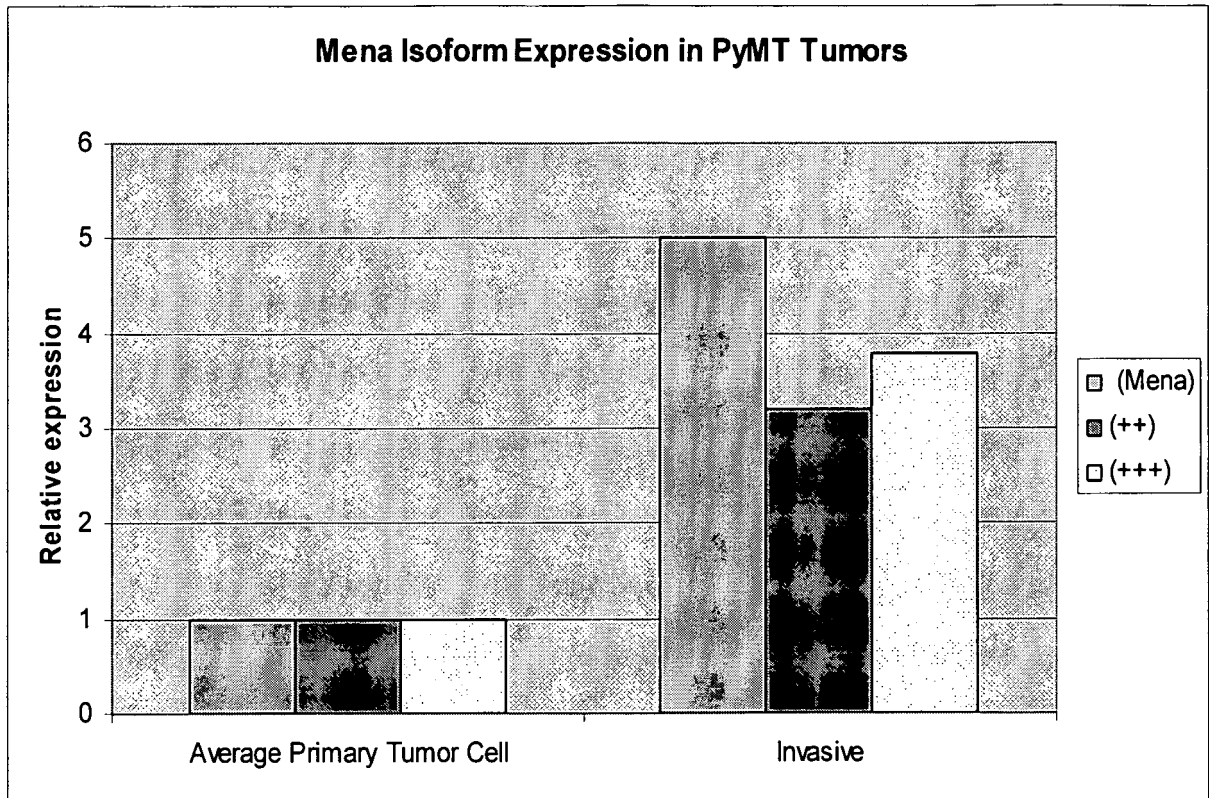


FIGURE 1B

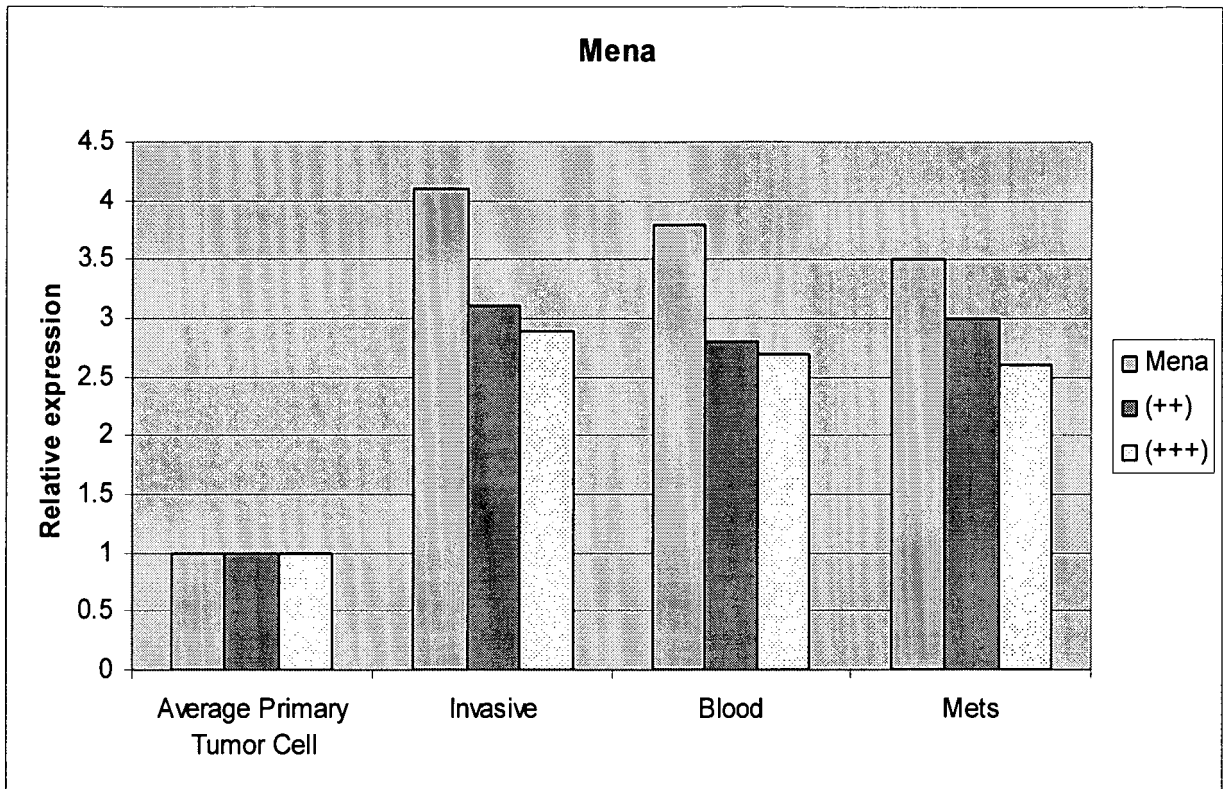
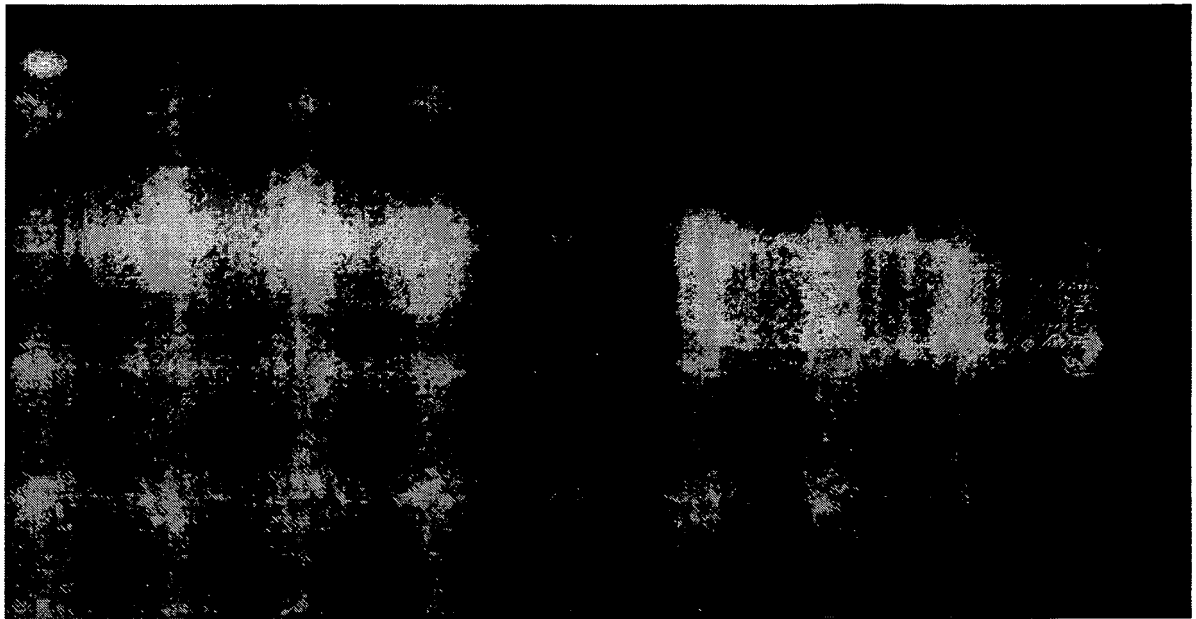


FIGURE 2



MDA 231

T47D

FIGURE 3

**A ++ nucleotide sequence**

Human: TTCTATTTAGGG  
Mouse: TTCTATTTAGGG  
Invasive: TTCTATTTAGGG  
SEQ ID NO:1

**++ inferred amino acid sequence**

Human: FYLG  
Mouse: FYLG  
Invasive: FYLG  
SEQ ID NO:2

**B +++ nucleotide sequence**

Human: GCCCAGAGCAAGGTTACTGCTACCCAGGACAGCACTAATTTGCGATGTATTTTCTGT  
Mouse: GCCCAGAGCAAGGTTACTGCTACGCAGGACAGCACTAATTTGCGATGTATTTTCTGT  
Invasive: GCCCAGAGCAAGGTTACTGCTACGCAGGACAGCACTAATTTGCGATGTATTTTCTGT  
SEQ ID NO:3

**+++ inferred amino acid sequence**

Human: AQS KVTATQDSTNLR CIFC  
Mouse: AQS KVTATQDSTNLR CIFC  
Invasive: AQS KVTATQDSTNLR CIFC  
SEQ ID NO:4

**FIGURE 4A-4B**

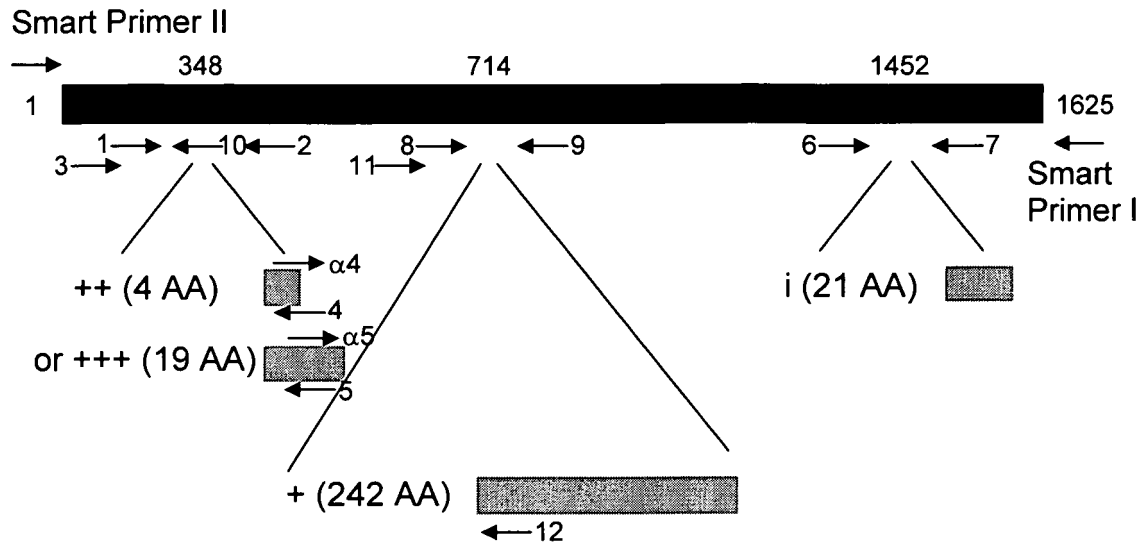


FIGURE 5

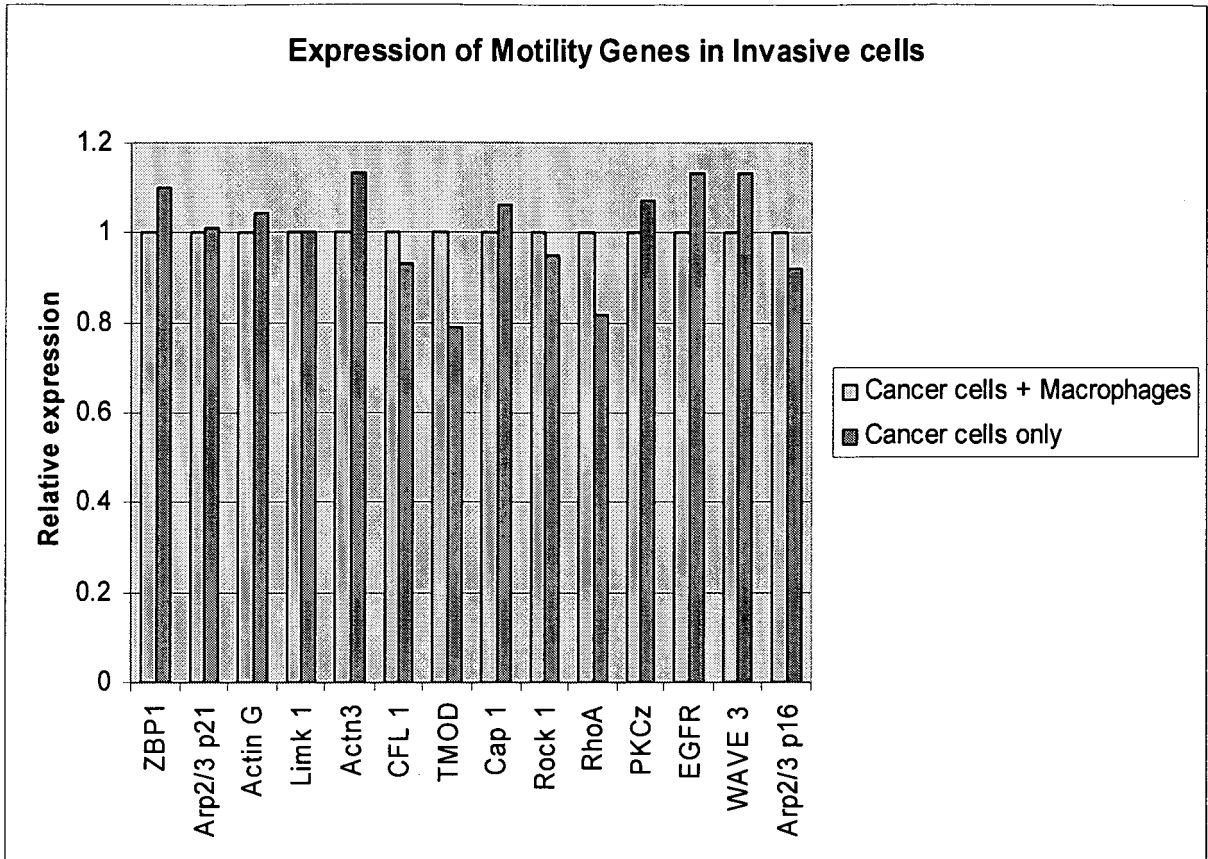


FIGURE 6

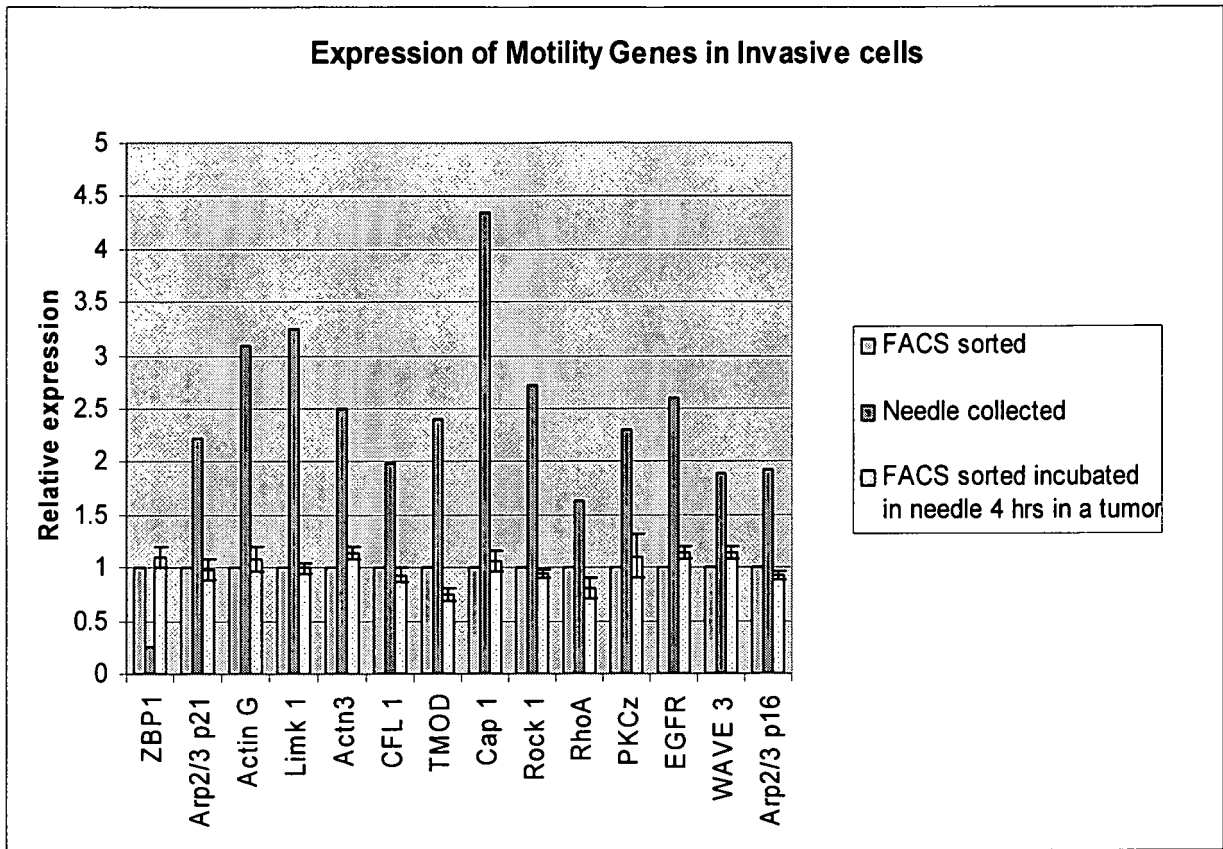


FIGURE 7

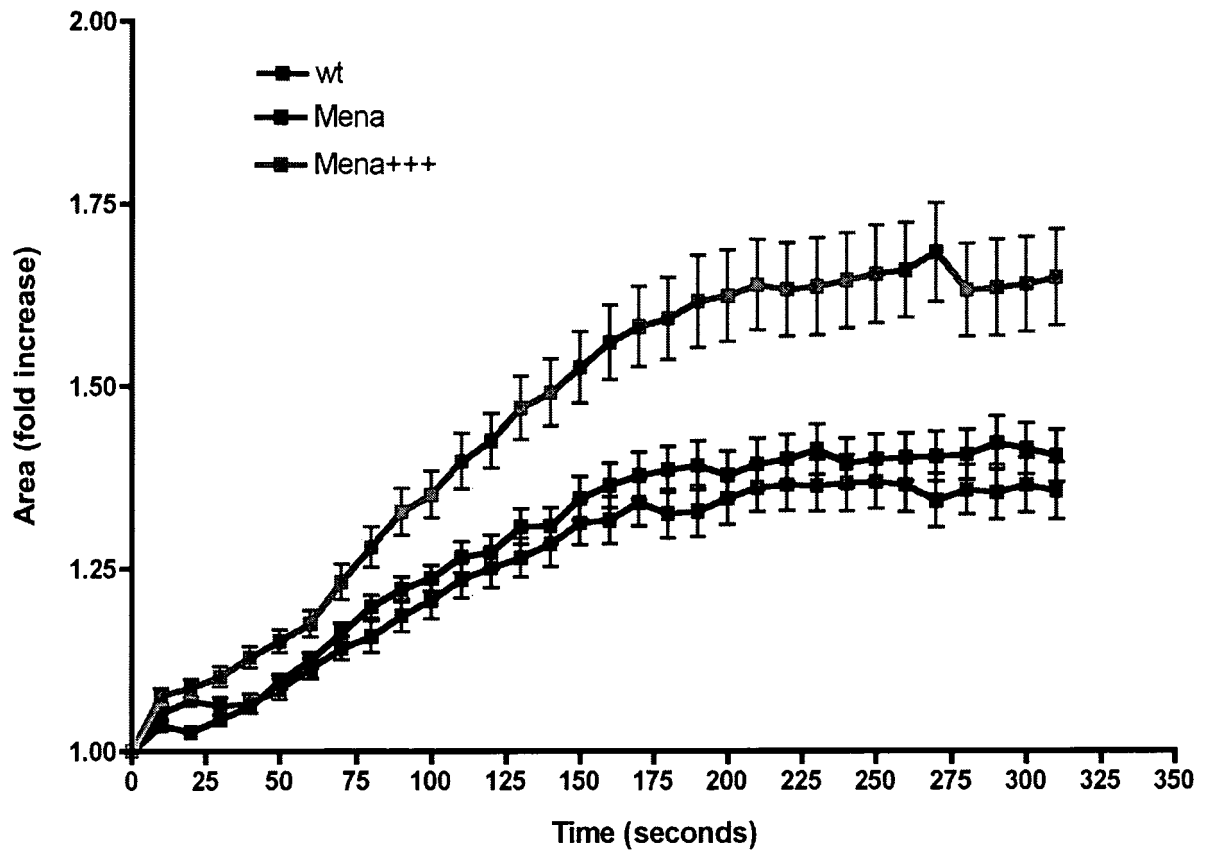


FIGURE 8

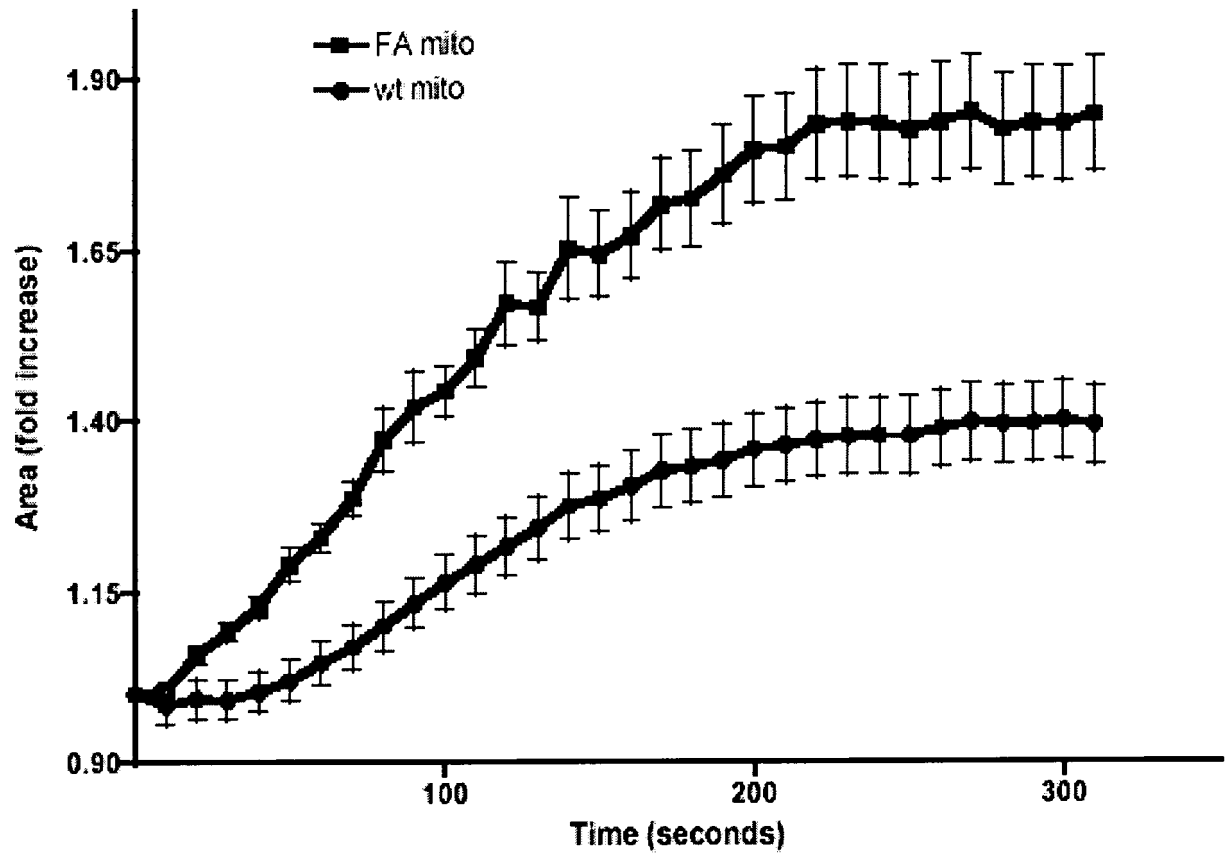


FIGURE 9

专利名称(译)	MENA的转移特异性剪接变体及其在肿瘤的诊断，预后和治疗中的用途		
公开(公告)号	<a href="#">EP2126566A2</a>	公开(公告)日	2009-12-02
申请号	EP2008713370	申请日	2008-01-31
[标]申请(专利权)人(译)	阿尔伯爱因斯坦医科叶希瓦大学 麻省理工学院 IFO REGINA ELENA癌症INST		
申请(专利权)人(译)	叶史瓦大学医学院爱因斯坦学校 麻省理工学院 IFO-REGINA ELENA癌症研究所		
当前申请(专利权)人(译)	麻省理工学院 阿尔伯特·爱因斯坦医学院的，Inc.的. IFO-REGINA ELENA癌症研究所		
[标]发明人	CONDEELIS JOHN S GOSWAMI SUMANTA GERTLER FRANK NISTICO PAOLA		
发明人	CONDEELIS, JOHN, S. GOSWAMI, SUMANTA GERTLER, FRANK NISTICO, PAOLA		
IPC分类号	G01N33/53 G01N33/567 G01N33/574 A61K31/713 C12Q1/68 G01N33/50		
CPC分类号	C12Q1/6886 A61K31/713 C12Q2600/106 C12Q2600/112 C12Q2600/136 G01N33/5011 G01N33/57415 G01N33/57419 G01N33/57434 G01N33/57438 G01N33/57496		
优先权	60/899303 2007-02-02 US		
其他公开文献	EP2126566B1 EP2126566A4		
外部链接	<a href="#">Espacenet</a>		

#### 摘要(译)

提供了用于转移性肿瘤的诊断，预后和治疗的方法和试剂盒，其中转移性肿瘤的特征在于Mena的剪接变体的过表达。