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(54) Title: TREATMENT OF ANGIOGENESIS DISORDERS

(57) Abstract: This invention concerns pathological angiogenesis and cancer, related treatment methods, and related compositions. Also disclosed are related diagnosis kits and methods.

TREATMENT OF ANGIOGENESIS DISORDERS

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority of U.S. Provisional Application No. 61/441,738, filed on February 11, 2011. The content of the application is incorporated herein by reference in its entirety.

FIELD OF INVENTION

This invention relates to treatments for angiogenesis disorders.

BACKGROUND OF INVENTION

Angiogenesis is a process of growth of new blood vessels and remodeling of preexisting blood vessels. It is vital for normal growth and development, as well as other physiological processes, such as wound healing. On the other hand, angiogenesis is also important in various pathological processes. For example, pathological angiogenesis is a fundamental step in the transition of tumors from a dormant state to a malignant one, characterized by the properties of anaplasia, invasiveness, and metastasis.

Metastatic progression of cancer is a daunting clinical challenge. Technological advances have allowed for the detection and treatment of some early stage neoplasm, however, total death rates from epithelial malignancies have remained essentially unchanged over the last forty years (seer.cancer.gov/csr/1975_2007/, National Institute of Health, 2007). It generally is believed that this is due to several factors, including molecular heterogeneity within cancer types, chemotherapeutic regimens of modest efficacy that were historically empirically derived, and a long-standing focus on the molecular drivers of primary tumor growth rather than metastatic progression.

Effective prevention or treatment of metastasis calls for understanding of molecular and cellular events, including angiogenesis, underlying this complex process (Talmadge, J. E. *et al., Cancer Res* 70 (14), 5649 (2010); Sleeman, J. *et al., Eur J Cancer* 46 (7), 1177 (2010); and Hurst, D. R., *et al., Cancer Res* 69 (19), 7495 (2009)). VEGF has been discovered as a promoter of tumorigenesis in primary tumors (Kim, K. J. *et al.*, Nature 362 (6423), 841 (1993)). Clinical trials have shown that VEGF inhibition can, in combination with chemotherapy, lengthen survival by 2-3 months in patients with stage IV colorectal or lung cancer (Hurwitz, H. *et al.,* N Engl J Med 350 (23), 2335 (2004);

Giantonio, B. J. *et al.,* J Clin Oncol 25 (12), 1539 (2007); and Sandler, A. *et al.,* N Engl J Med 355 (24), 2542 (2006)). However, VEGF inhibition has not proven beneficial for metastasis prevention in the adjuvant setting (Barugel, M. E., *et al.* Expert Rev Anticancer Ther 9 (12), 1829 (2009) and in recent pre-clinical metastasis models (Paez-Ribes, M. *et al.*, Cancer Cell 15 (3), 220 (2009) and Ebos, J. M. *et al.,* Cancer Cell 15 (3), 232 (2009)). While compensation by other unknown factors that promote metastatic angiogenesis has been proposed to underlie these outcomes, a number of investigators have sought to address metastasis via pathways other than angiogenesis. For example, WO 2009082744 described genes over-expressed in bone and lung metastases of breast cancer, where the genes were not related to angiogenesis. Others endeavored to identify factors that mediate metastatic angiogenesis. Yet, the success has been limited.

Thus, there is a need for agents and methods for regulating angiogenesis and for treating disorders characterized by pathological angiogenesis, including cancer.

SUMMARY OF INVENTION

This invention is based, at least in part, on an unexpected discovery of a new pathway that regulates endothelial recruitment and, in turn angiogenesis.

Accordingly, one aspect of this invention features a method for inhibiting endothelial recruitment, as well as angiogenesis, in a subject in need thereof. The method includes a step of administering to the subject a first agent that inhibits expression or activity of a first protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1. In one embodiment, the subject has an angiogenesis disorder, i.e., a disorder characterized by pathological angiogenesis, such as cancer, an eye disorder, or an inflammatory disorder. Examples of the cancer include metastatic cancer. The above-mentioned method can further include a step of administering to the subject a second agent that inhibits expression or activity of a second protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR. The aforementioned first agent or second agent can be an antibody (or an antigen-binding portion thereof.), a nucleic acid, a polypeptide, or a small molecule compound. In one example, the above antibody is an monoclonal that contains a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10 shown below.

In a second aspect, this invention features a method for treating metastatic cancer in a subject in need thereof. The method includes a step of administering to the subject a first agent that inhibits expression or activity of a first protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1, where the first agent inhibits angiogenesis. Examples of the cancer include breast cancer. The method can further include a step of administering to the subject a second agent that inhibits expression or activity of a second protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR. The first agent or second agent can be an antibody (or an antigen-binding portion thereof.), a nucleic acid, a polypeptide, or a small molecule compound. In one example, the above antibody is an monoclonal that contains a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10 shown below.

In a third aspect, this invention features an isolated nucleic acid having a sequence encoding an RNAi agent capable of inhibiting expression of a protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1. In one embodiment, the RNAi agent has a double-stranded structure having a first strand and a second strand; each of the first and second strands is between 19 and 30 nucleotides long; and the first strand is encoded by any one of SEQ ID NOs: 1-6 as listed in Table 5 below.

In a fourth aspect, this invention provides a composition having an agent that inhibits expression or activity of a protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1, where the agent can be an antibody (or an antigen-binding portion thereof.), a nucleic acid, a polypeptide, or a small molecule compound. In one example, the agent is the above-mentioned isolated nucleic acid. In another, the agent is an antibody or an antigen-binding portion thereof.

In a fifth aspect, this invention features a method for diagnosing a metastatic potential of cancer in a subject. The method includes steps of obtaining a first expression level for a first gene of the subject selected from the group consisting of IGFBP2, MERTK, and PITPNC1, and comparing the first expression level with a first predetermined level for the selected first gene. The subject is determined to have or be prone to develop metastatic cancer if the first expression level is greater than the first predetermined level. The first predetermined level can be obtained from a control subject

that is free of cancer. In one example, the method further includes steps of obtaining a second expression level for a second gene of the subject selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR; and comparing the second expression level with a second predetermined level for the selected second gene. The subject is determined to have or be prone to develop metastatic cancer if both the first expression level and the second expression level are greater than the first predetermined level and the second predetermined level, respectively. The second predetermined level can also be obtained from a control subject that is free of cancer.

The invention also features a method for inhibiting endothelial recruitment in a subject in need thereof. The method includes a step of administering to the subject a first agent that increases expression or activity of GAS6 (i.e. an activating agent of GAS6). The invention further features a composition having an agent that increases expression or activity of GAS6. In one example, the aforementioned agent has GAS6 activity. In another, the agent is an antibody (or an antigen-binding portion thereof), a nucleic acid, a polypeptide, or a small molecule compound. In one embodiment, the agent is a polypeptide having the sequence of GAS6.

In a yet another aspect, the invention features a kit for diagnosing a metastatic potential of cancer in a subject. The kit includes a first reagent that specifically binds to a first expression product (e.g., polypeptide or mRNA) of a first gene selected from the group consisting of IGFBP2, MERTK, and PITPNC1. The kit can further include a second reagent that specifically binds to a second expression product of a second gene selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

In a further aspect, the invention features a method for identifying genes and non-coding RNAs that regulate metastatic cancer colonization of any body tissue. The method includes a first step of generating a population of mammalian cancer cells with increased metastatic tissue colonization potential by performing serial rounds of a) transplantation of a population of labeled or unlabeled cancer cells into any living tissue of the body and then b) performing isolation of said labeled cancer cells from the tissue after metastatic colonization has occurred and then c) performing repeat transplantation of isolated labeled cancer cells into living tissue of the body. By performing serial rounds of transplantation,

isolation, and repeat transplantation of labeled cancer cells as described above, a population of labeled or unlabeled cancer cells with high metastatic tissue colonization potential is generated. The second step of the method includes transducing, transfecting, or otherwise introducing a population of one or more shRNA molecules into the population of cancer cells with high metastatic tissue colonization potential to generate a population of engineered cancer cells with high metastatic potential that express one or more shRNA molecules that reduce expression of one or more genes or non-coding RNAs. This the population of engineered cancer cells with high metastatic potential that express one or more shRNA molecules is then a) transplanted into any living tissue and then b) isolated from the living tissue after metastatic colonization has occurred. The presence, absence, or abundance of one or more of the transfected, transduced, or otherwise introduced shRNAs in the population of isolated post-transplant engineered cancer cells is then assessed by either microarray analysis, DNA sequencing technology, deep sequencing technology, or cloning. The reduction in levels of any single shRNA in the population of isolated cells relative to its representation prior to injection indicates that the shRNA's target gene is required for metastatic colonization of the tissue. The increase in levels of any single shRNA in the population of isolated cells relative to its representation prior to injection indicates that the shRNA's target antagonizes metastatic colonization of the tissue. The second step of this method could also include transducing, transfecting, or otherwise introducing a population of one or more RNAi molecules, microRNAs, or non-coding RNAs. Additionally, the second step could also include transducing, transfecting, or otherwise introducing a population of one or more sequences encoding protein coding genes. The population of engineered cancer cells with high metastatic potential that express one or more protein coding genes is then a) transplanted into any living tissue and then b) isolated from the living tissue after metastatic colonization has occurred. The presence, absence, or abundance of one or more of the transfected, transduced, or otherwise introduced coding genes in the population of isolated post-transplant engineered cancer cells is then assessed by either microarray analysis, DNA sequencing technology, deep sequencing technology, or cloning. The increase in the levels of any single gene in the population of isolated cells relative to its representation prior to injection indicates that the gene represents a target gene required for metastatic colonization of the tissue. The decrease in the levels of any single gene in the population of isolated cells relative to its

representation prior to injection indicates that the gene represents a target gene that antagonizes metastatic colonization of the tissue.

In a further aspect, the invention features a monoclonal antibody (e.g., a humanized or human monoclonal antibody) or an antigen-binding portion thereof that neutralizes IGFBP2 function by inhibiting IGFBP2 binding to IGF1. This antibody is capable of inhibiting endothelial recruitment by cancer cells, such as metastatic breast cancer cells or inhibiting pathological angiogenesis. This antibody is also capable of inhibiting tumor progression or tumor metastasis of cancer cells, such as human breast cancer, *in vivo*. In one example, the monoclonal antibody contains a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10 shown below.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

DESCRIPTION OF DRAWINGS

FIGs. 1a-f are diagrams and photographs showing that endogenous miR-126 suppresses metastatic colonization. **a,** Bioluminescence imaging of lung metastasis by poorly metastatic breast cancer cells upon miR-126 inhibition. 4 X 10^4 MDA-MB-231 cells expressing a short hairpin (miR-Zip) targeting miR-126 or the control hairpin were injected intravenously into immunodeficient NOD-SCID mice. Representative mice shown correspond to the MDA-MB-231/miR-126KD set (top) and MDA-MB-231/scrambled set (bottom) at day 49. Lung colonization was quantified through bioluminescence imaging. n=5; error bars represent s.e.m.; p-value based on a one-sided student's t-test at day 49. Lungs were extracted at day 49 and immuno-histochemically stained for human vimentin (right). **b**, Bioluminescence imaging of systemic metastasis by poorly metastatic breast cancer cells with inhibited miR-126 expression. 4 X 10^4 MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control hairpin were injected via intracardiac route into athymic nude mice. Representative mice shown correspond to the MDA-MB-231/miR-126KD set (top) and MDA-MB-231/scrambled set (bottom) at day 34. Whole body colonization was measured by bioluminescence and quantified. n=4; error bars represent s.e.m.; p-value based on a one-sided student's t-test at

day 34. **c**, The total number of metastatic foci were counted in mice injected intracardiac with MDA-MB-231/miR-126KD and MDA-MB-231/scrambled cells (top). Representative images of bone and brain metastatic nodules are shown (bottom). **d,** 5 X 10^5 MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control hairpin were injected into the mammary fat pads of immunodeficient mice. Tumor volumes were measured over time. n=15; error bars indicate s.e.m.; p-values based on a one-sided student's t-test at day 35. **e,** Extracted lungs from (a) were stained for human-vimentin and the size of each metastatic nodule was measured through image analysis using ImageJ. **f,** 4 X 10^4 Lm2 cells expressing a doxycycline inducible pre-miR-126 cassette were injected via the tail vein into NOD-SCID mice at day 0. At day 3, doxycycline (2 mg/ml) and sucrose (5%) were added to the drinking water in one group of mice and only 5% sucrose in the other. At day 48, the lungs were removed and immunohistochemically stained for human vimentin (right). Total number of nodules in each lung is shown to the left.

FIGs. 2a-2J are diagrams and photographs showing that endogenous miR-126 non-cell autonomously suppressed metastatic angiogenesis by metastatic breast cancer cells. a, Lung sections from FIG. 1a were histologically double-stained for human vimentin and MECA-32 or **b,** for vimentin and intravenously injected lectin. The border of each nodule was demarcated based on vimentin staining and the lectin/MECA-32 staining inside the metastatic nodule highlighted in black (lower panels). The area positive for lectin/MECA-32 staining within each nodule was then determined by using ImageJ software and presented as the area covered by lectin/MECA-32 stain per area of the given nodule (% vessel density). The distribution of % vessel density between the injected MDA-MB-231 control and miR-126 KD cells are shown in a cumulative fraction plot. n=8/group (resulting in a total 18 metastatic nodules in the control, and 68 in the miR-126 KD cells), p-value based on the Kolmogorov-Smirnov test. **c,** 5 X 10^4 LM2 cells expressing miR-126 or a control hairpin were seeded onto a HUVEC monolayer and adhesion was quantified. Images of cells that had adhered to the HUVEC monolayer were obtained and analyzed using ImageJ software. n=4; error bars represent s.e.m. **d,** Conditioned media from 5 X 10^5 LM2 cells expressing miR-126 or a control hairpin was obtained by incubating the cells with EGM-2 media for 24h. 2.5 X 10^4 HUVEC cells were seeded in triplicate, grown in the conditioned media and viable cells were counted at 5 days post seeding. n=3; error

bars represent s.e.m. **e,** 2 X 10^4 HUVEC cells were mixed with 1 X 10^4 LM2 cells that were transduced with miR-126 or a control hairpin, and tube formation by the HUVEC cells was assayed. Images of each well were obtained and the number of branch points in each image was quantified using MetaMorph software. n=3; error bars represent s.e.m. Scale bar represents 250μm. **f,** 2.5 X 10^4 MDA-MB-231 cells and LM2 cells were seeded in quadruplicate. Trans-well migration of 5 X 10^4 HUVEC cells towards the cancer cells was then assessed by counting the number of cells that had migrated to the basal side of the trans-well inserts in images obtained using ImageJ. n=4; error bars represent s.e.m., p-values were obtained using student's t-test. **g,** LM2 cells expressing miR-126 or the control hairpin, as well as MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control hairpin were subjected to the HUVEC recruitment assay. Images of the basal side of the inserts were obtained and cells counted using ImageJ software. n=4; error bars represent s.e.m., p-values were obtained using student's t-test. Representative images shown correspond to the LM2/miR-126OE or control set (top) and MDA-MB-231/miR-126KD or control set (bottom). Scale bar represents 100μm. **h,** CN34 Parental cells and CN34 LM1a cells were subjected to the HUVEC recruitment assay. n=4; error bars represent s.e.m., p-values obtained using student's t-test. **i,** CN34 LM1a cells expressing miR-126 or the control hairpin, as well as CN34 Parental cells expressing a short hairpin targeting miR-126 or the control hairpin, were subjected to the HUVEC recruitment assay. n=4; error bars represent s.e.m., p-values obtained using student's t-test. Representative images are shown. Scale bar represents 100μm. **j,** 5 X 10^5 Lm2 cells over-expressing miR-126 or the control hairpin, as well as 5 X 10^5 MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control hairpin were mixed 1:1 with matrigel and injected into the mammary fat pad. Size-matched tumors were analyzed for blood vessel density by immuno-histochemical staining for MECA-32. 5 individual fields were taken for each tumor and the percentage of each field covered by a thresholded MECA-32 staining are given as % vessel density. Quantification is shown on top and representative images shown below. n=4; error bars represent s.e.m., p-values obtained using student's t-test.

FIGs. 3a-3f are diagrams and photographs showing systematic identification of a miR-126 regulatory network that mediates metastatic endothelial recruitment. a, The miR-126 metastasis signature is comprised of genes over-expressed in metastatic cells, down-regulated by miR-126 OE, and induced by miR-126 KD. The heatmap represents

variance-normalized expression levels based on microarray and qPCR analyses. Colourmap corresponds to standard deviations change from the mean. **b-d,** Kaplan-Meier curves for the (b) UCSF breast cancer cohort (117 tumors), (c) NKI cohort (295 tumors), and (d) the combined NKI/MSK/UCSF cohort (494 tumors) depicting metastasis-free-survival of those patients whose primary cancers over-expressed the miR-126 eight gene signature (positive) and those that did not (negative). P-values based on the Mantel-Cox log-rank test. **e,** Luciferase reporter assays of miR-126 metastasis genes in MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control KD hairpin. Reporter constructs containing the luciferase gene upstream of the 3'UTR or coding sequences (CDS) of each miR-126 regulated gene were transfected into the various cell lines and luciferase activity was assayed at 30 hours post transfection. n=4; error bars represent s.e.m.; p-values were obtained using student's t-test. **f,** The miR-126 complementary regions of the 3'UTR/CDS constructs were mutated and the luciferase reporter assay was repeated with these constructs in MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control hairpin (right). n=4; error bars represent s.e.m.; p-values were obtained using student's t-test.

FIGs. 4a-4e are a set of diagrams and photographs showing that IGFBP2, PITPNC1 and MERTK promoted metastatic colonization and angiogenesis. **a,** 2.5 X 10^4 LM2 cells expressing hairpins targeting IGFBP2, MERTK, PITPNC1, SHMT2 or the control hairpin were seeded in quadruplicate. Trans-well migration of 5 X 10^4 HUVEC cells towards the cancer cells was then assessed. Images of cells that migrated through the trans-well inserts were obtained and analyzed using ImageJ software. n=4; error bars represent s.e.m., p-values obtained using a student's t-test. Representative images are shown. Scale bar represents 100μm. **b,** The relative expression levels of IGFBP2, MERTK or PITPNC1 in human breast tumor samples from stage I/II (n=53) as compared to stage III (n=29) or stage IV (n=9) patients were quantified from the OriGene TissueScan Breast Cancer arrays using qPCR. Error bars represent s.e.m., p-values obtained using student's t-test. **c-e,** Bioluminescence imaging of lung metastasis by lung metastatic breast cancer cells with inhibited expression of the various miR-126 regulated genes. 4 X 10^4 LM2 cells expressing the control hairpin or independent short hairpins targeting IGFBP2 (c), PITPNC1 (d) and MERTK (e) were injected intravenously into immunodeficient NOD-

SCID mice. Lung colonization was measured by bioluminescence imaging and quantified. n=5; error bars represent s.e.m.; p-value based on a one-sided student's t-test.

FIGs. 5a-5g are diagrams and photographs showing that IGFBP2 mediated endothelial recruitment by activating IGF1/IGF1R signaling in endothelial cells. **a,** IGFBP2 levels in conditioned media from HUVEC recruitment assays using MDA-MB-231 cells and LM2 cells (FIG. 2f) were quantified by ELISA. n=4; error bars represent s.e.m., p-values obtained using student's t-test. **b,** 2.5 X 10^4 MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control hairpin, as well as LM2 cells expressing miR-126 or the control hairpin, were seeded in quadruplicate. Trans-well recruitment of 5 X 10^4 HUVEC cells in the presence of 50 ng/ml IGFBP2 Ab or 50 ng/ml control IgG Ab towards the cancer cells was then assessed. Images of the basal side of the trans-well inserts were obtained and the number of cells that had migrated was quantified using ImageJ. n=4; error bars represent s.e.m., p-values obtained using student's t-test. Scale bar represents 100μm. **c,** 2.5 X 10^4 CN34 Par cells expressing a short hairpin targeting miR-126 or the control hairpin, as well as CN34 Lm1a cells expressing miR-126 or the control hairpin, were seeded in quadruplicate. Trans-well migration of 5 X 10^4 HUVEC cells in the presence of 50 ng/ml IGFBP2 Ab or 50 ng/ml control IgG Ab towards the cancer cells was then assessed. n=4; error bars represent s.e.m., p-values obtained using student's t-test. **d**, **e,** Trans-well recruitment of HUVEC cells incubated with 20 μg/ml IGF-1 blocking Ab (d), 40 μg/ml IGF-2 blocking Ab (d), 20 μg/ml IGF1R blocking Ab (e), 5 μg/ml IGF2R blocking Ab (e), or control IgG (d,e) towards MDA-MB-231 cells expressing a short hairpin targeting miR-126 or control hairpin was assayed. n=4; error bars represent s.e.m., p-values obtained using student's t-test. **f**, HUVEC and cancer cells were pretreated with 20 μg/ml IGF1R blocking Ab or control IgG Ab for 1 hour before trans-well recruitment of HUVEC cells towards cancer cells were assessed. n=4; error bars represent s.e.m., p-values obtained using student's t-test **g,** IGFBP2 gradient was simulated by mixing the given amounts of recombinant IGFBP2 protein with matrigel (1:1) in the bottom of a well. Chemotaxis of 1.5 X 10^5 HUVEC cells along the IGFBP2 gradient was then assessed by counting the number of cells that had migrated to the basal side of trans-well inserts after 20h using ImageJ software. n=4; error bars represent s.e.m., p-values obtained using student's t-test.

FIGs. 6a-6e are diagrams and photographs showing that MERTK mediated recruitment through GAS6. **a,** IGFBP2 levels in conditioned media from Lm2 cells expressing control hairpin or 2 independent hairpins against PITPNC1 as determined by ELISA. **b,** 2.5 X 10^4 MDA-MB-231 cells expressing a control hairpin or a hairpin targeting miR-126 were seeded in quadruplicate. Trans-well migration of 5 X 10^4 HUVEC cells towards the cancer cells in the presence of 1 ng/ml GAS6 and/or 10μg/ml MerFc was then assessed by counting the number of cells that had migrated to the basal side of porous inserts in images obtained using ImageJ. n=4; error bars represent s.e.m., p-values were obtained using student's t-test. **c,** IGFBP2 gradient in the presence of Gas6 and secreted MERTK was simulated by mixing rhIGFBP2 (520 ng), Gas6 (5 ng) and MerFc (10 ug) protein with matrigel (1:1) in the bottom of a well. Chemotaxis of 1.5 X 10^5 HUVEC cells along the gradient was then assessed by counting the number of cells that had migrated to the basal side of trans-well inserts after 20h using ImageJ software. n=4; error bars represent s.e.m., p-values obtained using student's t-test. **d**, Lung sections were double stained for vimentin and MECA-32. The border of each nodule was drawn based on human-vimentin staining and MECA-32 staining inside the metastatic nodule highlighted in black (lower panels). The area positive for MECA-32 staining within each nodule was then determined by using ImageJ and presented as the area covered by MECA-32 staining per area of the given nodule (% vessel density). The distribution of % vessel density between the injected LM2 cells expressing hairpins targeting IGFBP2, PITPNC1, MERTK or a control hairpin are shown in a cumulative fraction plot. n=4, p-value based on the Kolmogorov-Smirnov Test. **e,** Schematic of miR-126 regulation of endothelial recruitment and metastatic colonization through interaction with IGFBP2, PITPNC1 and MERTK.

FIG. 7 is a diagram showing that miR-Zip miRNA-anti-sense shRNA system stably inhibited miR-126 expression in MDA-MB-231 cells. MDA-MB-231 cells were transduced with lentivirus expressing either a miR-Zip construct that targets miR-126 or a scrambled version of the construct that does not target any known microRNA (SYSTEM BIOSCIENCES, Mountain View, CA). The expression levels of mature miR-126 were then tested using qPCR.

FIG. 8 is a set of photographs and a diagram showing that breast cancer cell-expressed miR-126 regulates perfusion in metastatic nodules. 4 X 10^4 MDA-MB-231 cells expressing a short hairpin targeting miR-126 or the control hairpin were injected

intravenously into immunodeficient NOD-SCID mice. At day 59, FITC labeled low molecular weight dextran (10.000 MW) solution was injected intravenously. The dextran molecules were allowed to circulate for 15 min before mice were euthanized and the lungs excised. Frozen section were prepared, and stained for human Vimentin in order to localize metastatic nodules, and the FITC signal inside the nodules was quantified with a constant threshold using ImageJ. n=5; error bars represent s.e.m., p-values obtained using student's t-test.

FIGs. 9a-9b are a set of diagrams showing that endogenous miR-126 did not suppress endothelial adhesion, proliferation, or tube formation. **a,** 5 X 10^4 MDA cells expressing miR-126 KD or control KD vector were seeded onto a HUVEC monolayer and adhesion was assessed. Images of cells that had adhered to the HUVEC monolayer were obtained and analyzed using ImageJ software. n=4; error bars represent s.e.m. **b,** Conditioned media from 5 X 10^5 MDA miR-126 KD or MDA control KD cells was obtained by incubating the cells with EGM-2 media for 24h. 2.5 X 10^4 HUVEC cells were seeded in triplicate, grown in the conditioned media and viable cells were counted at 5 days after seeding. n=3; error bars represent s.e.m. **c,** 2 X 10^4 HUVEC cells were mixed with 1 X 10^4 MDA miR-126 KD or MDA control KD cells, and tube formation by HUVEC cells was assayed. Images of each well were obtained and the number of branch points in each image was analyzed using MetaMorph software. n=3; error bars represent s.e.m.

FIGs. 10a-10c are a set of diagrams and diagrams showing that endogenous miR-126 regulated angiogenesis, but not CD45 positive lymphocyte and Mac-2 positive macrophage recruitment. a-c, 5 X 10^5 MDA cells expressing control hairpin or hairpin targeting miR-126 were mixed in 1:1 ratio with Matrigel and injected in the mammary fat pad. 5 min prior to sacrifice, biotinlyated lectin was injected in the tail vein. Size matched tumors were excised and functional blood vessels were detected by staining of the injected lectin (a), $CD45^+$ lymphocyte detected by anti-CD45 (b) and $Mac\text{-}2^+$ macrophages detected by anti-Mac-2 (c).

FIG. 11 is a Venn diagram showing the integrative experimental path that resulted in the identification of putative miR-126 target genes. Transcriptomic profiling of genes down-regulated by greater than 1.6 fold upon miR-126 over-expression were overlapped with genes up-regulated by more than 1.4 fold in metastatic LM2 cells as compared to the

parental MDA cells. This led to the identification of 23 potential miR-126 target genes. By qPCR, 8 of these 23 genes were modulated by miR-126 in both the MDA-MB-231 breast cancer cell line and the primary CN34 cell line. These 8 genes were functionally tested for direct regulation by miR-126 through luciferase reporter assays.

FIGs. 12a-12b are diagrams showing that miR-126 regulated IGFBP2 and MERTK through 3'UTR interactions and PITPNC1 and SHMT2 through CDS interactions. a, b, Luciferase reporter assays of the miR-126 metastasis gene set in MDA-MB-231 cells expressing a short hairpin targeting miR-126 as well as the control KD hairpin. Reporter constructs containing the luciferase gene upstream of the 3'UTR (a) or CDS (b) of ABCB9, IGFBP2, MERTK, PITPNC1, PSAT1, PYBG, SHMT2 and VIPR1 were transfected into the various cell lines and luciferase activity was assayed at 30 hours post transfection. n=4; error bars represent s.e.m.; p-values were obtained using student's t-test.

FIGs. 13a-13d are a set of diagrams showing that independent hairpins down-regulated the expression levels of IGFBP2, PITPNC1 and MERTK in LM2 cells. LM2 cells were transduced with lentivirus expressing a control hairpin or a short hairpin construct targeting IGFBP2, PITPNC1 or MERTK. The expression levels of the target genes were analyzed through qPCR.

FIG. 14 is a diagram showing proliferation analysis of miR-126 target genes. 2.5 X 10^4 LM2 cells expressing a control hairpin or short hairpins targeting IGFBP2, PITPNC1 or MERTK were seeded in triplicate and viable cells were counted at 5 days after seeding. n=3; error bars represent s.e.m.

FIG. 15 is a diagram showing that IGFBP2 promoted HUVEC migration. HUVEC cells were stimulated with the given amounts of recombinant human IGFBP2 protein and anti-IGF1R Ab (10μg/ml) for 40 min, trypsinized and 5 X 10^4 cells were seeded into a porous transwell insert. The cells were allowed to migrate for 24 hours before the number of cells that migrated across the membrane was quantified. n=6; error bars represent s.e.m.; p-values were obtained using student's t-test.

FIG. 16 is a photograph of Western blot analysis of MERTK in MDA-MB-231 cellular lysate and conditioned media, showing that ectodomain of MERTK was cleaved and secreted by MDA-MB-231 cells.

FIG. 17 is a set of photographs and diagrams showing that GFBP2, PITPNC1 and MERTK promoted metastatic angiogenesis. Lung sections were histologically double-

stained for human vimentin and intravenously injected lectin. Nodule borders were demarcated based on vimentin staining and lectin staining inside metastatic nodules. ImageJ was used to determine the area positive for lectin staining within each nodule. % vessel density represents the area covered by lectin staining per area of a given nodule. The distribution of % vessel density between the injected LM2 cells expressing the control hairpin or short hairpins targeting IGFBP2, PITPNC1 or MERTK are shown in a cumulative fraction plot. n=5. P-value based on the Kolmogorov-Smirnov test.

FIG. 18 is a diagram showing that endogenous miR-126 expression in HUVEC cells did not suppress recruitment of other HUVEC cells. An antagomiR targeting miR-126 or a control antagomiR were transfected into HUVEC cells before being subjected to the HUVEC recruitment assay. Images of the basal side of the inserts were obtained and cells counted using ImageJ software. n=4; error bars represent s.e.m.

FIG. 19 is a diagram showing expression of potential miR-126 targets in HUVEC cells with suppressed miR-126 levels. An antagomiR targeting miR-126 or a control antagomiR were transfected into HUVEC cells and the relative expression of potential targets in transfected cells were quantified using qPCR. Error bars represent s.e.m., p-values obtained using student's t-test.

FIG. 20 is a set of diagrams showing data from antibody-capture ELISA assays used to characterize binding properties of IGFBP2 neutralizing antibodies. The figure shows that supernatant from one of the hybridoma libraries (wo6663-1) generated from animals inoculated with recombinant IGFBP2 total peptide, contains antibodies that bind to IGFBP2 with high affinity.

FIG. 21 is a set of diagrams showing data from antibody capture ELISA assays used to characterize binding properties of IGFBP2 neutralizing antibodies. The figure shows that supernatant from hybridoma wo6663-1 contains antibodies that bind to IGFBP2 to neutralize IGF1 binding to IGFBP2. Also note that the antibodies from the hybridoma wo6663-1 bind specifically to IGFBP2, and do not bind other IGFBP family members (IGFBP3, IGFBP4).

FIG. 22 is a set of diagrams showing data from antibody capture ELISA assays used to characterize binding properties of IGFBP2 neutralizing monoclonal antibodies recovered from single hybridoma clones isolated from hybridoma library wo6663-1. Several of the IGFBP2 neutralizing monoclonal antibodies, including M1, M4, M6, M9,

M13, M14, M15, and M16, were able to bind IGFBP2 with high affinity and neutralize its binding to IGF1.

FIG. 23 is a diagram showing that a composition containing physiological concentrations of the monoclonal antibody M14 is capable of inhibiting endothelial recruitment by metastatic breast cancer cells. As in experiments described above, a trans-well migration assay was used to quantify endothelial recruitment by metastatic cells. Highly metastatic LM2 human breast cancer cells were placed in the bottom of a Boyden chamber, where their ability to recruit HUVECS through a porous trans-well insert could be assayed. The addition of a small physiologic concentration of M14 to the transwell was able to significantly inhibit the recruitment and migration (migrated cells per field) of HUVEC cells (50% reduction in migrated cells) versus the negative controls (IgG and M5 antibodies). Error bars represent s.e.m.

FIG. 24 is a diagram showing that a composition containing physiological concentrations of the monoclonal antibody M14 is capable of inhibiting breast cancer tumor progression in vivo in a mouse model. Bioluminescence imaging of mammary tumor growth by 2000 MDA-MB-231 human breast cancer cells in animals treated with either PBS or monoclonal antibody M14. At day 14, tumor progression was significantly inhibited by treatment with M14 (7 to 11 fold reduction in tumor progression) compared with the PBS treated mice. The signal is normalized to signal from Day 0. Significance is based on a two sided student T-test.

DETAILED DESCRIPTION OF THE INVENTION

The described invention provides reagents and methods for treating disorders characterized by pathological angiogenesis, such as metastasis.

As disclosed herein, a systematic analysis and focus on metastasis and metastatic angiogenesis led to the identification of a number of molecules, including secreted IGFBP2, the transferase PITPNC1, the kinase MERTK, and miR-126, as targets for therapeutic inhibition with the potential for treating metastatic cancer. A newly discovered pathway coordinates IGFBP2, PITPNC1, and MERTK—pro-angiogenic genes that correlate in expression with human metastasis. These genes represent regulators of metastatic endothelial recruitment and angiogenesis. For example, IGFBP2, a protein

secreted by metastatic cells, recruits endothelia by modulating IGF1-mediated activation of the IGF type-I Receptor on endothelial cells.

Endothelial recruitment is a process where endothelial cells or their progenitors are mobilized and homing to a site or region in a subject for generating new blood vessels or remodeling of preexisting blood vessels, i.e., angiogenesis. Inhibiting this process via the above-mentioned new pathway can be used to inhibit pathological angiogenesis, and thereby to treat disorders characterized by pathological angiogenesis, such as metastasis.

To inhibit endothelial recruitment and resulting angiogenesis in a subject in need thereof, one can administer to the subject an agent that inhibits expression or activity of a protein selected from IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR. Listed below are the amino acid sequences of these proteins. The agent can be a nucleic acid, a polypeptide, an antibody, or a small molecule compound.

IGFBP2

MLPRVGCPALPLPPPPLLPLLPLLLLLLGASGGGGGARAEVLFRCPPCTPERLAACGPPP
VAPPAAVAAVAGGARMPCAELVREPGCGCCSVCARLEGEACGVYTPRCGQGLRCYPHPGS
ELPLQALVMGEGTCEKRRDAEYGASPEQVADNGDDHSEGGLVENHVDSTMNMLGGGGSAG
RKPLKSGMKELAVFREKVTEQHRQMGKGGKHHLGLEEPKKLRPPPARTPCQQELDQVLER
ISTMRLPDERGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRGECWCVNPNTGKLIQGA
PTIRGDPECHLFYNEQQEARGVHTQRMQ

IGF1 (isoform 1)

MGKISSLPTQLFKCCFCDFLKVKMHTMSSSHLFYLALCLLTFTSSATAGPETLCGAELVD
ALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSARS
VRAQRHTDMPKTQKYQPPSTNKNTKSQRRKGSTFEERK

IGF1 (isoform 2)

MITPTVKMHTMSSSHLFYLALCLLTFTSSATAGPETLCGAELVDALQFVCGDRGFYFNKP
TGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSARSVRAQRHTDMPKTQKEV
HLKNASRGSAGNKNYRM

IGF1 (isoform 3)

MGKISSLPTQLFKCCFCDFLKVKMHTMSSSHLFYLALCLLTFTSSATAGPETLCGAELVD
ALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSARS
VRAQRHTDMPKTQKYQPPSTNKNTKSQRRKGWPKTHPGGEQKEGTEASLQIRGKKKEQRR
EIGSRNAECRGKKGK

IGF1 (isoform 4)

MGKISSLPTQLFKCCFCDFLKVKMHTMSSSHLFYLALCLLTFTSSATAGPETLCGAELVD
ALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSARS
VRAQRHTDMPKTQKEVHLKNASRGSAGNKNYRM

IGF1R

MKSGSGGGSPTSLWGLLFLSAALSLWPTSGEICGPGIDIRNDYQQLKRLENCTVIEGYLH
ILLISKAEDYRSYRFPKLTVITEYLLLFRVAGLESLGDLFPNLTVIRGWKLFYNYALVIF
EMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTVDWSLILDAVSNNYIVGNKPPKECGD
LCPGTMEEKPMCEKTTINNEYNYRCWTTNRCQKMCPSTCGKRACTENNECCHPECLGSCS
APDNDTACVACRHYYYAGVCVPACPPNTYRFEGWRCVDRDFCANILSAESSDSEGFVIHD
GECMQECPSGFIRNGSQSMYCIPCEGPCPKVCEEEKKTKTIDSVTSAQMLQGCTIFKGNL

LINIRRGNNIASELENFMGLIEVVTGYVKIRHSHALVSLSFLKNLRLILGEEQLEGNYSF
YVLDNQNLQQLWDWDHRNLTIKAGKMYFAFNPKLCVSEIYRMEEVTGTKGRQSKGDINTR
NNGERASCESDVLHFTSTTTSKNRIIITWHRYRPPDYRDLISFTVYYKEAPFKNVTEYDG
QDACGSNSWNMVDVDLPPNKDVEPGILLHGLKPWTQYAVYVKAVTLTMVENDHIRGAKSE
ILYIRTNASVPSIPLDVLSASNSSSQLIVKWNPPSLPNGNLSYYIVRWQRQPQDGYLYRH
NYCSKDKIPIRKYADGTIDIEEVTENPKTEVCGGEKGPCCACPKTEAEKQAEKEEAEYRK
VFENFLHNSIFVPRPERKRRDVMQVANTTMSSRSRNTTAADTYNITDPEELETEYPFFES
RVDNKERTVISNLRPFTLYRIDIHSCNHEAEKLGCSASNFVFARTMPAEGADDIPGPVTW
EPRPENSIFLKWPEPENPNGLILMYEIKYGSQVEDQRECVSRQEYRKYGGAKLNRLNPGN
YTARIQATSLSGNGSWTDPVFFYVQAKTGYENFIHLIIALPVAVLLIVGGLVIMLYVFHR
KRNNSRLGNGVLYASVNPEYFSAADVYVPDEWEVAREKITMSRELGQGSFGMVYEGVAKG
VVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTLVIME
LMTRGDLKSYLRSLRPEMENNPVLAPPSLSKMIQMAGEIADGMAYLNANKFVHRDLAARN
CMVAEDFTVKIGDFGMTRDIYETDYYRKGGKGLLPVRWMSPESLKDGVFTTYSDVWSFGV
VLWEIATLAEQPYQGLSNEQVLRFVMEGGLLDKPDNCPDMLFELMRMCWQYNPKMRPSFL
EIISSIKEEMEPGFREVSFYYSEENKLPEPEELDLEPENMESVPLDPSASSSSLPLPDRH
SGHKAENGPGPGVLVLRASFDERQPYAHMNGGRKNERALPLPQSSTC

MERTK

MGPAPLPLLLGLFLPALWRRAITEAREEAKPYPLFPGPFPGSLQTDHTPLLSLPHASGYQ
PALMFSPTQPGRPHTGNVAIPQVTSVESKPLPPLAFKHTVGHIILSEHKGVKFNCSISVP
NIYQDTTISWWKDGKELLGAHHAITQFYPDDEVTAIIASFSITSVQRSDNGSYICKMKIN
NEEIVSDPIYIEVQGLPHFTKQPESMNVTRNTAFNLTCQAVGPPEPVNIFWVQNSSRVNE
QPEKSPSVLTVPGLTEMAVFSCEAHNDKGLTVSKGVQINIKAIPSPPTEVSIRNSTAHSI
LISWVPGFDGYSPFRNCSIQVKEADPLSNGSVMIFNTSALPHLYQIKQLQALANYSIGVS
CMNEIGWSAVSPWILASTTEGAPSVAPLNVTVFLNESSDNVDIRWMKPPTKQQDGELVGY
RISHVWQSAGISKELLEEVGQNGSRARISVQVHNATCTVRIAAVTRGGVGPFSDPVKIFI
PAHGWVDYAPSSTPAPGNADPVLIIFGCFCGFILIGLILYISLAIRKRVQETKFGNAFTE
EDSELVVNYIAKKSFCRRAIELTLHSLGVSEELQNKLEDVVIDRNLLILGKILGEGEFGS
VMEGNLKQEDGTSLKVAVKTMKLDNSSQREIEEFLSEAACMKDFSHPNVIRLLGVCIEMS
SQGIPKPMVILPFMKYGDLHTYLLYSRLETGPKHIPLQTLLKFMVDIALGMEYLSNRNFL
HRDLAARNCMLRDDMTVCVADFGLSKKIYSGDYYRQGRIAKMPVKWIAIESLADRVYTSK
SDVWAFGVTMWEIATRGMTPYPGVQNHEMYDYLLHGHRLKQPEDCLDELYEIMYSCWRTD
PLDRPTFSVLRLQLEKLLESLPDVRNQADVIYVNTQLLESSEGLAQGSTLAPLDLNIDPD
SIIASCTPRAAISVVTAEVHDSKPHEGRYILNGGSEEWEDLTSAPSAAVTAEKNSVLPGE
RLVRNGVSWSHSSMLPLGSSLPDELLFADDSSEGSEVLM

PITPNC1 (isoform a)

MLLKEYRICMPLTVDEYKIGQLYMISKHSHEQSDRGEGVEVVQNEPFEDPHHGNGQFTEK
RVYLNSKLPSWARAVVPKIFYVTEKAWNYYPYTITEYTCSFLPKFSIHIETKYEDNKGSN
DTIFDNEAKDVEREVCFIDIACDEIPERYYKESEDPKHFKSEKTGRGQLREGWRDSHQPI
MCSYKLVTVKFEVWGLQTRVEQFVHKVVRDILLIGHRQAFAWVDEWYDMTMDEVREFERA
TQEATNKKIGIFPPAISISSIPLLPSSVRSAPSSAPSTPLSTDAPEFLSVPKDRPRKKSA
PETLTLPDPEKKATLNLPGMHSSDKPCRPKSE

PITPNC1 (isoform b)

MLLKEYRICMPLTVDEYKIGQLYMISKHSHEQSDRGEGVEVVQNEPFEDPHHGNGQFTEK
RVYLNSKLPSWARAVVPKIFYVTEKAWNYYPYTITEYTCSFLPKFSIHIETKYEDNKGSN
DTIFDNEAKDVEREVCFIDIACDEIPERYYKESEDPKHFKSEKTGRGQLREGWRDSHQPI
MCSYKLVTVKFEVWGLQTRVEQFVHKVVRDILLIGHRQAFAWVDEWYDMTMDDVREYEKN
MHEQTNIKVCNQHSSPVDDIESHAQTST

ABCB9

MRLWKAVVVTLAFMSVDICVTTAIYVFSHLDRSLLEDIRHFNIFDSVLDLWAACLYRSCL
LLGATIGVAKNSALGPRRLRASWLVITLVCLFVGIYAMVKLLLFSEVRRPIRDPWFWALF
VWTYISLGASFLLWWLLSTVRPGTQALEPGAATEAEGFPGSGRPPPEQASGATLQKLLSY
TKPDVAFLVAASFFLIVAALGETFLPYYTGRAIDGIVIQKSMDQFSTAVVIVCLLAIGSS
FAAGIRGGIFTLIFARLNIRLRNCLFRSLVSQETSFFDENRTGDLISRLTSDTTMVSDLV
SQNINVFLRNTVKVTGVVVFMFSLSWQLSLVTFMGFPIIMMVSNIYGKYYKRLSKEVQNA
LARASNTAEETISAMKTVRSFANEEEEAEVYLRKLQQVYKLNRKEAAAYMYYVWGSGLTL
LVVQVSILYYGGHLVISGQMTSGNLIAFIIYEFVLGDCMESVGSVYSGLMQGVGAAEKVF
EFIDRQPTMVHDGSLAPDHLEGRVDFENVTFTYRTRPHTQVLQNVSFSLSPGKVTALVGP

SGSGKSSCVNILENFYPLEGGRVLLDGKPISAYDHKYLHRVISLVSQEPVLFARSITDNI
SYGLPTVPFEMVVEAAQKANAHGFIMELQDGYSTETGEKGAQLSGGQKQRVAMARALVRN
PPVLILDEATSALDAESEYLIQQAIHGNLQKHTVLIIAHRLSTVEHAHLIVVLDKGRVVQ
QGTHQQLLAQGGLYAKLVQRQMLGLQPAADFTAGHNEPVANGSHKA

PSAT1 (isoform 1)

MDAPRQVVNFGPGPAKLPHSVLLEIQKELLDYKGVGISVLEMSHRSSDFAKIINNTENLV
RELLAVPDNYKVIFLQGGGCGQFSAVPLNLIGLKAGRCADYVVTGAWSAKAAEEAKKFGT
INIVHPKLGSYTKIPDPSTWNLNPDASYVYYCANETVHGVEFDFIPDVKGAVLVCDMSSN
FLSKPVDVSKFGVIFAGAQKNVGSAGVTVVIVRDDLLGFALRECPSVLEYKVQAGNSSLY
NTPPCFSIYVMGLVLEWIKNNGGAAAMEKLSSIKSQTIYEIIDNSQGFYVCPVEPQNRSK
MNIPFRIGNAKGDDALEKRFLDKALELNMLSLKGHRSVGGIRASLYNAVTIEDVQKLAAF
MKKFLEMHQL

PSAT1 (isoform 2)

MDAPRQVVNFGPGPAKLPHSVLLEIQKELLDYKGVGISVLEMSHRSSDFAKIINNTENLV
RELLAVPDNYKVIFLQGGGCGQFSAVPLNLIGLKAGRCADYVVTGAWSAKAAEEAKKFGT
INIVHPKLGSYTKIPDPSTWNLNPDASYVYYCANETVHGVEFDFIPDVKGAVLVCDMSSN
FLSKPVDVSKFGVIFAGAQKNVGSAGVTVVIVRDDLLGFALRECPSVLEYKVQAGNSSLY
NTPPCFSIYVMGLVLEWIKNNGGAAAMEKLSSIKSQTIYEIIDNSQGFYVSVGGIRASLY
NAVTIEDVQKLAAFMKKFLEMHQL

PYGB

MAKPLTDSEKRKQISVRGLAGLGDVAEVRKSFNRHLHFTLVKDRNVATPRDYFFALAHTV
RDHLVGRWIRTQQHYYERDPKRIYYLSLEFYMGRTLQNTMVNLGLQNACDEAIYQLGLDL
EELEEIEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYGIRYEFGIFNQKIVNGWQVEEA
DDWLRYGNPWEKARPEYMLPVHFYGRVEHTPDGVKWLDTQVVLAMPYDTPVPGYKNNTVN
TMRLWSAKAPNDFKLQDFNVGDYIEAVLDRNLAENISRVLYPNDNFFEGKELRLKQEYFV
VAATLQDIIRRFKSSKFGCRDPVRTCFETFPDKVAIQLNDTHPALSIPELMRILVDVEKV
DWDKAWEITKKTCAYTNHTVLPEALERWPVSMFEKLLPRHLEIIYAINQRHLDHVAALFP
GDVDRLRRMSVIEEGDCKRINMAHLCVIGSHAVNGVARIHSEIVKQSVFKDFYELEPEKF
QNKTNGITPRRWLLLCNPGLADTIVEKIGEEFLTDLSQLKKLLPLVSDEVFIRDVAKVKQ
ENKLKFSAFLEKEYKVKINPSSMFDVHVKRIHEYKRQLLNCLHVVTLYNRIKRDPAKAFV
PRTVMIGGKAAPGYHMAKLIIKLVTSIGDVVNHDPVVGDRLKVIFLENYRVSLAEKVIPA
ADLSQQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGAENLFIFGLRVEDVE
ALDRKGYNAREYYDHLPELKQAVDQISSGFFSPKEPDCFKDIVNMLMHHDRFKVFADYEA
YMQCQAQVDQLYRNPKEWTKKVIRNIACSGKFSSDRTITEYAREIWGVEPSDLQIPPPNI
PRD

SHMT2 (isoform 1)

MLYFSLFWAARPLQRCGQLVRMAIRAQHSNAAQTQTGEANRGWTGQESLSDSDPEMWELL
QREKDRQCRGLELIASENFCSRAALEALGSCLNNKYSEGYPGKRYYGGAEVVDEIELLCQ
RRALEAFDLDPAQWGVNVQPYSGSPANLAVYTALLQPHDRIMGLDLPDGGHLTHGYMSDV
KRISATSIFFESMPYKLNPKTGLIDYNQLALTARLFRPRLIIAGTSAYARLIDYARMREV
CDEVKAHLLADMAHISGLVAAKVIPSPFKHADIVTTTTHKTLRGARSGLIFYRKGVKAVD
PKTGREIPYTFEDRINFAVFPSLQGGPHNHAIAAVAVALKQACTPMFREYSLQVLKNARA
MADALLERGYSLVSGGTDNHLVLVDLRPKGLDGARAERVLELVSITANKNTCPGDRSAIT
PGGLRLGAPALTSRQFREDDFRRVVDFIDEGVNIGLEVKSKTAKLQDFKSFLLKDSETSQ
RLANLRQRVEQFARAFPMPGFDEH

SHMT2 (isoform 2)

MLYFSLFWAARPLQRCGQLVRMAIRAQHSNAAQTQTGEANRGWTGQESLSDSDPEMWELL
QREKDRQCRGLELIASENFCSRAALEALGSCLNNKYSEGYPGKRYYGGAEVVDEIELLCQ
RRALEAFDLDPAQWGVNVQPYSGSPANLAVYTALLQPHDRIMGLDLPDGGHLTHGYMSDV
KRISATSIFFESMPYKLNLALTARLFRPRLIIAGTSAYARLIDYARMREVCDEVKAHLLA
DMAHISGLVAAKVIPSPFKHADIVTTTTHKTLRGARSGLIFYRKGVKAVDPKTGREIPYT
FEDRINFAVFPSLQGGPHNHAIAAVAVALKQACTPMFREYSLQVLKNARAMADALLERGY
SLVSGGTDNHLVLVDLRPKGLDGARAERVLELVSITANKNTCPGDRSAITPGGLRLGAPA
LTSRQFREDDFRRVVDFIDEGVNIGLEVKSKTAKLQDFKSFLLKDSETSQRLANLRQRVE
QFARAFPMPGFDEH

SHMT2 (isoform 3)

MAIRAQHSNAAQTQTGEANRGWTGQESLSDSDPEMWELLQREKDRQCRGLELIASENFCS
RAALEALGSCLNNKYSEGYPGKRYYGGAEVVDEIELLCQRRALEAFDLDPAQWGVNVQPY
SGSPANLAVYTALLQPHDRIMGLDLPDGGHLTHGYMSDVKRISATSIFFESMPYKLNPKT
GLIDYNQLALTARLFRPRLIIAGTSAYARLIDYARMREVCDEVKAHLLADMAHISGLVAA
KVIPSPFKHADIVTTTTHKTLRGARSGLIFYRKGVKAVDPKTGREIPYTFEDRINFAVFP
SLQGGPHNHAIAAVAVALKQACTPMFREYSLQVLKNARAMADALLERGYSLVSGGTDNHL
VLVDLRPKGLDGARAERVLELVSITANKNTCPGDRSAITPGGLRLGAPALTSRQFREDDF
RRVVDFIDEGVNIGLEVKSKTAKLQDFKSFLLKDSETSQRLANLRQRVEQFARAFPMPGF
DEH

VIPR1

MRPPSPLPARWLCVLAGALAWALGPAGGQAARLQEECDYVQMIEVQHKQCLEEAQLENET
IGCSKMWDNLTCWPATPRGQVVVLACPLIFKLFSSIQGRNVSRSCTDEGWTHLEPGPYPI
ACGLDDKAASLDEQQTMFYGSVKTGYTIGYGLSLATLLVATAILSLFRKLHCTRNYIHMH
LFISFILRAAAVFIKDLALFDSGESDQCSEGSVGCKAAMVFFQYCVMANFFWLLVEGLYL
YTLLAVSFFSERKYFWGYILIGWGVPSTFTMVWTIARIHFEDYGCWDTINSSLWWIIKGP
ILTSILVNFILFICIIRILLQKLRPPDIRKSDSSPYSRLARSTLLLIPLFGVHYIMFAFF
PDNFKPEVKMVFELVVGSFQGFVVAILYCFLNGEVQAELRRKWRRWHLQGVLGWNPKYRH
PSGGSNGATCSTQVSMLTRVSPGARRSSSFQAEVSLV

An inhibitory agent (i.e., inhibitor) or an activating agent (i.e., activator) can be a nucleic acid, a polypeptide, an antibody, or a small molecule compound. Preferably, it is an isolated agent, but not an endogenous molecule (a micro RNA) in a cell of the subject. In one example, it excludes a micro RNA that is endogenous in human cells, e.g., miR-126, miR206, or/and miR-335. In another example, the inhibitory or activating agent functions at a level of transcription, mRNA stability, translation, protein stability/degradation, protein modification, and protein binding.

A nucleic acid refers to a DNA molecule (for example, but not limited to, a cDNA or genomic DNA), an RNA molecule (for example, but not limited to, an mRNA), or a DNA or RNA analog. A DNA or RNA analog can be synthesized from nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded. An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain

reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein.

The terms "RNA," "RNA molecule," and "ribonucleic acid molecule" are used interchangeably herein, and refer to a polymer of ribonucleotides. The term "DNA" or "DNA molecule" or deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally (e.g., by DNA replication or transcription of DNA, respectively). RNA can be post-transcriptionally modified. DNA and RNA also can be chemically synthesized. DNA and RNA can be single-stranded (i.e., ssRNA and ssDNA, respectively) or multi-stranded (e.g., double-stranded, i.e., dsRNA and dsDNA, respectively).

The nucleic acid sequence can encode a small interference RNA (e.g., an RNAi agent) that targets one or more of the above-mentioned genes and inhibits its expression or activity. The term "RNAi agent" refers to an RNA, or analog thereof, having sufficient sequence complementarity to a target RNA to direct RNA interference. Examples also include a DNA that can be used to make the RNA. RNA interference (RNAi) refers to a sequence-specific or selective process by which a target molecule (e.g., a target gene, protein or RNA) is down-regulated. Generally, an interfering RNA ("iRNA") is a double stranded short-interfering RNA (siRNA), short hairpin RNA (shRNA), or single-stranded micro-RNA (miRNA) that results in catalytic degradation of specific mRNAs, and also can be used to lower or inhibit gene expression.

The term "short interfering RNA" or "siRNA" (also known as "small interfering RNAs") refers to an RNA agent, preferably a double-stranded agent, of about 10-50 nucleotides in length, preferably between about 15-25 nucleotides in length, more preferably about 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, the strands optionally having overhanging ends comprising, for example 1, 2 or 3 overhanging nucleotides (or nucleotide analogs), which is capable of directing or mediating RNA interference. Naturally-occurring siRNAs are generated from longer dsRNA molecules (e.g., >25 nucleotides in length) by a cell's RNAi machinery (e.g., Dicer or a homolog thereof).

The term "miRNA" or "microRNA" refers to an RNA agent, preferably a single-stranded agent, of about 10-50 nucleotides in length, preferably between about 15-25 nucleotides in length, more preferably about 17, 18, 19, 20, 21, 22, 23, 24, or 25

nucleotides in length, which is capable of directing or mediating RNA interference. Naturally-occurring miRNAs are generated from stem-loop precursor RNAs (i.e., pre-miRNAs) by Dicer. The term "Dicer" as used herein, includes Dicer as well as any Dicer orthologue or homologue capable of processing dsRNA structures into siRNAs, miRNAs, siRNA-like or miRNA-like molecules. The term microRNA (or "miRNA") is used interchangeably with the term "small temporal RNA" (or "stRNA") based on the fact that naturally-occurring microRNAs (or "miRNAs") have been found to be expressed in a temporal fashion (e.g., during development).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region.

Thus, also within the scope of this invention is utilization of RNAi featuring degradation of RNA molecules (e.g., within a cell). Degradation is catalyzed by an enzymatic, RNA-induced silencing complex (RISC). A RNA agent having a sequence sufficiently complementary to a target RNA sequence (e.g., one or more of the above-mentioned genes) to direct RNAi means that the RNA agent has a homology of at least 50%, (e.g., 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or 100% homology) to the target RNA sequence so that the two are sufficiently complementary to each other to hybridize and trigger the destruction of the target RNA by the RNAi machinery (e.g., the RISC complex) or process. A RNA agent having a "sequence sufficiently complementary to a target RNA sequence to direct RNAi" also means that the RNA agent has a sequence sufficient to trigger the translational inhibition of the target RNA by the RNAi machinery or process. A RNA agent also can have a sequence sufficiently complementary to a target RNA encoded by the target DNA sequence such that the target DNA sequence is chromatically silenced. In other words, the RNA agent has a sequence sufficient to induce transcriptional gene silencing, *e.g.*, to down-modulate gene expression at or near the target DNA sequence, *e.g.*, by inducing chromatin structural changes at or near the target DNA sequence.

The above-mentioned polynucleotides can be delivered using polymeric, biodegradable microparticle or microcapsule delivery devices known in the art. Another way to achieve uptake of the polynucleotides is using liposomes, prepared by standard methods. The polynucleotide can be incorporated alone into these delivery vehicles or co-incorporated with tissue-specific antibodies. Alternatively, one can prepare a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that can bind to a receptor on target cells (Cristiano, *et al.*, 1995, J. Mol. Med. 73:479). Alternatively, tissue specific targeting can be achieved by the use of tissue-specific transcriptional regulatory elements that are known in the art. Delivery of naked DNA (i.e., without a delivery vehicle) to an intramuscular, intradermal, or subcutaneous site is another means to achieve *in vivo* expression.

siRNA, miRNA, and asRNA (antisense RNA) molecules can be designed by methods well known in the art. siRNA, miRNA, and asRNA molecules with homology sufficient to provide sequence specificity required to uniquely degrade any RNA can be designed using programs known in the art, including, but not limited to, those maintained on websites for AMBION, Inc. and DHARMACON, Inc. Systematic testing of several designed species for optimization of the siRNA, miRNA, and asRNA sequence can be routinely performed by those skilled in the art. Considerations when designing short interfering nucleic acid molecules include, but are not limited to, biophysical, thermodynamic, and structural considerations, base preferences at specific positions in the sense strand, and homology. These considerations are well known in the art and provide guidelines for designing the above-mentioned RNA molecules.

In one example, the polypeptide is an antibody. The term "antibody" refers to an immunoglobulin molecule or immunologically active portion thereof, i.e., an antigen-binding portion. Examples include, but are not limited to, a protein having at least one or two, heavy (H) chain variable regions (V_H), and at least one or two light (L) chain variable regions (V_L). The V_H and V_L regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized human immunoglobulin

genes include the kappa, lambda, alpha (IgA1 and IgA2), gamma (IgG1, IgG2, IgG3, and IgG4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes.

The term "antigen-binding portion" of an antibody (or "antibody portion") refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, or VIPR). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

Antibodies that specifically bind to one of the above-mentioned target protein can be made using methods known in the art. This antibody can be a polyclonal or a monoclonal antibody. Examples of such antibodies include those described in the working examples below. In one embodiment, the antibody can be recombinantly produced, e.g., produced by phage display or by combinatorial methods. In another embodiment, the antibody is a fully human antibody (e.g., an antibody made in a mouse which has been genetically engineered to produce an antibody from a human immunoglobulin sequence), a humanized antibody, or a non-human antibody, for example, but not limited to, a rodent

(mouse or rat), goat, primate (for example, but not limited to, monkey), rabbit, or camel antibody. Examples of methods to generate humanized version of antibodies include, but are not limited to, CDR grafting (Queen *et al.,* U.S. Pat. No. 5,585,089; Riechmann *et al.,* Nature 332:323 (1988)), chain shuffling (U.S. Pat. No. 5,565,332); and veneering or resurfacing (EP 592,106; EP 519,596); Padlan, Molecular Immunology 28(415):489-498 (1991); Studnicka *et al.,* Protein Engineering 7(6):805-814 (1994); Roguska. *et al.*, PNAS 91:969-973 (1994)). Examples of methods to generate fully human antibodies include, but are not limited to, generation of antibodies from mice that can express human immunoglobulin genes and use of phage-display technology to generate and screen human immunoglobulin gene libraries.

An "isolated antibody" is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, or VIPR is substantially free of antibodies that specifically bind antigens other than such an antigen). An isolated antibody that specifically binds the antigen may, however, have cross-reactivity to other antigens, such as IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, or VIPR molecules from other species. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

The term "human antibody", as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which

CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, e.g., a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The term "recombinant human antibody," as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, e.g., from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to in vitro mutagenesis (or, when an animal transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire in vivo.

As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by the heavy chain constant region genes.

The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen."

As used herein, the term "high affinity" for an IgG antibody refers to an antibody having a K_D of 10^{-7} M or less, preferably 10^{-8} M or less, more preferably 10^{-9} M or less

and even more preferably 10^{-10} M or less for a target antigen. However, "high affinity" binding can vary for other antibody isotypes. For example, "high affinity" binding for an IgM isotype refers to an antibody having a K_D of 10^{-7} M or less, more preferably 10^{-8} M or less.

In one example, a composition comprising a monoclonal antibody that neutralizes IGFBP2 function by inhibiting IGFBP2 binding to IGF1 is described. In one embodiment, this antibody can be a fully human antibody, a humanized antibody, or a non-human antibody, for example, but not limited to, a rodent (mouse or rat), goat, primate (for example, but not limited to, monkey), rabbit, or camel antibody. In one embodiment, one or more amino-acids of this monoclonal monoclonal antibody may be substituted in order to alter its physical properties. These properties include, but are not limited to, binding specificity, binding affinity, immunogenicity, and antibody isotype. Pharmaceutical compositions containing fully human or humanized versions of the above described antibodies can be used to treat disorders of pathological angiogenesis.

In one example, a composition comprising an IGFBP2 neutralizing antibody that inhibits IGF1 from binding to IGFBP2 inhibits breast cancer tumor progression and tumor burden in vivo. In this example, administration of the above described antibody reduced tumor burden of human breast cancer in vivo in a mouse model of human cancer.

Pharmaceutical compositions containing fully human or humanized versions of the above described antibodies can be used to inhibit breast cancer metastasis in human patients by inhibiting endothelial recruitment by metastatic cells. In another embodiment, pharmaceutical compositions containing fully human or humanized versions of these antibodies can be used to treat other types of vascular tumors. Typical vascularized tumors that can be treated with this composition include solid tumors, particularly carcinomas, which require a vascular component for the provision of oxygen and nutrients. Exemplary solid tumors include, but are not limited to, carcinomas of the lung, breast, bone, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, glioblastomas, neuroblastomas, Kaposi's sarcoma, and sarcomas.

In another embodiment, the polypeptide is a mutant form of the above-mentioned protein, which interferes with the above-mentioned pathway and therefore inhibits

endothelial recruitment and angiogenesis. The term "mutant" encompasses naturally occurring mutants and mutants created chemically and/or using recombinant DNA techniques. A mutant of one of the above-mentioned wild type polypeptide can be due to alteration, e.g., truncation, elongation, substitution, deletion, or insertion, of one or more amino acids. The alteration also can have a modified amino acid, such as one comprising a post-translational modification. The pro-angiogenic activity of a mutant, if any, is substantially lower than the activity of the wild type polypeptide by at least about 20% (e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%) as measured using an assay described herein or known in the art. One example is a polypeptide having the extracellular domain of IGF1-R, but lacking the intra-cellular domain. By competing for IGF-1, this mutant can inhibit the above-mentioned pathway and pro-angiogenic activity in a dominant-negative manner.

The amino acid compositions of the above-mentioned antibodies or polypeptides may vary with or without disrupting the ability (e.g., affinity) to bind to the respective antigens or targets, and trigger or inhibit the respective cellular response. For example, they can contain one or more conservative amino acid substitutions. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), β-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in, e.g., SEQ ID NO: 9 or 10 can be replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of the sequences, such as by saturation mutagenesis, and the resultant mutants can be screened for the ability to bind to the respective antigen and trigger the respective cellular response to identify mutants that retain the activity.

Compositions

Within the scope of this invention is a composition that contains a suitable carrier and one or more of the agents described above. The composition can be a pharmaceutical composition that contains a pharmaceutically acceptable carrier, a dietary composition that contains a dietarily acceptable suitable carrier, or a cosmetic composition that contains a cosmetically acceptable carrier.

The term "pharmaceutical composition" refers to the combination of an active agent with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vivo* or *ex vivo*. A "pharmaceutically acceptable carrier," after administered to or upon a subject, does not cause undesirable physiological effects. The carrier in the pharmaceutical composition must be "acceptable" also in the sense that it is compatible with the active ingredient and can be capable of stabilizing it. One or more solubilizing agents can be utilized as pharmaceutical carriers for delivery of an active compound. Examples of a pharmaceutically acceptable carrier include, but are not limited to, biocompatible vehicles, adjuvants, additives, and diluents to achieve a composition usable as a dosage form. Examples of other carriers include colloidal silicon oxide, magnesium stearate, cellulose, sodium lauryl sulfate, and D&C Yellow # 10.

The above-described composition, in any of the forms described above, can be used for treating disorders characterized by pathological angiogenesis. An effective amount refers to the amount of an active compound/agent that is required to confer a therapeutic effect on a treated subject. Effective doses will vary, as recognized by those skilled in the art, depending on the types of diseases treated, route of administration, excipient usage, and the possibility of co-usage with other therapeutic treatment.

A pharmaceutical composition of this invention can be administered parenterally, orally, nasally, rectally, topically, or buccally. The term "parenteral" as used herein refers to subcutaneous, intracutaneous, intravenous, intrmuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional, or intracranial injection, as well as any suitable infusion technique.

A sterile injectable composition can be a solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Such solutions include, but are not limited to, 1,3-butanediol, mannitol, water, Ringer's solution, and isotonic sodium chloride solution. In addition, fixed oils are conventionally employed as a solvent or suspending medium (e.g., synthetic mono- or diglycerides). Fatty acid, such as, but not limited to, oleic acid

and its glyceride derivatives, are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as, but not limited to, olive oil or castor oil, polyoxyethylated versions thereof. These oil solutions or suspensions also can contain a long chain alcohol diluent or dispersant such as, but not limited to, carboxymethyl cellulose, or similar dispersing agents. Other commonly used surfactants, such as, but not limited to, Tweens or Spans or other similar emulsifying agents or bioavailability enhancers, which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms also can be used for the purpose of formulation.

A composition for oral administration can be any orally acceptable dosage form including capsules, tablets, emulsions and aqueous suspensions, dispersions, and solutions. In the case of tablets, commonly used carriers include, but are not limited to, lactose and corn starch. Lubricating agents, such as, but not limited to, magnesium stearate, also are typically added. For oral administration in a capsule form, useful diluents include, but are not limited to, lactose and dried corn starch. When aqueous suspensions or emulsions are administered orally, the active ingredient can be suspended or dissolved in an oily phase combined with emulsifying or suspending agents. If desired, certain sweetening, flavoring, or coloring agents can be added.

Pharmaceutical compositions for topical administration according to the described invention can be formulated as solutions, ointments, creams, suspensions, lotions, powders, pastes, gels, sprays, aerosols, or oils. Alternatively, topical formulations can be in the form of patches or dressings impregnated with active ingredient(s), which can optionally comprise one or more excipients or diluents. In some preferred embodiments, the topical formulations include a material that would enhance absorption or penetration of the active agent(s) through the skin or other affected areas. The topical composition is useful for treating disorders in the skin, such as melanoma and certain inflammatory disorders.

A topical composition contains a safe and effective amount of a dermatologically acceptable carrier suitable for application to the skin. A “cosmetically acceptable” or “dermatologically-acceptable” composition or component refers a composition or component that is suitable for use in contact with human skin without undue toxicity, incompatibility, instability, allergic response, and the like. The carrier enables an active agent and optional component to be delivered to the skin at an appropriate

concentration(s). The carrier thus can act as a diluent, dispersant, solvent, or the like to ensure that the active materials are applied to and distributed evenly over the selected target at an appropriate concentration. The carrier can be solid, semi-solid, or liquid. The carrier can be in the form of a lotion, a cream, or a gel, in particular one that has a sufficient thickness or yield point to prevent the active materials from sedimenting. The carrier can be inert or possess dermatological benefits. It also should be physically and chemically compatible with the active components described herein, and should not unduly impair stability, efficacy, or other use benefits associated with the composition.

Treatment Methods

The described invention provides methods for treating in a subject an angiogenic disorder or a disorder of angiogenesis.

The terms "angiogenic disorder," "disorder of angiogenesis," and "angiogenesis disorder" are used interchangeably herein, and refer to a disorder characterized by pathological angiogenesis. A disorder characterized by pathological angiogenesis refers to a disorder where abnormal or aberrant angiogenesis, alone or in combination with others, contributes to causation, origination, or symptom of the disorder. Examples of this disorder include various cancers (e.g., vascularized tumors), eye disorders, inflammatory disorders, and others.

Typical vascularized tumors that can be treated with the method include solid tumors, particularly carcinomas, which require a vascular component for the provision of oxygen and nutrients. Exemplary solid tumors include, but are not limited to, carcinomas of the lung, breast, bone, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, glioblastomas, neuroblastomas, Kaposi's sarcoma, and sarcomas.

A number of disorders or conditions, other than cancer, also can be treated with the above-described method. Examples include arthritis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, age-related macular degeneration, Grave's disease, vascular restenosis (including restenosis following angioplasty), arteriovenous malformations (AVM), meningioma, hemangioma, neovascular glaucoma, chronic kidney disease, diabetic nephropathy, polycystic kidney disease, interstitial lung disease, pulmonary hypertension, chronic obstructive pulmonary disease (COPD), emphysema,

autoimmune hepatitis, chronic inflammatory liver disease, hepatic cirrhosis, cutaneous T-cell lymphoma, rosacea, and basal cell carcinoma.

Other treatment targets include those described in, e.g., US Applications 2009004297, 20090175791, and 20070161553, such as angiofibroma, atherosclerotic plaques, corneal graft neovascularization, hemophilic joints, hypertrophic scars, Osler-Weber syndrome, pyogenic granuloma retrolental fibroplasia, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, various other inflammatory diseases and disorders, and endometriosis.

A "subject" refers to a human and a non-human animal. Examples of a non-human animal include all vertebrates, e.g., mammals, such as non-human mammals, non-human primates (particularly higher primates), dog, rodent (e.g., mouse or rat), guinea pig, cat, and rabbit, and non-mammals, such as birds, amphibians, reptiles, etc. In one embodiment, the subject is a human. In another embodiment, the subject is an experimental animal or animal suitable as a disease model. A subject to be treated for a disorder can be identified by standard diagnosing techniques for the disorder.

Optionally, the subject can be examined for mutation, expression level, or activity level of one or more of the genes or proteins mentioned above by methods known in the art or described above before treatment. If the subject has a particular mutation in the gene, or if the gene expression or activity level is, for example, greater in a sample from the subject than that in a sample from a normal person, the subject is a candidate for treatment.

To confirm the inhibition or treatment, one can evaluate and/or verify the inhibition of endothelial recruitment or resulting angiogenesis using technology known in the art before and/or after the administering step. Exemplary technologies include angiography or arteriography, a medical imaging technique used to visualize the inside, or lumen, of blood vessels and organs of the body, can generally be done by injecting a radio-opaque contrast agent into the blood vessel and imaging using X-ray based techniques such as fluoroscopy.

"Treating" or "treatment" refers to administration of a compound or agent to a subject who has a disorder with the purpose to cure, alleviate, relieve, remedy, delay the onset of, prevent, or ameliorate the disorder, the symptom of the disorder, the disease state secondary to the disorder, or the predisposition toward the disorder.

An “effective amount” or “therapeutically effective amount” refers to an amount of the compound or agent that is capable of producing a medically desirable result in a treated subject. The treatment method can be performed *in vivo* or *ex vivo*, alone or in conjunction with other drugs or therapy. A therapeutically effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route.

The agent can be administered *in vivo* or *ex vivo*, alone or co-administered in conjunction with other drugs or therapy, i.e., a cocktail therapy. As used herein, the term "co-administration" or “co-administered” refers to the administration of at least two agent(s) or therapies to a subject. For example, in the treatment of tumors, particularly vascularized, malignant tumors, the agents can be used alone or in combination with, e.g., chemotherapeutic, radiotherapeutic, apoptopic, anti-angiogenic agents and/or immunotoxins or coaguligands.

In some embodiments, the co-administration of two or more agents/therapies is concurrent. In other embodiments, a first agent/therapy is administered prior to a second agent/therapy. Those of skill in the art understand that the formulations and/or routes of administration of the various agents/therapies used may vary.

In an *in vivo* approach, a compound or agent is administered to a subject. Generally, the compound is suspended in a pharmaceutically-acceptable carrier (such as, for example, but not limited to, physiological saline) and administered orally or by intravenous infusion, or injected or implanted subcutaneously, intramuscularly, intrathecally, intraperitoneally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, or intrapulmonarily.

The dosage required depends on the choice of the route of administration; the nature of the formulation; the nature of the patient's illness; the subject's size, weight, surface area, age, and sex; other drugs being administered; and the judgment of the attending physician. Suitable dosages are in the range of 0.01-100 mg/kg. Variations in the needed dosage are to be expected in view of the variety of compounds available and the different efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by i.v. injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art. Encapsulation of the compound

in a suitable delivery vehicle (e.g., polymeric microparticles or implantable devices) can increase the efficiency of delivery, particularly for oral delivery.

Diagnosis

The described invention also provides diagnosis kits and methods. A subject having cancer cells or a cells prone to tumorigenesis can be diagnosed based on the expression or activity of one or more of the above-described genes or polypeptides in a test sample from the subject. The polypeptide and nucleic acids can be used as markers to indicate the presence or absence of a cancer cell or cell prone to tumorigenesis. Diagnostic and prognostic assays of the described invention include methods for assessing the expression level of the polypeptide or nucleic acid.

The presence, level, or absence of the polypeptide or nucleic acid in a test sample can be evaluated by obtaining a test sample from a test subject and contacting the test sample with a compound or an agent capable of detecting the polypeptide or nucleic acid (e.g., mRNA probe, genomic cDNA probe, or cDNA probe). The "test sample" can include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. The level of expression of the gene can be measured in a number of ways, including, but not limited to, measuring the mRNA encoded by the gene; measuring the amount of polypeptide encoded by the gene; or measuring the activity of polypeptide encoded by the gene.

The level of mRNA corresponding to the gene in a cell can be determined both by *in situ* and by *in vitro* formats. Messenger RNA isolated from a test sample can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, PCR analyses, and probe arrays. For example, one diagnostic method for the detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid probe that can hybridize to the mRNA encoded by the gene. The probe can be a full-length nucleic acid, or a portion thereof, such as an oligonucleotide of at least 10 nucleotides in length and sufficient to specifically hybridize under stringent conditions to mRNA or genomic DNA.

In one format, mRNA (or cDNA prepared from it) is immobilized on a surface and contacted with the probes, for example, by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In another format, the probes are immobilized on a surface and the mRNA (or cDNA) is contacted

with the probes, for example, in a gene chip array. A skilled artisan can adapt known mRNA detection methods for detecting the level of mRNA.

The level of mRNA (or cDNA prepared from it) in a sample encoded by one or more of the above-mentioned genes can be evaluated with nucleic acid amplification, e.g., by standard PCR (U.S. Patent No. 4,683,202), RT-PCR (Bustin S. J Mol Endocrinol. 25:169-93, 2000), quantitative PCR (Ong Y. *et al.*, Hematology. 7:59-67, 2002), real time PCR (Ginzinger D. Exp Hematol. 30:503-12, 2002), and *in situ* PCR (Thaker V. Methods Mol Biol. 115:379-402, 1999), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques known in the art. As used herein, "amplification primers" are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule having the nucleotide sequence flanked by the primers.

For *in situ* methods, a cell or tissue sample can be prepared and immobilized on a support, such as, but not limited to, a glass slide, and then contacted with a probe that can hybridize to genomic DNA on chromosomes or mRNA that encodes the corresponding polypeptide.

In another embodiment, the methods of the described invention further include contacting a control sample with a compound or agent capable of detecting mRNA, or genomic DNA, and comparing the presence of mRNA or genomic DNA in the control sample with the presence of mRNA or genomic DNA in the test sample.

The above-described nucleic acid-based diagnostic methods can provide qualitative and quantitative information to determine whether a subject has or is predisposed to a disease associated with aberrant gene expression and aberrant angiogenesis, e.g., cancers.

A variety of methods can be used to determine the level of one or more of the above-mentioned polypeptide. In general, these methods include contacting an agent that selectively binds to the polypeptide, such as an antibody, to evaluate the level of polypeptide in a sample. Antibodies can be polyclonal, or monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) also can be used. In another embodiment, the antibody bears a detectable label. The term "labeled", with regard to the

probe or antibody, is intended to encompass direct labeling of the probe or antibody by physically linking a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with a detectable substance. For example, an antibody with a rabbit Fc region can be indirectly labeled using a second antibody directed against the rabbit Fc region, wherein the second antibody is coupled to a detectable substance. Examples of detectable substances are provided herein. Appropriate detectable substance or labels include, but are not limited to, radio isotopes (for example, but not limited to, ^{125}I, ^{131}I, ^{35}S, ^{3}H, or ^{32}P), enzymes (for example, but not limited to, alkaline phosphatase, horseradish peroxidase, luciferase, or β-glactosidase), fluorescent moieties or proteins (for example, but not limited to, fluorescein, rhodamine, phycoerythrin, GFP, or BFP), or luminescent moieties (for example, but not limited to, Qdot™ nanoparticles by the Quantum Dot Corporation, Palo Alto, CA).

The detection methods can be used to detect one or more of the above-mentioned polypeptide in a biological sample *in vitro* as well as *in vivo*. *In vitro* techniques for detection of the polypeptide include ELISAs, immuno-precipitations, immunofluorescence, EIA, RIA, and Western blotting analysis. *In vivo* techniques for detection of the polypeptide include introducing into a subject a labeled antibody. For example, the antibody can be labeled with a detectable substance as described above. The presence and location of the detectable substance in a subject can be detected by standard imaging techniques.

The diagnostic methods described herein can identify subjects having, or at risk of developing, a disease or disorder associated with aberrant expression or activity of one or more of the above-mentioned polypeptides. As described herein, examples of such a disease or disorder include those described above.

The prognostic assays described herein can be used to determine whether a subject is suitable to be administered with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disorder, such as cancer. For example, such assays can be used to determine whether a subject can be administered with a cytotoxic drug to treat the disorder.

Information obtained from practice of the above assays is useful in prognostication, identifying progression of, and clinical management of diseases and other deleterious conditions affecting an individual's health status. In some embodiments, the

foregoing diagnostic assays provide information useful in prognostication, identifying progression of and management of malignancies (cancers) that are characterized by abnormal, pathological angiogenesis. The information more specifically assists the clinician in designing chemotherapeutic or other treatment regimes to eradicate such malignancies from the body of an afflicted mammal, e.g., a human.

EXAMPLE 1: Methods and Materials

This example describes general methods and materials used in Examples 2-8.

Cell Culture

All cell lines were propagated as described in Tavazoie, S. F. *et al.*, *Nature* 451 (7175), 147 (2008). 293T cells were cultured with DMEM media supplemented with 10% FBS; H29 cells were cultured with DMEM media supplemented with 10% FBS, 20 ng/mL doxycycline, 2 μg/mL puromycin, and 0.3 mg/mL G418; and HUVEC cells were cultured with EGM-2 media (CC-3162, Lonza, Basel, Switzerland). The MDA-MB-231 and CN34 breast cancer cell line and its metastatic derivatives LM2, BoM2 and Lm1a are described in Minn, A. J. *et al., Nature* 436 (7050), 518 (2005).

Animal Studies

All animal work was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University. Age-matched female NOD/SCID mice (6-8 week old) were used for both orthotopic mammary fat pad tumor initiation assays (Minn, A. J. *et al., Nature* 436 (7050), 518 (2005) and for lung metastasis assays (Tavazoie, S. F. *et al.*, *Nature* 451 (7175), 147 (2008)). Eight-week old age-matched female athymic mice were used for systemic metastasis assays (Kang, Y. *et al.*, *Cancer Cell* 3 (6), 537 (2003) and Yin, J. J. *et al.*, *J Clin Invest* 103 (2), 197 (1999)).

Inducible miR-126 expression was obtained by cloning pre-miR-126 into the tet-ON containing pTripz vector (Thermo Scientific, Huntsville, AL). At day 3, 2 mg/ml doxycycline (Sigma Aldrich) was added to the drinking water containing 5% sucrose. Control mice were given drinking water with 5% sucrose.

Generation of Lentivirus, Retrovirus, Knockdown and Over-expressing Cells

For generation of lentivirus, $1x10^6$ 293T cells were seeded onto a 10 cm plate and incubated for 24h. Twelve micrograms of vector K (Gag/Pol), 6 μg of vector A (Env) and 12 μg of the appropriate shRNA plasmid were then co-transfected into the 293T cells

using 40 μL of TRANSIT®-293 transfection reagent (MIR 2700, MIRUS BIO LLC, Madison, WI). After 16h, the media was replaced with fresh antibiotic-free DMEM supplemented with 10% FBS. After another 24h, the virus was harvested by spinning for 5 min at 1,500g before being filtered through a 0.45 μm filter. For generation of retrovirus, H29 cells were seeded onto a 10-cm plate and allowed to grow to 90% confluence. Ten micrograms of the appropriate plasmid was then transfected into H29 cells using 60 μl of LIPOFECTAMINE™ 2000 transfection reagent (11668-019, INVITROGEN by LIFE TECHNOLOGIES, Carlsbad, CA). After 16h, the media were replaced with fresh antibiotic-free DMEM supplemented with 10% FBS. After another 48h, the virus was harvested by spinning for 5 min at 1,500g and filtered through a 0.45 μm filter. Two milliliters of the appropriate virus was used to transduce 50K cancer cells in the presence of 10 μg/mL of polybrene (TR-1003-G, MILLIPORE, Billerica, A). After 24h, the media was changed to DMEM supplemented with 10% FBS and 2 μg/mL puromycin (lentivirus) or 10 μg/mL blasticidin for selection. After another 72h, the cells were washed and allowed to grow in D10F and tested for knock down of the gene of interest by qPCR.

Endothelial Recruitment

Cancer cells (25,000) were seeded into 24-well plates approximately 24h before the start of the recruitment assay. HUVEC cells were serum starved in EGM-2 media supplemented with 0.2% FBS for 24 hours. The HUVEC cells were then labeled with CELLTRACKER Red CMTPX dye (C34552, INVITROGEN) for 45 min and rescued in EGM-2 media supplemented with 2% FBS for 30 min. Meanwhile, cancer cells were washed with PBS and 1 mL 0.2% FBS EGM-2 media was added to each well. Each well was then fitted with a 3.0 μm HTS FLUROBLOCK Insert (351151, BD FALCON, San Jose, CA). For antibody experiments, the appropriate concentration of each antibody was then added to each well: 50 ng/mL anti-IGFBP2 (AF674, R&D SYSTEMS, Minneapolis, MN), 20 μg/mL anti-IGF-1 (AF-291-NA, R&D SYSTEMS), 40 μg/mL anti-IGF-2 (MAB292, R&D SYSTEMS), 20 μg/mL anti-IGF1R (MAB391, R&D SYSTEMS), 5 μg/mL anti-IGF2R (AF2447, R&D Systems) and anti-IgG (AB-108-C, R&D SYSTEMS). For endothelial recruitment assays that require pre-incubation with antibodies, either HUVEC cells or cancer cells were then incubated with 20 μg/mL anti-IGF1R or control IgG antibody for 1h and washed once with PBS. The HUVEC cells were then serum starved for 1h before being resuspended 0.2% FBS EGM-2 at 100K HUVECs per mL.

The resuspension (0.5 mL) was then added into each FLUOROBLOCK insert and the recruitment assay was allowed to proceed for 16h. After completion of the assay, FLUOROBLOCK inserts were fixed with 4% paraformaldehyde for 15 min and mounted onto slides with VECTASHIELD mounting media (H-1000, VECTOR LABORATORIES, Burlingame, CA). Three images of each insert were taken and the images were analyzed using IMAGEJ (NIH).

Chemotaxis Assay

Matrigel (250 µl, BD BIOSCIENCES, #356231) containing given amounts of bovine serum albumin (A2153, Sigma Aldrich), rhIGFBP2 (674-B2, R&D Systems), rhGas6 (885-GS, R&D Systems), anti-IGF1R (MAB391, R&D Systems) and MerFc (891-MR-100, R&D Systems) were allowed to solidify at the bottom of a 24 well plate for 30 min before 250 µl HUVEC media containing 0.2% FBS were added. A 3.0-µm HTS Fluroblock Insert (351151, BD Falcon) was then placed in each well. HUVEC cells were labeled with CellTracker Red CMTPX dye (C34552, Invitrogen) before resuspending 300K HUVECs per mL of 0.2% FBS EGM-2. 0.5 mL of the resuspension was then added into each Fluoroblock insert and the assay allowed proceeding for 20h. The inserts were then fixed for 15 min in 4% paraformaldehyde and mounted onto slides with VectaShield mounting media (H-1000, Vector Laboratories). 5 fields of the basal side of each insert were then imaged and the images were analyzed using ImageJ (NIH).

Migration Assay

HUVEC cells were grown to 90% confluence and stimulated in the given concentrations of bovine serum albumin (Sigma Aldrich, #A2153), rhIGFBP2 (674-B2, R&D Systems) and anti-IGF1R (MAB391, R&D Systems) in HUVEC media containing 0.2% FBS for 40 min at 37°C. The cells were then trypsinized and 50K cells were added into HTS Fluroblock Inserts (351151, BD Falcon). After 24 hours in 37°C with 5% CO_2, the inserts were removed, the membrane excised and fixed in 4% paraformaldehyde. HUVEC cells that had migrated to the basal side of the membrane were visualized with DAPI and counted in 5 fields per membrane using Image J (NIH).

Endothelial Adhesion

HUVEC cells were seeded on a 6-cm plate and allowed to grow to confluence. Cancer cells were serum starved in DMEM media supplemented with 0.2% FBS for 30 min, labeled with CELLTRACKER Green CMFDA dye (C7025, Invitrogen) for 45 min

and incubated in DMEM media supplemented with 10% FBS for 30 min. Cancer cells were then trypsinized and resuspended in 10% FBS/DMEM to 10K cells/mL. Five milliliters of the resuspension was then added to each plate of HUVECs and the plate was incubated at 37°C for 10 min. The plates were then washed gently with PBS and fixed with 4% paraformaldehyde for 15 min. Each plate was then treated with 1 mL of PBS and 6 images were taken from each plate. The number of cancer cells adherent to the HUVEC cells were then quantified using IMAGEJ.

Endothelial Proliferation

Cancer cells ($1x10^6$) were seeded to a 10-cm plate and allowed to grow for 24h. The cancer cells were then washed gently with PBS and EGM-2 media supplemented with 2% FBS was added to each plate. The conditioned EGM-2 media was collected after 24h. HUVEC cells (25K) were seeded in triplicate in a 6-well plate and allowed to grow for 16h. The HUVEC cells were then washed gently with PBS and 3 mL conditioned EGM-2 media was added to each well. After 48h, the conditioned media was replaced with another 3 mL of conditioned media. After another 48h, the cells were trypsinized and counted using a haemocytometer.

Tube Formation Assay

Tube formation assay was performed according to manufacturer's protocol (354149, BD BIOCOAT™ ANGIOGENESIS SYSTEM – Endothelial Cell Tube Formation). Briefly, HUVEC cells were serum starved in EGM-2 media supplemented with 0.2% FBS for 24 hours. The HUVEC cells were then labeled with CELLTRACKER Red CMTPX dye (C34552, INVITROGEN) for 45 min and subsequently treated in EGM-2 media supplemented with 2% FBS for 30 min. Meanwhile, the tube formation assay plate, which was in 96-well format, was incubated at 37°C for 30 min. The cancer cells and HUVEC cells were trypsinized and resuspended at 400K/mL and 800K/mL respectively in EGM-2 media supplemented with 2% FBS. The cancer cell and HUVEC cell suspensions were then mixed at a 1:1 ratio and 50 µl of each mixture was seeded into each well of the tube formation assay plate. The assay plate was incubated at 37°C for 16h. Images of each well were taken and the images were processed using METAMORPH analysis software (MOLECULAR DEVICES, Inc.) to obtain the number of branch points per image.

Analysis of miRNA and mRNA Expression

Total RNA was extracted from various cell lines using the MIRVANA kit (AM1560, APPLIED BIOSYSTEMS, Austin, TX). TAQMAN microRNA assay (4427975-0002228, APPLIED BIOSYSTEMS) was used to quantify expression levels of mature miRNA as described in Tavazoie, S. F. *et al.*, *Nature* 451 (7175), 147 (2008). For quantification of mRNA, 400 ng of total RNA were reverse transcribed using the cDNA First-Strand Synthesis Kit (18080-051, INVITROGEN). Approximately 4 ng of the resulting cDNA was then mixed with SYBR green PCR MASTER MIX (4309155, APPLIED BIOSYSTEMS) and appropriate primers (Table 1). Quantitative mRNA expression data was obtained using an ABI PRISM 7900HT Real-Time PCR System (APPLIED BIOSYSTEMS). Smad4 was used as an endogenous control for normalization. Expression analysis of human breast cancers at various disease stages was performed using the TISSUESCAN qPCR Array Breast Cancer Panels 2 and 3 (BCRT102 & BCRT103, ORIGENE, Rockville, MD).

Table 1 SYBR green qPCR primers

Gene	Forward	Reverse
ABCB9	GACCTTCACCTACCGCACTC	CACAGGAGCTCTTCCCACTG
BEX2	GCCCCGAAAGTAGGAAGC	CTCCATTACTCCTGGGCCTAT
BGLAP	GGCGCTACCTGTATCAATGG	TCAGCCAACTCGTCACAGTC
CA12	CCAAGGCTACAATCTGTCTGC	GGGCAGGTTCAGCTTCACT
GDF15	CCGGATACTCACGCCAGA	AGAGATACGCAGGTGCAGGT
GEM	GACAGCATGGACAGCGACT	AACCATCAGGGTTCGTTCAT
IGFBP2	CCAAGAAGCTGCGACCAC	GGGATGTGCAGGGAGTAGAG
ITGB4	TCAGCCTCTCTGGGACCTT	TATCCACACGGACACACTCC
KIAA0746	GTTGTCTGTGCAGATGTACGC	TAGCAGGGCCAGGTTAAAAA
KLF4	GCCGCTCCATTACCAAGA	TCTTCCCCTCTTTGGCTTG
MARS	AACAACCTGGGCAACTTCAT	ACCATCTCAGGCACATAGCC
MERTK	GGAGACAGGACCAAAGC	GGGCAATATCCACCATGAAC
PADI4	AAGTGCAAGCTGACCATCTG	GCCGATCTCCATTTCATCC
PHGDH	TGGTGGAAAAGCAGAACCTT	AACAATAAGGCCTTCACAGTCC
PITPNC1	GCGCTACTACAAAGAATCTGAGG	GAGCACATGATAGGCTGATGAC
PSAT1	TCTTGTGCGGGAATTGCTA	AAGGGGACAGCACTGAACTG
PYGB	TCCAGGGTCCTGTATCCAAA	CCACGAAGTACTCCTGCTTCA
RGC32	TGCTGATCTTGACAAAACTTTAGC	GCAGGTCCTCGGAACTTTCT
SHMT2	GAGGGAGAAGGACAGGCAGT	CTCGGCTGCAGAAGTTCTCT

SMAD4	TGGCCCAGGATCAGTAGGT	CATCAACACCAATTCCAGCA
THBD	AATTGGGAGCTTGGGAATG	TGAGGACCTGATTAAGGCTAGG
TNFSF4	GTATCCTCGAATTCAAAGTATCAAAGT	CTGAGTTGTTCTGCACCTTCA
VIPR1	CTGTCCCCTCATCTTCAAGC	CAGCTGCGGCTTACATTG

miR-126 Target Prediction

Potential miR-126 targets were identified by using 3 sets of microarray profiles: LM2 control cells relative to LM2 cells over-expressing miR-126 (GSE No. 23905) and 2 replicate arrays of MDA and LM2 cells (GSE No. 23904 and Minn, A. J. *et al., Nature* 436 (7050), 518 (2005). With these arrays, the following criteria were used to identify possible miR-126 targets genes: (1) Genes down-regulated more than 1.6 fold upon miR-126 over-expression in LM2 cells and (2) Genes up-regulated by more than 1.4 fold in one of the two LM2 versus MDA arrays. All potential targets were subsequently verified by qPCR.

Luciferase Reporter Assay

Luciferase reporter assay was performed as described in Tavazoie, S. F. *et al.*, *Nature* 451 (7175), 147 (2008). Briefly the full-length 3'UTR's and CDS's of ABCB9, IGFBP2, MERTK, PITPNC1, PSAT1, PYGB, SHMT2 and VIPR1 were cloned into the psiCheck2 dual luciferase reporter vector (C8021, PROMEGA, Madison, WI). Listed below are the sequences of the CDS's and 3'UTR's.

ABCB9 CDS

ATGCGGCTGTGGAAGGCGGTGGTGGTGACTTTGGCCTTCATGAGTGTGGACATCTGCGTGACCACG
GCCATCTATGTCTTCAGCCACCTGGACCGCAGCCTCCTGGAGGACATCCGCCACTTCAACATCTTT
GACTCGGTGCTGGATCTCTGGGCAGCCTGCCTGTACCGCAGCTGCCTGCTGCTGGGAGCCACCATT
GGTGTGGCCAAGAACAGTGCGCTGGGGCCCCGGCGGCTGCGGGCCTCGTGGCTGGTCATCACCCTC
GTGTGCCTCTTCGTGGGCATCTATGCCATGGTGAAGCTGCTGCTCTTCTCAGAGGTGCGCAGGCCC
ATCCGGGACCCCTGGTTTTGGGCCCTGTTCGTGTGGACGTACATTTCACTCGGCGCATCCTTCCTG
CTCTGGTGGCTGCTGTCCACCGTGCGGCCAGGCACCCAGGCCCTGGAGCCAGGGGCGGCCACCGAG
GCTGAGGGCTTCCCTGGGAGCGGCCGGCCACCGCCCGAGCAGGCGTCTGGGGCCACGCTGCAGAAG
CTGCTCTCCTACACCAAGCCCGACGTGGCCTTCCTCGTGGCCGCCTCCTTCTTCCTCATCGTGGCA
GCTCTGGGAGAGACCTTCCTGCCCTACTACACGGGCCGCGCCATTGATGGCATCGTCATCCAGAAA
AGCATGGATCAGTTCAGCACGGCTGTCGTCATCGTGTGCCTGCTGGCCATTGGCAGCTCATTTGCC
GCAGGTATTCGGGGCGGCATTTTTACCCTCATATTTGCCAGACTGAACATTCGCCTTCGAAACTGT
CTCTTCCGCTCACTGGTGTCCCAGGAGACAAGCTTCTTTGATGAGAACCGCACAGGGGACCTCATC
TCCCGCCTGACCTCGGACACCACCATGGTCAGCGACCTGGTCTCCCAGAACATCAATGTCTTCCTG
CGGAACACAGTCAAGGTCACGGGCGTGGTGGTCTTCATGTTCAGCCTCTCATGGCAGCTCTCCTTG
GTCACCTTCATGGGCTTCCCCATCATCATGATGGTGTCCAACATCTACGGCAAGTACTACAAGAGG
CTCTCCAAAGAGGTCCAGAATGCCCTGGCCAGAGCGAGCAACACGGCGGAGGAGACCATCAGTGCC
ATGAAGACTGTCCGGAGCTTCGCCAATGAGGAGGAGGAGGCAGAGGTGTACCTGCGGAAGCTGCAG
CAGGTGTACAAGCTGAACAGGAAGGAGGCAGCTGCCTACATGTACTACGTCTGGGGCAGCGGGCTC
ACACTGCTGGTGGTCCAGGTCAGCATCCTCTACTACGGGGGCCACCTTGTCATCTCAGGCCAGATG
ACCAGCGGCAACCTCATCGCCTTCATCATCTACGAGTTTGTCCTGGGAGATTGTATGGAGTCCGTG
GGCTCCGTCTACAGTGGCCTGATGCAGGGAGTGGGGGCTGCTGAGAAGGTGTTCGAGTTCATCGAC

CGGCAGCCGACCATGGTGCACGATGGCAGCTTGGCCCCCGACCACCTGGAGGGCCGGGTGGACTTT
GAGAATGTGACCTTCACCTACCGCACTCGGCCCCACACCCAGGTCCTGCAGAATGTCTCCTTCAGC
CTGTCCCCCGGCAAGGTGACGGCCCTGGTGGGGCCCTCGGGCAGTGGGAAGAGCTCCTGTGTCAAC
ATCCTGGAGAACTTCTACCCCCTGGAGGGGGGCCGGGTGCTGCTGGACGGCAAGCCCATCAGCGCC
TACGACCACAAGTACTTGCACCGTGTGATCTCCCTGGTGAGCCAGGAGCCCGTGCTGTTCGCCCGC
TCCATCACGGATAACATCTCCTACGGCCTGCCCACTGTGCCTTTCGAGATGGTGGTGGAGGCCGCA
CAGAAGGCCAATGCCCACGGCTTCATCATGGAACTCCAGGACGGCTACAGCACAGAGACAGGGGAG
AAGGGCGCCCAGCTGTCAGGTGGCCAGAAGCAGCGGGTGGCCATGGCCCGGGCTCTGGTGCGGAAC
CCCCCAGTCCTCATCCTGGATGAAGCCACCAGCGCTTTGGATGCCGAGAGCGAGTATCTGATCCAG
CAGGCCATCCATGGCAACCTGCAGAAGCACACGGTACTCATCATCGCGCACCGGCTGAGCACCGTG
GAGCACGCGCACCTCATTGTGGTGCTGGACAAGGGCCGCGTAGTGCAGCAGGGCACCCACCAGCAG
CTGCTGGCCCAGGGCGGCCTCTACGCCAAGCTGGTGCAGCGGCAGATGCTGGGGCTTCAGCCCGCC
GCAGACTTCACAGCTGGCCACAACGAGCCTGTAGCCAACGGCAGTCACAAGGCCTGA

ABCB9 3'UTR

TGGGGGGCCCCTGCTTCTCCCGGTGGGGCAGAGGACCCGGTGCCTGCCTGGCAGATGTGCCCACGG
AGGCCCCCAGCTGCCCTCCGAGCCCAGGCCTGCAGCACTGAAAGACGACCTGCCATGTCCCATGGA
TCACCGCTTCCTGCATCTTGCCCCTGGTCCCTGCCCCATTCCCAGGGCACTCCTTACCCCTGCTGC
CCTGAGCCAACGCCTTCACGGACCTCCCTAGCCTCCTAAGCAAAGGTAGAGCTGCCTTTTTAAACC
TAGGTCTTACCAGGGTTTTTACTGTTTGGTTTGAGGCACCCCAGTCAACTCCTAGATTTCAAAAAC
CTTTTTCTAATTGGGAGTAATGGCGGGCACTTTCACCAAGATGTTCTAGAAACTTCTGAGCCAGGA
GTGAATGGCCCTTCCTTAGTAGCCTGGGGGATGTCCAGAGACTAGGCCTCTCCCCTTTACCCCTCC
AGAGAAGGGGCTTCCCTGTCCCGGAGGGAGACACGGGGAACGGGATTTTCCGTCTCTCCCTCTTGC
CAGCTCTGTGAGTCTGGCCAGGGCGGGTAGGGAGCGTGGAGGGCATCTGTCTGCCATCGCCCGCTG
CCAATCTAAGCCAGTCTCACTGTGAACCACACGAAACCTCAACTGGGGGAGTGAGGGGCTGGCCAG
GTCTGGAGGGGCCTCAGGGGTGCCCCCAGCCCGGCACCCAGCGCTTTCGCCCCTCGTCCACCCACC
CCTGGCTGGCAGCCTCCCTCCCCACACCCGCCCCTGTGCTCTGCTGTCTGGAGGCCACGTGGATGT
TCATGAGATGCATTCTCTTCTGTCTTTGGTGGATGGGATGGTGGCAAAGCCCAGGATCTGGCTTTG
CCAGAGGTTGCAACATGTTGAGAGAACCCGGTCAATAAAGTGTACTACCTCTTACCCCTAA

IGFBP2 CDS

ATGCTGCCGAGAGTGGGCTGCCCCGCGCTGCCGCTGCCGCCGCCGCCGCTGCTGCCGCTGCTGCTG
CTGCTACTGGGCGCGAGTGGCGGCGGCGGCGGGGCGCGCGCGGAGGTGCTGTTCCGCTGCCCGCCC
TGCACACCCGAGCGCCTGGCCGCCTGCGGGCCCCCGCCGGTTGCGCCGCCCGCCGCGGTGGCCGCA
GTGGCCGGAGGCGCCCGCATGCCATGCGCGGAGCTCGTCCGGGAGCCGGGCTGCGGCTGCTGCTCG
GTGTGCGCCCGGCTGGAGGGCGAGGCGTGCGGCGTCTACACCCCGCGCTGCGGCCAGGGGCTGCGC
TGCTATCCCCACCCGGGCTCCGAGCTGCCCCTGCAGGCGCTGGTCATGGGCGAGGGCACTTGTGAG
AAGCGCCGGGACGCCGAGTATGGCGCCAGCCCGGAGCAGGTTGCAGACAATGGCGATGACCACTCA
GAAGGAGGCCTGGTGGAGAACCACGTGGACAGCACCATGAACATGTTGGGCGGGGGAGGCAGTGCT
GGCCGGAAGCCCCTCAAGTCGGGTATGAAGGAGCTGGCCGTGTTCCGGGAGAAGGTCACTGAGCAG
CACCGGCAGATGGGCAAGGGTGGCAAGCATCACCTTGGCCTGGAGGAGCCCAAGAAGCTGCGACCA
CCCCCTGCCAGGACTCCCTGCCAACAGGAACTGGACCAGGTCCTGGAGCGGATCTCCACCATGCGC
CTTCCGGATGAGCGGGGCCCTCTGGAGCACCTCTACTCCCTGCACATCCCCAACTGTGACAAGCAT
GGCCTGTACAACCTCAAACAGTGCAAGATGTCTCTGAACGGGCAGCGTGGGGAGTGCTGGTGTGTG
AACCCCAACACCGGGAAGCTGATCCAGGGAGCCCCCACCATCCGGGGGGACCCCGAGTGTCATCTC
TTCTACAATGAGCAGCAGGAGGCTCGCGGGGTGCACACCCAGCGGATGCAGTAG

IGFBP2 3'UTR

ACCGCAGCCAGCCGGTGCCTGGCGCCCCTGCCCCCCGCCCCTCTCCAAACACCGGCAGAAAACGGA
GAGTGCTTGGGTGGTGGGTGCTGGAGGATTTTCCAGTTCTGACACACGTATTTATATTTGGAAAGA
GACCAGCACCGAGCTCGGCACCTCCCCGGCCTCTCTCTTCCCAGCTGCAGATGCCACACCTGCTCC
TTCTTGCTTTCCCCGGGGGAGGAAGGGGGTTGTGGTCGGGGAGCTGGGGTACAGGTTTGGGGAGGG
GGAAGAGAAATTTTTATTTTTGAACCCCTGTGTCCCTTTTGCATAAGATTAAAGGAAGGAAAAGTA
A

MERTK CDS

ATGGGGCCGGCCCCGCTGCCGCTGCTGCTGGGCCTCTTCCTCCCCGCGCTCTGGCGTAGAGCTATC
ACTGAGGCAAGGGAAGAAGCCAAGCCTTACCCGCTATTCCCGGGACCTTTTCCAGGGAGCCTGCAA

ACTGACCACACACCGCTGTTATCCCTTCCTCACGCCAGTGGGTACCAGCCTGCCTTGATGTTTTCA
CCAACCCAGCCTGGAAGACCACATACAGGAAACGTAGCCATTCCCCAGGTGACCTCTGTCGAATCA
AAGCCCCTACCGCCTCTTGCCTTCAAACACACAGTTGGACACATAATACTTTCTGAACATAAAGGT
GTCAAATTTAATTGCTCAATCAGTGTACCTAATATATACCAGGACACCACAATTTCTTGGTGGAAA
GATGGGAAGGAATTGCTTGGGGCACATCATGCAATTACACAGTTTTATCCAGATGATGAAGTTACA
GCAATAATCGCTTCCTTCAGCATAACCAGTGTGCAGCGTTCAGACAATGGGTCGTATATCTGTAAG
ATGAAAATAAACAATGAAGAGATCGTGTCTGATCCCATCTACATCGAAGTACAAGGACTTCCTCAC
TTTACTAAGCAGCCTGAGAGCATGAATGTCACCAGAAACACAGCCTTCAACCTCACCTGTCAGGCT
GTGGGCCCGCCTGAGCCCGTCAACATTTTCTGGGTTCAAAACAGTAGCCGTGTTAACGAACAGCCT
GAAAAATCCCCCTCCGTGCTAACTGTTCCAGGCCTGACGGAGATGGCGGTCTTCAGTTGTGAGGCC
CACAATGACAAAGGGCTGACCGTGTCCAAGGGAGTGCAGATCAACATCAAAGCAATTCCCTCCCCA
CCAACTGAAGTCAGCATCCGTAACAGCACTGCACACAGCATTCTGATCTCCTGGGTTCCTGGTTTT
GATGGATACTCCCCGTTCAGGAATTGCAGCATTCAGGTCAAGGAAGCTGATCCGCTGAGTAATGGC
TCAGTCATGATTTTTAACACCTCTGCCTTACCACATCTGTACCAAATCAAGCAGCTGCAAGCCCTG
GCTAATTACAGCATTGGTGTTTCCTGCATGAATGAAATAGGCTGGTCTGCAGTGAGCCCTTGGATT
CTAGCCAGCACGACTGAAGGAGCCCCATCAGTAGCACCTTTAAATGTCACTGTGTTTCTGAATGAA
TCTAGTGATAATGTGGACATCAGATGGATGAAGCCTCCGACTAAGCAGCAGGATGGAGAACTGGTG
GGCTACCGGATATCCCACGTGTGGCAGAGTGCAGGGATTTCCAAAGAGCTCTTGGAGGAAGTTGGC
CAGAATGGCAGCCGAGCTCGGATCTCTGTTCAAGTCCACAATGCTACGTGCACAGTGAGGATTGCA
GCCGTCACCAGAGGGGGAGTTGGGCCCTTCAGTGATCCAGTGAAAATATTTATCCCTGCACACGGT
TGGGTAGATTATGCCCCCTCTTCAACTCCGGCGCCTGGCAACGCAGATCCTGTGCTCATCATCTTT
GGCTGCTTTTGTGGATTTATTTTGATTGGGTTGATTTTATACATCTCCTTGGCCATCAGAAAAAGA
GTCCAGGAGACAAAGTTTGGGAATGCATTCACAGAGGAGGATTCTGAATTAGTGGTGAATTATATA
GCAAAGAAATCCTTCTGTCGGCGAGCCATTGAACTTACCTTACATAGCTTGGGAGTCAGTGAGGAA
CTACAAAATAAACTAGAAGATGTTGTGATTGACAGGAATCTTCTAATTCTTGGAAAAATTCTGGGT
GAAGGAGAGTTTGGGTCTGTAATGGAAGGAAATCTTAAGCAGGAAGATGGGACCTCTCTGAAAGTG
GCAGTGAAGACCATGAAGTTGGACAACTCTTCACAGCGGGAGATCGAGGAGTTTCTCAGTGAGGCA
GCGTGCATGAAAGACTTCAGCCACCCAAATGTCATTCGACTTCTAGGTGTGTGTATAGAAATGAGC
TCTCAAGGCATCCCAAAGCCCATGGTAATTTTACCCTTCATGAAATACGGGGACCTGCATACTTAC
TTACTTTATTCCCGATTGGAGACAGGACCAAAGCATATTCCTCTGCAGACACTATTGAAGTTCATG
GTGGATATTGCCCTGGGAATGGAGTATCTGAGCAACAGGAATTTTCTTCATCGAGATTTAGCTGCT
CGAAACTGCATGTTGCGAGATGACATGACTGTCTGTGTTGCGGACTTCGGCCTCTCTAAGAAGATT
TACAGTGGCGATTATTACCGCCAAGGCCGCATTGCTAAGATGCCTGTTAAATGGATCGCCATAGAA
AGTCTTGCAGACCGAGTCTACACAAGTAAAAGTGATGTGTGGGCATTTGGCGTGACCATGTGGGAA
ATAGCTACGCGGGGAATGACTCCCTATCCTGGGGTCCAGAACCATGAGATGTATGACTATCTTCTC
CATGGCCACAGGTTGAAGCAGCCCGAAGACTGCCTGGATGAACTGTATGAAATAATGTACTCTTGC
TGGAGAACCGATCCCTTAGACCGCCCCACCTTTTCAGTATTGAGGCTGCAGCTAGAAAAACTCTTA
GAAAGTTTGCCTGACGTTCGGAACCAAGCAGACGTTATTTACGTCAATACACAGTTGCTGGAGAGC
TCTGAGGGCCTGGCCCAGGGCTCCACCCTTGCTCCACTGGACTTGAACATCGACCCTGACTCTATA
ATTGCCTCCTGCACTCCCCGCGCTGCCATCAGTGTGGTCACAGCAGAAGTTCATGACAGCAAACCT
CATGAAGGACGGTACATCCTGAATGGGGGCAGTGAGGAATGGGAAGATCTGACTTCTGCCCCCTCT
GCTGCAGTCACAGCTGAAAAGAACAGTGTTTTACCGGGGGAGAGACTTGTTAGGAATGGGGTCTCC
TGGTCCCATTCGAGCATGCTGCCCTTGGGAAGCTCATTGCCCGATGAACTTTTGTTTGCTGACGAC
TCCTCAGAAGGCTCAGAAGTCCTGATGTGA

MERTK 3'UTR

GGAGAGGTGCGGGGAGACATTCCAAAAATCAAGCCAATTCTTCTGCTGTAGGAGAATCCAATTGTA
CCTGATGTTTTTGGTATTTGTCTTCCTTACCAAGTGAACTCCATGGCCCCAAAGCACCAGATGAAT
GTTGTTAAGTAAGCTGTCATTAAAAATACATAATATATATTTATTTAAAGAGAAAAAATATGTGTA
TATCATGGAAAAAGACAAGGATATTTTAATAAAACATTACTTATTTCATTTCACTTATCTTGCATA
TCTTAAAATTAAGCTTCAGCTGCTCCTTGATATTAACATTTGTACAGAGTTGAAGTTGTTTTTTCA
AGTTCTTTTCTTTTTCATGACTATTAAATGTAAAAATATTTGTAAAATGAAATGCCATATTTGACT
TGGCTTCTGGTCTTGATGTATTTGATAAGAATGATTCATTCAATGTTTAAAGTTGTATAACTGATT
AATTTTCTGATATGGCTTCCTAATAAAATATGAATAAGGAAG

PITPNC1 isoform A CDS

ATGCTGCTGA AAGAGTACCG GATCTGCATG CCGCTCACCG TAGACGAGTA CAAAATTGGA
CAGCTGTACA TGATCAGCAA ACACAGCCAT GAACAGAGTG ACCGGGGAGA AGGGGTGGAG

GTCGTCCAGA ATGAGCCCTT TGAGGACCCT CACCATGGCA ATGGGCAGTT CACCGAGAAG
CGGGTGTATC TCAACAGCAA ACTGCCTAGT TGGGCTAGAG CTGTTGTCCC CAAAATATTT
TATGTGACAG AGAAGGCTTG GAACTATTAT CCCTACACAA TTACAGAATA CACATGTTCC
TTTCTGCCGA AATTCTCCAT TCATATAGAA ACCAAGTATG AGGACAACAA AGGAAGCAAT
GACACCATTT TCGACAATGA AGCCAAAGAC GTGGAGAGAG AAGTTTGCTT TATTGATATT
GCCTGCGATG AAATTCCAGA GCGCTACTAC AAAGAATCTG AGGATCCTAA GCACTTCAAG
TCAGAGAAGA CAGGACGGGG ACAGTTGAGG GAAGGCTGGA GAGATAGTCA TCAGCCTATC
ATGTGCTCCT ACAAGCTGGT GACTGTGAAG TTTGAGGTCT GGGGGCTTCA GACCAGAGTG
GAACAATTTG TACACAAGGT GGTCCGAGAC ATTCTGCTGA TTGGACATAG ACAGGCTTTT
GCATGGGTTG ATGAGTGGTA TGACATGACA ATGGATGAAG TCCGAGAATT TGAACGAGCC
ACTCAGGAAG CCACCAACAA GAAAATCGGC ATTTTCCCAC CTGCAATTTC TATCTCCAGC
ATCCCCCTGC TGCCTTCTTC CGTCCGCAGT GCGCCTTCTA GTGCTCCATC CACCCCTCTC
TCCACAGACG CACCCGAATT TCTGTCCGTT CCCAAAGATC GGCCCCGGAA AAAGTCTGCC
CCAGAAACTC TCACACTTCC AGACCCTGAG AAAAAAGCCA CCCTGAATTT ACCCGGCATG
CACTCTTCAG ATAAGCCATG TCGGCCCAAA TCTGAGTAA

PITPNC1 isoform B CDS

ATGCTGCTGAAAGAGTACCGGATCTGCATGCCGCTCACCGTAGACGAGTACAAAATTGGACAGCTG
TACATGATCAGCAAACACAGCCATGAACAGAGTGACCGGGGAGAAGGGGTGGAGGTCGTCCAGAAT
GAGCCCTTTGAGGACCCTCACCATGGCAATGGGCAGTTCACCGAGAAGCGGGTGTATCTCAACAGC
AAACTGCCTAGTTGGGCTAGAGCTGTTGTCCCCAAAATATTTTATGTGACAGAGAAGGCTTGGAAC
TATTATCCCTACACAATTACAGAATACACATGTTCCTTTCTGCCGAAATTCTCCATTCATATAGAA
ACCAAGTATGAGGACAACAAAGGAAGCAATGACACCATTTTCGACAATGAAGCCAAAGACGTGGAG
AGAGAAGTTTGCTTTATTGATATTGCCTGCGATGAAATTCCAGAGCGCTACTACAAAGAATCTGAG
GATCCTAAGCACTTCAAGTCAGAGAAGACAGGACGGGGACAGTTGAGGGAAGGCTGGAGAGATAGT
CATCAGCCTATCATGTGCTCCTACAAGCTGGTGACTGTGAAGTTTGAGGTCTGGGGGCTTCAGACC
AGAGTGGAACAATTTGTACACAAGGTGGTCCGAGACATTCTGCTGATTGGACATAGACAGGCTTTT
GCATGGGTTGATGAGTGGTATGATATGACAATGGATGATGTTCGGGAATACGAGAAAAACATGCAT
GAACAAACCAACATAAAAGTTTGCAATCAGCATTCCTCCCCTGTGGATGACATAGAGAGTCATGCC
CAAACAAGTACATGA

PITPNC1 3'UTR

CAATGGATGAAGTCCGAGAATTTGAACGAGCCACTCAGGAAGCCACCAACAAGAAAATCGGCATTT
TCCCACCTGCAATTTCTATCTCCAGCATCCCCCTGCTGCCTTCTTCCGTCCGCAGTGCGCCTTCTA
GTGCTCCATCCACCCCTCTCTCCACAGACGCACCCGAATTTCTGTCCGTTCCCAAAGATCGGCCCC
GGAAAAAGTCTGCCCCAGAAACTCTCACACTTCCAGACCCTGAGAAAAAAGCCACCCTGAATTTAC
CCGGCATGCACTCTTCAGATAAGCCATGTCGGCCCAAATCTGAGTAACTTTATATAAATATCTCAT
GGGGTTTTATATTTTCATTTGTTGTTGTTGTTTTTTTTTAAGAATCTTCTGATAGAGAAAAAGACT
GCTTTGTCACTCAAACATGTTCCTTCGACCTTTCAGTGTGCATGTGACTCAGTAACTTCACATAGA
ATATGATTCCCTAAGTATGCTACACAGCATCATATTAGATGTAAGATGTAAGACTTGCAAAGGACA
GAAGGAATCTTCTGTAACCACATAGCTGTATGCCAGAGAGGAAGCCTTGTTATTGGGCATTTGATG
AGGTTTGGCATGGACTTCAAGGATAAATGAATGAAAACTTTGCACCACTTTTGTTACAAGGTACGG
TAGAAAATAGTGAAGTCAGTTTCCTCTCATCAAATCTAAAATTCTCCAAAATACTCTCAGGCATAA
CATACTTAGCTGTTAAATTTTGAACTGCTAATTACTAATACTTGAATACCAATAGTTACTGAGATT
CCTATTTTGTGGTTAGTCTGACTCAGGATTTGGAGCCTAATTAACTCTAAACTTTTGAAAATTTTA
ATCATCAAGCTATAGAGGCTCCAAGTGCAATTAATAATAACTCATTTATACCTTCCACAGAATTTA
ATAAAGATTCTACTTGTTTCTGTCTTTTAA

PSAT1 CDS

ATGGACGCCCCCAGGCAGGTGGTCAACTTTGGGCCTGGTCCCGCCAAGCTGCCGCACTCAGTGTTG
TTAGAGATACAAAAGGAATTATTAGACTACAAAGGAGTTGGCATTAGTGTTCTTGAAATGAGTCAC
AGGTCATCAGATTTTGCCAAGATTATTAACAATACAGAGAATCTTGTGCGGGAATTGCTAGCTGTT
CCAGACAACTATAAGGTGATTTTTCTGCAAGGAGGTGGGTGCGGCCAGTTCAGTGCTGTCCCCTTA
AACCTCATTGGCTTGAAAGCAGGAAGGTGTGCTGACTATGTGGTGACAGGAGCTTGGTCAGCTAAG
GCCGCAGAAGAAGCCAAGAAGTTTGGGACTATAAATATCGTTCACCCTAAACTTGGGAGTTATACA
AAAATTCCAGATCCAAGCACCTGGAACCTCAACCCAGATGCCTCCTACGTGTATTATTGCGCAAAT
GAGACGGTGCATGGTGTGGAGTTTGACTTTATACCCGATGTCAAGGGAGCAGTACTGGTTTGTGAC

ATGTCCTCAAACTTCCTGTCCAAGCCAGTGGATGTTTCCAAGTTTGGTGTGATTTTTGCTGGTGCC
CAGAAGAATGTTGGCTCTGCTGGGGTCACCGTGGTGATTGTCCGTGATGACCTGCTGGGGTTTGCC
CTCCGAGAGTGCCCCTCGGTCCTGGAATACAAGGTGCAGGCTGGAAACAGCTCCTTGTACAACACG
CCTCCATGTTTCAGCATCTACGTCATGGGCTTGGTTCTGGAGTGGATTAAAAACAATGGAGGTGCC
GCGGCCATGGAGAAGCTTAGCTCCATCAAATCTCAAACAATTTATGAGATTATTGATAATTCTCAA
GGATTCTACGTTTGTCCAGTGGAGCCCCAAAATAGAAGCAAGATGAATATTCCATTCCGCATTGGC
AATGCCAAAGGAGATGATGCTTTAGAAAAAAGATTTCTTGATAAAGCTCTTGAACTCAATATGTTG
TCCTTGAAAGGGCATAGGTCTGTGGGAGGCATCCGGGCCTCTCTGTATAATGCTGTCACAATTGAA
GACGTTCAGAAGCTGGCCGCCTTCATGAAAAAATTTTTGGAGATGCATCAGCTATGA

PSAT1 3'UTR

ACACATCCTAACCAGGATATACTCTGTTCTTGAACAACATACAAAGTTTAAAGTAACTTGGGGATG
GCTACAAAAAGTTAACACAGTATTTTTCTCAAATGAACATGTTTATTGCAGATTCTTCTTTTTTGA
AAGAACAACAGCAAAACATCCACAACTCTGTAAAGCTGGTGGGACCTAATGTCACCTTAATTCTGA
CTTGAACTGGAAGCATTTTAAGAAATCTTGTTGCTTTTCTAACAAATTCCCGCGTATTTTGCCTTT
GCTGCTACTTTTTCTAGTTAGATTTCAAACTTGCCTGTGGACTTAATAATGCAAGTTGCGATTAAT
TATTTCTGGAGTCATGGGAACACACAGCACAGAGGGTAGGGGGGCCCTCTAGGTGCTGAATCTACA
CATCTGTGGGGTCTCCTGGGTTCAGCGGCTGTTGATTCAAGGTCAACATTGACCATTGGAGGAGTG
GTTTAAGAGTGCCAGGCGAAGGGCAAACTGTAGATCGATCTTTATGCTGTTATTACAGGAGAAGTG
ACATACTTTATATATGTTTATATTAGCAAGGTCTGTTTTTAATACCATATACTTTATATTTCTATA
CATTTATATTTCTAATAATACAGTTATCACTGATATATGTAGACACTTTTAGAATTTATTAAATCC
TTGACCTTGTGCATTATAGCATTCCATTAGCAAGAGTTGTACCCCCTCCCCAGTCTTCGCCTTCCT
CTTTTTAAGCTGTTTTATGAAAAAGACCTAGAAGTTCTTGATTCATTTTTACCATTCTTTCCATAG
GTAGAAGAGAAAGTTGATTGGTTGGTTGTTTTTCAATTATGCCATTAAACTAAACATTTCTGTTAA
ATTACCCTATCCTTTGTTCTCTACTGTTTTCTTTGTAATGTATGACTACGAGAGTGATACTTTGCT
GAAAAGTCTTTCCCCTATTGTTTATCTATTGTCAGTATTTTATGTTGAATATGTAAAGAACATTAA
AGTCCTAAAACATCTAA

PYGB CDS

ATGGCGAAGCCGCTGACGGACAGCGAGAAGCGGAAGCAGATCAGCGTGCGCGGCCTGGCGGGGCTA
GGCGACGTGGCCGAGGTGCGGAAGAGCTTCAACCGGCACTTGCACTTCACGCTGGTCAAGGACCGC
AATGTGGCCACGCCCCGCGACTACTTCTTCGCGCTGGCGCACACGGTGCGCGACCACCTCGTGGGC
CGCTGGATCCGCACGCAGCAGCACTACTACGAGCGCGACCCCAAGCGCATTTATTATCTTTCCCTG
GAATTCTACATGGGTCGCACGCTGCAGAACACGATGGTGAACCTGGGCCTTCAGAATGCCTGCGAT
GAAGCCATCTATCAGTTGGGGTTAGACTTGGAGGAACTCGAGGAGATAGAAGAAGATGCTGGCCTT
GGGAATGGAGGCCTGGGGAGGCTGGCAGCGTGTTTCCTTGACTCAATGGCTACCTTGGGCCTGGCA
GCATACGGCTATGGAATCCGCTATGAATTTGGGATTTTTAACCAGAAGATTGTCAATGGCTGGCAG
GTAGAGGAGGCCGATGACTGGCTGCGCTACGGCAACCCCTGGGAGAAAGCGCGGCCTGAGTATATG
CTTCCCGTGCACTTCTACGGACGCGTGGAGCACACCCCCGACGGCGTGAAGTGGCTGGACACACAG
GTGGTGCTGGCCATGCCCTACGACACCCCAGTGCCCGGCTACAAGAACAACACCGTCAACACCATG
CGGCTGTGGTCCGCCAAGGCTCCCAACGACTTCAAGCTGCAGGACTTCAACGTGGGAGACTACATC
GAGGCGGTCCTGGACCGGAACTTGGCTGAGAACATCTCCAGGGTCCTGTATCCAAATGATAACTTC
TTTGAGGGGAAGGAGCTGCGGCTGAAGCAGGAGTACTTCGTGGTGGCCGCCACGCTCCAGGACATC
ATCCGCCGCTTCAAGTCGTCCAAGTTCGGCTGCCGGGACCCTGTGAGAACCTGTTTCGAGACGTTC
CCAGACAAGGTGGCCATCCAGCTGAACGACACCCACCCCGCCCTCTCCATCCCTGAGCTCATGCGG
ATCCTGGTGGACGTGGAGAAGGTGGACTGGGACAAGGCCTGGGAAATCACGAAGAAGACCTGTGCA
TACACCAACCACACTGTGCTGCCTGAGGCCTTGGAGCGCTGGCCCGTGTCCATGTTTGAGAAGCTG
CTGCCGCGGCACCTGGAGATAATCTATGCCATCAACCAGCGGCACCTGGACCACGTGGCCGCGCTG
TTTCCCGGCGATGTGGACCGCCTGCGCAGGATGTCTGTGATCGAGGAGGGGGACTGCAAGCGGATC
AACATGGCCCACCTGTGTGTGATTGGGTCCCATGCTGTCAATGGTGTGGCGAGGATCCACTCGGAG
ATCGTGAAACAGTCGGTCTTTAAGGATTTTTATGAACTGGAGCCAGAGAAGTTCCAGAATAAGACC
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AAAATTGGGGAGGAGTTCCTGACTGACCTGAGCCAGCTGAAGAAGCTGCTGCCGCTGGTCAGTGAC
GAGGTGTTCATCAGGGACGTGGCCAAGGTCAAACAGGAGAACAAGCTCAAGTTCTCGGCCTTCCTG
GAGAAGGAGTACAAGGTGAAGATCAACCCCTCCTCCATGTTCGATGTGCATGTGAAGAGGATCCAC
GAGTACAAGCGGCAGCTGCTCAACTGCCTGCACGTCGTCACCCTGTACAATCGAATCAAGAGAGAC
CCGGCCAAGGCTTTTGTGCCCAGGACTGTTATGATTGGGGGCAAGGCAGCGCCCGGTTACCACATG
GCCAAGCTGATCATCAAGTTGGTCACCTCCATCGGCGACGTCGTCAATCATGACCCAGTTGTGGGT

GACAGGTTGAAAGTGATCTTCCTGGAGAACTACCGTGTGTCCTTGGCTGAGAAAGTGATCCCGGCC
GCTGATCTGTCGCAGCAGATCTCCACTGCAGGCACCGAGGCCTCAGGCACAGGCAACATGAAGTTC
ATGCTCAACGGGGCCCTCACCATCGGCACCATGGACGGCGCCAACGTGGAGATGGCCGAGGAGGCC
GGGGCCGAGAACCTCTTCATCTTCGGCCTGCGGGTGGAGGATGTCGAGGCCTTGGACCGGAAAGGG
TACAATGCCAGGGAGTACTACGACCACCTGCCCGAGCTGAAGCAGGCCGTGGACCAGATCAGCAGT
GGCTTTTTTTCTCCCAAGGAGCCAGACTGCTTCAAGGACATCGTGAACATGCTGATGCACCATGAC
AGGTTCAAGGTGTTTGCAGACTATGAAGCCTACATGCAGTGCCAGGCACAGGTGGACCAGCTGTAC
CGGAACCCCAAGGAGTGGACCAAGAAGGTCATCAGGAACATCGCCTGCTCGGGCAAGTTCTCCAGT
GACCGGACCATCACGGAGTATGCACGGGAGATCTGGGGTGTGGAGCCCTCCGACCTGCAGATCCCG
CCCCCCAACATCCCCCGGGACTAG

PYGB 3'UTR

GCACACCCTGCCTTGGCGGGACCAGCGGGCATTTGTTTTCTTGCTGACTTTGCACCTCCTTTTTTC
CCCAAACACTTTGCCAGCCACTGGTGGTCCCTGCTTTTCTGAGTACCATGTTTCCAGGAGGGGCCA
TGGGGGTCAGGGTGGTTTTGAGAGAGCAGGGTAAGGAAGGAATGTGCTAGAAGTGCTCCTAGTTTC
TTGTAAAGGAAGCCAGAGTTGACAGTACAAAGGGTCGTGGCCAGCCCTGCAGCTTCAGCACCTGCC
CCACCCAGAGTGGGAGTCAGGTGGAGCCACCTGCTGGGCTCCCCCAGAACTTTGCACACATCTTGC
TATGTATTAGCCGATGTCTTTAGTGTTGAGCCTCTGGATTCTGGGGTCTGGGCCAGTGGCCATAGT
GAAGCCTGGGAATGAGTGTTACTGCAGCATCTGGGCTGCCAGCCACAGGGAAGGGCCAAGCCCCAT
GTAGCCCCAGTCATCCTGCCCAGCCCTGCCTCCTGGCCATGCCGGGAGGGGTCGGATCCTCTAGGC
ATCGCCTTCACAGCCCCCTGCCCCCTGCCCTCTGTCCTGGCTCTGCACCTGGTATATGGGTCATGG
ACCCAGATGGGGCTTTCCCTTTGTAGCCATCCAATGGGCATTGTGTGGGTGCTTGGAACCCGGGAT
GACTGAGGGGGACACTGGAGTGGGTGCTTGTGTCTGCTGTCTCAGAGGCCTTGGTCAGGATGAAGT
TGGCTGACACAGCTTAGCTTGGTTTTGCTTATTCAAAAGAGAAAATAACTACACATGGAAATGAAA
CTAGCTGAAGCCTTTTCTTGTTTTAGCAACTGAAAATTGTACTTGGTCACTTTTGTGCTTGAGGAG
GCCCATTTTCTGCCTGGCAGGGGGCAGGTCTGTGCCCTCCCGCTGACTCCTGCTGTGTCCTGAGGT
GCATTTCCTGTTGTACACACAAGGGCCAGGCTCCATTCTCCCTCCCTTTCCACCAGTGCCACAGCC
TCGTCTGGAAAAAGGACCAGGGGTCCCGGAGGAACCCATTTGTGCTCTGCTTGGACAGCAGGCCTG
GCACTGGGAGGTGGGGGTGAGCCCCTCACAGCCTTGCCCCTCCCCAAGGCTGGCAACCTGCCTCCC
ATTGCCCAAGAGAGAGGGCAGGGAACAGGCTACTGTCCTTCCCTGTGGAATTGCCGAGAAATCTAG
CACCTTGCATGCTGGATCTGGGCTGCGGGGAGGCTCTTTTTCTCCCTGGCCTCCAGTGCCCACCAG
GAGGATCTGCGCACGGTGCACAGCCCACCAGAGCACTACAGCCTTTTATTGAGTGGGGCAAGTGCT
GGGCTGTGGTCGTGCCCTGACAGCATCTTCCCCAGGCAGCGGCTCTGTGGAGGAGGCCATACTCCC
CTAGTTGGCCACTGGGGCCACCACCCTGACCACCACTGTGCCCCTCATTGTTACTGCCTTGTGAGA
TAAAAACTGATTAAACCTTTGTGGCTGTGGTTGGCTGA

SHMT2 CDS

ATGCTGTACTTCTCTTTGTTTTGGGCGGCTCGGCCTCTGCAGAGATGTGGGCAGCTGGTCAGGATG
GCCATTCGGGCTCAGCACAGCAACGCAGCCCAGACTCAGACTGGGGAAGCAAACAGGGGCTGGACA
GGCCAGGAGAGCCTGTCGGACAGTGATCCTGAGATGTGGGAGTTGCTGCAGAGGGAGAAGGACAGG
CAGTGTCGTGGCCTGGAGCTCATTGCCTCAGAGAACTTCTGCAGCCGAGCTGCGCTGGAGGCCCTG
GGGTCCTGTCTGAACAACAAGTACTCGGAGGGTTATCCTGGCAAGAGATACTATGGGGGAGCAGAG
GTGGTGGATGAAATTGAGCTGCTGTGCCAGCGCCGGGCCTTGGAAGCCTTTGACCTGGATCCTGCA
CAGTGGGGAGTCAATGTCCAGCCCTACTCCGGGTCCCCAGCCAACCTGGCCGTCTACACAGCCCTT
CTGCAACCTCACGACCGGATCATGGGGCTGGACCTGCCCGATGGGGGCCATCTCACCCACGGCTAC
ATGTCTGACGTCAAGCGGATATCAGCCACGTCCATCTTCTTCGAGTCTATGCCCTATAAGCTCAAC
CCCAAAACTGGCCTCATTGACTACAACCAGCTGGCACTGACTGCTCGACTTTTCCGGCCACGGCTC
ATCATAGCTGGCACCAGCGCCTATGCTCGCCTCATTGACTACGCCCGCATGAGAGAGGTGTGTGAT
GAAGTCAAAGCACACCTGCTGGCAGACATGGCCCACATCAGTGGCCTGGTGGCTGCCAAGGTGATT
CCCTCGCCTTTCAAGCACGCGGACATCGTCACCACCACTACTCACAAGACTCTTCGAGGGGCCAGG
TCAGGGCTCATCTTCTACCGGAAAGGGGTGAAGGCTGTGGACCCCAAGACTGGCCGGGAGATCCCT
TACACATTTGAGGACCGAATCAACTTTGCCGTGTTCCCATCCCTGCAGGGGGGCCCCCACAATCAT
GCCATTGCTGCAGTAGCTGTGGCCCTAAAGCAGGCCTGCACCCCCATGTTCCGGGAGTACTCCCTG
CAGGTTCTGAAGAATGCTCGGGCCATGGCAGATGCCCTGCTAGAGCGAGGCTACTCACTGGTATCA
GGTGGTACTGACAACCACCTGGTGCTGGTGGACCTGCGGCCCAAGGGCCTGGATGGAGCTCGGGCT
GAGCGGGTGCTAGAGCTTGTATCCATCACTGCCAACAAGAACACCTGTCCTGGAGACCGAAGTGCC
ATCACACCGGGCGGCCTGCGGCTTGGGGCCCCAGCCTTAACTTCTCGACAGTTCCGTGAGGATGAC

TTCCGGAGAGTTGTGGACTTTATAGATGAAGGGGTCAACATTGGCTTAGAGGTGAAGAGCAAGACT
GCCAAGCTCCAGGATTTCAAATCCTTCCTGCTTAAGGACTCAGAAACAAGTCAGCGTCTGGCCAAC
CTCAGGCAACGGGTGGAGCAGTTTGCCAGGGCCTTCCCCATGCCTGGTTTTGATGAGCATTGA

SHMT2 3'UTR

AGGCACCTGGGAAATGAGGCCCACAGACTCAAAGTTACTCTCCTTCCCCCTACCTGGGCCAGTGAA
ATAGAAAGCCTTTCTATTTTTTGGTGCGGGAGGGAAGACCTCTCACTTAGGGCAAGAGCCAGGTAT
AGTCTCCCTTCCCAGAATTTGTAACTGAGAAGATCTTTTCTTTTTCCTTTTTTTGGTAACAAGACT
TAGAAGGAGGGCCCAGGCACTTTCTGTTTGAACCCCTGTCATGATCACAGTGTCAGAGACGCGTCC
TCTTTCTTGGGGAAGTTGAGGAGTGCCCTTCAGAGCCAGTAGCAGGCAGGGGTGGGTAGGCACCCT
CCTTCCTGTTTTTATCTAATAAAATGCTAACCTGCCCTGAGTTTCCATTACTGTGGGTGGGGTTCC
CCTGGGCCAAACAGTGATTTGTCTCCCTCAATGTGTACACCGCTCCGCTCCCACCACCGCTACCAC
AAGGACCCCCGGGGCTGCAGCCTCCTCTTTCTGTCTCTGATCAGAGCCGACACCAGACGTGATTAG
CAGGCGCAGCAAATTCAATTTGTTAAATGAAATTGTATTTTG

VIPR1 CDS

ATGCGCCCGCCAAGTCCGCTGCCCGCCCGCTGGCTATGCGTGCTGGCAGGCGCCCTCGCCTGGGCC
CTTGGGCCGGCGGGCGGCCAGGCGGCCAGGCTGCAGGAGGAGTGTGACTATGTGCAGATGATCGAG
GTGCAGCACAAGCAGTGCCTGGAGGAGGCCCAGCTGGAGAATGAGACAATAGGCTGCAGCAAGATG
TGGGACAACCTCACCTGCTGGCCAGCCACCCCTCGGGGCCAGGTAGTTGTCTTGGCCTGTCCCCTC
ATCTTCAAGCTCTTCTCCTCCATTCAAGGCCGCAATGTAAGCCGCAGCTGCACCGACGAAGGCTGG
ACGCACCTGGAGCCTGGCCCGTACCCCATTGCCTGTGGTTTGGATGACAAGGCAGCGAGTTTGGAT
GAGCAGCAGACCATGTTCTACGGTTCTGTGAAGACCGGCTACACCATTGGCTACGGCCTGTCCCTC
GCCACCCTTCTGGTCGCCACAGCTATCCTGAGCCTGTTCAGGAAGCTCCACTGCACGCGGAACTAC
ATCCACATGCACCTCTTCATATCCTTCATCCTGAGGGCTGCCGCTGTCTTCATCAAAGACTTGGCC
CTCTTCGACAGCGGGGAGTCGGACCAGTGCTCCGAGGGCTCGGTGGGCTGTAAGGCAGCCATGGTC
TTTTTCCAATATTGTGTCATGGCTAACTTCTTCTGGCTGCTGGTGGAGGGCCTCTACCTGTACACC
CTGCTTGCCGTCTCCTTCTTCTCTGAGCGGAAGTACTTCTGGGGGTACATACTCATCGGCTGGGGG
GTACCCAGCACATTCACCATGGTGTGGACCATCGCCAGGATCCATTTTGAGGATTATGGGTGCTGG
GACACCATCAACTCCTCACTGTGGTGGATCATAAAGGGCCCCATCCTCACCTCCATCTTGGTAAAC
TTCATCCTGTTTATTTGCATCATCCGAATCCTGCTTCAGAAACTGCGGCCCCCAGATATCAGGAAG
AGTGACAGCAGTCCATACTCAAGGCTAGCCAGGTCCACACTCCTGCTGATCCCCCTGTTTGGAGTA
CACTACATCATGTTCGCCTTCTTTCCGGACAATTTTAAGCCTGAAGTGAAGATGGTCTTTGAGCTC
GTCGTGGGGTCTTTCCAGGGTTTTGTGGTGGCTATCCTCTACTGCTTCCTCAATGGTGAGGTGCAG
GCGGAGCTGAGGCGGAAGTGGCGGCGCTGGCACCTGCAGGGCGTCCTGGGCTGGAACCCCAAATAC
CGGCACCCGTCGGGAGGCAGCAACGGCGCCACGTGCAGCACGCAGGTTTCCATGCTGACCCGCGTC
AGCCCAGGTGCCCGCCGCTCCTCCAGCTTCCAAGCCGAAGTCTCCCTGGTCTGA

VIPR1 3'UTR

CCACCAGGATCCCAGGGGCCCAAGGCGGCCCCTCCCGCCCCTTCCCACTCACCCCGGCAGACGCCG
GGGACAGAGGCCTGCCCGGGCGCGGCCAGCCCCGGCCCTGGGCTCGGAGGCTGCCCCCGGCCCCCT
GGTCTCTGGTCCGGACACTCCTAGAGAACGCAGCCCTAGAGCCTGCCTGGAGCGTTTCTAGCAAGT
GAGAGAGATGGGAGCTCCTCTCCTGGAGGATTGCAGGTGGAACTCAGTCATTAGACTCCTCCTCCA
AAGGCCCCCTACGCCAATCAAGGGCAAAAAGTCTACATACTTTCATCCTGACTCTGCCCCCTGCTG
GCTCTTCTGCCCAATTGGAGGAAAGCAACCGGTGGATCCTCAAACAACACTGGTGTGACCTGAGGG
CAGAAAGGTTCTGCCCGGGAAGGTCACCAGCACCAACACCACGGTAGTGCCTGAAATTTCACCATT
GCTGTCAAGTTCCTTTGGGTTAAGCATTACCACTCAGGCATTTGACTGAAGATGCAGCTCACTACC
CTATTCTCTCTTTACGCTTAGTTATCAGCTTTTTAAAGTGGGTTATTCTGGAGTTTTTGTTTGGAG
AGCACACCTATCTTAGTGGTTCCCCACCGAAGTGGACTGGCCCCTGGGTCAGTCTGGTGGGAGGAC
GGTGCAACCCAAGGACTGAGGGACTCTGAAGCCTCTGGGAAATGAGAAGGCAGCCACCAGCGAATG
CTAGGTCTCGGACTAAGCCTACCTGCTCTCCAAGTCTCAGTGGCTTCATCTGTCAAGTGGGATCTG
TCACACCAGCCATACTTATCTCTCTGTGCTGTGGAAGCAACAGGAATCAAGAGCTGCCCTCCTTGT
CCACCCACCTATGTGCCAACTGTTGTAACTAGGCTCAGAGATGTGCACCCATGGGCTCTGACAGAA
AGCAGATACCTCACCCTGCTACACATACAGGATTTGAACTCAGATCTGTCTGATAGGAATGTGAAA
GCACGGACTCTTACTGCTAACTTTTGTGTATCGTAACCAGCCAGATCCTCTTGGTTATTTGTTTAC
CACTTGTATTATTAATGCCATTATCCCTGAATCCCCCTTGCCACCCCACCCTCCCTGGAGTGTGGC
TGAGGAGGCCTCCATCTCATGTATCATCTGGATAGGAGCCTGCTGGTCACAGCCTCCTCTGTCTGC

CCTTCACCCCAGTGGCCACTCAGCTTCCTACCCACACCTCTGCCAGAAGATCCCCTCAGGACTGCA
ACAGGCTTGTGCAACAATAAATGTTGGCTTGGA

MDA-MB-231 cells expressing either a control hairpin or a hairpin targeting miR-126 were transfected with the respective specific reporter construct. Thirty hours after transfection, the cells were lysed and the ratio of renilla to firefly luciferase expression was determined using the dual luciferase assay (E1910, PROMEGA). Cloning primer sequences are shown in Table 2 below.

Table 2 Cloning Primers

Gene	Forward	Reverse
ABCB9 3'UTR	CCGGCCCTCGAGTGGGGGGCCCCTGCTTCTCC	CCGGCCGCGGCCGCTTAGGGGTAAGAGGTAGTAC
ABCB9 CDS	CCGGCCCTCGAGATGCGGCTGTGGAAGGCGGT	CCGGCCGCGGCCGCTCAGGCCTTGTGACTGCCGT
IGFBP2 3'UTR	CCGGCCCTCGAGACCGCAGCCAGCCGGTGCCT	CCGGCCGCGGCCGCTTACTTTTCCTTCCTTTAAT
IGFBP2 CDS	CCGGCCCTCGAG ATGCTGCCGAGAGTGGGCTG	CCGGCCGCGGCCGCCTACTGCATCCGCTGGGTGT
MERTK 3'UTR	CCGGCCCTCGAGGGAGAGGTGCGGGGAGACAT	CCGGCCGCGGCCGCCTTCCTTATTCATATTTTAT
MERTK CDS	CCGGCCCTCGAGATGGGGCCGGCCCCGCTGCC	CCGGCCGCGGCCGCTCACATCAGGACTTCTGAGC
PITPNC1 3'UTR	CCGGCCCTCGAG CAATGGATGAAGTCCGAGAA	CCGGCCGCGGCCGCTTAAAAGACAGAAACAAGTA
PITPNC1 CDS	CCGGCCCTCGAG ATGCTGCTGAAAGAGTACCG	CCGGCCGCGGCCGCTCATGTACTTGTTTGGGCAT
PSAT1 3'UTR	CCGGCCCTCGAGACACATCCTAACCAGGATAT	CCGGCCGCGGCCGCTTAGATGTTTTAGGACTTTA
PSAT1 CDS	CCGGCCGCGGCCGCTCATAGCTGATGCATCTCCA	CCGGCCCTCGAGATGGACGCCCCCAGGCAGGT
PYGB 3'UTR	CCGGCCCTCGAGGCACACCCTGCCTTGGCGGG	CCGGCCGCGGCCGCTCAGCCAACCACAGCCACAA
PYGB CDS	CCGGCCGTTTAAACATGGCGAAGCCGCTGACGGA	CCGGCCGCGGCCGCCTAGTCCCGGGGGATGTTGG
SHMT2 3'UTR	CCGGCCCTCGAGAGGCACCTGGGAAATGAGGC	CCGGCCGCGGCCGCCAAAATACAATTTCATTTAA
SHMT2 CDS	CCGGCCCTCGAGATGCTGTACTTCTCTTTGTT	CCGGCCGCGGCCGCTCAATGCTCATCAAAACCAG
VIPR CDS	CCGGCCGTTTAAACTCAGACCAGGGAGACTTCGG	CCGGCCCTCGAGATGCGCCCGCCAAGTCCGCT
VIPR1 3'UTR	CCGGCCCTCGAGCCACCAGGATCCCAGGGGCC	CCGGCCGCGGCCGCTCCAAGCCAACATTTATTGT

Potential miR-126 sites in genes were identified by alignment to the complementary miR-126 sequence 5-TTACTCACGGTACGA-3, and mutagenesis was performed using the QUICKCHANGE Multi Site-Directed Mutagenesis Kit (200514, AGILENT TECHNOLOGIES, Santa Clara, CA). Based on the UCSC genome browser the 3'UTR of MERTK was mutated at position 5 (GTT to CAC), the 3'UTR of IGFBP2 was mutated at

position 246 (GGT to CAC), the CDS of PITPNC1 was mutated at position 709 (TAC to GTA) from the start codon and the CDS of SHMT2 was mutated at position 1126 (GGT to CAC). Mutagenesis primers are in shown in Table 3 below.

Table 3 Mutagenesis Primers

Gene	Forward
IGFBP2 3'UTR	AAGGGGGTTGTGGTCGGGGAGCTGGCACACAGGTTTGGGGAGGGGGAAGAGAA
MERTK 3'UTR	ATTCTAGGCGATCGCTCGAGGGAGACACGCGGGGAGACATTCCAAAAATCAAG
PITPNC1 CDS	TATGACAATGGATGATGTTCGGGAAGTAGAGAAAAACATGCATGAACAAACCA
SHMT2 CDS	GCGAGGCTACTCACTGGTATCAGGTCACACTGACAACCACCTGGTGCTGGTGG
	Reverse
IGFBP2 3'UTR	TTCTCTTCCCCCTCCCCAAACCTGTGTGCCAGCTCCCCGACCACAACCCCCTT
MERTK 3'UTR	CTTGATTTTTGGAATGTCTCCCCGCGTGTCTCCCTCGAGCGATCGCCTAGAAT
PITPNC1 CDS	TGGTTTGTTCATGCATGTTTTTCTCTACTTCCCGAACATCATCCATTGTCATA
SHMT2 CDS	CCACCAGCACCAGGTGGTTGTCAGTGTGACCTGATACCAGTGAGTAGCCTCGC

Cancer Cell Proliferation

LM2 cells (2.5 X 10^4) expressing a control hairpin or short hairpins targeting IGFBP2, PITPNC1 or MERTK were seeded in triplicate in 6 well plates and viable cells were counted at 5 days after seeding.

Histology

Lungs were prepared by perfusion fixation with 4% paraformaldehyde infused through the vascular system and through the trachea. After excision, the lungs were placed in 4% paraformaldehyde overnight and embedded in paraffin. Five minutes prior to fixation, 100 mg biotinylated lectin (B-1175, VECTOR LABORATORIES) was injected into the circulation via the tail vein. Five-micrometer thick paraffin sections were stained with primary antibodies against MECA-32 (Developmental Studies Hybridoma Bank, The University of Iowa, IA), Vimentin (VP-V684, VECTOR LABORATORIES) and with FITC labeled Avidin (B-1175, VECTOR LABORATORIES) for the detection of injected biotinylated lectin. Primary antibodies were detected using various Alexa Flour dye-conjugated secondary antibodies. Fluorescence was obtained using a ZEISS laser scanning confocal microscope (LSM 510). To determine the vascularisation of metastatic nodules, the MECA-32 and lectin signals were quantified using IMAGEJ while the metastatic nodules' extents were determined through co-staining with human vimentin.

The collective area covered by vessels was determined by subtracting background (rolling ball radius of 1 pixel) and by using a pre-determined threshold as cut-off. Vessel density is given as the percentage of area covered by the blood vessels compared to the total area of the metastatic nodule. A metastatic nodule was defined by an area positive for vimentin staining with a total area above 2000 μm^2.

Mammary fat pad tumors were excised and submerged into 4% paraformaldehyde for 24 hours. The fixed tissue was embedded in paraffin and sectioned in 5 μm thick slices. Immuno-detection were performed using antibodies directed towards MECA-32 (Developmental Studies Hybridoma Bank), Mac-2 (CL8942AP, Cederlane, Burlington) and CD45 (550539, BD Biosciences). Detection of primary antibodies was performed using various biotinylated secondary antibodies (Vector Laboratories). The signal was subsequently amplified using the ABC kit (Vector Laboratories), and detected using DAB (3,3´-diaminodbenzidine). Before mounting the slides were counterstained with hematoxilin.

Dextran permeability was determined as described in Arnold *et al.*, 2010 *Dis Model Mech* 3 (1-2), 57 (2010) with slight modifications. Briefly, an intravenous bolus of 10 mg/ml rhodamine B labeled low molecular weight Dextran (1 x 10^4 kDa: D1824, INVITROGEN) in sterile PBS was infused. Fifteen minutes later, the mice were anaesthetized and the lungs were perfused with OCT, removed and frozen on dry ice. Ten-micrometer section was cut and the dextran permeability inside metastatic nodules - as determined by vimentin staining - was measured by fluorescence microscopy. Using IMAGEJ, a preset threshold was used to determine the levels of dextran permeability. The results are presented as the mean percentage of the thresholded area inside the metastatic nodule.

ELISA

IGBFP2 levels in conditioned media were determined using an IGFBP2 ELISA (AAHBLG-1-2, RAYBIOTECH, Norgross, GA).

Western Blotting

Cellular lysates from MDA-MB-231 cells were prepared by lysing cells in 1 ml ice-cold RIPA buffer containing protease inhibitors (ROCHE, Mannheim, Germany). Conditioned media were prepared by incubating MDA-MB-231 cells in serum free media for 24 hours. The media was then concentrated twenty times by spin filtering. 40 μg

protein was subsequently separated on a 4-12% SDS-PAGE, and transferred to a PVDF membrane. A monoclonal antibody against human MERTK (CVO-311, CAVEO THERAPEUTICS, Aurora, CO) was used to detect MERTK.

Metastasis Free Survival Analysis

Upon identifying the eight miR-126 regulated genes through an integrative analysis, it was determined whether the expression of these genes in aggregate correlates with human clinical metastasis. Published microarray data of series from UCSF46, NKI47, and MSKCC13 were used to obtain probe-level expression values. For genes that were represented by multiple probes, probes that displayed sufficient signal intensity as well as the highest coefficient of variation (most informative) in an independent dataset were used. Each breast cancer was classified as miR-126 signature positive if the sum of the Zscores for the expression values of the 8 genes was greater than the mean of the population. Kaplan-Meier metastasis-free survival curves were generated using GRAPHPAD PRISM 5 software (GRAPHPAD Software, Inc., LA Jolla, CA). Statistical significance for differences between survival curves of patients was determined using the Mantel-Cox log-rank test using GRAPHPAD Prism 5 software.

Vessel Density Analysis

The Kolmogorov-Smirnov test was used to determine the significance of difference in the blood vessel density for both MECA-32 and lectin staining using the publicly available software at physics.csbsju.edu/stats/KS-test.html.

EXAMPLE 2: Endogenous Mir-126 Suppressed Systemic Metastatic Colonization

In this example, assays were carried out to analyze metastatic progression in the setting of miR-126 loss-of-function. This enabled one to compare *in vivo* metastatic events between control and miR-126 knockdown (KD) cells and to reveal the influence of endogenous miR-126 on metastatic colonization.

A MDA-231 breast cancer cell line was generated in which miR-126 was stably knocked down (94% knock down; FIG. 7) using the miR-Zip anti-sense hairpin microRNA inhibition system. miR-126 KD and control KD cells were injected into immunodeficient mice and evaluated for metastatic colonization capacity in tail-vein colonization assays. miR-126 silencing in poorly metastatic cells increased lung metastatic colonization by 4.2 fold ($P = 0.0073$) as assessed by quantitative bioluminescence imaging (FIG. 1a) and dramatically increased metastatic colonization on gross histology (FIG. 1a).

Intracardiac injection of MDA miR-126 KD and control KD cells further revealed endogenous miR-126 to suppress systemic metastasis as evidenced by enhanced colonization of multiple organs such as brain and bone in the setting of miR-126 knockdown (FIG. 1b-c; $P = 0.0232$(b), $P = 0.0119$(c)).

Next, assays were carried out to examine to what extent the dramatic increase in metastatic colonization observed with miR-126 inhibition was due to the effect of miR-126 on tumor growth. To this end, miR-126 KD and control KD cells were injected into the mammary fat pads of immunodeficient mice and monitored tumor volume. miR-126 inhibition led to a modest increase (39.4%) in tumor volume (FIG. 1d) that was an order of magnitude smaller than the effect of miR-126 inhibition on metastasis enhancement—indicating that the effect of miR-126 on metastasis is not simply a result of its effect on tumor growth suppression.

To better understand the role of this miRNA on metastatic colonization, the numbers and sizes of all metastases were quantified through image analysis of lungs from control and miR-126 KD mice (FIG. 1e). This revealed a substantial increase in the total number of metastatic nodules in miR-126 KD lungs relative to control lungs (13.6±3.2 *versus* 4.9±1.8; $P = 0.03$). This increase was noted for both small and large nodules (FIG. 1e) and mirrored the increase in the number of metastases to other organs (FIG. 1c). Importantly, the increase in nodule number was more pronounced for smaller nodule sizes relative to larger ones, consistent with primarily an increase in the initiation of metastases rather than an increase in the growth of established metastases. Without being bound by theory, if miR-126 silencing provides a metastatic initiation advantage for cells as they initiate metastases in the metastatic niche, its induction in the initial phase of metastasis formation should reduce the number of metastatic nodules. To test this, miR-126 expression was induced in metastatic breast cancer cells (LM2) displaying silencing of this miRNA using a conditional tet-on system. Consistent with this, restoring miR-126 expression to LM2 cells after they have extravasated in the lung (Day 3) significantly reduced metastatic colonization (FIG. 1f). Thus, restoring miR-126 expression at this early phase of metastasis initiation in the niche significantly reduced the number of metastasis nodules visualized at day 49.

The above findings demonstrated that miR-126 silencing enhances the efficiency of metastasis formation leading to a larger number of metastases. The findings thus

revealed endogenous miR-126 to be a suppressor of metastatic initiation and metastatic colonization.

EXAMPLE 3: miR-126 Suppresses Metastatic Endothelial Recruitment by Breast Cancer Cells

The above findings suggest that miR-126 silencing can provide metastatic cells and incipient metastases an advantage during metastatic colonization. While considering the basis of this advantage, it was noted that miR-126 knockdown metastases displayed higher vessel densities on microscopic visualization of lung H&E tissue sections. To quantify this, co-immunostaining was performed for human vimentin, which labels MDA-231 breast cancer cells, and the endothelial marker MECA-32, which allowed one to quantify the endothelial density within metastatic nodules in lungs of mice injected with either control or miR-126 KD breast cancer cells. Image analysis and quantification revealed metastases derived from miR-126 KD cells to have a significantly higher endothelial density (FIG. 2a; 35% increase; $P = 0.02$).

To determine if the enhanced endothelial density in miR-126 KD metastases represents functional vessels, sugar-binding lectin was injected into the circulation of mice prior to lung extractions, and subsequently stained for the injected lectin. Lectin cytochemistry revealed that miR-126 knockdown metastases displayed increased density of functional blood vessels (FIG. 2b; 33% increase; $P = 0.001$).

Finally, it was sought to determine if miR-126 regulates hemodynamic perfusion to metastases through intravenous perfusion and subsequent visualization of low-molecular weight dextran (1×10^4 kDa). Indeed, miR-126 KD metastases displayed significantly increased perfusion relative to control metastases (FIG. 8; $P = 0.02$).

Thus, these independent and complementary methods reveal that miR-126 suppresses *in vivo* functional metastatic angiogenesis and perfusion. These findings are consistent with miR-126 silencing providing metastases a selective advantage in angiogenic progression.

EXAMPLE 4: mir-126 Suppresses Cancer Endothelial Recruitment *In Vitro*

In this example, it was sought to determine the cellular basis for the miR-126 dependent angiogenesis phenotype observed.

The ability of miR-126 to regulate various cancer-endothelial interactions such as endothelial adhesion, endothelial proliferation, and tube-formation was analyzed in LM2

metastatic cells (originally derived from the poorly metastatic MDA-231 population, Minn, A. J. *et al., Nature* 436 (7050), 518 (2005)) in co-culture with human umbilical vein endothelial cells (HUVECs). Restoring miR-126 expression to LM2 cells, which display silencing of miR-126, did not suppress adhesion of metastatic cells to endothelial cells (FIG. 2c), proliferation of endothelial cells (FIG. 2d), or tube formation as assessed by automated quantification of branch points (FIG. 2e). Consistent with this, inhibition of miR-126 in MDA-231 cells did not enhance these angiogenic phenotypes either (FIGs. 9a-c).

The role of miR-126 in regulation of recruitment of endothelial cells to metastatic cells was investigated. Metastatic LM2 cells placed in the bottom of a Boyden chamber strongly recruited HUVECS through a porous trans-well insert and displayed a significantly enhanced ability to recruit endothelia compared to their poorly metastatic parental line (FIG. 2f). Endothelial recruitment by metastatic cells was strongly inhibited (47% reduction) by miR-126 over-expression (FIG. 2g). Conversely, knockdown of miR-126 in the poorly metastatic parental MDA-231 population significantly increased endothelial recruitment (146% increase; FIG. 2g). The CN34LM1a line, a highly lung metastatic derivative that was previously obtained through in-vivo selection of the CN34 primary malignant population (Tavazoie *et al., Nature* 451 (7175), 147 (2008)) (an independent primary malignant population obtained from the pleural fluid of a patient with metastatic breast cancer Gupta *et al., Nature* 446 (7137), 765 (2007)), also displayed significantly enhanced endothelial recruitment capacity compared to its poorly metastatic parental line (FIG. 2h). Both gain- and loss-of-function experiments revealed miR-126 to significantly suppress endothelial recruitment by the CN34 population as well (FIG. 2i). The findings reveal enhanced endothelial recruitment capacity to be a key feature of metastatic breast cancer populations and identify endogenous miR-126 as a major regulator of this process.

Next, it was sought to determine if endogenous miR-126 can selectively regulate endothelial recruitment to breast cancer cells independent of their location. Metastatic breast cancer cells expressing a control hairpin or over-expressing miR-126 were thus implanted into the mammary fat pads of mice. Metastatic cells, which display silenced miR-126 expression, displayed higher vessel density in the mammary gland relative to poorly metastatic cells. Endothelial recruitment to metastatic cells in the mammary fat

pad was inhibited by miR-126 expression (FIG. 2j), while miR-126 knockdown in poorly metastatic cells significantly increased endothelial recruitment to and functional vessel content of breast tumors growing in mammary fat pads as determined by meca-32 staining (FIG. 2j) and lectin staining (FIG. 10a) respectively. This recruitment effect was selective to endothelial cells as miR-126 silencing did not increase leukocyte density (FIG. 10b) or macrophage density (FIG. 16c) in mammary tumors.

The above findings revealed that miR-126 selectively regulates endothelial recruitment to breast cancer cells independent of their anatomic location.

EXAMPLE 5: mir-126 Regulon Promotes Endothelial Recruitment

In this example, a systematic search was conducted to identify the molecular targets of miR-126 that mediate endothelial recruitment and metastatic colonization. Specifically, transcriptomic analysis of LM2 cells over-expressing miR-126 was performed and global transcript alterations to poorly metastatic MDA-231 cells and highly metastatic LM2 cells were compared.

Without being bound by theory, it was hypothesized that, given the role of miR-126 in inhibiting metastasis, the biological mediators of miR-126 display increased expression in metastatic cells and that they would be suppressed by this miRNA. A set of 23 genes were identified as they were suppressed upon miR-126 over-expression (>1.6-fold; FIG. 11; Table 4), and up-regulated (>1.4-fold) in metastatic cells relative to the parental MDA-231 line (FIG. 11).

Of these genes, 14 were validated to be significantly changed by quantitative real-time PCR (qPCR) of MDA-231 control and miR-126KD cells as well as LM2 control and miR-126 over-expressing cells. To further increase the confidence of this list, the expression of these genes in the metastatic derivatives of the independent CN34 line was tested, and 8 genes were identified as displaying significantly increased expression in multiple metastatic CN34 derivatives relative to their parental line (FIG. 3a).

The contribution of these 8 genes to human metastasis was ascertained by determining whether their over-expression in primary human breast cancers correlates with distal metastasis-free survival. Patients whose primary breast cancers displayed their over-expression were significantly more likely to develop distal metastases and experienced shorter metastasis-free survival than those whose cancers did not over-express these genes (FIG. 3b-d). This association displayed significance in the UCSF (n=117; $P <$

0.0165), NKI (n=295; $P < 0.0005$), and the combined MSK/NKI/UCSF cohorts (n=494; $P < 0.0004$). Thus, miR-126 suppressed the expression of a set of eight genes that are positively and strongly correlated with human metastatic relapse.

Next, assays were carried out to identify the direct targets of miR-126. To this end, the 3'-untranslated regions (3'-UTR's) and coding sequences (CDS's) of all eight miR-126 regulated genes were cloned and used to generate luciferase fusion constructs. Luciferase reporter assays with this entire set revealed miR-126 to regulate the expression of IGFBP2 and MERTK through interactions with their 3'-UTR's and PITPNC1 and SHMT2 through interactions with their coding regions as knockdown of endogenous miR-126 in MDA-231 cells enhanced expression of these luciferase fusion genes (FIG. 3e and FIG. 12). Mutation of miR-126 complementary sequences in the 3'-UTR's of IGFBP2 and MERTK abolished miR-126 mediated regulation of luciferase expression (FIG. 3f), while mutation of the CDS's of PIPNC1 and SHMT2 abolished miRNA mediated targeting (FIG. 3f).

Table 4 Fold reduction by miR-126 in LM2 cells

Gene Name	Fold	Gene Name	Fold	Gene Name	Fold	Gene Name	Fold
GDF15	-4.15	CTAGE5	-1.93	PRKAR1A	-1.80	KIAA0746	-1.71
RARA	-3.53	CDA	-1.93	CHAC1	-1.80	PADI4	-1.71
P8	-2.98	FLJ46385	-1.92	SCD	-1.80	BEX2	-1.71
RPS6KA2	-2.54	RALGPS2	-1.92	PCK2	-1.80	TAF13	-1.70
C20orf100	-2.47	BDNFOS	-1.91	CDC42BPB	-1.79	KLF4	-1.70
C12orf39	-2.38	MBNL1	-1.91	DSCR1	-1.79	DLG1	-1.70
HERPUD1	-2.37	MKX	-1.91	TCF7L2	-1.79	DDEFL1	-1.70
CTH	-2.36	LPIN1	-1.90	TNRC6C	-1.79	MID1IP1	-1.70
LOC23117	-2.35	DNAJB9	-1.90	TncRNA	-1.78	LOC124220	-1.70
LOC23117	-2.35	TncRNA	-1.90	CLDN23	-1.78	C10orf58	-1.70
ASNS	-2.35	BCL2L1	-1.90	GPR153	-1.78	CDKN1C	-1.70
RGC32	-2.33	DNAJB9	-1.90	KRTHA4	-1.78	DTX3	-1.70
CTH	-2.33	ENTH	-1.89	SCD	-1.78	SETD5	-1.70
NRP1	-2.28	S100A5	-1.89	VIPR1	-1.78	SLC7A11	-1.69
RIT1	-2.26	CST4	-1.89	SLC1A4	-1.77	WSB1	-1.69
HMGA1	-2.24	TRIB3	-1.89	PNPLA3	-1.77	KIAA1618	-1.69
DDIT3	-2.20	PHLDA1	-1.89	PPP1R11	-1.77	PYGB	-1.69
MBNL1	-2.20	RGNEF	-1.89	CFLAR	-1.77	CSNK1A1	-1.69
SUPT6H	-2.16	GFPT1	-1.88	NSF	-1.77	THBD	-1.68
LPIN1	-2.15	TMTC2	-1.88	ABHD4	-1.77	CG012	-1.68
ZNF451	-2.12	TPARL	-1.87	SOCS2	-1.77	DDX17	-1.68
THBD	-2.10	INHBB	-1.87	TACSTD2	-1.76	BGLAP	-1.68
ITGB4	-2.10	FASN	-1.87	SESN2	-1.76	MAGI1	-1.68
BHLHB8	-2.09	CALB2	-1.86	CTNNB1	-1.76	WARS	-1.68
SLCO4C1	-2.09	IGFBP2	-1.86	MAP1LC3B	-1.76	LOC283050	-1.68
AFF4	-2.07	SLC6A9	-1.86	LOC165186	-1.76	AQP3	-1.68
ATP6V0D2	-2.05	PLAT	-1.86	FLJ20054	-1.75	LOC400581	-1.68
KRT19	-2.05	SIN3B	-1.86	ZNF69	-1.74	CYLN2	-1.68

Gene Name	Fold	Gene Name	Fold	Gene Name	Fold	Gene Name	Fold
SMAD3	-2.04	S100A6	-1.85	TNFSF4	-1.74	CD97	-1.68
ARHGAP5	-2.04	WSB1	-1.85	LOC441453	-1.74	CNTNAP3	-1.67
DNAJB9	-2.04	C20orf18	-1.85	MARS	-1.74	PDE2A	-1.67
ATF3	-2.03	HMGCS1	-1.85	LOC647135	-1.74	AOF1	-1.67
LOC440092	-2.03	MBNL1	-1.85	ACSL3	-1.74	IDS	-1.67
RIT1	-2.03	MBNL1	-1.85	SCD	-1.74	SCD	-1.67
ZNF499	-2.02	WHSC1L1	-1.85	SERINC2	-1.73	SHMT2	-1.67
ATXN1	-2.02	NCF2	-1.85	ZCCHC7	-1.73	RNF10	-1.67
CST6	-2.01	MERTK	-1.84	ETNK1	-1.73	CRLF3	-1.67
WBP2	-2.00	PFAAP5	-1.84	CHRM3	-1.73	PSAT1	-1.67
ZFAND3	-2.00	RTN4	-1.83	DCAMKL1	-1.73	FNBP1	-1.67
FLJ38717	-1.99	LARP6	-1.83	C20orf119	-1.73	LOC554203	-1.66
LOC158160	-1.99	TRIB3	-1.83	CDKN1C	-1.73	MYADM	-1.66
PITPNC1	-1.99	RAB37	-1.83	CXorf33	-1.72	ATXN1	-1.66
JMJD1C	-1.99	LOC399959	-1.83	LPIN1	-1.72	CA12	-1.66
PRO2852	-1.98	SYTL1	-1.82	GEM	-1.72	SF3B4	-1.66
AGR2	-1.97	SDF2L1	-1.82	KIAA0746	-1.72	KHDRBS1	-1.66
SLC7A5	-1.94	RPH3AL	-1.82	LOC115648	-1.72	EGFR	-1.66
NSF	-1.94	OGDH	-1.82	TIA1	-1.72	FRMD5	-1.65
BCL2L1	-1.94	CDYL	-1.81	FLJ10120	-1.71	ZNF252	-1.65
KIAA1267	-1.93	RHOQ	-1.81	DUSP5	-1.71	FNBP1	-1.65
NT5C2	-1.93	ITGB4	-1.81	RNF12	-1.71	TNKS2	-1.65
C9orf3	-1.65	C14orf118	-1.61				
AOF1	-1.65	PIAS1	-1.61				
PDP2	-1.65	PXN	-1.61				
MLLT10	-1.65	C14orf118	-1.61				
WIRE	-1.65	PIAS1	-1.61				
ATXN1	-1.65	FLJ43663	-1.65				
WARS	-1.65	SOS2	-1.61				
RAB5B	-1.64	FLJ43663	-1.60				
SQLE	-1.64	HCRP1	-1.60				
SCNN1A	-1.64	LOC646916	-1.60				
C14orf78	-1.64	NUP43	-1.60				
SHMT2	-1.63	PEBP1	-1.60				
PSCD3	-1.63	FLJ23556	-1.60				
LOC643998	-1.63	NRP1	-1.60				
PHGDH	-1.63	JUP	-1.60				
HEXA	-1.63						
CDRT4	-1.63						
ACTN4	-1.63						
C6orf155	-1.63						
EXT1	-1.63						
JDP2	-1.63						
LSS	-1.63						
PITPNC1	-1.63						
C20orf18	-1.63						
CLDN7	-1.63						
NPC1	-1.62						
IDH1	-1.62						
THBD	-1.62						
GSTM4	-1.62						
ATP5C1	-1.62						
PMM1	-1.62						
C9orf5	-1.62						

Gene Name	Fold	Gene Name	Fold	Gene Name	Fold	Gene Name	Fold
COL8A2	-1.62						
CST1	-1.62						
MAGI1	-1.62						
G6PD	-1.62						
FOSL1	-1.61						
RASD1	-1.61						
PITX1	-1.61						
P2RY2	-1.61						
HYOU1	-1.61						
CSF2RA	-1.61						
SLC16A4	-1.61						
SQLE	-1.61						
EFHD2	-1.61						
ABCB9	-1.61						
SYDE1	-1.61						
MAGI1	-1.61						
SLC7A11	-1.61						
HSPA5	-1.61						

Thus, the binding protein IGF-binding protein 2, the receptor kinase MERTK, the phosphatidylinositol transfer protein PITPNC1, and the hydroxymethyltranferase enzyme SHMT2 comprise a set of direct targets of miR-126 in human breast cancer.

EXAMPLE 6: IGFBP2, PITPNC1, and MERTK Promote Endothelial Recruitment and Metastasis

In this example, assays were carried out to examine if any of the miR-126 target genes regulate the recruitment of endothelial cells by cancer cells. Of these four genes, knockdown of IGFBP2, MERTK, or PITPNC1 using independent short hairpins significantly suppressed the ability of metastatic LM2 cells to recruit endothelial cells (FIG. 4a and FIG. 13). Importantly, knockdown of these genes did not result in a significant decrease in cell proliferation (FIG. 14).

Given the robust effects of the miR-126 target genes on endothelial recruitment, it was examined whether the expression levels of these genes individually correlate with metastatic propensity of human cancers. The expression levels of each of these genes were thus analyzed through qPCR in an entirely independent set of 96 human breast cancers for which cDNAs were available.

Patients with stage III and stage IV breast cancers display local metastatic dissemination and distal metastases, respectively, and collectively comprise those that develop distal relapse at much higher rates than stage I and II patients. Interestingly, expression levels of IGFBP2 ($P < 0.0003$), MERTK ($P < 0.002$), and PITPNC1 ($P < 0.004$) were individually significantly increased in primary cancers of stage III and IV

patients relative to stage I and II patients (FIG. 4b). Given their requirement for endothelial recruitment by metastatic cells, as well as their direct targeting by miR-126, it was sought to determine if any of the miR-126 target genes are required for metastatic colonization.

It was found that, importantly, knockdown of IGFBP2 using independent short hairpins significantly suppressed metastatic colonization to the lung (sh_1: 10-fold; sh_2: 6.25 fold; FIG. 4c). In addition, knockdown of PITPNC1 and MERTK also strongly inhibited metastatic colonization (PITPNC1sh_1: 7.69-fold; PITPNC1sh_2: 4.55-fold, FIG. 4d; MERTKsh_1: 3.91-fold; MERTK1sh_2: 3.08-fold, FIG 4e). shRNA sequences used are listed in Table 5 below.

These findings revealed that the miR-126 direct target genes IGFBP2, PITPNC1 and MERTK are each individually required for endothelial recruitment and metastatic colonization and individually correlate in expression with human metastatic progression.

Table 5 shRNA sequences

Gene	Sequence
IGFBP2_sh1	CCGGCCAGTTCTGACACACGTATTTCTCGAGAAATACGTGTGTCAGAACTGGTTTTT (SEQ ID NO: 1)
IGFBP2_sh2	CCGGCAGGTTGCAGACAATGGCGATCTCGAGATCGCCATTGTCTGCAACCTGTTTTT (SEQ ID NO: 2)
MERTK_sh1	CCGGGCTTCTGGTCTTGATGTATTTCTCGAGAAATACATCAAGACCAGAAGCTTTTT (SEQ ID NO: 3)
MERTK_sh2	CCGGCCTGCATACTTACTTACTTTACTCGAGTAAAGTAAGTAAGTATGCAGGTTTTT (SEQ ID NO: 4)
PITPNC1_sh1	CCGGCGGGTGTATCTCAACAGCAAACTCGAGTTTGCTGTTGAGATACACCCGTTTTTG (SEQ ID NO: 5)
PITPNC1_sh2	CCGGCAATGGATGAAGTCCGAGAATCTCGAGATTCTCGGACTTCATCCATTGTTTTTG (SEQ ID NO: 6)
shSHMT2	CCGGCCGGAGAGTTGTGGACTTTATCTCGAGATAAAGTCCACAACTCTCCGGTTTTTG (SEQ ID NO: 7)
shcontrol	CCGGCAACAAGATGAAGAGCACCAACTC-GAGTTGGTGCTCTTCATCTTGTTGTTTTT (SEQ ID NO: 8)

EXAMPLE 7: IGFBP2 Mediates Recruitment through IGF1/IGF1R Activation of Endothelial Cells

Of the miR-126 targets, IGFBP2 is a secreted factor and, as such, poised to mediate inter-cellular communication between metastatic cancer cells and endothelial cells. Thus, it was examined if metastatic cells secrete increased levels of IGFBP2. It was

found that, indeed, ELISA analysis revealed metastatic LM2 cells to secrete 2.1-fold higher levels of this factor than the poorly metastatic MDA-231 parental line (FIG. 5a).

Members of the IGFBP family exert their effects by interacting with various insulin-like growth factors (IGF's) and modulate their binding to IGF receptors (Baxter, R. C., Horm Res 42 (4-5), 140 (1994) and Jones, J. I. *et al.* Endocr Rev 16 (1), 3 (1995). To determine if metastatic endothelial recruitment is mediated through secreted IGFBP2, IGFBP2 binding to the IGF's was inhibited by means of incubation with neutralizing IGFBP2 antibody.

It was found that antibody-mediated inhibition of IGFBP2 in a trans-well recruitment assay significantly inhibited metastatic cell endothelial recruitment to levels comparable to that obtained with miR-126 over-expression (FIG. 5b) and also prevented miR-126 dependent recruitment (FIG. 5b). Thus, this effect was specific to the miR-126/IGFBP2 pathway, as inhibition of endothelial recruitment by IGFBP2 antibody was occluded upon miR-126 over-expression (FIG. 5b). Antibody-mediated inhibition of IGFBP2 also suppressed endothelial recruitment by the CNLM1A derivative of the independent CN34 malignant line and resulted in a statistically significant reduction in miR-126-dependent endothelial recruitment (FIG. 5c). These findings revealed secreted IGFBP2 to be an inter-cellular signalling mediator for miR-126-dependent endothelial recruitment by metastatic cells.

IGFBP2 was known to bind both IGF1 and IGF2 in the extracellular space and modulate their signaling activity (Jones, J. I. *et al. Endocr Rev* 16 (1), 3 (1995); Arai, T., *et al. Endocrinology* 137 (11), 4571 (1996); Rajaram, S., *et al. Endocr Rev* 18 (6), 801 (1997); and Hoflich, A. *et al., FEBS Lett* 434 (3), 329 (1998)). To determine which IGF mediates miR-126-dependent endothelial recruitment, cells were treated with blocking antibodies against IGF1, IGF2, or with immunoglobulin control. Antibody-mediated inhibition of IGF1, but not IGF2, significantly reduced endothelial recruitment resulting from miR-126 knockdown (FIG. 5d).

Next, it was sought to determine the receptor through which the miR-126-dependent endothelial recruitment is being mediated. Inhibition of the IGF type-1 receptor (IGF1R) by incubation with IGF1R blocking antibody significantly reduced endothelial recruitment resulting from miR-126 knockdown, while IGF2R neutralization had no effect

(FIG. 5e). These findings demonstrated that the miR-126/IGFBP2/IGF1 pathway activates IGF1R on endothelial cells.

To be certain that the miR-126-dependent recruitment was mediated through IGF1R on endothelial cells—rather than on cancer cells—HUVEC endothelial or cancer cells were pre-incubated with the IGF1R antibody prior to the endothelial recruitment assay. This revealed that only IGF1R antibody pre-incubation of endothelial cells inhibited miR-126 mediated endothelial recruitment as there was no effect on recruitment upon pre-incubation with the cancer cells (FIG. 5f).

The above findings are consistent with metastatic endothelial recruitment resulting from the secretion of the miR-126 target gene IGFBP2, which binds IGF1 in the extracellular space and enhances IGF1-dependent activation of the IGF1 receptor on endothelial cells. Enhanced IGF1R activation on endothelial cells in turn stimulates endothelial migration towards metastatic breast cancer cells. Consistent with this model, recombinant IGFBP2 protein was sufficient, in a dose-dependent way, to promote endothelial chemotaxis (FIG. 5g) and migration (FIG. 15) in an IGF1R dependent manner.

EXAMPLE 8: MERTK Mediates Recruitment through GAS6

In this example, assays were carried out to investigate the mechanisms by which the other miR-126 target genes PITPNC1 and MERTK mediate endothelial recruitment.

Given the identification of IGFBP2 as a secreted miR-126-dependent factor that mediates this phenotype, the role PITPNC1 or MERTK in the regulation of the secretion of this factor from cancer cells was investigated. It was found that knockdown of PITPNC1 using independent hairpins reduced IGFBP2 secretion from breast cancer cells (FIG. 6a)—consistent with PITPNC1 regulation of endothelial recruitment being in part mediated through positive regulation of IGFBP2 secretion. Knockdown of MERTK, however, did not lead to decreased IGFBP2 secretion, suggesting an IGFBP2 independent pathway by which this miR-126 target gene mediates recruitment.

To determine the mechanism by which the MERTK receptor mediates recruitment, assays were carried out to test the impact of its soluble ligand GAS6 on cancer-mediated endothelial recruitment. Adding recombinant GAS6 to the co-culture system—at a physiological concentration found in human serum (Balogh, I. et al., *Arterioscler Thromb Vasc Biol* 25 (6), 1280 (2005)—potently reduced miR-126 dependent recruitment (FIG. 6b), suggesting that GAS6 acts as an inhibitor of endothelial recruitment. MERTK

receptor exists in both membrane bound and soluble forms, where the extracellular domain has been cleaved and thus generally is believed to act as a decoy receptor to negatively regulate MERTK receptor activation on cells expressing it (Sather, S. et al., *Blood* (3), 1026 (2007). Soluble MERTK was detected in conditioned media of MDA-MB-231 cells (FIG. 16). Without being bound by theory, soluble MERTK released from cancer cells may promote endothelial recruitment through binding and inhibition of GAS6. Consistent with this, the addition of recombinant soluble form of the MERTK extracellular domain (MerFc) suppressed exogenous as well as serum GAS6-mediated inhibition of endothelial recruitment by cancer cells (FIG. 6b). Importantly, this effect was miR-126 dependent (FIG. 6b). These findings suggest that secreted MERTK from metastatic cells acts as a decoy receptor for GAS6, thereby reducing the suppressive effects of GAS6 on endothelial cell recruitment. Listed below are amino acids sequences of three GAS6 isoforms.

Isoform 1

MAPSLSPGPAALRRAPQLLLLLLAAECALAALLPAREATQFLRPRQRRAFQVFEEAKQGH
LERECVEELCSREEAREVFENDPETDYFYPRYLDCINKYGSPYTKNSGFATCVQNLPDQC
TPNPCDRKGTQACQDLMGNFFCLCKAGWGGRLCDKDVNECSQENGGCLQICHNKPGSFHC
SCHSGFELSSDGRTCQDIDECADSEACGEARCKNLPGSYSCLCDEGFAYSSQEKACRDVD
ECLQGRCEQVCVNSPGSYTCHCDGRGGLKLSQDMDTCEDILPCVPFSVAKSVKSLYLGRM
FSGTPVIRLRFKRLQPTRLVAEFDFRTFDPEGILLFAGGHQDSTWIVLALRAGRLELQLR
YNGVGRVTSSGPVINHGMWQTISVEELARNLVIKVNRDAVMKIAVAGDLFQPERGLYHLN
LTVGGIPFHEKDLVQPINPRLDGCMRSWNWLNGEDTTIQETVKVNTRMQCFSVTERGSFY
PGSGFAFYSLDYMRTPLDVGTESTWEVEVVAHIRPAADTGVLFALWAPDLRAVPLSVALV
DYHSTKKLKKQLVVLAVEHTALALMEIKVCDGQEHVVTVSLRDGEATLEVDGTRGQSEVS
AAQLQERLAVLERHLRSPVLTFAGGLPDVPVTSAPVTAFYRGCMTLEVNRRLLDLDEAAY
KHSDITAHSCPPVEPAAA

Isoform 2

MDTCEDILPCVPFSVAKSVKSLYLGRMFSGTPVIRLRFKRLQPTRLVAEFDFRTFDPEGI
LLFAGGHQDSTWIVLALRAGRLELQLRYNGVGRVTSSGPVINHGMWQTISVEELARNLVI
KVNRDAVMKIAVAGDLFQPERGLYHLNLTVGGIPFHEKDLVQPINPRLDGCMRSWNWLNG
EDTTIQETVKVNTRMQCFSVTERGSFYPGSGFAFYSLDYMRTPLDVGTESTWEVEVVAHI
RPAADTGVLFALWAPDLRAVPLSVALVDYHSTKKLKKQLVVLAVEHTALALMEIKVCDGQ
EHVVTVSLRDGEATLEVDGTRGQSEVSAAQLQERLAVLERHLRSPVLTFAGGLPDVPVTS
APVTAFYRGCMTLEVNRRLLDLDEAAYKHSDITAHSCPPVEPAAA

Isoform 3

MFSGTPVIRLRFKRLQPTRLVAEFDFRTFDPEGILLFAGGHQDSTWIVLALRAGRLELQL
RYNGVGRVTSSGPVINHGMWQTISVEELARNLVIKVNRDAVMKIAVAGDLFQPERGLYHL
NLTVGGIPFHEKDLVQPINPRLDGCMRSWNWLNGEDTTIQETVKVNTRMQCFSVTERGSF
YPGSGFAFYSLDYMRTPLDVGTESTWEVEVVAHIRPAADTGVLFALWAPDLRAVPLSVAL
VDYHSTKKLKKQLVVLAVEHTALALMEIKVCDGQEHVVTVSLRDGEATLEVDGTRGQSEV
SAAQLQERLAVLERHLRSPVLTFAGGLPDVPVTSAPVTAFYRGCMTLEVNRRLLDLDEAA
YKHSDITAHSCPPVEPAAA

To determine whether recombinant forms of IGFBP2 and MERTK, which are expressed by metastatic cells, and GAS6, which is present in human serum, are sufficient to regulate endothelial chemotaxis, trans-well chemotactic assays were performed for

quantifying the chemotactic migration of endothelial cells towards these factors. Recombinant GAS6 at low, physiological doses inhibited endothelial chemotaxis towards recombinant IGFBP2 (FIG. 6c). Importantly, recombinant soluble MERTK ectodomain abrogated the GAS6 suppressive effect on endothelial chemotaxis (FIG. 6c). Pre-incubation of endothelial cells with GAS6 did not affect endothelial migration, suggesting that GAS6 inhibits chemotactic migration. These findings reveal that IGFBP2 mediates a positive migratory and chemotactic signal to endothelial cells through the IGF type-1 receptor, while soluble MERTK receptor antagonizes an inhibitory chemotactic signal mediated by GAS6.

Given the roles of IGFPB2, PITPNC1, and MERTK in endothelial recruitment in vitro and metastatic colonization *in vivo*, assays were carried out to examine if these genes regulate *in vivo* endothelial recruitment. To this end, MECA-32 staining was performed on lungs from mice injected with control and knockdown breast cancer cells to quantify endothelial recruitment *in vivo* as measured by metastatic vessel density. Inhibiting of IGFPB2, PITPNC1, and MERTK individually using independent short hairpins significantly reduced metastatic endothelial density (FIG. 6d; $P < 0.0001$ and $P = 0.002$ for shIGFBP2, $P = 0.01$ and $P = 0.02$ for shPITPNC1, and $P < 0.0001$ and $P = 0.005$ for shMERTK). Additionally, lectin perfusion and cytochemistry revealed a significant reduction in functional metastatic vessel content as well (FIG. 17). Thus, the miR-126 target genes IGFBP2, PITPNC1 and MERTK are individually required for metastatic endothelial recruitment in vivo.

The above findings, comprising both cancer cell mediated endothelial recruitment and recombinant protein-mediated recruitment assays in vitro as well as in vivo analyses, demonstrated that cancer-expressed IGFBP2 and MERTK are necessary and sufficient for mediating endothelial recruitment and relay parallel pathways emanating from metastatic cancer cells (FIG. 6e).

EXAMPLE 9 miRNA Regulon That Mediates Metastatic Angiogenesis

The above-described findings revealed that a miRNA expressed in cancer cells can non-cell-autonomously regulate the complex process of metastatic endothelial recruitment and vascular perfusion through the coordinate regulation of IGFBP2, MERTK, and PITPNC1 - a novel set of angiogenesis and metastasis genes.

It was found that the increased expression of these metastatic angiogenesis genes endows highly metastatic breast cancer cells with enhanced endothelial recruitment capacity relative to poorly metastatic cells. Metastatic cells over-expressing these genes are able to more readily establish blood vessels needed for effective colonization. Although the requirement for all of these three genes in metastatic endothelial recruitment was demonstrated, one of them, i.e., secreted IGFBP2, is a trans-cellular mediator of this phenotype.

Additionally, it was discovered the IGF1 signaling pathway—modulated by IGFBP2 secreted from cancer cells and culminating in IGF1R activation on endothelial cells—as a mediator of metastatic-cell endothelial recruitment and have identified miR-126 in cancer cells as a regulator of this pathway. Although roles of IGF1 and IGF2 in organismal and cellular growth have been reported (Laviola, L., *et al. Curr Pharm Des* 13 (7), 663 (2007) and Varela-Nieto, I., *et al. Curr Pharm Des* 13 (7), 687 (2007).), the ubiquitous expression of these growth factors and their receptors in various tissues and their requirements for normal physiology limit their therapeutic application (Varela-Nieto, I., *et al. Curr Pharm Des* 13 (7), 687 (2007)).

IGFBP2 is one of 16 members of the IGFBP family; see Schmid, C., Cell Biol Int 19 (5), 445 (1995); Hwa, V., *et al.* Endocr Rev 20 (6), 761 (1999); and Firth, S. M. *et al.* Endocr Rev 23 (6), 824 (2002). Identification of IGFBP2 as a promoter of metastasis, its over-expression in metastatic human breast cancer, and the robust effect of its antibody-mediated inhibition on endothelial recruitment by metastatic cells provides a specific handle for therapeutic targeting of the IGF pathway in breast cancer progression and cancer angiogenesis.

While IGFBP2 was identified as a positive regulator of endothelial recruitment through its activation of a positive regulator of this process (IGF1), MERTK was also discovered as a promoter of recruitment through its inhibition of a negative regulator of endothelial chemotaxis (GAS6). Thus a single miRNA can control a complex phenotype by modulating both positive and negative regulators of a phenomenon.

Subsequent to its identification as a metastasis suppressor miRNA, miR-126, which is developmentally expressed in endothelial cells, was genetically targeted in mice. It was found that miR-126 deletion led to partial embryonic lethality, loss of vascular integrity, and hemorrhage (Wang, S. *et al.*, *Dev Cell* 15 (2), 261 (2008).). Endothelial-

expressed miR-126 was thus found to be a promoter of normal developmental angiogenesis in mouse and zebrafish systems (Nicoli, S. *et al.,* Nature 464 (7292), 1196 (2010) and Fish, J. E. *et al.,* Dev Cell 15 (2), 272 (2008).)

In view of its role as an angiogenesis promoter, it was unexpected that miR-126 also suppressed angiogenesis in, e.g., breast cancer, as disclosed herein. It was unexpected that miR-126 could act in at least two different ways. On one hand, it acts in a cell-type specific fashion to suppress pathologic angiogenesis as disclosed in this application. As disclosed in this application, miR-126 suppressed pathologic endothelial migration to metastases. On the other hand, while in development miR-126 expression maintains vessel integrity. Indeed, endothelial miR-126 was shown to regulate developmental angiogenesis through targeting of Spred-1 and PIK3R2, genes that were not significantly regulated by miR-126 in breast cancer cells (Wang, S. *et al.*, *Dev Cell* 15 (2), 261 (2008) and Fish, J. E. *et al.,* Dev Cell 15 (2), 272 (2008).). See Table 6. Conversely, it was found that miR-126 inhibition in endothelial cells does not enhance endothelial recruitment by endothelial cells (FIG. 18) as it does in breast cancer cells. Consistent with this, miR-126 inhibition in endothelial cells did not alter the expression of PITPNC1, MERTK, or IGFBP2, while it did increase the expression of established endothelial miR-126 targets SPRED1 and PIK3R2 (FIG. 19).

Table 6

Gene Name	LM2	LM2 miR-126 OE	Fold
SPRED1	979	851	-1.1030
PIK3R2	2188	1513	-1.2634

EXAMPLE 10 Identifying Genes or Non-Coding RNAs That Regulate Metastatic Cancer Colonization of Any Body Tissue

This example describes two approaches for identifying a gene or a non-coding RNA that regulates metastatic cancer colonization of a body tissue

1. Lenti-miR Approach

Transduction of lenti-miR library into cells and injection into animals

The lenti-miR library (SYSTEM BIOSCIENCES, Cat # PMIRHPLVAHT-1) was used in this approach. This library consists of a pool of lentivirus containing precursor microRNAs representative of the entire human genome. Parental populations of the SW620 and LS174T cell-lines (2 x 10^5 cells) were transduced with the library at a

multiplicity of infection (MOI) of 1 to obtain a heterogeneous pool of parental cells with individual cells over-expressing different microRNA. Each microRNA precursor was represented at approximately 50X after transduction. Four days after transduction, a half-portion of the transduced cells were set aside and genomic DNA extracted using Qiagen DNeasy kit. This was the reference pool of genomic DNA prior to the selective pressure of liver colonization. The remaining half population was injected into the livers of NOD/SCID mice. 3-5 weeks after injection, genomic DNA was extracted from the tumors that formed in the livers. Transductions and injections were performed in replicates for both cell-lines.

Identification of microRNAs modulating liver colonization

Lenti-miR derived microRNA precursors were recovered from genomic DNA by PCR amplification in the linear range using library-specific, T7 promoter-containing primers (forward primer: 5'-GAAATTAATACGACTCACTATAGGGCCTGGAGACGCCATCCAC GCTG -3'; reverse primer: 5': GATGTGCGCTCTGCCCACTGAC-3') on the reference genomic DNA and tumor genomic DNA. Four PCR reactions using 400ng of genomic DNA as template were performed and pooled per sample to ensure adequate representation of transduced precursor microRNAs.

The resulting PCR amplicons were a composite of different precursor microRNAs with T7 promoter sequences and were used as templates for *in vitro* transcription to obtain a biotinylated precursor library. The biotinylated library obtained from the reference pool and tumors were labeled with Cy3 and Cy5 respectively and hybridized to a microarray designed to detect the microRNA sequences (Genosensor). A dye-swap experiment was performed to control for dye-bias.

The ratio of the abundance of each microRNA precursor between the reference pool and after selective pressure during liver colonization was calculated after normalization of microarray signal. microRNAs that became over-represented in the tumor population compared to the reference pool were considered as promoters and microRNAs that were under-represented, suppressors of liver colonization.

2. Lentiplex Approach

Transduction of lentiplex library into cells and injection into animals

The lentiplex whole-genome shRNA library (SIGMA-ALDRICH, Cat # SHPH01) was used in this approach. This library is a pooled library of lentivirus containing approximately 150,000 shRNAs targeting the whole human genome, with each gene being targeted by 3-5 independent shRNAs.

Parental populations of the cell-lines SW620, LS174T and WiDR (2 x 10^6 cells) were transduced with the library at a MOI of 1, resulting in a pool of heterogeneous population, with individual cells expressing a single shRNA. Each shRNA was transduced at approximately 100X representation. 48hrs after transduction; the transduced cells were selected with puromycin for 48hrs to remove untransduced cells. After antibiotic selection, the remaining cells were allowed to recover for a week prior to subsequent experiments. A half-portion of the selected cells were set aside and genomic DNA extracted. This was the reference pool of genomic DNA prior to the selective pressure of liver colonization. The remaining half population was injected into the livers of NOD/SCID mice. 3-5 weeks after injection, genomic DNA was extracted from the tumors that formed in the livers. Transductions and injections were performed in replicates for all three cell-lines.

Identification of novel genes modulating liver colonization through whole genome pooled shRNA screen

To recover a complex pool of shRNA library sequences from the genomic DNA, a PCR approach followed by Solexa deep sequencing of PCR amplicons were used. An initial PCR amplification was performed on 500ng of genomic DNA using primers (forward primer: 5'-TGGACTATCATATGCTTACCGTAACT-3'; reverse primer: 5'-AAAGAGGAT CTCTGTCCCTGT-3') specific for the virus vector, followed by primers with sequences required for Solexa deep sequencing (forward primer: 5'-AATGATACGGCGACCACCGAG ATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGTATTCTTGGCTTTATAT ATCTTGTGGAAAGGAC-3'; reverse primer: 5'- CAAGCAGAAGACGGCATACG AGCTCTTCCGATCTGGATGAATACTGCCATTTGTCTCGAGGTCGA-3') to obtain amplicons containing the shRNA sequences. Ten PCR reactions equivalent to 5ug of genomic DNA were performed for each set of genomic DNA and the products pooled for sequencing to ensure adequate representation of shRNAs.

The pooled amplicons represent a composite of genome-wide shRNA sequences and deep sequencing was performed to determine the representation of each shRNA species in reference pool compared to the pool amplified from tumors. The count for each shRNA species was normalized against the total number of sequences obtained and their gene targets identified by matching to a database provided by Sigma. Gene targets whose shRNAs which became over-represented in the tumor pool are considered suppressors of liver colonization and vice versa. To account for non-specific effects of shRNA-silencing, only gene targets hit identified by three or more independent shRNA "hits" were considered as putative suppressors or promoters.

EXAMPLE 11 Monoclonal antibody that neutralizes IGFBP2 function inhibited endothelial recruitment by metastatic human breast cancer cells.

This example demonstrates a monoclonal antibody that inhibits endothelial recruitment by metastatic breast cancer cells by binding to IGFBP2 and inhibiting the interaction (binding) of IGF1 to IGFBP2. By blocking IGF1 binding to IGFBP2, this monoclonal antibody is capable of inhibiting endothelial recruitment by metastatic human breast cancer cells. The methods used to generate neutralizing antibodies to IGFBP2 are those commonly known in the art.

In short, mice were immunized with recombinant IGFBP2 total peptide to generate a polyclonal antibody response. Next, hybridomas libraries were generated by fusion of B cells isolated from the immunimized mice to myeloma cell lines. Supernatant from these hybridomas were then isolated in order to screen and identify those hybridoma cells generating antibodies that bind IGFBP2 with high affinity, using antibody capture competitive ELISA assays (FIG. 20). Once identified, hybridomas generating antibodies with high affinity to IGFBP2 were screened in order to identify those that generate antibodies capable of inhibiting IGFBP2 from binding IGF1, using antibody capture competitive ELISA assays (FIG. 21). Hybridoma library wo6663-1 contained antibodies that bound to IGFBP2 to neutralize IGF1 binding, without binding to other IGFBP family members IGFBP3 and IGFBP4 (FIGs. 20 and 21). To isolate single clones (monoclonal) hybridoma cells, seperation and screening was performed on hybridoma library wo6663-1. 2000 single hybridoma clones (monoclonal) were screened from this library to identify those that generated monoclonal antibodies that bound to IGFBP2 with high affinity to

neutralize IGF1 binding . The table in FIG. 22 lists antibody-capture ELISA competitor assay data of several monoclonal antibodies isolated from the above screen, many that had affinity to IGFBP2 and were capable of inhibiting IGF1 binding to IGFBP2 (FIG. 22), including the monoclonal antibody IGFBP2_14 (M14) (dashed box in FIG. 22). These IGFBP2 neutralizing monoclonal antibodies were then screened to identify those capable of inhibiting endothelial recruitment by metastatic cells using trans-well endothelial migration assays. The monoclonal antibody IGFBP2_M14 (M14) inhibited endothelial recruitment by human metastatic breast cancer cells:

To identify monoclonal antibodies that could inhibit endothelial recruitment, the IGFBP2 neutralizing monoclonal antibodies generated in the above screen were tested in an in vitro endothelial recruitment assay using transwells. Highly metastatic LM2 human breast cancer cells were placed in the bottom of a Boyden chamber, where their ability to recruit HUVECS through a porous trans-well insert could be assayed. Small physiologic concentration of IGFBP2 neutralizing antibodies (including M1, M4, M6, M9, M13, M14, M15, and M16 (from FIG. 22)) were added to the transwells individually in physiologic concentrations. Of all antibodies tested, M14 (dashed box in FIG. 22) was able to significantly inhibit the recruitment (migrated cells/field) of HUVEC cells (50% reduction in migrated cells) versus the negative controls antibodies IgG and M5 (FIG. 23). This demonstrates the ability of the monoclonal antibody M14 to inhibit human endothelial cell recruitment by human metastatic cancer cells (FIG. 23).

To further characterize M14, the heavy chain and light chain variable regions of the antibody were sequenced. The amino acid sequence of both the heavy chain and light chain variable regions of M14 are presented in Table 7.

Table 7

M14 heavy chain variable region amino acid sequence	QVQLEQSGGGLVQPGGSLKLSCGASGFTFSDYYMYWIRQTPEKRLEWVAYISNG GGITYYPDTVKGRFTISRDNAKNTLYLQMSRLKSEDTAVYYCVRRSDGSWFVYW GQGTLVTVSA (SEQ ID NO: 9)
M14 light chain variable region amino acid sequence	DIVITQSPSSLAVSVGEKVTLSCKSSQSLLYSSNQKNCLAWYQQKPGQSPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQYYSYLTFGAGTKLEL KRADAAPTVS (SEQ ID NO:10)

EXAMPLE 12 Monoclonal antibody M14 inhibited tumor progression of human breast cancer in vivo.

This example demonstrates that the IGFBP2 neutralizing antibody M14 is capable of inhibiting tumor progression and tumor metastasis in vivo in a mouse model of human breast cancer.

To test whether monoclonal antibody M14 was able to reduce tumor burden and inhibit tumor progression in vivo, 2000 luciferase expressing MDA-MB-231 human breast cancer cells were mixed in a 1:1 ratio with growth factor reduced matrigel and injected bilaterally in the mammary fat pads of NOD-SCID mice. Immediately after injection, luciferin was injected and the cancer cell bioluminescence signal was quantified to establish a Day 0 baseline signal of tumor burden. The mice were then separated randomly into two groups: a control group which were treated with PBS alone, and an M14 group treated with M14 monoclonal antibody. Intraperitoneal injections of PBS and M14 antibody (250 micrograms) were given immediately on Day 0 to mice in each group respectively, and then subsequently, injections were given biweekly. Tumor burden in both the M14 treated and PBS treated control mice were followed twice a week by bioluminensence from the luciferase reporter. At day 14, tumor progression was significantly inhibited by treatment with M14 (7 to 11 fold reduction in tumor progression) compared with the PBS treated mice (FIG. 24).

The foregoing example and description of the preferred embodiments should be taken as illustrating, rather than as limiting the present invention as defined by the claims. All publications cited herein are hereby incorporated by reference in their entirety. As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. Such variations are not regarded as a departure from the scope of the invention, and all such variations are intended to be included within the scope of the following claims.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for inhibiting endothelial recruitment in a subject in need thereof, comprising administering to the subject a first agent that inhibits expression or activity of a first protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1.

2. The method of claim 1, wherein the subject has a disorder characterized by pathological angiogenesis.

3. The method of claim 2, wherein the disorder is cancer, an eye disorder, or an inflammatory disorder.

4. The method of claim 3, wherein the cancer is metastatic cancer.

5. The method of any of claims 1-4, further comprising administering to the subject a second agent that inhibits expression or activity of a second protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

6. The method of claim 5, further comprising administering to the subject a third agent that inhibits expression or activity of a third protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

7. The method of any of claims 1-6, wherein the first agent, second agent, or third agent is an antibody, a nucleic acid, a polypeptide, or a small molecule compound.

8. A method for treating metastatic cancer in a subject in need thereof, comprising administering to the subject a first agent that inhibits expression or activity of a first protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1, wherein the first agent inhibits angiogenesis.

9. The method of claim 8, wherein the cancer is breast cancer.

10. The method of any of claims 8-9, further comprising administering to the subject a second agent that inhibits expression or activity of a second protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

11. The method of claim 10, further comprising administering to the subject a third agent that inhibits expression or activity of a third protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

12. The method of any of claims 8-11, wherein the first agent, second agent, or third agent is an antibody, a nucleic acid, a polypeptide, or a small molecule compound.

13. The method of any of claims 1-12, further comprising administering to the subject chemotherapy, surgery, or radiotherapy.

14. An isolated nucleic acid comprising a sequence encoding an RNAi agent capable of inhibiting expression of a protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1.

15. The nucleic acid of claim 14, wherein the RNAi agent comprises a double-stranded structure having a first strand and a second strand, said first and second strands each being between 19 and 30 nucleotides long, and wherein the first strand is encoded by any one of SEQ ID NOs: 1-6.

16. A composition comprising an agent that inhibits expression or activity of a protein selected from the group consisting of IGFBP2, MERTK, and PITPNC1, wherein the agent is an antibody, a nucleic acid, a polypeptide, or a small molecule compound and wherein the first agent inhibits angiogenesis.

17. The composition of claim 16, wherein the agent is an isolated nucleic acid of any of claims 14-15.

18. The composition of claim 16, wherein the agent is an antibody.

19. A method for diagnosing a metastatic potential of cancer in a subject, comprising obtaining a first expression level for a first gene of the subject selected from the group consisting of IGFBP2, MERTK, and PITPNC1, and comparing the first expression level with a first predetermined level for the selected first gene, wherein the subject is determined to have or be prone to develop metastatic cancer if the first expression level is greater than the first predetermined level

20. The method of claim 19, wherein the first predetermined level is obtained from a control subject that is free of cancer.

21. The method of claim 19, further comprising obtaining a second expression level for a second gene of the subject selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR; and comparing the second expression level with a second predetermined level for the selected second gene.

22. A kit for diagnosing a metastatic potential of cancer in a subject, comprising a first reagent that specifically binds to a first expression product of a first gene selected from the group consisting of IGFBP2, MERTK, and PITPNC1.

23. The kit of claim 22, further comprising a second reagent that specifically binds to a second expression product of a second gene selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIP.

24. A method for inhibiting endothelial recruitment in a subject in need thereof, comprising administering to the subject a first agent that increases expression or activity of GAS6.

25. The method of claim 24, wherein the subject has a disorder characterized by pathological angiogenesis.

26. The method of claim 25, wherein the disorder is cancer, an eye disorder, or an inflammatory disorder.

27. The method of claim 26, wherein the cancer is metastatic cancer.

28. The method of any of claims 24-27, further comprising administering to the subject a second agent that inhibits expression or activity of a second protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

29. The method of claim 28, further comprising administering to the subject a third agent that inhibits expression or activity of a third protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

30. The method of any of claims 24-29, wherein the first agent, second agent, or third agent is an antibody, a nucleic acid, a polypeptide, or a small molecule compound.

31. The method of any of claims 24-30, wherein the first agent has GAS6 activity.

32. The method of any of claims 24-31, wherein the first agent is GAS6.

33 The method of any of claims 24-31, wherein the first agent is a polypeptide fragment of GAS6.

34. A method for treating metastatic cancer in a subject in need thereof, comprising administering to the subject a first agent that increase expression or activity of GAS6, wherein the first agent inhibits angiogenesis.

35. The method of claim 34, wherein the cancer is breast cancer.

36. The method of any of claims 34-35, further comprising administering to the subject a second agent that inhibits expression or activity of a second protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

37. The method of claim 36, further comprising administering to the subject a third agent that inhibits expression or activity of a third protein selected from the group consisting of IGFBP2, IGF1, IGF1R, MERTK, PITPNC1, ABCB9, PSAT1, PYGB, SHMT2, and VIPR.

38. The method of any of claims 34-37, wherein the first agent, second agent, or third agent is an antibody, a nucleic acid, a polypeptide, or a small molecule compound.

39. The method of any of claims 34-38, wherein the first agent has GAS6 activity.

40. The method of any of claims 34-39, wherein the first agent is GAS6.

41. The method of any of claims 34-39, wherein the first agent is a polypeptide fragment of GAS6.

42. The method of any of claims 24-41, further comprised administering to the subject chemotherapy, surgery, or radiotherapy.

43. A composition comprising an agent that increases expression or activity of GAS6.

44. The composition of claim 43, wherein said agent has GAS6 activity.

45. The composition of claim 43, wherein said agent is GAS6.

46. The composition of claim 43, wherein said agent is a polypeptide fragment of GAS6.

47. A method for generating a population of mammalian cancer cells with increased metastatic tissue colonization potential, comprising performing serial rounds of transplantation, isolation, and repeat transplantation of a population of labeled or unlabeled cancer cells into a living tissue.

48. A method for identifying a gene or a non-coding RNA that regulates metastatic cancer colonization of a body tissue, comprising,

a) introducing one or more shRNA, RNAi, microRNA, or non-coding RNA molecules into a population of starting cancer cells to generate a population of engineered cancer cells;

b) transplanting said population of engineered cancer cells into a tissue of the body;

c) isolating transplanted cells to obtain a population of isolated cancer cells; and

d) assessing the quantity of the shRNA, RNAi, microRNA, or non-coding RNA molecule or protein encoding gene in the population of isolated cancer cells,

wherein a decrease in the quantity of said shRNA, RNAi, microRNA, or non-coding RNA in the population of isolated cells relative to its quantity prior to transplantation indicates that the target gene or genes of said shRNA, RNAi, microRNA, or non-coding RNAs represents a gene required for metastatic colonization of said tissue.

49. A method for identifying a gene or a non-coding RNA that regulates metastatic cancer colonization of a body tissue, comprising

a) introducing one or more DNA molecules that encode a protein encoding gene into a population of starting cancer cells to generate a population of engineered cancer cells;

b) transplanting said population of engineered cancer cells into a tissue of the body;

c) isolating transplanted cells to obtain a population of isolated cancer cells; and

d) assessing the quantity of the protein encoding gene in the population of isolated cancer cells,

wherein an increase in the quantity of said protein encoding gene in the population of isolated cells relative to its quantity prior to transplantation indicates that the protein encoding gene is required for metastatic colonization of said tissue.

50. A composition comprising a population of metastatic human colon cancer cells with an increased potential to colonize the liver.

51. The method of claim 48 or 49, wherein the population of starting cancer cells is obtained according to the method of claim 47.

52. The method of claim 48 or 49, wherein the assessing step is performed using microarray analysis, DNA sequencing technology, deep sequencing technology, or cloning analysis.

53. The composition of claim 16, wherein the agent is a monoclonal antibody or an antigen-binding portion thereof.

54. The composition of claim 16, wherein the agent is a humanized monoclonal antibody or an antigen-binding portion thereof.

55. The composition of claim 16, wherein the agent is a human monoclonal antibody or an antigen-binding portion thereof.

56. The composition of claim 16, wherein the agent is a monoclonal antibody that inhibits IGFBP2 function or an antigen-binding portion thereof.

57. The composition of claim 16, wherein the agent is a monoclonal antibody that inhibits IGFBP2 from binding to IGF1 or an antigen-binding portion thereof.

58. The composition of claim 16, wherein the agent is a monoclonal antibody that inhibits endothelial recruitment or an antigen-binding portion thereof.

59. The composition of claim 16, wherein the agent is a monoclonal antibody that inhibits pathological angiogenesis or an antigen-binding portion thereof.

60. The composition of claim 16, wherein the agent is a monoclonal antibody that inhibits tumor progression or an antigen-binding portion thereof.

61. The composition of claim 16, wherein the agent is a monoclonal antibody that inhibits tumor metastasis or an antigen-binding portion thereof.

62. The composition of claim 16, wherein the agent is an antibody that inhibits breast cancer metastasis or an antigen-binding portion thereof.

63. The monoclonal antibody of claim 57, wherein the monoclonal antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10.

64. The monoclonal antibody of claim 63, where one or more amino acids within the antibody are substituted in order to alter the affinity, specificity, immunogenicity, or isotype of the antibody.

65. The method of any claims 1-13, wherein the first agent is a monoclonal antibody that inhibits IGFBP2 from binding to IGF1 or an antigen-binding portion thereof.

66. The method of any claims 1-13, wherein the first agent is the monoclonal antibody of claim 63 or an antigen-binding portion thereof.

67. The method of any claims 1-13, wherein the first an agent is the monoclonal antibody of claim 64 or an antigen-binding portion thereof.

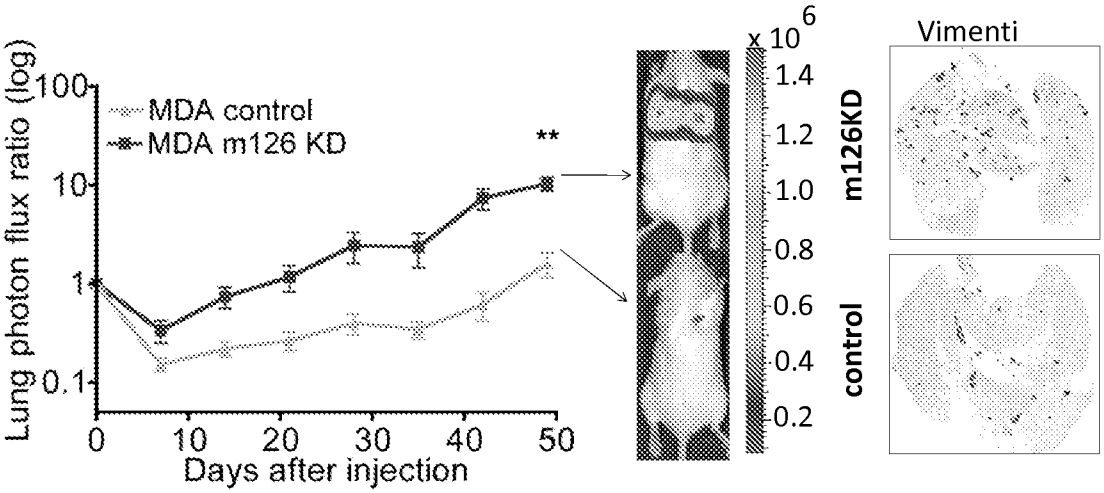


FIG. 1A

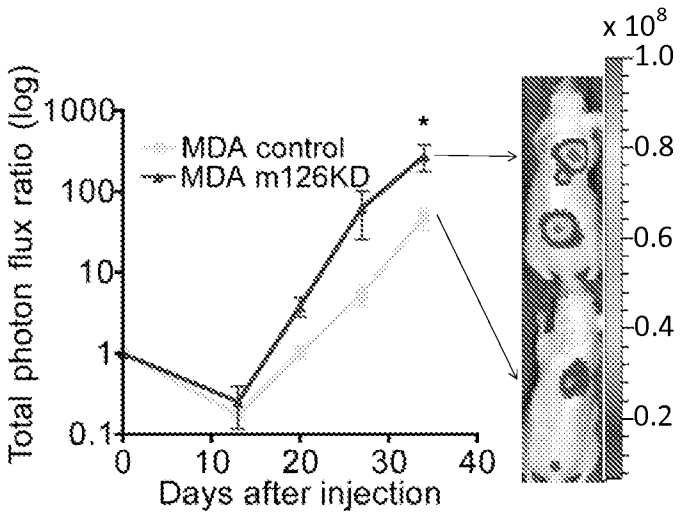


FIG. 1B

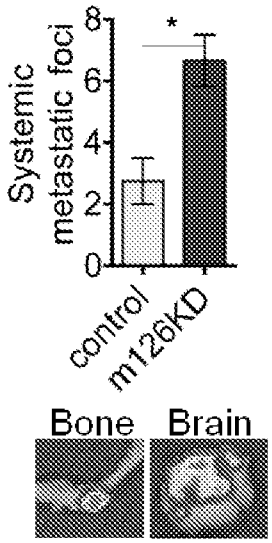


FIG. 1C

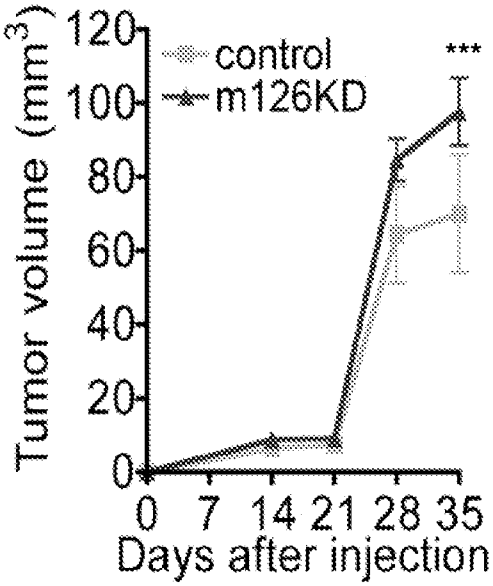


FIG. 1D

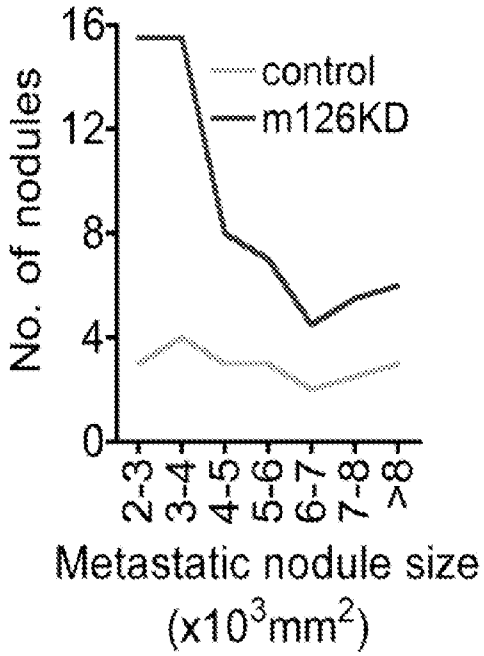


FIG. 1E

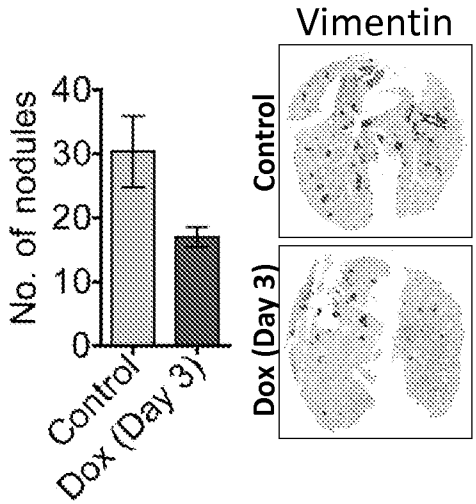


FIG. 1F

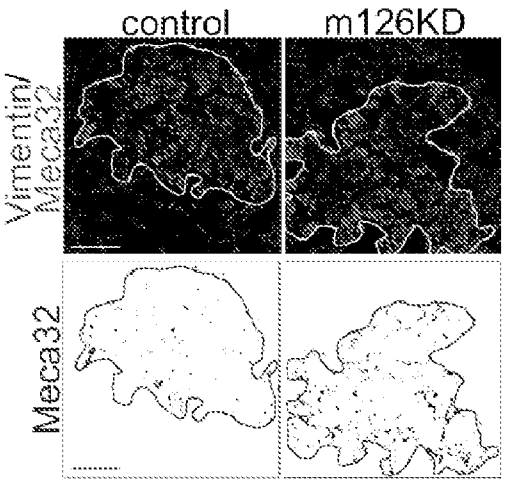


FIG. 2A

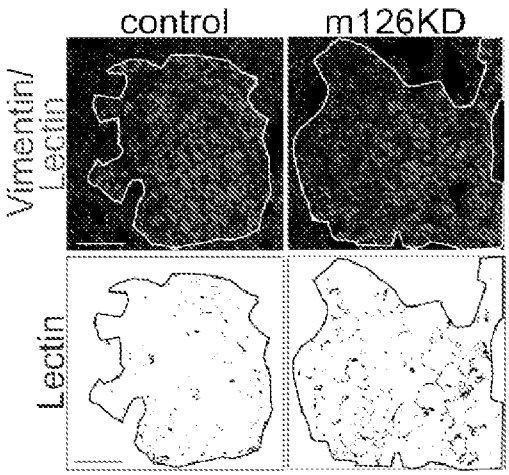


FIG. 2B

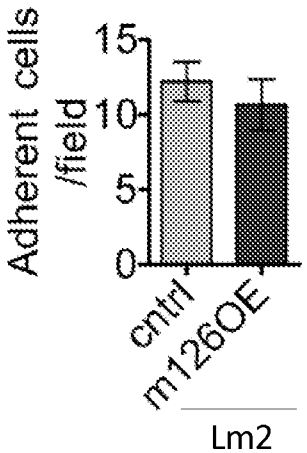


FIG. 2C

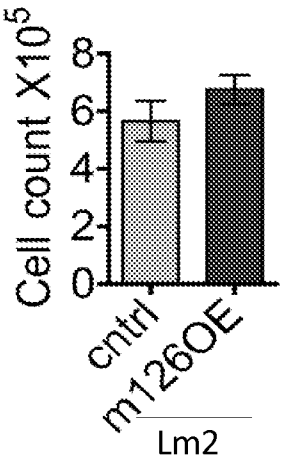


FIG. 2D

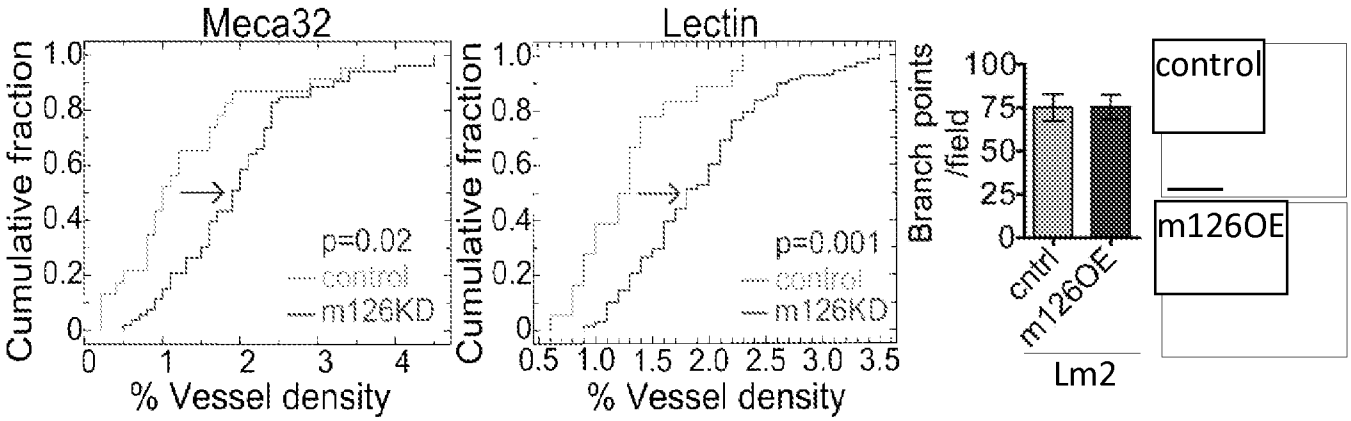


FIG. 2E

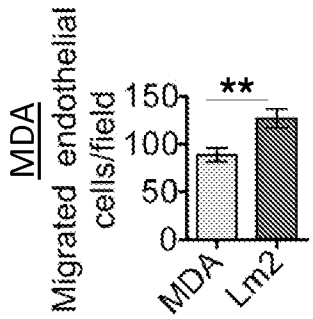


FIG. 2F

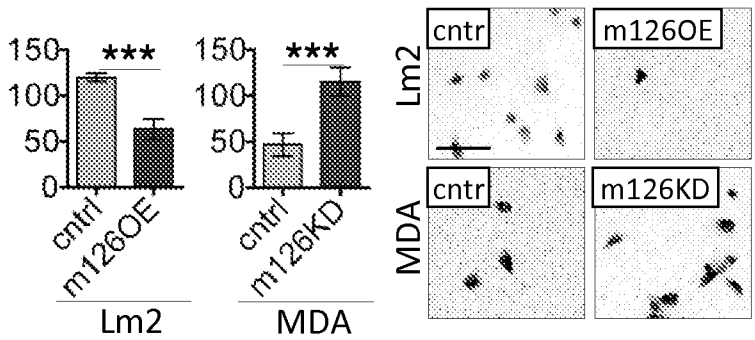


Fig. 2G

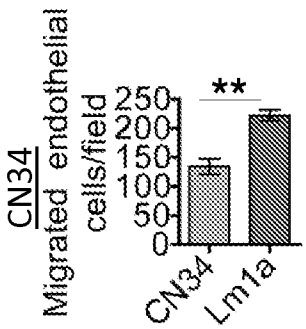


FIG. 2H

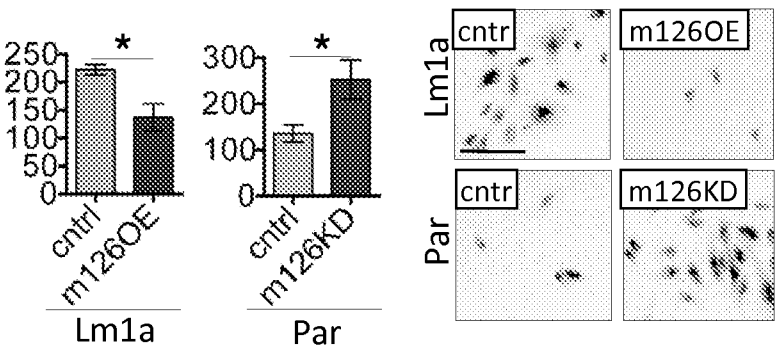
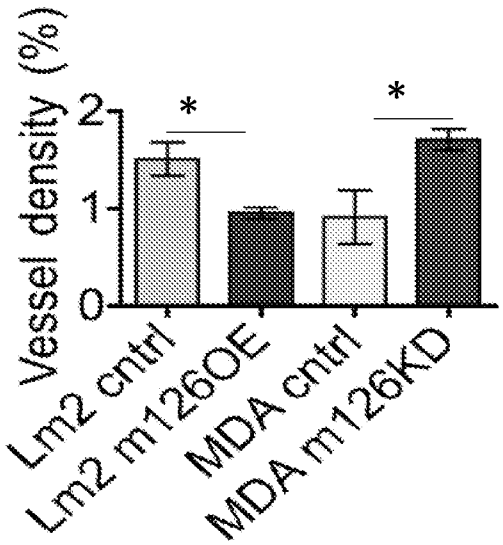


Fig. 2I



MECA-32

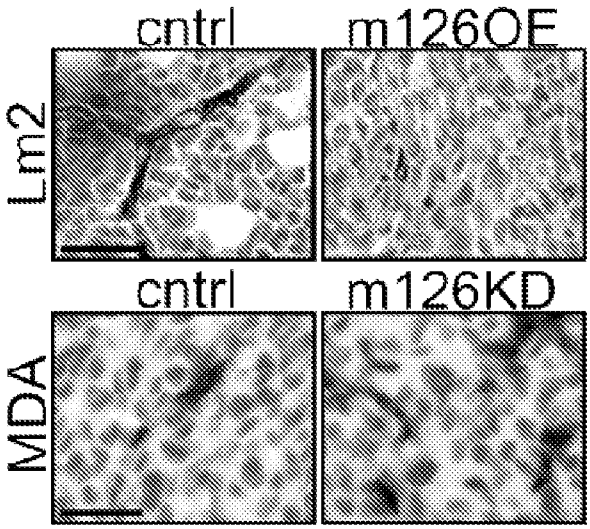


FIG. 2J

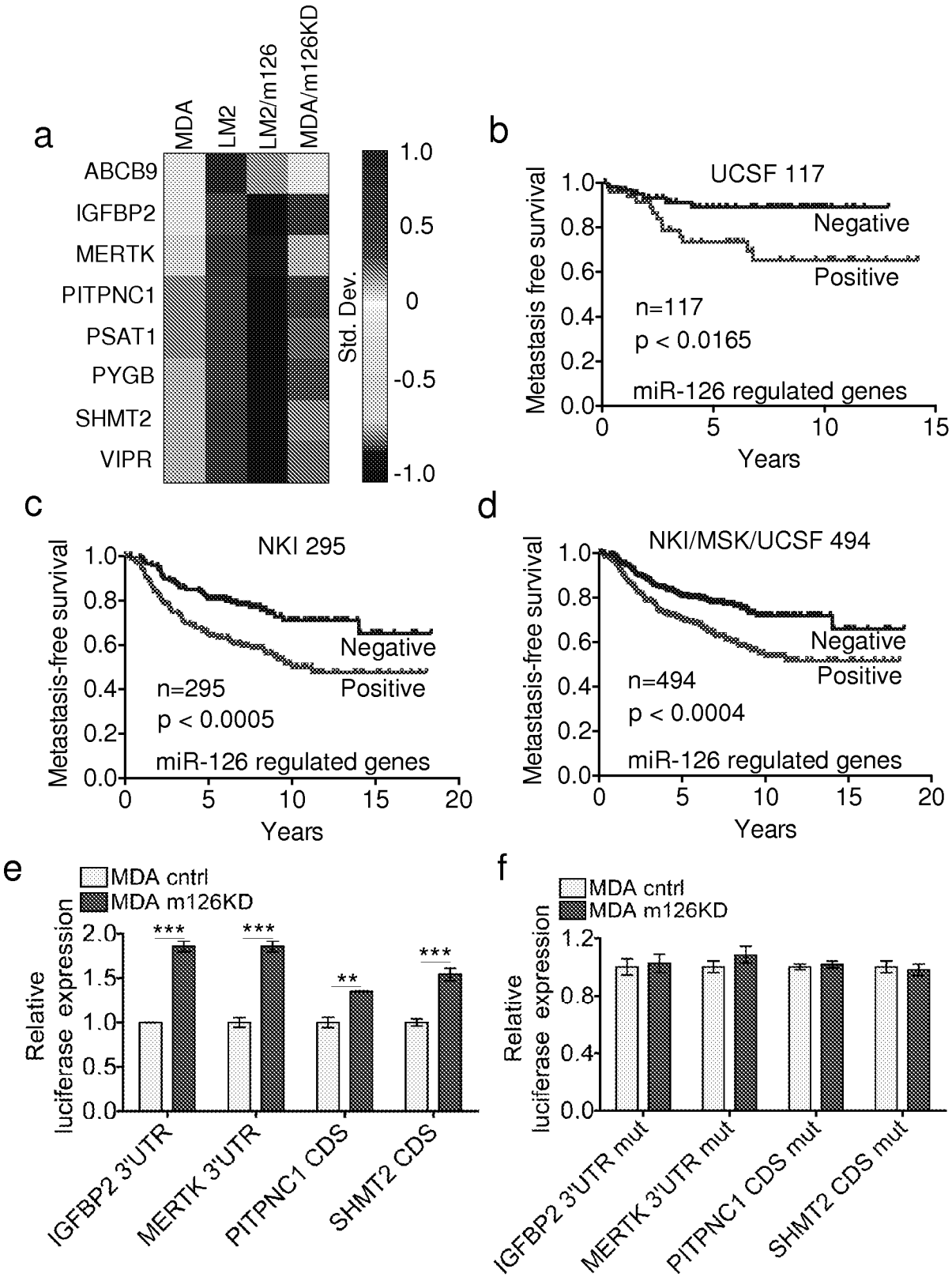

a
MDA
LM2
LM2/m126
MDA/m126KD
ABCB9
IGFBP2
MERTK
PITPNC1
PSAT1
PYGB
SHMT2
VIPR
Std. Dev.
1.0
0.5
0
-0.5
-1.0
b
UCSF 117
Metastasis free survival
Negative
Positive
n=117
p < 0.0165
miR-126 regulated genes
Years
c
NKI 295
Metastasis-free survival
Negative
Positive
n=295
p < 0.0005
miR-126 regulated genes
Years
d
NKI/MSK/UCSF 494
Metastasis-free survival
Negative
Positive
n=494
p < 0.0004
miR-126 regulated genes
Years
e
MDA cntrl
MDA m126KD
Relative luciferase expression
***
***
**
***
IGFBP2 3'UTR
MERTK 3'UTR
PITPNC1 CDS
SHMT2 CDS
f
MDA cntrl
MDA m126KD
Relative luciferase expression
IGFBP2 3'UTR mut
MERTK 3'UTR mut
PITPNC1 CDS mut
SHMT2 CDS mut

FIG. 3

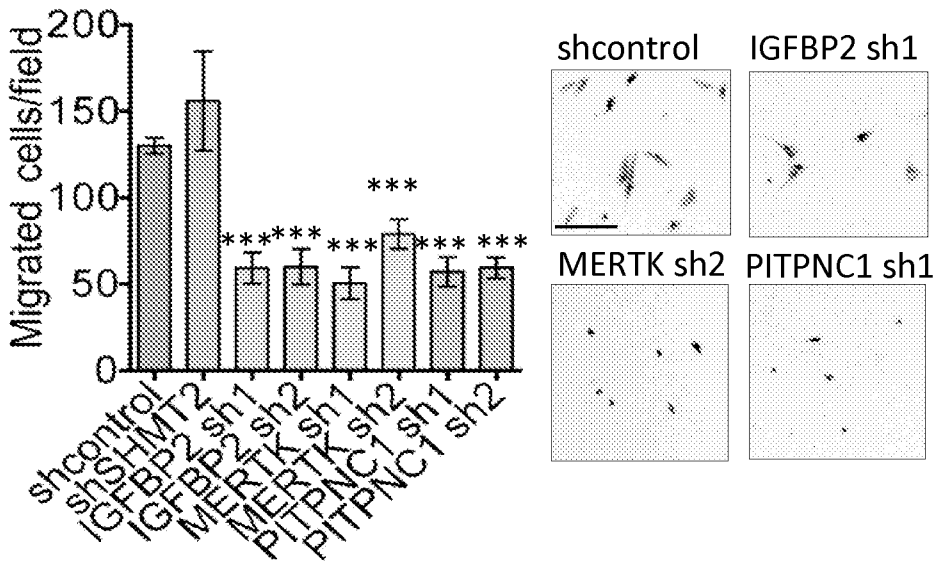


FIG. 4A

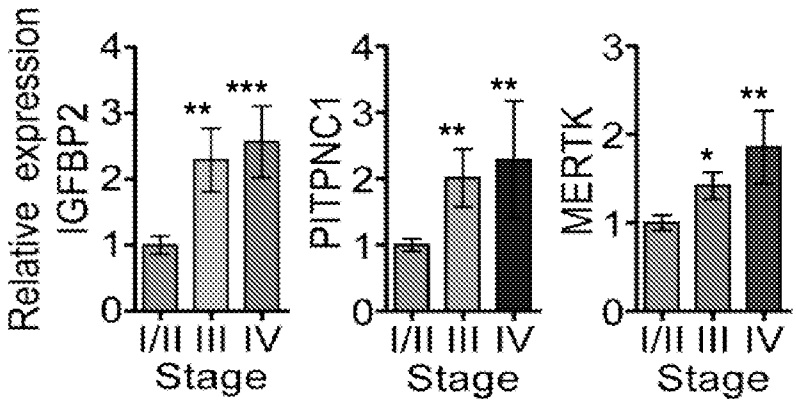


FIG. 4B

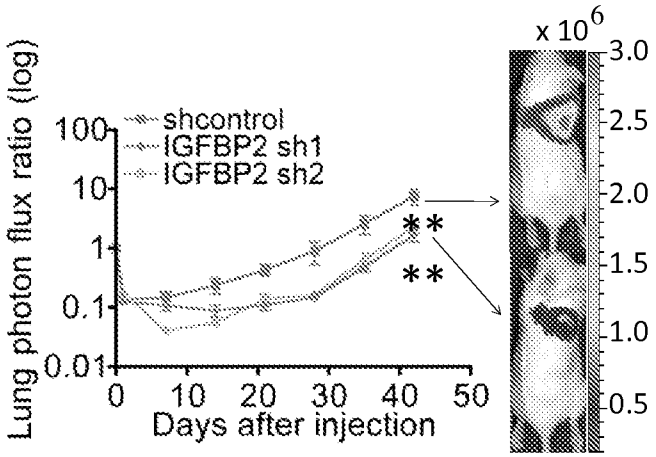


FIG. 4C

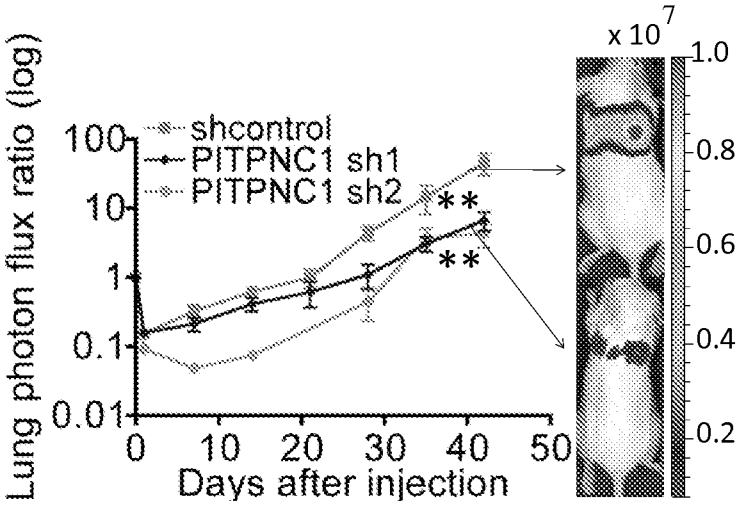

Lung photon flux ratio (log)
100
10
1
0.1
0.01
shcontrol
PITPNC1 sh1
PITPNC1 sh2
**
**
0
10
20
30
40
50
Days after injection
x 10^7
1.0
0.8
0.6
0.4
0.2

FIG. 4D

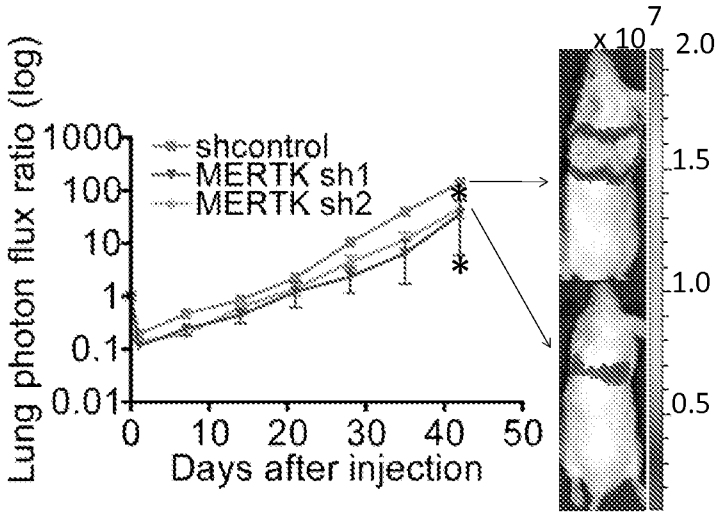

Lung photon flux ratio (log)
1000
100
10
1
0.1
0.01
shcontrol
MERTK sh1
MERTK sh2
*
*
0
10
20
30
40
50
Days after injection
x 10^7
2.0
1.5
1.0
0.5

FIG. 4E

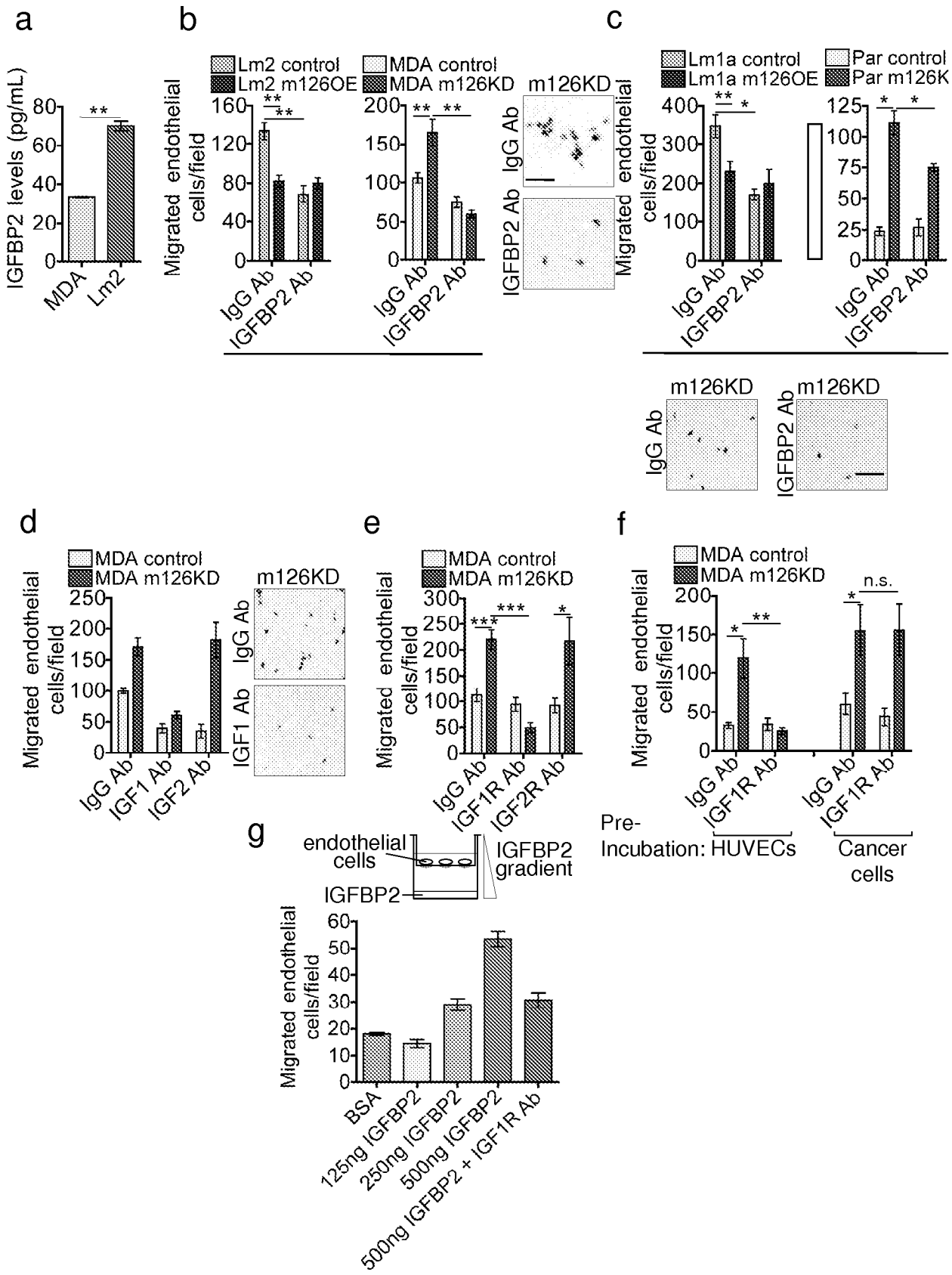

a
IGFBP2 levels (pg/mL)
MDA
Lm2
b
Migrated endothelial cells/field
Lm2 control
Lm2 m126OE
MDA control
MDA m126KD
IgG Ab
IGFBP2 Ab
m126KD
c
Lm1a control
Lm1a m126OE
Par control
Par m126K
d
IGF1 Ab
IGF2 Ab
e
IGF1R Ab
IGF2R Ab
f
n.s.
Pre-Incubation:
HUVECs
Cancer cells
g
endothelial cells
IGFBP2
IGFBP2 gradient
BSA
125ng IGFBP2
250ng IGFBP2
500ng IGFBP2
500ng IGFBP2 + IGF1R Ab

FIG. 5

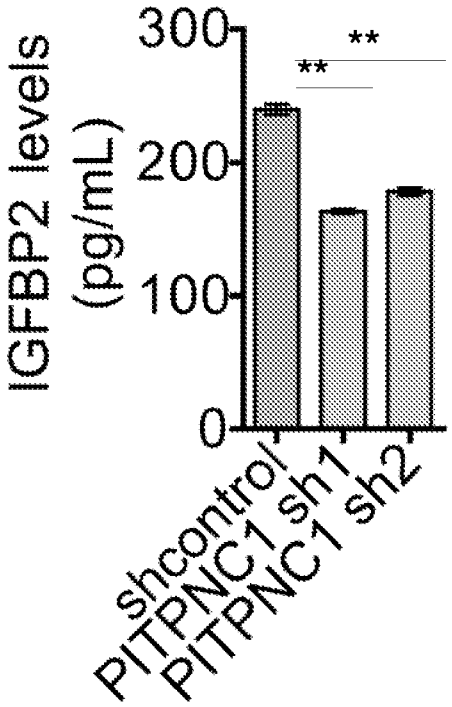


FIG. 6A

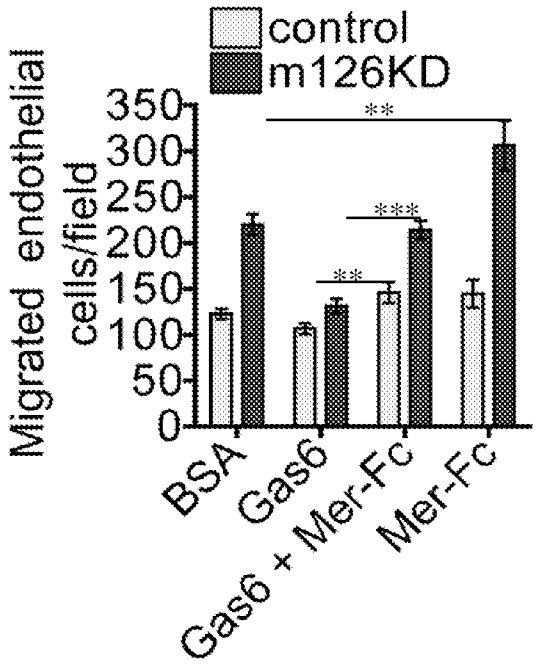


FIG. 6B

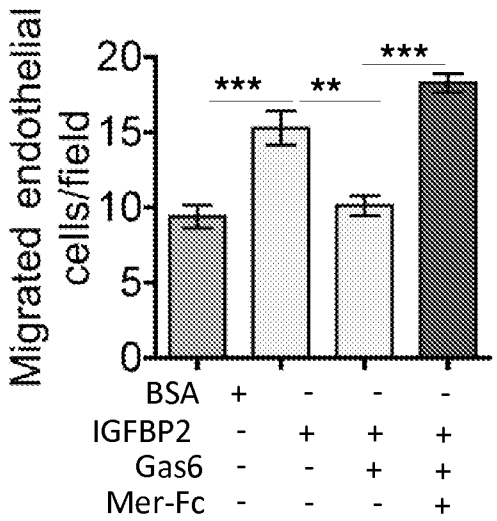


FIG. 6C

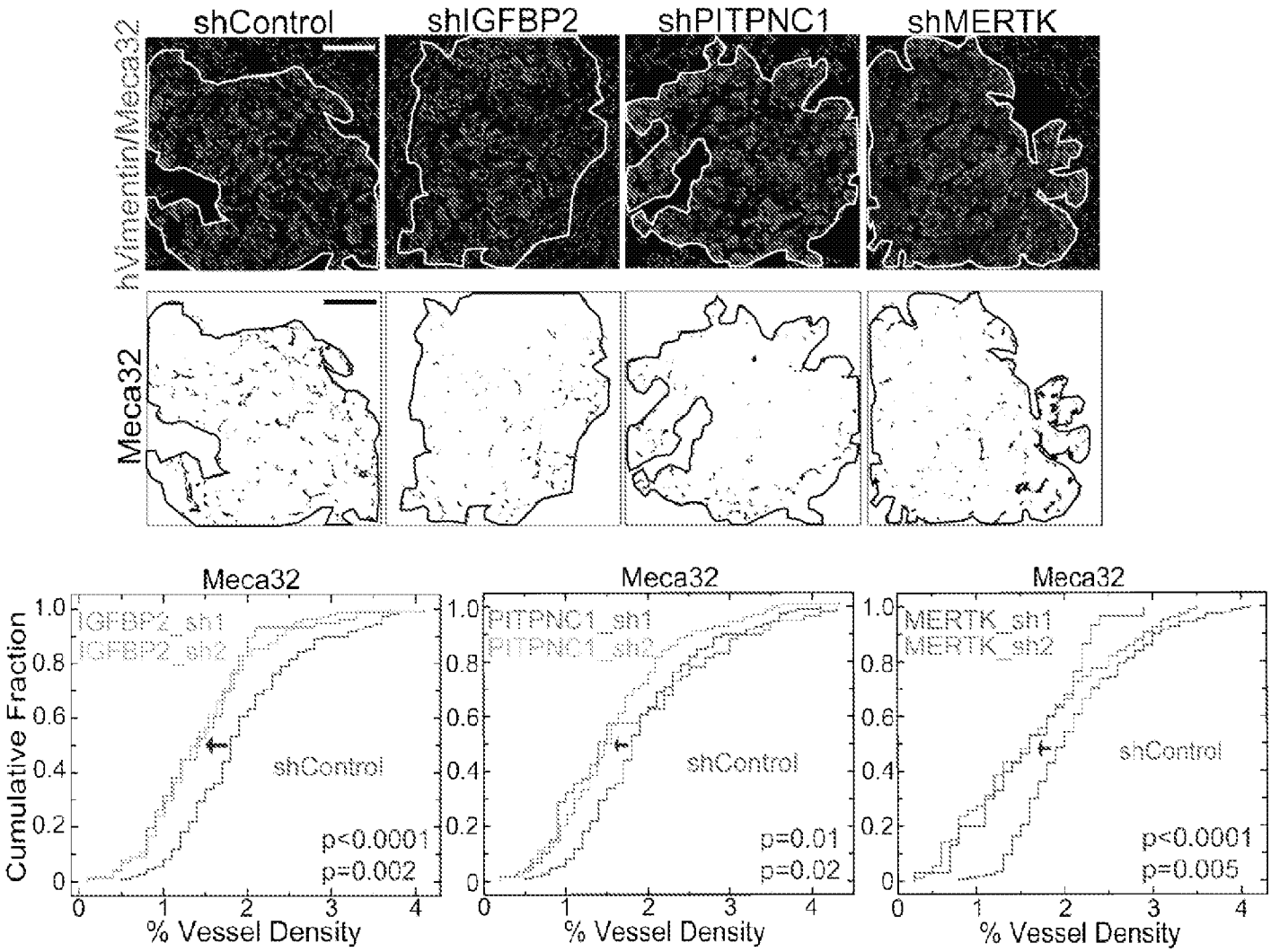

shControl
shIGFBP2
shPITPNC1
shMERTK
hVimentin/Meca32
Meca32
Meca32
Meca32
Meca32
Cumulative Fraction
IGFBP2_sh1
IGFBP2_sh2
shControl
p<0.0001
p=0.002
% Vessel Density
PITPNC1_sh1
PITPNC1_sh2
shControl
p=0.01
p=0.02
% Vessel Density
MERTK_sh1
MERTK_sh2
shControl
p<0.0001
p=0.005
% Vessel Density

FIG. 6D

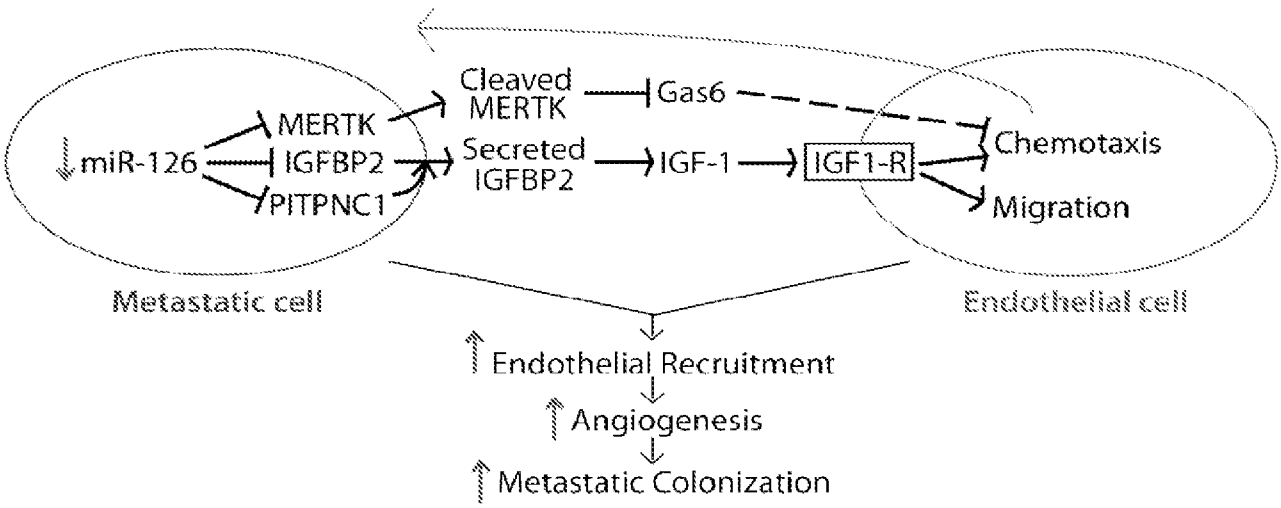

miR-126
MERTK
IGFBP2
PITPNC1
Cleaved MERTK
Gas6
Secreted IGFBP2
IGF-1
IGF1-R
Chemotaxis
Migration
Metastatic cell
Endothelial cell
Endothelial Recruitment
Angiogenesis
Metastatic Colonization

FIG. 6E

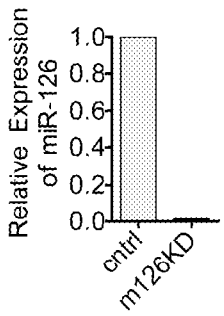


FIG. 7

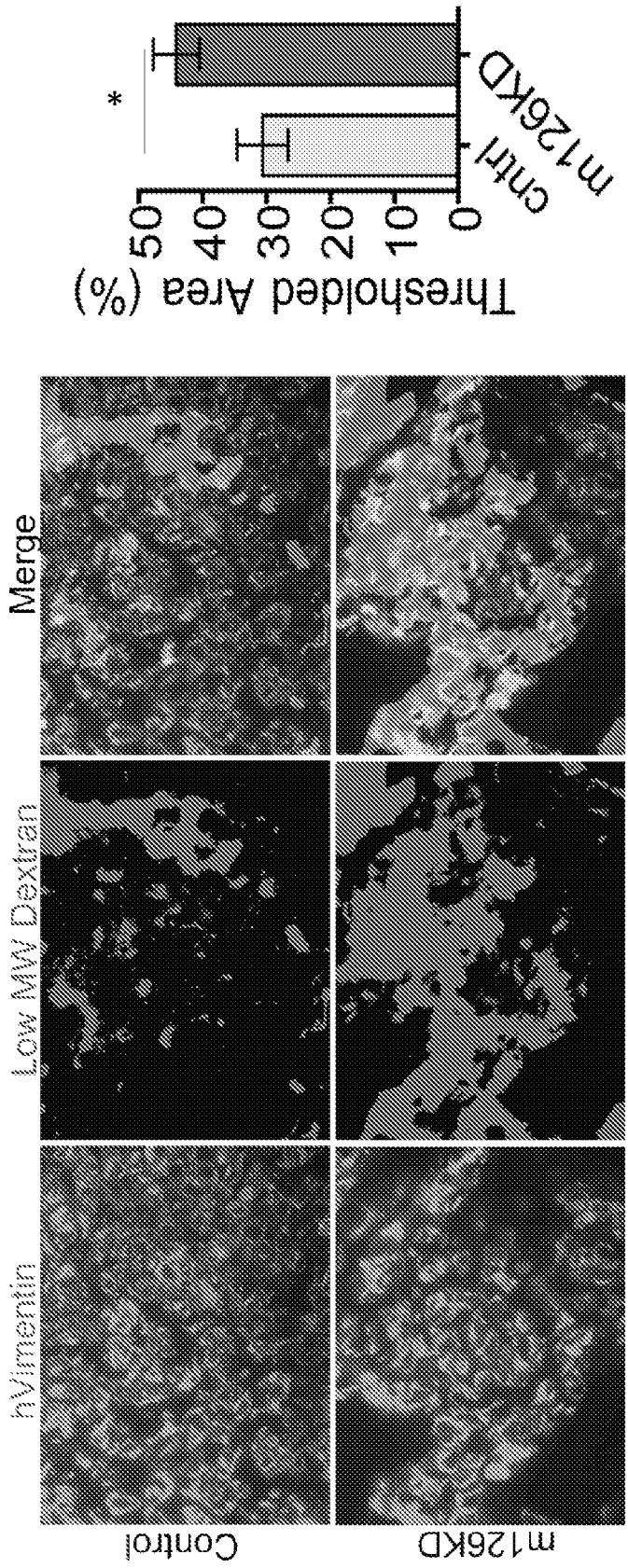

Thresholded Area (%)
0
10
20
30
40
50
*
cntrl
m126KD
Merge
Low MW Dextran
hVimentin
Control
m126KD

FIG. 8

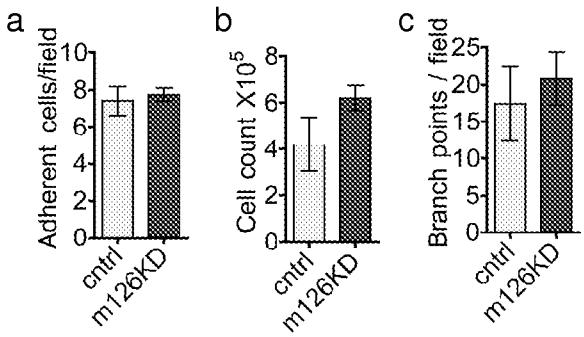

a
Adherent cells/field
10
8
6
4
2
0
cntrl
m126KD
b
Cell count X10^5
8
6
4
2
0
cntrl
m126KD
c
Branch points / field
25
20
15
10
5
0
cntrl
m126KD

FIG. 9

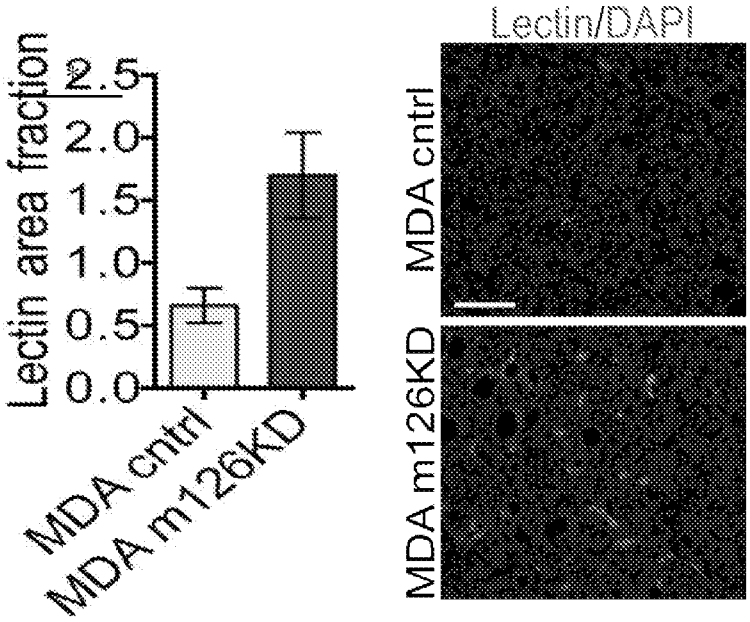


FIG. 10A

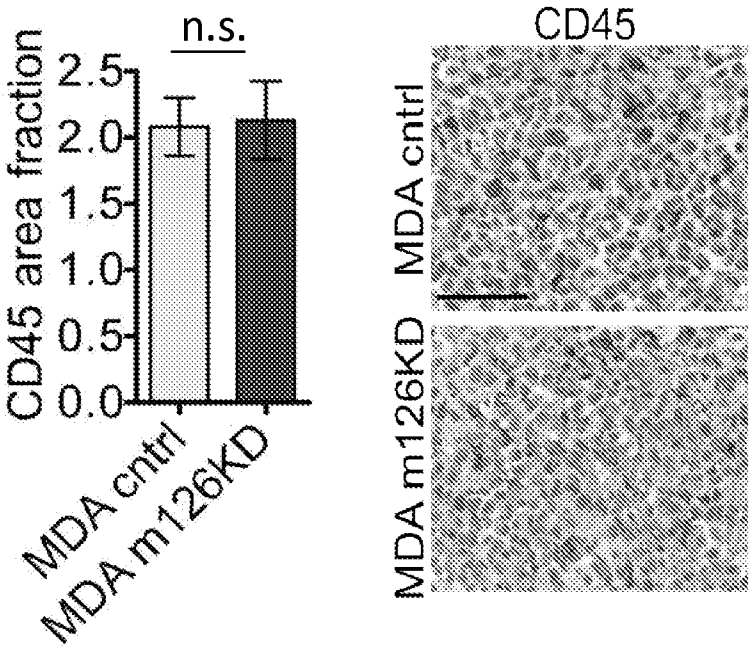


FIG. 10B

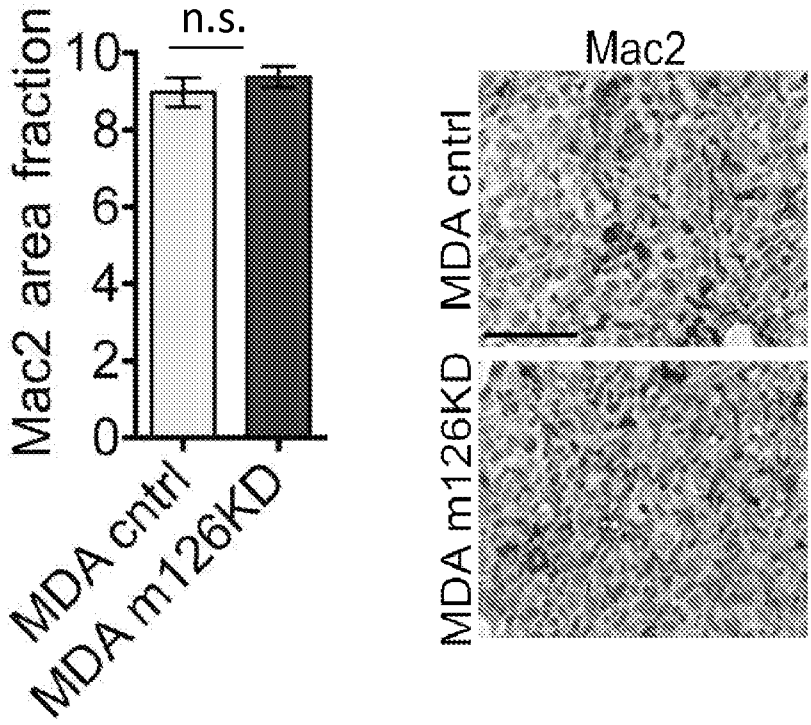

n.s.
Mac2 area fraction
10
8
6
4
2
0
MDA cntrl
MDA m126KD
Mac2
MDA cntrl
MDA m126KD

FIG. 10C

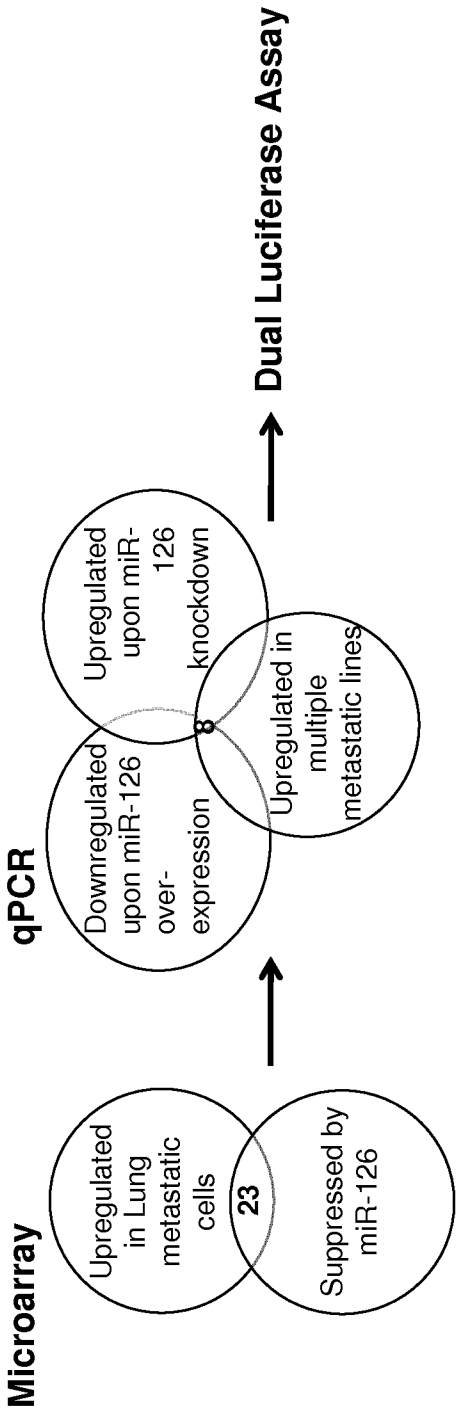

Microarray
Upregulated in Lung metastatic cells
23
Suppressed by miR-126
qPCR
Downregulated upon miR-126 over-expression
8
Upregulated upon miR-126 knockdown
Upregulated in multiple metastatic lines
Dual Luciferase Assay

FIG. 11

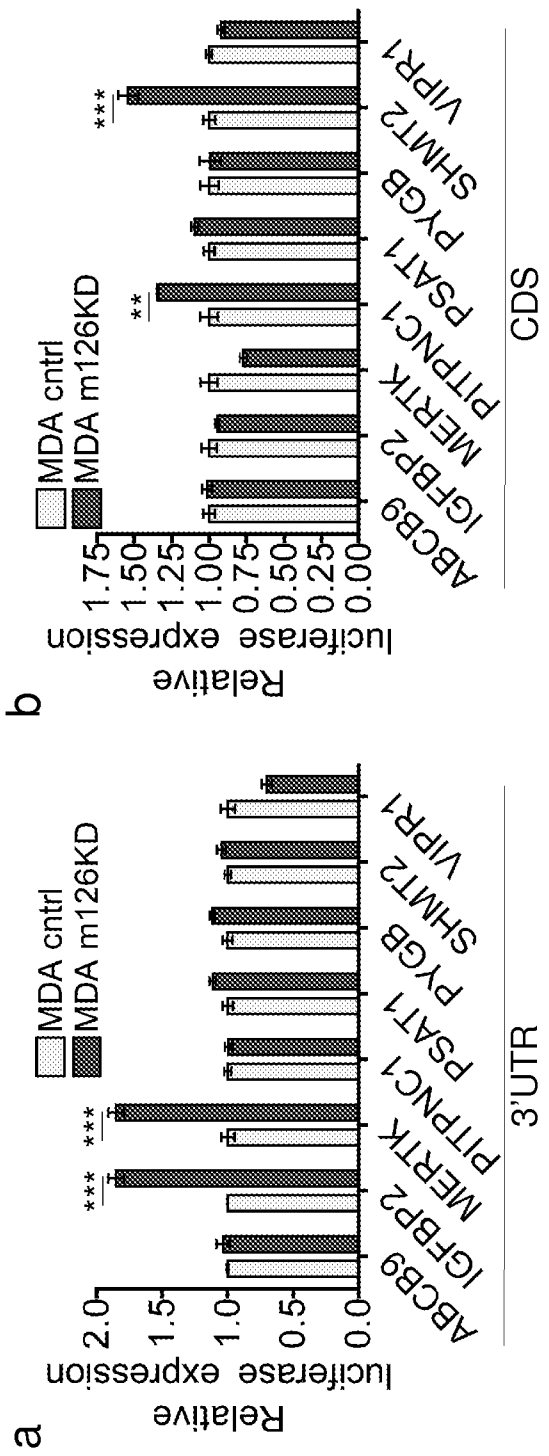

a
Relative luciferase expression
2.0
1.5
1.0
0.5
0.0
MDA cntrl
MDA m126KD
***
***
ABCB9
IGFBP2
MERTK
PITPNC1
PSAT1
PYGB
SHMT2
VIPR1
3'UTR
b
Relative luciferase expression
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
MDA cntrl
MDA m126KD
**
***
ABCB9
IGFBP2
MERTK
PITPNC1
PSAT1
PYGB
SHMT2
VIPR1
CDS

FIG. 12

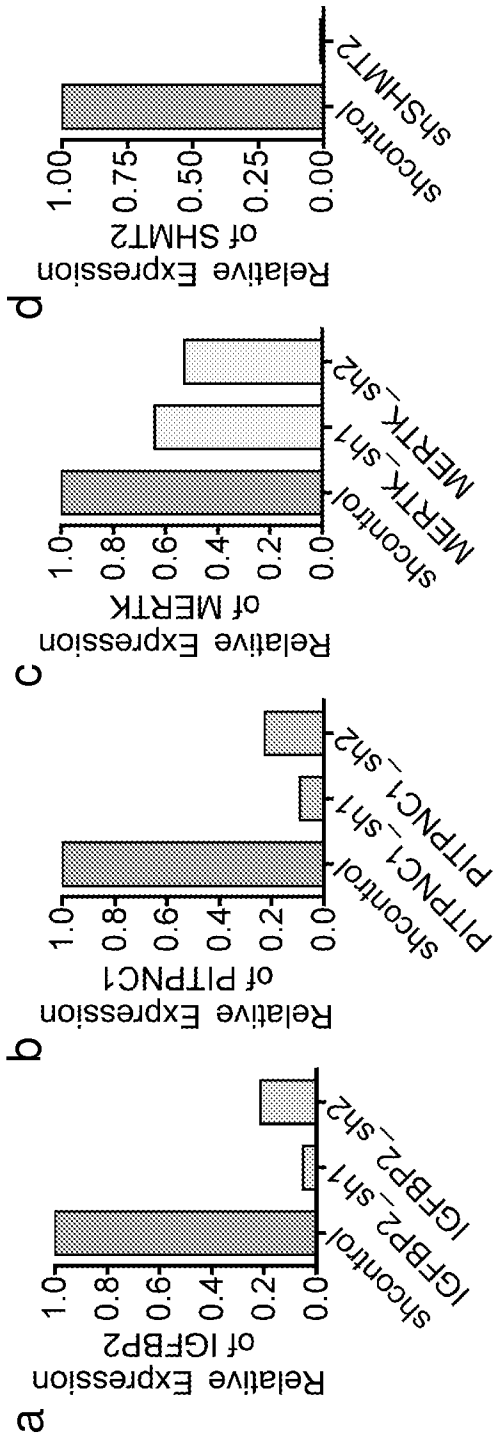

a
Relative Expression of IGFBP2
1.0
0.8
0.6
0.4
0.2
0.0
shcontrol
IGFBP2_sh1
IGFBP2_sh2
b
Relative Expression of PITPNC1
shcontrol
PITPNC1_sh1
PITPNC1_sh2
c
Relative Expression of MERTK
shcontrol
MERTK_sh1
MERTK_sh2
d
Relative Expression of SHMT2
1.00
0.75
0.50
0.25
0.00
shcontrol
shSHMT2

FIG. 13

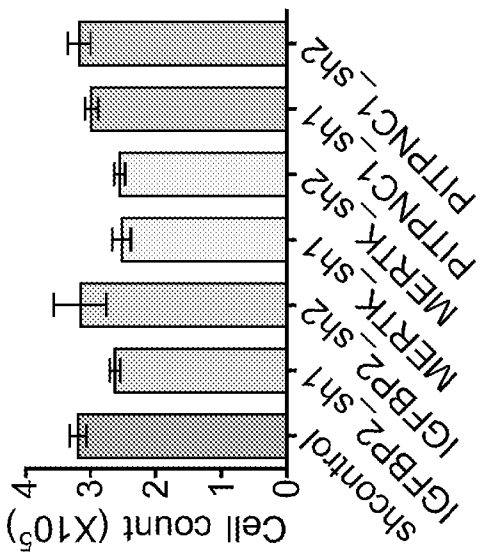

Cell count (X10^5)
0
1
2
3
4
shcontrol
IGFBP2_sh1
IGFBP2_sh2
MERTK_sh1
MERTK_sh2
PITPNC1_sh1
PITPNC1_sh2

FIG 14

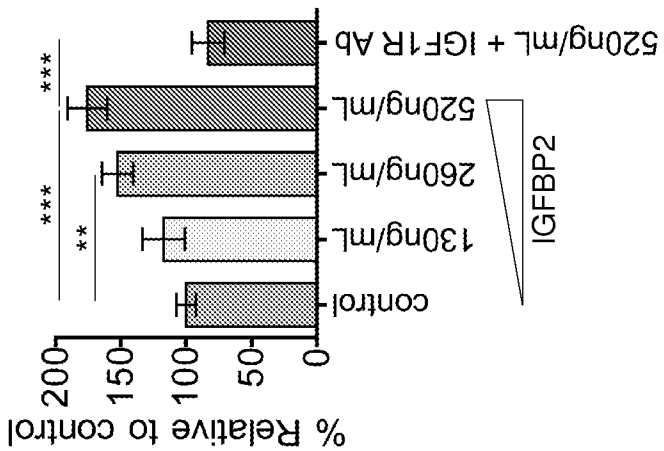

% Relative to control
0
50
100
150
200
control
130ng/mL
260ng/mL
520ng/mL
520ng/mL + IGF1R Ab
IGFBP2
**
***
***

FIG. 15

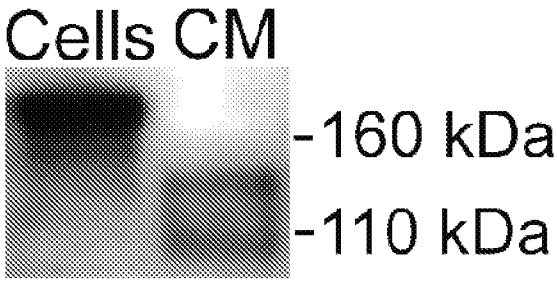


FIG. 16

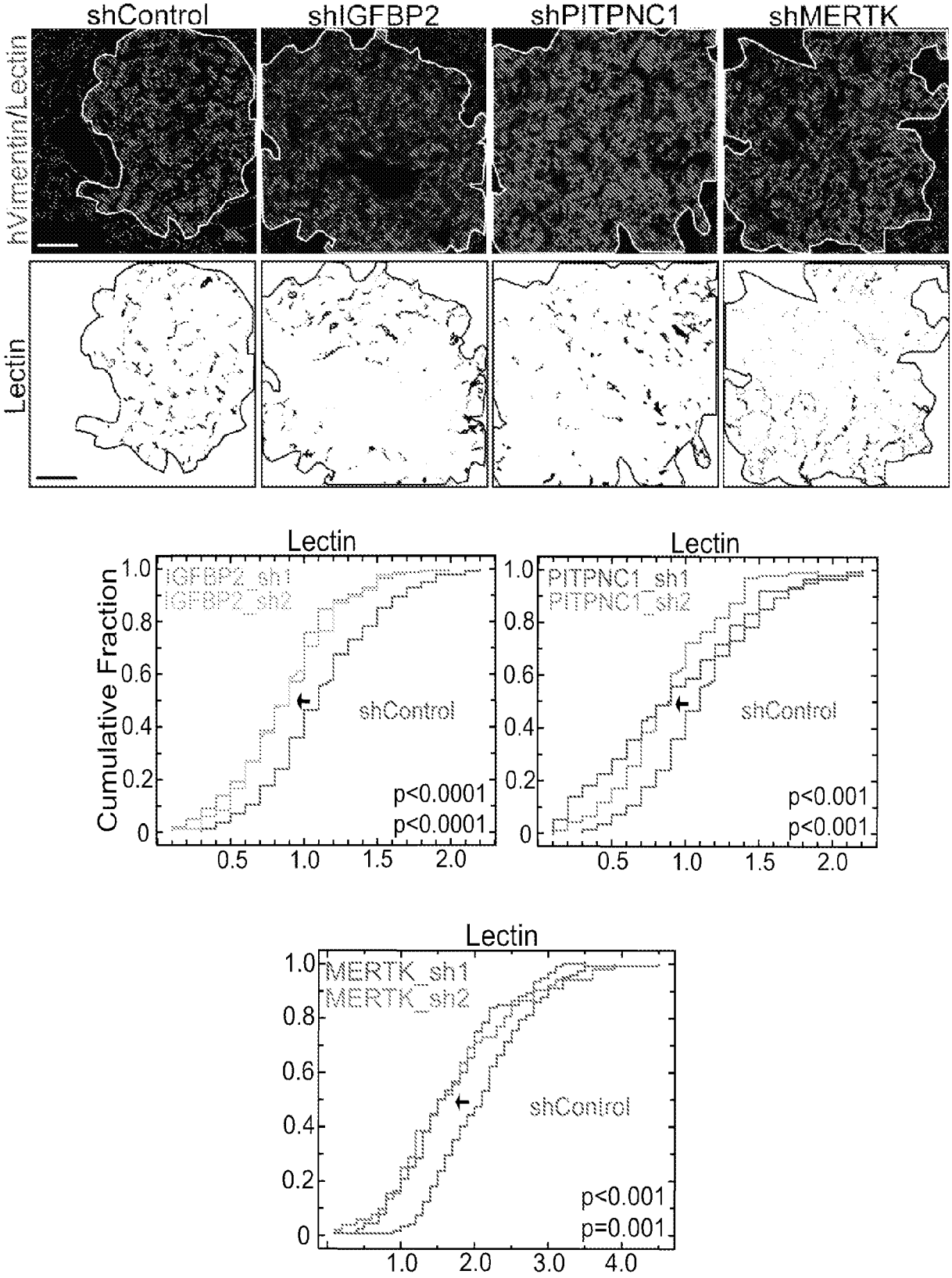

shControl
shIGFBP2
shPITPNC1
shMERTK
hVimentin/Lectin
Lectin
Lectin
Cumulative Fraction
IGFBP2_sh1
IGFBP2_sh2
shControl
p<0.0001
p<0.0001
Lectin
PITPNC1_sh1
PITPNC1_sh2
shControl
p<0.001
p<0.001
Lectin
MERTK_sh1
MERTK_sh2
shControl
p<0.001
p=0.001

FIG. 17

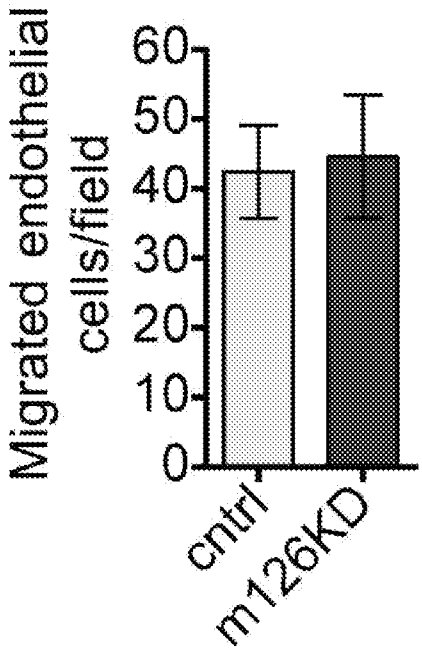

Migrated endothelial cells/field
60
50
40
30
20
10
0
cntrl
m126KD

FIG. 18

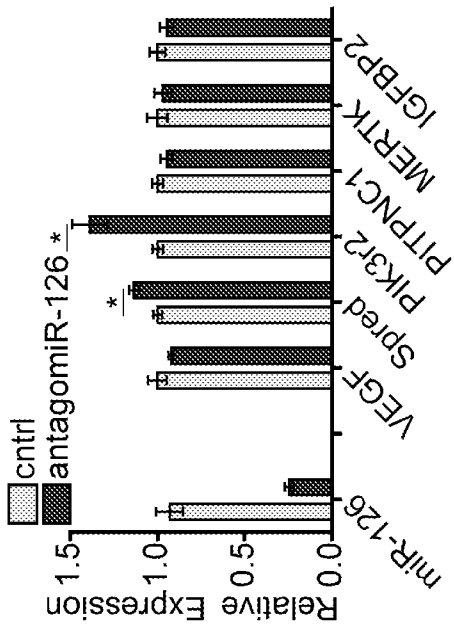
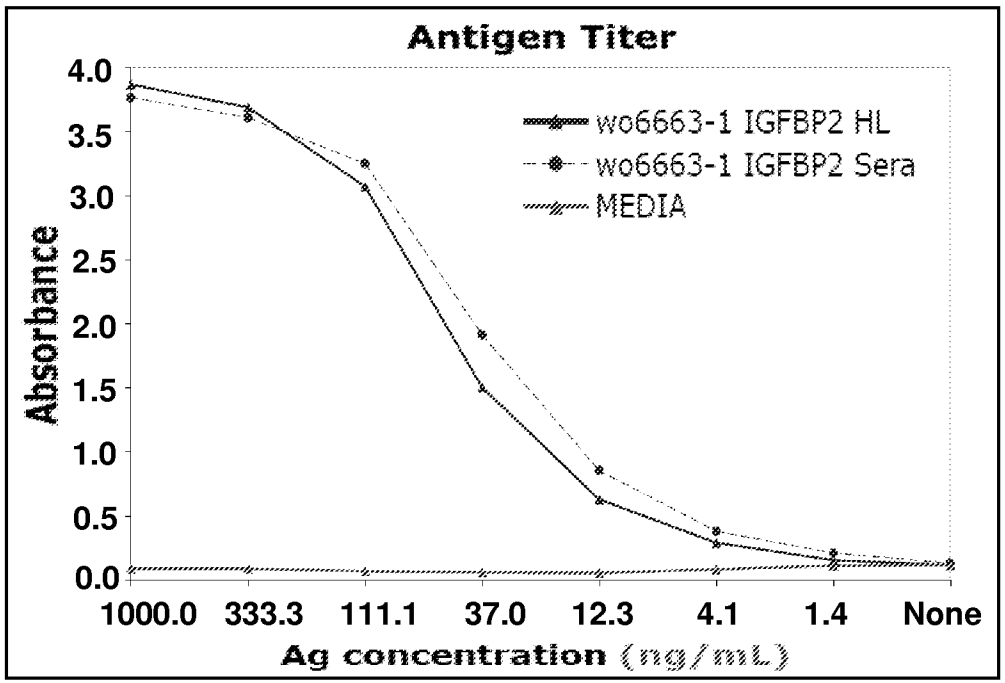

cntrl
antagomiR-126
Relative Expression
1.5
1.0
0.5
0.0
*
*
miR-126
VEGF
Spred
PIK3r2
PITPNC1
MERTK
IGFBP2

FIG. 19

Ag Concentration	wo6663-1 IGFBP2 HL	wo6663-1 IGFBP2 Sera	AVERAGE MEDIA	HT MEDIA	HT MEDIA
1000.0	3.8654	3.762	0.0888	0.075	0.1026
333.3	3.6839	3.6066	0.0893	0.0623	0.1162
111.1	3.0672	3.2468	0.0697	0.0564	0.0829
37.0	1.5044	1.9111	0.0631	0.0627	0.0635
12.3	0.6277	0.8578	0.0567	0.0576	0.0557
4.1	0.2915	0.3796	0.0863	0.0758	0.0968
1.4	0.1556	0.2108	0.1110	0.1101	0.1119
None	0.1196	0.1322	0.1186	0.1228	0.1143



Coating Ab ID: Gt Anti-Mouse IgG

Ab ID: IGFBP2 HL Supnt

Ab ID: IGFBP2 Mice Sera m1-m3 day 70

Ag ID: IGFBP2-biotin

Detecting Reagent ID: SA-HRP

Assay Summary: Coat EIA/RIA polystyrene plate with coating Ab--->Block--->Primary Ab--->Biotinylated-Ag--->SA-HRP--->Stop and read.

FIG. 20

Competitor Ag Concentration	IGFBP2 HL vs. IGF-I	IGFBP2 HL vs. IGFBP3	IGFBP2 HL vs. IGFBP4	Media vs. IGF-I	Media vs. IGFBP3	Media vs. IGFBP4
5000.0	3.3238	3.2873	3.2493	0.5187	0.5171	0.5
2500.0	3.3069	3.2855	3.2905	0.5272	0.5414	0.5108
1250.0	3.3431	3.2769	3.3108	0.5143	0.5398	0.53
625.0	3.4375	3.3597	3.347	0.4893	0.5307	0.5504
312.5	3.4756	3.4014	3.3937	0.5004	0.5013	0.5144
156.3	3.5041	3.4262	3.4286	0.4916	0.496	0.5322
78.1	3.4833	3.39	3.4193	0.5234	0.5183	0.5384
None	3.4463	3.4077	3.3936	0.5344	0.5485	0.5277

Coating Ab ID; Gt Anti-Mouse IgG

Ab ID; IGFBP2 HL Supnt

Ag ID; IGFBP2-biotin

Detecting Reagent ID; SA-HRP

Competitor ID; IGF-I (PeproTech #100-11)

Competitor ID; IGFBP3 (PeproTech #100-08)

Competitor ID; IGFBP4 (PeproTech #350-05B)

Assay Summary: Coat EIA/RIA polystyrene plate with coating Ab--->Block--->Primary Ab--->Biotinylated-Ag--->SA-HRP--->Stop and read.

FIG. 21

T.C. Designation	T.C. Well	O.D. by ELISA vs. IGFBP2-Biotin	O.D. by ELISA vs. IGFBP2-Biotin + IGF-1	Ratio
	1B1	0.86	0.59	1.44
IGFBP2_M1	1C2	2.53	1.82	1.39
	1D9	0.82	0.53	1.54
IGFBP2_M2	1A11	0.90	0.64	1.41
	1E11	0.76	0.52	1.46
IGFBP2_M3	2F6	0.80	0.61	1.30
IGFBP2_M4	2H7	2.42	2.09	1.16
IGFBP2_M5	3D2	0.10	0.39	0.26
IGFBP2_M6	3B3	2.27	1.99	1.14
IGFBP2_M7	3A4	1.65	0.73	2.26
IGFBP2_M8	3E7	0.56	0.09	5.98
IGFBP2_M9	4H2	2.43	2.14	1.14
	4E4	0.71	0.60	1.19
IGFBP2_M10	4C8	0.85	0.72	1.17
	4D8	0.72	0.61	1.19
	4A9	0.75	0.59	1.27
IGFBP2_M11	4F10	0.86	0.70	1.22
	5B1	0.83	0.70	1.18
IGFBP2_M12	5D1	0.89	0.82	1.08
	5F2	0.72	0.64	1.13
	5H2	0.76	0.68	1.13
	5H4	0.75	0.64	1.17
	5B5	0.83	0.71	1.17
IGFBP2_M13	5E5	2.37	2.09	1.13
	5F5	0.72	0.60	1.21
	5D7	0.84	0.74	1.14
	5A8	0.81	0.66	1.24
	6D2	0.84	0.70	1.19
IGFBP2_M14	6B4	2.39	2.12	1.13
IGFBP2_M15	6H9	2.43	2.16	1.12
IGFBP2_M16	6C12	2.36	2.11	1.12
	7E4	0.76	0.66	1.15
	7G4	0.73	0.61	1.20
	7A7	0.82	0.70	1.16
IGFBP2_M17	7E7	0.78	0.61	1.27
	7E9	0.72	0.62	1.16
	8H3	0.87	0.83	1.05
	8A10	0.74	0.74	1.00
IGFBP2_M18	8C10	0.56	0.80	0.70
IGFBP2_M19	10H1	0.89	0.76	1.17
	10E3	0.76	0.66	1.14
IGFBP2_M20	10A5	2.42	2.22	1.09
	10F8	0.86	0.79	1.08
	10C9	0.82	0.74	1.12

Assay Summary: Coat EIA/RIA polystyrene plate with coating Ab--->Block--->Primary Ab--->Biotinylated-Ag--->SA-HRP--->Stop and read.

Controls		Avg. O.D. by ELISA vs. IGFBP2-Biotin	Avg. O.D. by ELISA vs. IGFBP2-Biotin + IGF-1
Positive control	Hyb Lib	2.43	2.17
Positive control	Pos. Sera	2.76	2.45
Negative control	HT Media	0.11	0.10

Coating Ab ID:	**Gt Anti-Mouse IgG (Fc spec)**
Ab ID:	**IGFBP2 Hybridoma Library Supernatant**
Ab ID:	**IGFBP2 Mice Sera m1-m3**
Ag ID:	**IGFBP2-biotin**
Comp Ag ID:	**IGF-I (Peprotech #100-110)**
Detecting Reagent ID:	**SA-HRP**

FIG. 22

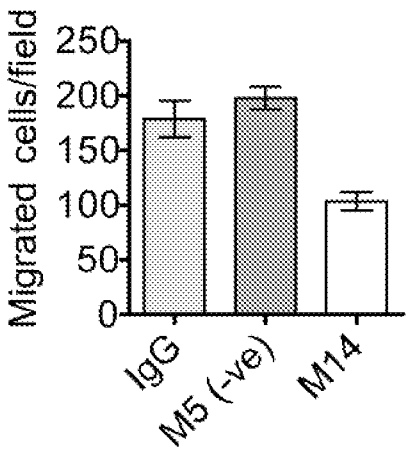


FIG. 23

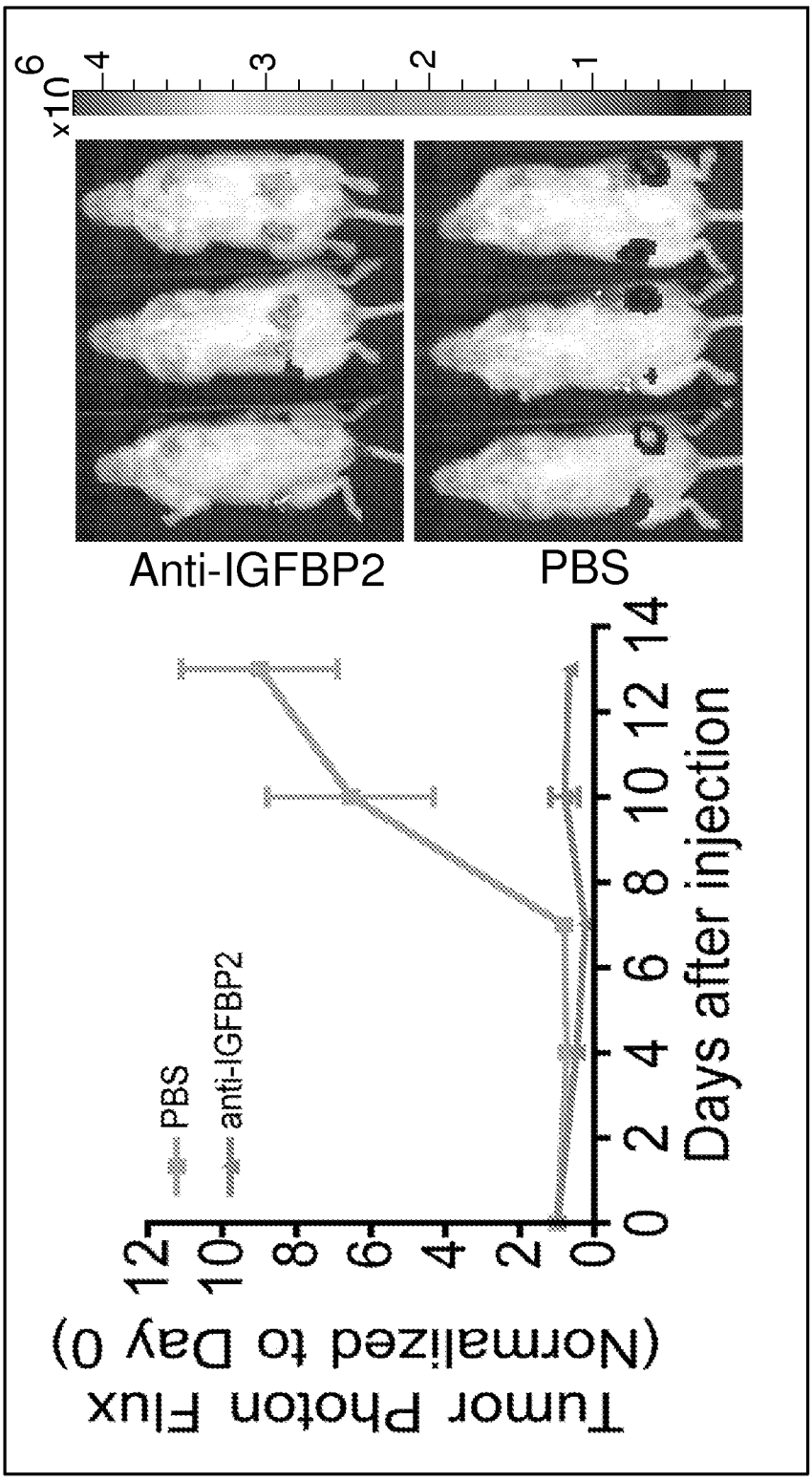

x10^6
4
3
2
1
Anti-IGFBP2
PBS
Tumor Photon Flux
(Normalized to Day 0)
12
10
8
6
4
2
0
0 2 4 6 8 10 12 14
Days after injection
PBS
anti-IGFBP2

FIG. 24