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(54) Title: MBMS AS MODIFIERS OF BRANCHING MORPHOGENESIS AND METHODS OF USE

(57) Abstract: Human MBM genes are identified as modulators of branching morphogenesis, and thus are therapeutic targets for disorders associated with defective branching morphogenesis function. Methods for identifying modulators of branching morphogenesis, comprising screening for agents that modulate the activity of MBM are provided.

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MBMS AS MODIFIERS OF BRANCHING MORPHOGENESIS AND METHODS OF USE

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent application 60/539,835 filed 1/28/2004. The contents of the prior application are hereby incorporated in their entirety.

BACKGROUND OF THE INVENTION

[0002] Several essential organs (e.g., lungs, kidney, lymphatic system and vasculature) are made up of complex networks of tube-like structures that serve to transport and exchange fluids, gases, nutrients and waste. The formation of these complex branched networks occurs by the evolutionarily conserved process of branching morphogenesis, in which successive ramification occurs by sprouting, pruning and remodeling of the network. During human embryogenesis, blood vessels develop via two processes: vasculogenesis, whereby endothelial cells are born from progenitor cell types; and angiogenesis, in which new capillaries sprout from existing vessels.

[0003] Branching morphogenesis encompasses many cellular processes, including proliferation, survival/apoptosis, migration, invasion, adhesion, aggregation, inflammation, and matrix remodeling. Numerous cell types contribute to branching morphogenesis, including endothelial, epithelial and smooth muscle cells, and monocytes. Gene pathways that modulate the branching process function both within the branching tissues as well as in other cells, e.g., certain monocytes can promote an angiogenic response even though they may not directly participate in the formation of the branch structures.

[0004] An increased level of angiogenesis is central to several human disease pathologies, including inflammatory disease, rheumatoid arthritis, diabetic retinopathy, and, significantly, to the growth, maintenance and metastasis of solid tumors (for detailed reviews see Liotta LA et al, 1991 Cell 64:327-336; Folkman J., 1995 Nature Medicine 1:27-31; Hanahan D and Folkman J, 1996 Cell 86:353-364). Impaired angiogenesis figures prominently in other human diseases, including heart disease, chronic inflammatory disease, stroke, infertility, ulcers and scleroderma.

[0005] The transition from dormant to active blood vessel formation involves modulating the balance between angiogenic stimulators and inhibitors. Under certain pathological

circumstances an imbalance arises between local inhibitory controls and angiogenic inducers resulting in excessive angiogenesis, while under other pathological conditions an imbalance leads to insufficient angiogenesis. This delicate equilibrium of pro- and anti-angiogenic factors is regulated by a complex interaction between the extracellular matrix, endothelial cells, smooth muscle cells, and various other cell types, as well as environmental factors such as oxygen demand within tissues. The lack of oxygen (hypoxia) in and around wounds and solid tumors is thought to provide a key driving force for angiogenesis by regulating a number of angiogenic factors, including Hypoxia Induced Factor alpha (HIF1 alpha) (Richard DE et al., Biochem Biophys Res Commun. 1999 Dec 29;266(3):718-22). HIF1 in turn regulates expression of a number of growth factors including Vascular Endothelial Growth Factor (VEGF) (Connolly DT, J Cell Biochem 1991 Nov;47(3):219-23). Various VEGF ligands and receptors are vital regulators of endothelial cell proliferation, survival, vessel permeability and sprouting, and lymphangiogenesis (Neufeld G et al., FASEB J 1999 Jan;13(1):9-22; Stacker SA et al., Nature Medicine 2001 7:186-191; Skobe M, et al., Nature Medicine 2001 7:192-198; Makinen T, et al., Nature Medicine 2001 7:199-205).

[0006] Most known angiogenesis genes, their biochemical activities, and their organization into signaling pathways are employed in a similar fashion during angiogenesis in human, mouse and Zebrafish, as well as during branching morphogenesis of the *Drosophila* trachea. Accordingly, *Drosophila* tracheal development and zebrafish vascular development provide useful models for studying mammalian angiogenesis (Sutherland D et al., Cell 1996, 87:1091-101; Roush W, Science 1996, 274:2011; Skaer H., Curr Biol 1997, 7:R238-41; Metzger RJ, Krasnow MA. Science. 1999. 284:1635-9; Roman BL, and Weinstein BM. Bioessays 2000, 22:882-93).

[0007] Angiogenesis is associated with numerous inflammatory conditions such as atherosclerosis, arthritis, retinopathy and tumor growth. Recent evidence suggests that inflammation exists in a mutually dependent association with angiogenesis (Dvorak HF et al et al. (1995) Am J Pathol. 146:1029-1039; Jackson et al. (1997) Faseb J. 11:457-465). During inflammatory processes, newly formed vessels supply the inflamed tissues with nutrients and oxygen allowing the transport of inflammatory cells.

[0008] The ability to manipulate and screen the genomes of model organisms such as *Drosophila* and zebrafish provides a powerful means to analyze biochemical processes that, due to significant evolutionary conservation of genes, pathways, and cellular processes, have direct relevance to more complex vertebrate organisms.

[0009] Short life cycles and powerful forward and reverse genetic tools available for both Zebrafish and *Drosophila* allow rapid identification of critical components of pathways controlling branching morphogenesis. Given the evolutionary conservation of gene sequences and molecular pathways, the human orthologs of model organism genes can be utilized to modulate branching morphogenesis pathways, including angiogenesis.

[0010] All references cited herein, including patents, patent applications, publications, and sequence information in referenced Genbank identifier numbers, are incorporated herein in their entireties.

SUMMARY OF THE INVENTION

[0011] We have discovered genes that modify branching morphogenesis in zebrafish *Danio rerio*, and identified their human orthologs, hereinafter referred to as modifier of branching morphogenesis (MBM). The invention provides methods for utilizing these branching morphogenesis modifier genes and polypeptides to identify MBM-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired branching morphogenesis function and/or MBM function. Preferred MBM-modulating agents specifically bind to MBM polypeptides and restore branching morphogenesis function. Other preferred MBM-modulating agents are nucleic acid modulators such as antisense oligomers and RNAi that repress MBM gene expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

[0012] MBM modulating agents may be evaluated by any convenient *in vitro* or *in vivo* assay for molecular interaction with an MBM polypeptide or nucleic acid. In one embodiment, candidate MBM modulating agents are tested with an assay system comprising an MBM polypeptide or nucleic acid. Agents that produce a change in the activity of the assay system relative to controls are identified as candidate branching morphogenesis modulating agents. The assay system may be cell-based or cell-free. MBM-modulating agents include MBM related proteins (e.g. dominant negative mutants, and biotherapeutics); MBM -specific antibodies; MBM -specific antisense oligomers and other nucleic acid modulators; and chemical agents that specifically bind to or interact with MBM or compete with MBM binding partner (e.g. by binding to an MBM binding partner). In one specific embodiment, a small molecule modulator is identified using a kinase assay. In specific embodiments, the screening assay system is selected from a binding assay, an apoptosis assay, a cell proliferation assay, an angiogenesis assay, a

hypoxic induction assay, a tubulogenesis assay, a cell adhesion assay, and a sprouting assay.

[0013] In another embodiment of the invention, the assay system comprises cultured cells or a non-human animal expressing MBM, and the assay system detects an agent-biased change in branching morphogenesis, including angiogenesis. Events detected by cell-based assays include cell proliferation, cell cycling, apoptosis, tubulogenesis, cell migration, and response to hypoxic conditions. For assays that detect tubulogenesis or cell migration, the assay system may comprise the step of testing the cellular response to stimulation with at least two different pro-angiogenic agents. Alternatively, tubulogenesis or cell migration may be detected by stimulating cells with an inflammatory angiogenic agent. In specific embodiments, the animal-based assay is selected from a matrix implant assay, a xenograft assay, a hollow fiber assay, or a transgenic tumor assay.

[0014] In another embodiment, candidate branching morphogenesis modulating agents that have been identified in cell-free or cell-based assays are further tested using a second assay system that detects changes in an activity associated with branching morphogenesis. In a specific embodiment, the second assay detects an agent-biased change in an activity associated with angiogenesis. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating branching morphogenesis, including increased or impaired angiogenesis or solid tumor metastasis.

[0015] The invention further provides methods for modulating the MBM function and/or branching morphogenesis in a mammalian cell by contacting the mammalian cell with an agent that specifically binds an MBM polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to a mammalian animal predetermined to have a pathology associated branching morphogenesis.

DETAILED DESCRIPTION OF THE INVENTION

[0016] Genetic screens were designed to identify modifiers of branching morphogenesis in *zebrafish*. We used a screen based on antisense technologies to identify genes whose disruption produced vascular defects in zebrafish. Briefly, and as further described in the Examples, one-cell stage embryos were treated with antisense morpholino oligonucleotides (PMOs) that targeted a large number of predicted zebrafish genes.

Treated animals were fixed at the larval stage, and alkaline phosphatase staining was used to visualize blood vessel formation. Antisense knock-down of modifier genes produced specific vascular defects. Accordingly, other vertebrate orthologs of these modifiers, and preferably the human orthologs, MBM genes (i.e., nucleic acids and polypeptides) are attractive drug targets for the treatment of pathologies associated with a defective branching morphogenesis signaling pathway, such as cancer. Table 1 (Example III) lists the modifiers and their orthologs.

[0017] In vitro and in vivo methods of assessing MBM function are provided herein. Modulation of the MBM or their respective binding partners is useful for understanding the association of branching morphogenesis and its members in normal and disease conditions and for developing diagnostics and therapeutic modalities for branching morphogenesis related pathologies. MBM-modulating agents that act by inhibiting or enhancing MBM expression, directly or indirectly, for example, by affecting an MBM function such as enzymatic (e.g., catalytic) or binding activity, can be identified using methods provided herein. MBM modulating agents are useful in diagnosis, therapy and pharmaceutical development.

[0018] As used herein, branching morphogenesis encompasses the numerous cellular process involved in the formation of branched networks, including proliferation, survival/apoptosis, migration, invasion, adhesion, aggregation and matrix remodeling. As used herein, pathologies associated with branching morphogenesis encompass pathologies where branching morphogenesis contributes to maintaining the healthy state, as well as pathologies whose course may be altered by modulation of the branching morphogenesis.

Nucleic acids and polypeptides of the invention

[0019] Sequences related to MBM nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) or RefSeq number), shown in Table 1 and in the appended sequence listing.

[0020] The term "MBM polypeptide" refers to a full-length MBM protein or a functionally active fragment or derivative thereof. A "functionally active" MBM fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type MBM protein, such as antigenic or immunogenic activity, enzymatic activity, ability to bind natural cellular substrates, etc. The functional activity of MBM proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons,

Inc., Somerset, New Jersey) and as further discussed below. In one embodiment, a functionally active MBM polypeptide is an MBM derivative capable of rescuing defective endogenous MBM activity, such as in cell based or animal assays; the rescuing derivative may be from the same or a different species. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of an MBM, such as a kinase domain or a binding domain. Protein domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2). Methods for obtaining MBM polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of an MBM. In further preferred embodiments, the fragment comprises the entire functionally active domain.

[0021] The term "MBM nucleic acid" refers to a DNA or RNA molecule that encodes an MBM polypeptide. Preferably, the MBM polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with human MBM. Methods of identifying orthlogs are known in the art. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994, Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *zebrafish*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the

term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

[0022] A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

[0023] Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, Advances in Applied Mathematics 2:482-489; database: European Bioinformatics Institute; Smith and Waterman, 1981, J. of Molec.Biol., 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.; W.R. Pearson, 1991, Genomics 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff (Dayhoff: Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 Nucl. Acids Res. 14(6):6745-6763). The Smith-Waterman

algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated, the "Match" value reflects "sequence identity."

[0024] Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of an MBM. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of an MBM under high stringency hybridization conditions that are: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 μg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 μg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate).

[0025] In other embodiments, moderately stringent hybridization conditions are used that are: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

[0026] Alternatively, low stringency conditions can be used that are: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

Isolation, Production, Expression, and Mis-expression of MBM Nucleic Acids and Polypeptides

[0027] MBM nucleic acids and polypeptides, are useful for identifying and testing agents that modulate MBM function and for other applications related to the involvement of MBM in branching morphogenesis. MBM nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods. For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of an MBM protein for assays used to assess MBM function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (e.g., Higgins SJ and Hames BD (eds.) Protein Expression: A Practical Approach, Oxford University Press Inc., New York 1999; Stanbury PF et al., Principles of Fermentation Technology, 2nd edition, Elsevier Science, New York, 1995; Doonan S (ed.) Protein Purification Protocols, Humana Press, New Jersey, 1996; Coligan JE et al, Current Protocols in Protein Science (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant MBM is expressed in a cell line known to have defective branching morphogenesis function. The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

[0028] The nucleotide sequence encoding an MBM polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native MBM gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with

bacteriophage, plasmid, or cosmid DNA. An isolated host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

[0029] To detect expression of the MBM gene product, the expression vector can comprise a promoter operably linked to an MBM gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the MBM gene product based on the physical or functional properties of the MBM protein in *in vitro* assay systems (*e.g.* immunoassays).

[0030] The MBM protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (i.e. it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

[0031] Once a recombinant cell that expresses the MBM gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). Alternatively, native MBM proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

[0032] The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of MBM or other genes associated with branching morphogenesis. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

Genetically modified animals

[0033] Animal models that have been genetically modified to alter MBM expression may be used in *in vivo* assays to test for activity of a candidate branching morphogenesis modulating agent, or to further assess the role of MBM in a branching morphogenesis

process such as apoptosis or cell proliferation. Preferably, the altered MBM expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal MBM expression. The genetically modified animal may additionally have altered branching morphogenesis expression (e.g. branching morphogenesis knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice or rats), among others. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4:761-763.) or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

[0034] Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al., and Hogan, B., Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sandford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, Science (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et al.*, A Universal Marker for Transgenic Insects (1999) Nature 402:370-371; for transgenic Zebrafish see Lin S., Transgenic Zebrafish, Methods Mol Biol. (2000);136:375-3830); for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, Experientia (1991) 47:897-905; for transgenic rats see Hammer *et al.*, Cell (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) Nature 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

[0035] In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous MBM gene that

results in a decrease of MBM function, preferably such that MBM expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse MBM gene is used to construct a homologous recombination vector suitable for altering an endogenous MBM gene in the mouse genome. Detailed methodologies for homologous recombination in mice are available (see Capecchi, Science (1989) 244:1288-1292; Joyner *et al.*, Nature (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, Science (1989) 244:1281-1288; Simms *et al.*, Bio/Technology (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene that has been knocked out (Claesson MH et al., (1994) Scan J Immunol 40:257-264; Declerck PJ et al., (1995) J Biol Chem. 270:8397-400).

[0036] In another embodiment, the transgenic animal is a "knock-in" animal having an alteration in its genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the MBM gene, e.g., by introduction of additional copies of MBM, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the MBM gene. Such regulatory sequences include inducible, tissue-specific, and constitutive promoters and enhancer elements. The knock-in can be homozygous or heterozygous.

[0037] Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*, PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of Saccharomyces cerevisiae (O'Gorman et al.

(1991) Science 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the transgene, and for sequential deletion of vector sequences in the same cell (Sun X et al (2000) Nat Genet 25:83-6).

[0038] The genetically modified animals can be used in genetic studies to further elucidate branching morphogenesis, as animal models of disease and disorders implicating defective branching morphogenesis function, and for *in vivo* testing of candidate therapeutic agents, such as those identified in screens described below. The candidate therapeutic agents are administered to a genetically modified animal having altered MBM function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that receive placebo treatment, and/or animals with unaltered MBM expression that receive candidate therapeutic agent.

[0039] In addition to the above-described genetically modified animals having altered MBM function, animal models having defective branching morphogenesis function (and otherwise normal MBM function), can be used in the methods of the present invention. For example, a branching morphogenesis knockout mouse can be used to assess, *in vivo*, the activity of a candidate branching morphogenesis modulating agent identified in one of the *in vitro* assays described below. Preferably, the candidate branching morphogenesis modulating agent when administered to a model system with cells defective in branching morphogenesis function, produces a detectable phenotypic change in the model system indicating that the branching morphogenesis function is restored, i.e., the cells exhibit normal branching morphogenesis.

Modulating Agents

[0040] The invention provides methods to identify agents that interact with and/or modulate the function of MBM and/or branching morphogenesis. Modulating agents identified by the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with branching morphogenesis, as well as in further analysis of the MBM protein and its contribution to branching morphogenesis. Accordingly, the invention also provides methods for modulating branching morphogenesis comprising the step of specifically modulating MBM activity by administering an MBM-interacting or -modulating agent.

[0041] As used herein, an "MBM-modulating agent" is any agent that modulates MBM function, for example, an agent that interacts with MBM to inhibit or enhance MBM

activity or otherwise affect normal MBM function. MBM function can be affected at any level, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a preferred embodiment, the MBM - modulating agent specifically modulates the function of the MBM. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the MBM polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the MBM. These phrases also encompass modulating agents that alter the interaction of the MBM with a binding partner, substrate, or cofactor (e.g. by binding to a binding partner of an MBM, or to a protein/binding partner complex, and altering MBM function). In a further preferred embodiment, the MBM- modulating agent is a modulator of branching morphogenesis (e.g. it restores and/or upregulates branching morphogenesis function) and thus is also a branching morphogenesis-modulating agent.

[0042] Preferred MBM-modulating agents include small molecule compounds; MBM-interacting proteins, including antibodies and other biotherapeutics; and nucleic acid modulators such as antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

Small molecule modulators

[0043] Small molecules are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight up to 10,000, preferably up to 5,000, more preferably up to 1,000, and most preferably up to 500 daltons. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the MBM protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for MBM–modulating activity. Methods

for generating and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

[0044] Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with branching morphogenesis. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

Protein Modulators

[0045] Specific MBM-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to branching morphogenesis and related disorders, as well as in validation assays for other MBM-modulating agents. In a preferred embodiment, MBM-interacting proteins affect normal MBM function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, MBM-interacting proteins are useful in detecting and providing information about the function of MBM proteins, as is relevant to branching morphogenesis related disorders, such as cancer (e.g., for diagnostic means).

[0046] An MBM-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with an MBM, such as a member of the MBM pathway that modulates MBM expression, localization, and/or activity. MBM-modulators include dominant negative forms of MBM-interacting proteins and of MBM proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous MBM-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3rd, Trends Genet (2000) 16:5-8).

[0047] An MBM-interacting protein may be an exogenous protein, such as an MBM-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory; Harlow and Lane (1999) Using antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). MBM antibodies are further discussed below.

[0048] In preferred embodiments, an MBM-interacting protein specifically binds an MBM protein. In alternative preferred embodiments, an MBM-modulating agent binds an MBM substrate, binding partner, or cofactor.

Antibodies

[0049] In another embodiment, the protein modulator is an MBM specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify MBM modulators. The antibodies can also be used in dissecting the portions of the MBM pathway responsible for various cellular responses and in the general processing and maturation of the MBM.

[0050] Antibodies that specifically bind MBM polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of MBM polypeptide, and more preferably, to human MBM. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab').sub.2 fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Epitopes of MBM which are particularly antigenic can be selected, for example, by routine screening of MBM polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Nati. Acad. Sci. U.S.A. 78:3824-28; Hopp and Wood, (1983) Mol. Immunol. 20:483-89; Sutcliffe et al., (1983) Science 219:660-66) to the amino acid sequence of an MBM. Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 M^{-1} to 10^{10} M^{-1}, or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of MBM or substantially purified fragments thereof. If MBM fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of an MBM protein. In a particular embodiment, MBM-specific antigens and/or immunogens are coupled to carrier proteins that stimulate the immune

response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

[0051] The presence of MBM-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding MBM polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

[0052] Chimeric antibodies specific to MBM polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat. Nos. 5,530,101, 5,585,089, 5,693,762, and 6,180,370).

[0053] MBM-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No. 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

[0054] Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As

used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

[0055] The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989) 4:131-134). A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

[0056] When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg –to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml to about10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

Nucleic Acid Modulators

[0057] Other preferred MBM-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit MBM

activity. Preferred nucleic acid modulators interfere with the function of the MBM nucleic acid such as DNA replication, transcription, translocation of the MBM RNA to the site of protein translation, translation of protein from the MBM RNA, splicing of the MBM RNA to yield one or more mRNA species, or catalytic activity which may be engaged in or facilitated by the MBM RNA.

[0058] In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to an MBM mRNA to bind to and prevent translation, preferably by binding to the 5′ untranslated region. MBM-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

[0059] In another embodiment, the antisense oligomer is a phosphothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits, each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphodiamidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. :7:187-95; US Pat. No. 5,235,033; and US Pat No. 5,378,841).

[0060] Alternative preferred MBM nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-

245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498; Novina CD and Sharp P. 2004 Nature 430:161-164; Soutschek J et al 2004 Nature 432:173-178).

[0061] Nucleic acid modulators are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Nucleic acid modulators are also used, for example, to distinguish between functions of various members of a biological pathway. For example, antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, an MBM-specific nucleic acid modulator is used in an assay to further elucidate the role of the MBM in branching morphogenesis, and/or its relationship to other members of the pathway. In another aspect of the invention, an MBM-specific antisense oligomer is used as a therapeutic agent for treatment of branching morphogenesis-related disease states.

[0062] Zebrafish is a particularly useful model for the study of branching morphogenesis using antisense oligomers. For example, PMOs are used to selectively inactive one or more genes *in vivo* in the Zebrafish embryo. By injecting PMOs into Zebrafish at the 1-16 cell stage candidate targets emerging from the *Drosophila* screens are validated in this vertebrate model system. In another aspect of the invention, PMOs are used to screen the Zebrafish genome for identification of other therapeutic modulators of branching morphogenesis. In a further aspect of the invention, an MBM-specific antisense oligomer is used as a therapeutic agent for treatment of pathologies associated with branching morphogenesis.

Assay Systems

[0063] The invention provides assay systems and screening methods for identifying specific modulators of MBM activity. As used herein, an "assay system" encompasses all

the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the MBM nucleic acid or protein. In general, secondary assays further assess the activity of an MBM modulating agent identified by a primary assay and may confirm that the modulating agent affects MBM in a manner relevant to branching morphogenesis. In some cases, MBM modulators will be directly tested in a secondary assay.

[0064] In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising an MBM polypeptide or nucleic acid with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. kinase activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates MBM activity, and hence branching morphogenesis. The MBM polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

Primary Assays

[0065] The type of modulator tested generally determines the type of primary assay.

Primary assays for small molecule modulators

[0066] For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding), transcriptional activity (*e.g.*, using a reporter gene), enzymatic activity (*e.g.*, via a property of the substrate), activity of second messengers, immunogenicty and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide

range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular molecular event detected.

[0067] Cell-based screening assays usually require systems for recombinant expression of MBM and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when MBM-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the MBM protein may be assayed by various known methods such as substrate processing (e.g. ability of the candidate MBM-specific binding agents to function as negative effectors in MBM-expressing cells), binding equilibrium constants (usually at least about 10^7 M^{-1}, preferably at least about 10^8 M^{-1}, more preferably at least about 10^9 M^{-1}), and immunogenicity (e.g. ability to elicit MBM specific antibody in a heterologous host such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

[0068] The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of an MBM polypeptide, a fusion protein thereof, or to cells or membranes bearing the polypeptide or fusion protein. The MBM polypeptide can be full length or a fragment thereof that retains functional MBM activity. The MBM polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The MBM polypeptide is preferably human MBM, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay detects candidate agent-based modulation of MBM interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has MBM –specific binding activity, and can be used to assess normal MBM gene function.

[0069] Suitable assay formats that may be adapted to screen for MBM modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, Curr Opin Chem Biol (1998) 2:597-603; Sundberg SA, Curr Opin Biotechnol 2000, 11:47-53). In one preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved

fluorescence, and fluorescence resonance energy transfer. These systems offer means to monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.,* Selvin PR, Nat Struct Biol (2000) 7:730-4; Fernandes PB, *supra*; Hertzberg RP and Pope AJ, Curr Opin Chem Biol (2000) 4:445-451).

[0070] A variety of suitable assay systems may be used to identify candidate MBM and branching morphogenesis modulators (e.g. U.S. Pat. No. 6,165,992 (kinase assays); U.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); and U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434 (angiogenesis assays), among others). Specific preferred assays are described in more detail below.

[0071] **Kinase assays.** In some preferred embodiments the screening assay detects the ability of the test agent to modulate the kinase activity of an MBM polypeptide. In further embodiments, a cell-free kinase assay system is used to identify a candidate branching morphogenesis modulating agent, and a secondary, cell-based assay, such as an apoptosis or hypoxic induction assay (described below), may be used to further characterize the candidate branching morphogenesis modulating agent. Many different assays for kinases have been reported in the literature and are well known to those skilled in the art (e.g. U.S. Pat. No. 6,165,992; Zhu et al., Nature Genetics (2000) 26:283-289; and WO0073469). Radioassays, which monitor the transfer of a gamma phosphate are frequently used. For instance, a scintillation assay for p56 (lck) kinase activity monitors the transfer of the gamma phosphate from gamma -^{33}P ATP to a biotinylated peptide substrate; the substrate is captured on a streptavidin coated bead that transmits the signal (Beveridge M *et al.*, J Biomol Screen (2000) 5:205-212). This assay uses the scintillation proximity assay (SPA), in which only radio-ligand bound to receptors tethered to the surface of an SPA bead are detected by the scintillant immobilized within it, allowing binding to be measured without separation of bound from free ligand.

[0072] Other assays for protein kinase activity may use antibodies that specifically recognize phosphorylated substrates. For instance, the kinase receptor activation (KIRA) assay measures receptor tyrosine kinase activity by ligand stimulating the intact receptor in cultured cells, then capturing solubilized receptor with specific antibodies and quantifying phosphorylation via phosphotyrosine ELISA (Sadick MD, Dev Biol Stand (1999) 97:121-133).

[0073] Another example of antibody based assays for protein kinase activity is TRF (time-resolved fluorometry). This method utilizes europium chelate-labeled anti-phosphotyrosine antibodies to detect phosphate transfer to a polymeric substrate coated onto microtiter plate wells. The amount of phosphorylation is then detected using time-resolved, dissociation-enhanced fluorescence (Braunwalder AF, et al., Anal Biochem 1996 Jul 1;238(2):159-64).

[0074] Yet other assays for kinases involve uncoupled, pH sensitive assays that can be used for high-throughput screening of potential inhibitors or for determining substrate specificity. Since kinases catalyze the transfer of a gamma-phosphoryl group from ATP to an appropriate hydroxyl acceptor with the release of a proton, a pH sensitive assay is based on the detection of this proton using an appropriately matched buffer/indicator system (Chapman E and Wong CH (2002) Bioorg Med Chem. 10:551-5).

[0075] **Apoptosis assays.** Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., et al., 1998, Blood 15:4730-41). Other cell-based apoptosis assays include the caspase-3/7 assay and the cell death nucleosome ELISA assay. The caspase 3/7 assay is based on the activation of the caspase cleavage activity as part of a cascade of events that occur during programmed cell death in many apoptotic pathways. In the caspase 3/7 assay (commercially available Apo-ONE™ Homogeneous Caspase-3/7 assay from Promega, cat# 67790), lysis buffer and caspase substrate are mixed and added to cells. The caspase substrate becomes fluorescent when cleaved by active caspase 3/7. The nucleosome ELISA assay is a general cell death assay known to those skilled in the art, and available commercially (Roche, Cat# 1774425). This assay is a quantitative sandwich-enzyme-immunoassay which uses monoclonal antibodies directed against DNA and histones respectively, thus specifically determining amount of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Mono and oligonucleosomes are enriched in the cytoplasm during apoptosis due to the fact that DNA fragmentation occurs several hours before the plasma membrane breaks down, allowing for accumalation in the cytoplasm. Nucleosomes are not present in the cytoplasmic fraction of cells that are not undergoing apoptosis. An apoptosis assay

system may comprise a cell that expresses an MBM, and that optionally has defective branching morphogenesis function. A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate branching morphogenesis modulating agents. In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate branching morphogenesis modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether MBM function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express MBM relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the MBM plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

[0076] **Cell proliferation and cell cycle assays.** Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, Int. J. Cancer 38, 369; Campana *et al.*, 1988, J. Immunol. Meth. 107, 79), or by other means.

[0077] Cell proliferation is also assayed via phospho-histone H3 staining, which identifies a cell population undergoing mitosis by phosphorylation of histone H3. Phosphorylation of histone H3 at serine 10 is detected using an antibody specfic to the phosphorylated form of the serine 10 residue of histone H3. (Chadlee,D.N. 1995, J. Biol. Chem 270:20098-105). Cell Proliferation may also be examined using [^{3}H]-thymidine incorporation (Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [^{3}H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter). Another proliferation assay uses the dye Alamar Blue (available from Biosource International), which fluoresces when reduced in living cells and provides an indirect measurement of cell number (Voytik-Harbin SL et al., 1998, In Vitro Cell Dev Biol Anim 34:239-46). Yet another proliferation assay, the MTS assay, is based on in vitro cytotoxicity assessment of industrial chemicals, and uses the soluble tetrazolium salt,

MTS. MTS assays are commercially available, for example, the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Cat.# G5421).

[0078] Cell proliferation may also be assayed by colony formation in soft agar (Sambrook et al., Molecular Cloning, Cold Spring Harbor (1989)). For example, cells transformed with MBM are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

[0079] Cell proliferation may also be assayed by measuring ATP levels as indicator of metabolically active cells. Such assays are commercially available, for example Cell Titer-Glo™, which is a luminescent homogeneous assay available from Promega.

[0080] Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray JW et al. (1986) Int J Radiat Biol Relat Stud Phys Chem Med 49:237-55). Cells transfected with an MBM may be stained with propidium iodide and evaluated in a flow cytometer (available from Becton Dickinson), which indicates accumulation of cells in different stages of the cell cycle.

[0081] Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses an MBM, and that optionally has defective branching morphogenesis function. A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no test agent is added, identify candidate branching morphogenesis modulating agents. In some embodiments of the invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate branching morphogenesis modulating agents that is initially identified using another assay system such as a cell-free assay system. A cell proliferation assay may also be used to test whether MBM function plays a direct role in cell proliferation or cell cycle. For example, a cell proliferation or cell cycle assay may be performed on cells that over- or under-express MBM relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the MBM plays a direct role in cell proliferation or cell cycle.

[0082] **Angiogenesis.** Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and

tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses an MBM, and that optionally has defective branching morphogenesis function. A test agent can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate branching morphogenesis modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate branching morphogenesis modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether MBM function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express MBM relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the MBM plays a direct role in angiogenesis. U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434, among others, describe various angiogenesis assays.

[0083] Hypoxic induction. The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glyolytic enzymes and VEGF. Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with MBM in hypoxic conditions (such as with 0.1% O2, 5% CO2, and balance N2, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses an MBM, and that optionally has defective branching morphogenesis function. A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate branching morphogenesis modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate branching morphogenesis modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether MBM function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express MBM relative to wild type cells. Differences in hypoxic response

compared to wild type cells suggests that the MBM plays a direct role in hypoxic induction.

[0084] **Cell adhesion.** Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents. Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2× final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

[0085] Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF , and allowed to adhere to the monolayers in the presence of candidate agents. Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

[0086] High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., Bioconjug Chem. 2001 May-Jun;12(3):346-53).

[0087] **Tubulogenesis.** Tubulogenesis assays monitor the ability of cultured cells, generally endothelial cells, to form tubular structures on a matrix substrate, which generally simulates the environment of the extracellular matrix. Exemplary substrates include Matrigel™ (Becton Dickinson), an extract of basement membrane proteins

containing laminin, collagen IV, and heparin sulfate proteoglycan, which is liquid at 4° C and forms a solid gel at 37° C. Other suitable matrices comprise extracellular components such as collagen, fibronectin, and/or fibrin. Cells are stimulated with a pro-angiogenic stimulant, and their ability to form tubules is detected by imaging. Tubules can generally be detected after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Tube formation assays are well known in the art (e.g., Jones MK et al., 1999, Nature Medicine 5:1418-1423). These assays have traditionally involved stimulation with serum or with the growth factors FGF or VEGF. Serum represents an undefined source of growth factors. In a preferred embodiment, the assay is performed with cells cultured in serum free medium, in order to control which process or pathway a candidate agent modulates. Moreover, we have found that different target genes respond differently to stimulation with different pro-angiogenic agents, including inflammatory angiogenic factors such as TNF-alpa. Thus, in a further preferred embodiment, a tubulogenesis assay system comprises testing an MBM's response to a variety of factors, such as FGF, VEGF, phorbol myristate acetate (PMA), TNF-alpha, ephrin, etc.

[0088] **Cell Migration.** An invasion/migration assay (also called a migration assay) tests the ability of cells to overcome a physical barrier and to migrate towards pro-angiogenic signals. Migration assays are known in the art (e.g., Paik JH et al., 2001, J Biol Chem 276:11830-11837). In a typical experimental set-up, cultured endothelial cells are seeded onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The matrix generally simulates the environment of the extracellular matrix, as described above. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic stimuli. Migration is generally assayed after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Migration is assessed as the number of cells that crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific, South San Francisco, CA), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and migration is detected using fluorescent readings, for instance using the Falcon HTS FluoroBlok (Becton Dickinson). While some migration is observed in the absence of stimulus, migration is greatly increased in response to pro-angiogenic factors. As described above, a preferred assay system for migration/invasion assays comprises testing

an MBM's response to a variety of pro-angiogenic factors, including tumor angiogenic and inflammatory angiogenic agents, and culturing the cells in serum free medium.

[0089] **Sprouting assay.** A sprouting assay is a three-dimensional *in vitro* angiogenesis assay that uses a cell-number defined spheroid aggregation of endothelial cells ("spheroid"), embedded in a collagen gel-based matrix. The spheroid can serve as a starting point for the sprouting of capillary-like structures by invasion into the extracellular matrix (termed "cell sprouting") and the subsequent formation of complex anastomosing networks (Korff and Augustin, 1999, J Cell Sci 112:3249-58). In an exemplary experimental set-up, spheroids are prepared by pipetting 400 human umbilical vein endothelial cells into individual wells of a nonadhesive 96-well plates to allow overnight spheroidal aggregation (Korff and Augustin: J Cell Biol 143: 1341-52, 1998). Spheroids are harvested and seeded in 900μl of methocel-collagen solution and pipetted into individual wells of a 24 well plate to allow collagen gel polymerization. Test agents are added after 30 min by pipetting 100 μl of 10-fold concentrated working dilution of the test substances on top of the gel. Plates are incubated at 37°C for 24h. Dishes are fixed at the end of the experimental incubation period by addition of paraformaldehyde. Sprouting intensity of endothelial cells can be quantitated by an automated image analysis system to determine the cumulative sprout length per spheroid.

Primary assays for antibody modulators

[0090] For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the MBM protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for detecting MBM-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

[0091] In some cases, screening assays described for small molecule modulators may also be used to test antibody modulators.

Primary assays for nucleic acid modulators

[0092] For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance MBM gene expression, preferably mRNA expression. In general, expression analysis comprises comparing MBM expression in like populations of

cells (*e.g.*, two pools of cells that endogenously or recombinantly express MBM) in the presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that MBM mRNA expression is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, *eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with specific antibodies or antisera directed against either the MBM protein or specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

[0093] In some cases, screening assays described for small molecule modulators, particularly in assay systems that involve MBM mRNA expression, may also be used to test nucleic acid modulators.

Secondary Assays

[0094] Secondary assays may be used to further assess the activity of MBM-modulating agent identified by any of the above methods to confirm that the modulating agent affects MBM in a manner relevant to branching morphogenesis. As used herein, MBM-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with MBM.

[0095] Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express MBM) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate MBM–modulating agent results in changes in branching morphogenesis in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the branching morphogenesis or interacting pathways.

Cell-based assays

[0096] Cell based assays may use a variety of mammalian cell types. Preferred cells are capable of branching morphogenesis processes and are generally endothelial cells. Exemplary cells include human umbilical vein endothelial cells (HUVECs), human renal microvascular endothelial cells (HRMECs), human dermal microvascular endothelial cells (HDMECs), human uterine microvascular endothelial cells, human lung microvascular endothelial cells, human coronary artery endothelial cells, and immortalized microvascular cells, among others. Cell based assays may rely on the endogenous expression of MBM and/or other genes, such as those involved in branching morphogenesis, or may involve recombinant expression of these genes. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

[0097] Cell-based assays may detect a variety of events associated with branching morphogenesis and angiogenesis, including cell proliferation, apoptosis, cell migration, tube formation, sprouting and hypoxic induction, as described above.

Animal Assays

[0098] A variety of non-human animal models of branching morphogenesis, including angiogenesis, and related pathologies may be used to test candidate MBM modulators. Animal assays may rely on the endogenous expression of MBM and/or other genes, such as those involved in branching morphogenesis, or may involve engineered expression of these genes. In some cases, MBM expression or MBM protein may be restricted to a particular implanted tissue or matrix. Animal assays generally require systemic delivery of a candidate modulator, such as by oral administration, injection (intravenous, subcutaneous, intraperitoneous), bolus administration, etc.

[0099] In a preferred embodiment, branching morphogenesis activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal branching morphogenesis are used to test the candidate modulator's affect on MBM in Matrigel® assays. Matrigel® is an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4° C, but rapidly forms a solid gel at 37° C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells which over-express the MBM. The mixture is then injected subcutaneously(SC) into female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral

(PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit). Hemoglobin content of the gel is found to correlate the degree of neovascularization in the gel.

[0100] In another preferred embodiment, the effect of the candidate modulator on MBM is assessed via tumorigenicity assays. In one example, a xenograft comprising human cells from a pre-existing tumor or a tumor cell line known to be angiogenic is used; exemplary cell lines include A431, Colo205, MDA-MB-435, A673, A375, Calu-6, MDA-MB-231, 460, SF763T, or SKOV3tp5. Tumor xenograft assays are known in the art (see, e.g., Ogawa K et al., 2000, Oncogene 19:6043-6052). Xenografts are typically implanted SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the MBM endogenously are injected in the flank, 1 x 10^5 to 1 x 10^7 cells per mouse in a volume of 100 μL using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100 mg. Candidate modulator is delivered IV, SC, IP, or PO by bolus administration. Depending upon the pharmacokinetics of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors maybe utilized for biomarker identification or further analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

[0101] In another preferred embodiment, tumorogenicity is monitored using a hollow fiber assay, which is described in U.S. Pat No. US 5,698,413. Briefly, the method comprises implanting into a laboratory animal a biocompatible, semi-permeable encapsulation device containing target cells, treating the laboratory animal with a candidate modulating agent, and evaluating the target cells for reaction to the candidate modulator. Implanted cells are generally human cells from a pre-existing tumor or a tumor cell line known to be angiogenic. After an appropriate period of time, generally around six days, the implanted samples are harvested for evaluation of the candidate modulator. Tumorogenicity and modulator efficacy may be evaluated by assaying the quantity of viable cells present in the macrocapsule, which can be determined by tests known in the art, for example, MTT dye

conversion assay, neutral red dye uptake, trypan blue staining, viable cell counts, the number of colonies formed in soft agar, the capacity of the cells to recover and replicate in vitro, etc. Other assays specific to angiogenesis, as are known in the art and described herein, may also be used.

[0102] In another preferred embodiment, a tumorogenicity assay use a transgenic animal, usually a mouse, carrying a dominant oncogene or tumor suppressor gene knockout under the control of tissue specific regulatory sequences; these assays are generally referred to as transgenic tumor assays. In a preferred application, tumor development in the transgenic model is well characterized or is controlled. In an exemplary model, the "RIP1-Tag2" transgene, comprising the SV40 large T-antigen oncogene under control of the insulin gene regulatory regions is expressed in pancreatic beta cells and results in islet cell carcinomas (Hanahan D, 1985, Nature 315:115-122; Parangi S et al, 1996, Proc Natl Acad Sci USA 93: 2002-2007; Bergers G et al, 1999, Science 284:808-812). An "angiogenic switch," occurs at approximately five weeks, as normally quiescent capillaries in a subset of hyperproliferative islets become angiogenic. The RIP1-TAG2 mice die by age 14 weeks. Candidate modulators may be administered at a variety of stages, including just prior to the angiogenic switch (e.g., for a model of tumor prevention), during the growth of small tumors (e.g., for a model of intervention), or during the growth of large and/or invasive tumors (e.g., for a model of regression). Tumorogenicity and modulator efficacy can be evaluating life-span extension and/or tumor characteristics, including number of tumors, tumor size, tumor morphology, vessel density, apoptotic index, etc.

Diagnostic and therapeutic uses

[0103] Specific MBM-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in branching morphogenesis, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating branching morphogenesis in a cell, preferably a cell pre-determined to have defective or impaired branching morphogenesis function (e.g. due to overexpression, underexpression, or misexpression of branching morphogenesis, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates MBM activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the branching morphogenesis function is restored. The phrase "function is restored", and equivalents, as used herein, means that the desired phenotype is achieved,

or is brought closer to normal compared to untreated cells. For example, with restored branching morphogenesis function, cell proliferation and/or progression through cell cycle may normalize, or be brought closer to normal relative to untreated cells. The invention also provides methods for treating disorders or disease associated with impaired branching morphogenesis function by administering a therapeutically effective amount of an MBM -modulating agent that modulates branching morphogenesis. The invention further provides methods for modulating MBM function in a cell, preferably a cell pre-determined to have defective or impaired MBM function, by administering an MBM -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired MBM function by administering a therapeutically effective amount of an MBM -modulating agent.

[0104] The discovery that MBM is implicated in branching morphogenesis provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in branching morphogenesis and for the identification of subjects having a predisposition to such diseases and disorders.

[0105] Various expression analysis methods can be used to diagnose whether MBM expression occurs in a particular sample, including Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR, and microarray analysis. (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, *eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-47). Tissues having a disease or disorder implicating defective branching morphogenesis signaling that express an MBM, are identified as amenable to treatment with an MBM modulating agent. In a preferred application, the branching morphogenesis defective tissue overexpresses an MBM relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial MBM cDNA sequences as probes, can determine whether particular tumors express or overexpress MBM. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of MBM expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

[0106] Various other diagnostic methods may be performed, for example, utilizing reagents such as the MBM oligonucleotides, and antibodies directed against an MBM, as described above for: (1) the detection of the presence of MBM gene mutations, or the detection of either over- or under-expression of MBM mRNA relative to the non-disorder

state; (2) the detection of either an over- or an under-abundance of MBM gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by MBM.

[0107] Kits for detecting expression of MBM in various samples, comprising at least one antibody specific to MBM, all reagents and/or devices suitable for the detection of antibodies, the immobilization of antibodies, and the like, and instructions for using such kits in diagnosis or therapy are also provided.

[0108] Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease or disorder in a patient that is associated with alterations in MBM expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for MBM expression; c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of the disease or disorder. Preferably, the disease is cancer. The probe may be either DNA or protein, including an antibody.

EXAMPLES

[0109] The following experimental section and examples are offered by way of illustration and not by way of limitation.

I. Analysis of vasculature defects associated with modifier loss of function

[0110] Wild type, one-cell stage embryos from the Tübingen strain were treated with antisense morpholino oligonucleotide (PMOs) that targeted the 5'UTR and/or start codon of predicted zebrafish genes. PMOs were dissolved at a concentration of 3 mg/mL in injection buffer (0.4 mM $MgSO_4$, 0.6 mM $CaCl_2$, 0,7 mM KCl, 58 mM NaCl, 25 mM Hepes [pH 7,6]); a total of 1.5 nL (= 4.5 ng) was injected into zebrafish embryos at the 1-cell stage.

[0111] Larvae were fixed at 4 days post fertilization (dpf) in 4% para-formaldehyde in phosphate-buffered saline (PBS) for 30 minutes. Fixed larvae were dehydrated in methanol and stored over night at -20°C. After permeabilization in acetone (30 minutes at -20°C), embryos were washed in PBS and incubated in the staining buffer (100 mM Tris-HCl [pH 9.5], 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20) for 45 minutes. Staining reaction was started by adding 2.25 µl nitro blue tetrazolium (NBT, Sigma) and 1.75 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) per ml of staining buffer (stock

solutions: 75 mg/ml NBT in 70% N,N-dimethylformamide, 50 mg/ml BCIP in N,N-dimethylformamide).

[0112] The fixed specimens were scanned for changes in blood vessel formation, in particular, for any pro-angiobenic, anti-angiogenic, vasculogenic or vessel patterning phenotypes, among others. Other phenotypic changes resulting from the PMO treatment were also noted. Hits were "Confirmed" when the phenotype was seen for 2nd time in an independent injection of the PMO. Hits were "Characterized" when phenotype was seen for a 3rd time by angiography, to visualize the vascular anatomy. Orthologs of the modifiers are referred to herein as MBM.

II. Zebrafish "Negative" & "Positive" Secondary Assays for Morpholino (PMO) Screen Hits

[0113] Zebrafish "Negative" secondary assays are used to determine whether the effects seen on the vasculature with the morpholino knockdown is a primary effect on the vasculature vs. a secondary effect caused by a general patterning defect. Zebrafish "Positive" secondary assays provide pathway and/or mechanistic information about the gene target as well as cell and tissue specificity of its activity.

[0114] Negative assay #1 - Patterning vs. vascular defects. Whole mount stains are done with muscle-specific antibody mAb MF20 (acto-myosin) to evaluate whether there is a general patterning defect caused by the gene knockdown.

[0115] Negative assay #2 - Neuronal vs. vascular defects. Whole mount stains with a neuronal-specific antibody (anti-acetylated tubulin) to evaluate whether there is a underlying neuronal patterning defect that may cause a secondary vascular phentoype.

[0116] Negative assay #3 - Tissue dystrophic or necrotic vs. vascular defects. Live observation of morphology under Nomarski optics (at day 1-4 of development following PMO injection) to evaluate the extent of tissue apoptosis/necrosis induced by gene knockdown.

[0117] Negative assay #4 - Vascular or Hematopoietic Marker Expression (in situ hybridization). In situ hybridization w/ fli1 gene, which stains developing vessels, is done at day 2 of development to evaluate whether the phenotype observed at day 4 results from a vascular development defect vs. vascular maintenance defect.

[0118] Positive assay #5: Anti-Angiogenesis pathway interactions with *VEGF-Receptor (KDR)* and with Target gene PMOs. Target gene PMO with PMO to knockdown the KDR

(VEGFR2) gene are co-injected to evaluate whether the target functions in the VEGF pathway.

III. Analysis of Table 1

[0119] BLAST analysis (Altschul et al., *supra*) was employed to identify orthologs of *zebrafish* modifiers. The columns "MBM symbol", and "MBM name aliases " provide a symbol and the known name abbreviations for the Targets, where available, from Genbank. "MBM RefSeq_NA or GI_NA", "MBM GI_AA", "MBM NAME", and "MBM Description" provide the reference DNA sequences for the MBMs as available from National Center for Biology Information (NCBI), MBM protein Genbank identifier number (GI#), MBM name, and MBM description, all available from Genbank, respectively. The length of each amino acid is in the "MBM Protein Length" column.

[0120] Names and Protein sequences of *zebrafish* modifiers of branching morphogenesis are represented in the "Modifier Name" and "Modifier GI_AA" column by GI#, respectively.

Table1

MBM symbol	MBM name aliases	MBM RefSeq_NA or GI_NA	NA SEQ ID NO	MBM GI_AA	AA SEQ ID NO	MBM NAME	MBM Description	MBM Protein length	Modifier Name	Modifier GI_AA
CDKL2	CDC2-related kinase|CDKL2|KKIAMRE|P56|cyclin-dependent kinase-like 2 (CDC2-related kinase)|p56 KKIAMRE protein kinase|serine/threonine protein kinase KKIAMRE	NM_003948	1	4505569	5	cyclin-dependent kinase-like 2 (CDC2-related kinase)	protein kinase	493	Dr_cdkl1	0
CDKL3	CDKL3|NKIAMRE|cyclin-dependent kinase-like 3|serine-threonine protein kinase NKIAMRE	NM_016508	2	7706059	6	cyclin-dependent kinase-like 3	protein serine/threonine kinase	455	Dr_cdkl1	0

LOC130736	CDKL4|Hsap15509439|LOC130736|LOC344387|na|similar to Serine/threonine-protein kinase KKIALRE (Cyclin-dependent kinase-like 1)	XM_293029	3	37546887	7	similar to Serine/threonine-protein kinase KKIALRE (Cyclin-dependent kinase-like 1)	na	485	Dr_cdkl1	0
CDKL5	CDKL5|STK9|cyclin-dependent kinase-like 5|serine/threonine kinase 9	NM_003159	4	4507281	8	cyclin-dependent kinase-like 5	protein serine/threonine kinase	1030	Dr_cdkl1	0

IV. High-Throughput In Vitro Fluorescence Polarization Assay

[0121] Fluorescently-labeled MBM peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of MBM activity.

V. High-Throughput In Vitro Binding Assay.

[0122] ^{33}P-labeled MBM peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM $MgCl_2$, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate branching morphogenesis modulating agents.

VI. Immunoprecipitations and Immunoblotting

[0123] For coprecipitation of transfected proteins, 3×10^6 appropriate recombinant cells containing the MBM proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with

phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at 15,000 × g for 15 min. The cell lysate is incubated with 25 μl of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

[0124] After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

VII. Kinase assay

[0125] A purified or partially purified MBM is diluted in a suitable reaction buffer, e.g., 50 mM Hepes, pH 7.5, containing magnesium chloride or manganese chloride (1-20 mM) and a peptide or polypeptide substrate, such as myelin basic protein or casein (1-10 μg/ml). The final concentration of the kinase is 1-20 nM. The enzyme reaction is conducted in microtiter plates to facilitate optimization of reaction conditions by increasing assay throughput. A 96-well microtiter plate is employed using a final volume 30-100 μl. The reaction is initiated by the addition of ^{33}P-gamma-ATP (0.5 μCi/ml) and incubated for 0.5 to 3 hours at room temperature. Negative controls are provided by the addition of EDTA, which chelates the divalent cation ($Mg2^{+}$ or Mn^{2+}) required for enzymatic activity. Following the incubation, the enzyme reaction is quenched using EDTA. Samples of the reaction are transferred to a 96-well glass fiber filter plate (MultiScreen, Millipore). The filters are subsequently washed with phosphate-buffered saline, dilute phosphoric acid (0.5%) or other suitable medium to remove excess radiolabeled ATP. Scintillation cocktail is added to the filter plate and the incorporated radioactivity is quantitated by scintillation counting (Wallac/Perkin Elmer). Activity is defined by the amount of radioactivity detected following subtraction of the negative control reaction value (EDTA quench).

VIII. Expression analysis

[0126] All cell lines used in the following experiments are NCI (National Cancer Institute) lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues are obtained from Impath, UC Davis, Clontech, Stratagene, Ardais, Genome Collaborative, and Ambion.

[0127] TaqMan analysis is used to assess expression levels of the disclosed genes in various samples.

[0128] RNA is extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/μl. Single stranded cDNA is then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of Applied Biosystems (Foster City, CA).

[0129] Primers for expression analysis using TaqMan assay (Applied Biosystems, Foster City, CA) are prepared according to the TaqMan protocols, and the following criteria: a) primer pairs are designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product. Expression analysis is performed using a 7900HT instrument.

[0130] Taqman reactions are carried out following manufacturer's protocols, in 25 μl total volume for 96-well plates and 10 μl total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis is prepared using a universal pool of human cDNA samples, which is a mixture of cDNAs from a wide variety of tissues so that the chance that a target will be present in appreciable amounts is good. The raw data are normalized using 18S rRNA (universally expressed in all tissues and cells).

[0131] For each expression analysis, tumor tissue samples are compared with matched normal tissues from the same patient. A gene is considered overexpressed in a tumor when the level of expression of the gene is 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue is not available, a universal pool of cDNA samples is used instead. In these cases, a gene is considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the average of all normal samples from the same tissue type is greater than 2 times the standard deviation of all normal samples (i.e., Tumor – average(all normal samples) > 2 x STDEV(all normal samples)).

[0132] A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression analysis as described above, by antibody directed to the gene target, or by any other available detection method.

IX. Proliferation Assay

[0133] Human umbilical endothelial cells (HMVEC) are maintained at 37°C in flasks or plates coated with 1.5% porcine skin gelatin (300 bloom, Sigma) in Growth medium (Clonetics Corp.) supplemented with 10-20% fetal bovine serum (FBS, Hyclone). Cells are grown to confluency and used up to the seventh passage. Stimulation medium consists of 50% Sigma 99 media and 50% RPMI 1640 with L-glutamine and additional supplementation with 10 μg/ml insulin-transferrin-selenium (Gibco BRL) and 10% FBS. Cell growth is stimulated by incubation in Stimulation medium supplemented with 20 ng/ml of VEGF. Cell culture assays are carried out in triplicate. Cells are transfected with a mixture of 10 μg of pSV7d expression vectors carrying the MBM or the MBM coding sequences and 1 μg of pSV2 expression vector carrying the neo resistance gene with the Lipofectin reagent (Life Technologies, Inc.). Stable integrants are selected using 500 μg/ml G418; cloning was carried out by colony isolation using a Pasteur pipette. Transformants are screened by their ability to specifically bind iodinated VEGF. Proliferation assays are performed on growth-arrested cells seeded in 24-well cluster plates. The cell monolayers are incubated in serum-free medium with the modulators and 1 μCi of [3H]thymidine (47 Ci/mmol) for 4 h. The insoluble material is precipitated for 10 min with 10% trichloroacetic acid, neutralized, and dissolved in 0.2 M NaOH, and the radioactivity is counted in a scintillation counter.

WHAT IS CLAIMED IS:

1. A method of identifying a candidate branching morphogenesis modulating agent, said method comprising the steps of:

(a) providing an assay system comprising an MBM polypeptide or nucleic acid;

(b) contacting the assay system with a test agent under conditions whereby, but for the presence of the test agent, the system provides a reference activity; and

(c) detecting a test agent-biased activity of the assay system, wherein a difference between the test agent-biased activity and the reference activity identifies the test agent as a candidate branching morphogenesis modulating agent.

2. The method of Claim 1 wherein the assay system includes a screening assay comprising an MBM polypeptide, and the candidate test agent is a small molecule modulator.

3. The method of Claim 2 wherein the screening assay is a kinase assay.

4. The method of Claim 1 wherein the assay system includes a binding assay comprising an MBM polypeptide and the candidate test agent is an antibody.

5. The method of Claim 1 wherein the assay system includes an expression assay comprising an MBM nucleic acid and the candidate test agent is a nucleic acid modulator.

6. The method of Claim 5 wherein the nucleic acid modulator is an antisense oligomer.

7. The method of Claim 6 wherein the nucleic acid modulator is a PMO.

8. The method of Claim 1 wherein the assay system comprises cultured cells or a non-human animal expressing MBM,

and wherein the assay system includes an assay that detects an agent-biased change in branching morphogenesis

9. The method of Claim 8 wherein the branching morphogenesis is angiogenesis.

10. The method of Claim 8 wherein the assay system comprises cultured cells.

11. The method of Claim 10 wherein the assay detects an event selected from the group consisting of cell proliferation, cell cycling, apoptosis, tubulogenesis, cell migration, cell sprouting and response to hypoxic conditions.

12. The method of Claim 10 wherein the assay detects tubulogenesis or cell migration or cell sprouting, and wherein the assay system comprises the step of testing the cellular response to stimulation with at least two different pro-angiogenic agents.

13. The method of Claim 10 wherein the assay detects tubulogenesis or cell migration, and wherein cells are stimulated with an inflammatory angiogenic agent.

14. The method of Claim 8 wherein the assay system comprises a non-human animal.

15. The method of Claim 14 wherein the assay system includes a matrix implant assay, a xenograft assay, a hollow fiber assay, or a transgenic tumor assay.

16. The method of Claim 15 wherein the assay system includes a transgenic tumor assay that includes a mouse comprising a RIP1-Tag2 transgene.

17. The method of Claim 1, comprising the additional steps of:

(d) providing a second assay system comprising cultured cells or a non-human animal expressing MBM ,

(e) contacting the second assay system with the test agent of (b) or an agent derived therefrom under conditions whereby, but for the presence of the test agent or agent derived therefrom, the system provides a reference activity; and

(f) detecting an agent-biased activity of the second assay system,

wherein a difference between the agent-biased activity and the reference activity of the second assay system confirms the test agent or agent derived therefrom as a candidate branching morphogenesis modulating agent,

and wherein the second assay system includes a second assay that detects an agent-biased change in an activity associated with branching morphogenesis.

18. The method of Claim 17 wherein second assay detects an agent-biased change in an activity associated with angiogenesis.

19. The method of Claim 17 wherein the second assay system comprises cultured cells.

20. The method of Claim 19 wherein the second assay detects an event selected from the group consisting of cell proliferation, cell cycling, apoptosis, tubulogenesis, cell migration, cell sprouting and response to hypoxic conditions.

21. The method of Claim 20 wherein the second assay detects tubulogenesis or cell migration or cell sprouting, and wherein the second assay system comprises the step of testing the cellular response to stimulation with at least two different pro-angiogenic agents.

22. The method of Claim 20 wherein the assay detects tubulogenesis or cell migration, and wherein cells are stimulated with an inflammatory angiogenic agent.

23. The method of Claim 17 wherein the assay system comprises a non-human animal.

24. The method of Claim 23 wherein the assay system includes a matrix implant assay, a xenograft assay, a hollow fiber assay, or a transgenic tumor assay.

25. The method of Claim 24 wherein the assay system includes a transgenic tumor assay that includes a mouse comprising a RIP1-Tag2 transgene.

26. A method of modulating branching morphogenesis in a mammalian cell comprising contacting the cell with an agent that specifically binds an MBM polypeptide or nucleic acid.

27. The method of Claim 26 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with branching morphogenesis.

28. The method of Claim 26 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.

29. The method of Claim 26 wherein the branching morphogenesis is angiogenesis

30. The method of Claim 29 wherein tumor cell proliferation is inhibited.

31. A method for diagnosing a disease in a patient comprising:
    (a) obtaining a biological sample from the patient;
    (b) contacting the sample with a probe for MBM expression;
    (c) comparing results from step (b) with a control; and
    (d) determining whether step (c) indicates a likelihood of disease.

32. The method of claim 31 wherein said disease is cancer.

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tccaggtccg aggcacttcc atgtgcccga cactccaggt ccgaggcact gatgctttca 3180

gctgcccaac ccagcaatcc gggttctctt tcttcgtgag acacgttatg agggaagccc 3240

tgattcacag ggcccaggta aaccaagctg cgctcctgac ataccatgag aatgcggcac 3300

tgacgggcaa gtgacttctg caagcctgcg gctggtccca atgccctgaa tcacctctct 3360

catggaagaa ccaattaaca ccaatgaatc aaccaaaac 3399

<210> 5
<211> 493
<212> PRT
<213> Homo sapiens

<400> 5

Met Glu Lys Tyr Glu Asn Leu Gly Leu Val Gly Glu Gly Ser Tyr Gly
1 5 10 15

Met Val Met Lys Cys Arg Asn Lys Asp Thr Gly Arg Ile Val Ala Ile
20 25 30

Lys Lys Phe Leu Glu Ser Asp Asp Asp Lys Met Val Lys Lys Ile Ala
35 40 45

Met Arg Glu Ile Lys Leu Leu Lys Gln Leu Arg His Glu Asn Leu Val
50 55 60

Asn Leu Leu Glu Val Cys Lys Lys Lys Lys Arg Trp Tyr Leu Val Phe
65 70 75 80

Glu Phe Val Asp His Thr Ile Leu Asp Asp Leu Glu Leu Phe Pro Asn
85 90 95

Gly Leu Asp Tyr Gln Val Val Gln Lys Tyr Leu Phe Gln Ile Ile Asn
100 105 110

Gly Ile Gly Phe Cys His Ser His Asn Ile Ile His Arg Asp Ile Lys
115 120 125

Pro Glu Asn Ile Leu Val Ser Gln Ser Gly Val Val Lys Leu Cys Asp
130 135 140

Phe Gly Phe Ala Arg Thr Leu Ala Ala Pro Gly Glu Val Tyr Thr Asp
145 150 155 160

Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Leu Leu Val Gly Asp
165 170 175

Val Lys Tyr Gly Lys Ala Val Asp Val Trp Ala Ile Gly Cys Leu Val
180 185 190

Thr Glu Met Phe Met Gly Glu Pro Leu Phe Pro Gly Asp Ser Asp Ile
195 200 205

Asp Gln Leu Tyr His Ile Met Met Cys Leu Gly Asn Leu Ile Pro Arg
210 215 220

His Gln Glu Leu Phe Asn Lys Asn Pro Val Phe Ala Gly Val Arg Leu
225 230 235 240

Pro Glu Ile Lys Glu Arg Glu Pro Leu Glu Arg Arg Tyr Pro Lys Leu
245 250 255

Ser Glu Val Val Ile Asp Leu Ala Lys Lys Cys Leu His Ile Asp Pro
260 265 270

Asp Lys Arg Pro Phe Cys Ala Glu Leu Leu His His Asp Phe Phe Gln
275 280 285

Met Asp Gly Phe Ala Glu Arg Phe Ser Gln Glu Leu Gln Leu Lys Val
290 295 300

Gln Lys Asp Ala Arg Asn Val Ser Leu Ser Lys Lys Ser Gln Asn Arg
305 310 315 320

Lys Lys Glu Lys Glu Lys Asp Asp Ser Leu Val Glu Glu Arg Lys Thr
325 330 335

Leu Val Val Gln Asp Thr Asn Ala Asp Pro Lys Ile Lys Asp Tyr Lys
340 345 350

Leu Phe Lys Ile Lys Gly Ser Lys Ile Asp Gly Glu Lys Ala Glu Lys
355 360 365

Gly Asn Arg Ala Ser Asn Ala Ser Cys Leu His Asp Ser Arg Thr Ser
370 375 380

His Asn Lys Ile Val Pro Ser Thr Ser Leu Lys Asp Cys Ser Asn Val
385 390 395 400

Ser Val Asp His Thr Arg Asn Pro Ser Val Ala Ile Pro Pro Leu Thr
405 410 415

His Asn Leu Ser Ala Val Ala Pro Ser Ile Asn Ser Gly Met Gly Thr
420 425 430

Glu Thr Ile Pro Ile Gln Gly Tyr Arg Val Asp Glu Lys Thr Lys Lys
435 440 445

Cys Ser Ile Pro Phe Val Lys Pro Asn Arg His Ser Pro Ser Gly Ile
450 455 460

Tyr Asn Ile Asn Val Thr Thr Leu Val Ser Gly Pro Pro Leu Ser Asp
465 470 475 480

Asp Ser Gly Ala Asp Leu Pro Gln Met Glu His Gln His
485 490

<210> 6
<211> 455
<212> PRT
<213> Homo sapiens

<400> 6

Met Glu Met Tyr Glu Thr Leu Gly Lys Val Gly Glu Gly Ser Tyr Gly
1 5 10 15

Thr Val Met Lys Cys Lys His Lys Asn Thr Gly Gln Ile Val Ala Ile
20 25 30

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Lys Ile Phe Tyr Glu Arg Pro Glu Gln Ser Val Asn Lys Ile Ala Met
        35                  40                  45

Arg Glu Ile Lys Phe Leu Lys Gln Phe His His Glu Asn Leu Val Asn
    50                  55                  60

Leu Ile Glu Val Phe Arg Gln Lys Lys Lys Ile His Leu Val Phe Glu
65                  70                  75                  80

Phe Ile Asp His Thr Val Leu Asp Glu Leu Gln His Tyr Cys His Gly
                85                  90                  95

Leu Glu Ser Lys Arg Leu Arg Lys Tyr Leu Phe Gln Ile Leu Arg Ala
            100                 105                 110

Ile Asp Tyr Leu His Ser Asn Asn Ile Ile His Arg Asp Ile Lys Pro
        115                 120                 125

Glu Asn Ile Leu Val Ser Gln Ser Gly Ile Thr Lys Leu Cys Asp Phe
    130                 135                 140

Gly Phe Ala Arg Thr Leu Ala Ala Pro Gly Asp Ile Tyr Thr Asp Tyr
145                 150                 155                 160

Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Leu Val Leu Lys Asp Thr
                165                 170                 175

Ser Tyr Gly Lys Pro Val Asp Ile Trp Ala Leu Gly Cys Met Ile Ile
            180                 185                 190

Glu Met Ala Thr Gly Asn Pro Tyr Leu Pro Ser Ser Ser Asp Leu Asp
        195                 200                 205

Leu Leu His Lys Ile Val Leu Lys Val Gly Asn Leu Ser Pro His Leu
    210                 215                 220

Gln Asn Ile Phe Ser Lys Ser Pro Ile Phe Ala Gly Val Val Leu Pro
225                 230                 235                 240

Gln Val Gln His Pro Lys Asn Ala Arg Lys Lys Tyr Pro Lys Leu Asn
                245                 250                 255

Gly Leu Leu Ala Asp Ile Val His Ala Cys Leu Gln Ile Asp Pro Ala
            260                 265                 270

Asp Arg Ile Ser Ser Ser Asp Leu Leu His His Glu Tyr Phe Thr Arg
        275                 280                 285

Asp Gly Phe Ile Glu Lys Phe Met Pro Glu Leu Lys Ala Lys Leu Leu
    290                 295                 300
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Gln Glu Ala Lys Val Asn Ser Leu Ile Lys Pro Lys Glu Ser Ser Lys
305 310 315 320

Glu Asn Glu Leu Arg Lys Asp Glu Arg Lys Thr Val Tyr Thr Asn Thr
325 330 335

Leu Leu Ser Ser Ser Val Leu Gly Glu Glu Ile Glu Lys Glu Lys Lys
340 345 350

Pro Lys Glu Ile Lys Val Arg Val Ile Lys Val Lys Gly Gly Arg Gly
355 360 365

Asp Ile Ser Glu Pro Lys Lys Lys Glu Tyr Glu Gly Gly Leu Gly Gln
370 375 380

Gln Asp Ala Asn Glu Asn Val His Pro Met Ser Pro Asp Thr Lys Leu
385 390 395 400

Val Thr Ile Glu Pro Pro Asn Pro Ile Asn Pro Ser Thr Asn Cys Asn
405 410 415

Gly Leu Lys Glu Asn Pro His Cys Gly Gly Ser Val Thr Met Pro Pro
420 425 430

Ile Asn Leu Thr Asn Ser Asn Leu Met Ala Ala Asn Leu Ser Ser Asn
435 440 445

Leu Phe His Pro Ser Val Arg
450 455

<210> 7
<211> 485
<212> PRT
<213> Homo sapiens

<400> 7

Met Gly Leu Val Val Lys Ser Leu Leu Met Arg Glu Ala Ala Arg Asn
1 5 10 15

Gln Gln Phe Gln Lys Leu Glu Ser Lys Tyr His Glu Asp Ile Asn Gly
20 25 30

Leu Met Asp Leu Tyr Ile Ser Leu Met Leu Gln Ala Arg Pro Arg Gly
35 40 45

Tyr Thr His Gln Glu Glu Lys Asp Gln Gly Trp Gly Cys Gln Arg Gln
50 55 60

Gly Asn Pro Leu Arg Lys Pro Gln Cys Pro Ala Arg Pro Ser Val Pro
65 70 75 80

Val Ala Arg Glu Thr Leu Arg Arg Phe Arg Trp Leu Arg Ser Arg Trp
85 90 95

Val His Gly Ala Ser Ser Glu Arg His Ala Arg Glu Leu Gly Leu Gly
100 105 110

Leu Phe Gly Ala Ala Arg Ser Phe Val Thr Val Ala Leu Trp Val Gln
115 120 125

Leu Arg Ile Pro Val Ala Gly Thr Lys Lys Ser Ser Pro Lys Glu Lys
130 135 140

Arg Asp Arg Leu Pro Leu Arg Trp Pro Leu Glu Gly Lys Gly Arg Ile
145 150 155 160

Arg Gly Gly Ser Thr Asp Pro Tyr Glu Lys Leu Ala Lys Thr Gly Glu
165 170 175

Gly Ser Tyr Gly Val Val Phe Lys Cys Arg Asn Lys Thr Ser Gly Gln
180 185 190

Val Val Ala Val Lys Lys Phe Val Glu Ser Glu Asp Asp Pro Val Val
195 200 205

Lys Lys Ile Ala Leu Arg Glu Ile Arg Met Leu Lys Gln Leu Lys His
210 215 220

Pro Asn Leu Val Asn Leu Ile Glu Val Phe Arg Arg Lys Arg Lys Met
225 230 235 240

His Leu Val Phe Glu Tyr Cys Asp His Thr Leu Leu Asn Glu Leu Glu
245 250 255

Arg Asn Pro Asn Gly Met Asp Ser Asp Ser Ile Val Gly Arg Leu Arg
260 265 270

Ser Val Ala Asp Arg Ser Arg Leu Ala Pro Lys Asn Asn Gln Ile Met
275 280 285

Ile Leu Gln Cys Ile His Arg Asp Ile Lys Pro Glu Asn Ile Leu Ile
290 295 300

Thr Lys Gln Gly Ile Ile Lys Ile Cys Asp Phe Gly Phe Ala Gln Ile
305 310 315 320

Leu Ile Pro Gly Asp Ala Tyr Thr Asp Tyr Val Ala Thr Arg Trp Tyr
325 330 335

Arg Ala Pro Glu Leu Leu Val Gly Asp Thr Gln Tyr Gly Ser Ser Val
340 345 350

Asp Ile Trp Ala Ile Gly Cys Val Phe Ala Glu Leu Leu Thr Gly Gln
355 360 365

Pro Leu Trp Pro Gly Lys Ser Asp Val Asp Gln Leu Tyr Leu Ile Ile
370 375 380

Arg Thr Leu Gly Lys Leu Ile Pro Arg His Gln Ser Ile Phe Lys Ser
385 390 395 400

Asn Gly Phe Phe His Gly Ile Ser Ile Pro Glu Pro Glu Asp Met Glu
405 410 415

Thr Leu Glu Glu Lys Phe Ser Asp Val His Pro Val Ala Leu Asn Phe
420 425 430

Met Lys Gly Cys Leu Lys Met Asn Pro Asp Asp Arg Leu Thr Cys Ser
435 440 445

Gln Leu Leu Glu Ser Ser Tyr Phe Asp Ser Phe Gln Glu Ala Gln Ile
450 455 460

Lys Arg Lys Ala Arg Asn Glu Gly Arg Asn Arg Arg Arg Gln Gln Val
465 470 475 480

Leu Pro Leu Lys Ser
485

<210> 8
<211> 1030
<212> PRT
<213> Homo sapiens

<400> 8

Met Lys Ile Pro Asn Ile Gly Asn Val Met Asn Lys Phe Glu Ile Leu
1 5 10 15

Gly Val Val Gly Glu Gly Ala Tyr Gly Val Val Leu Lys Cys Arg His
20 25 30

Lys Glu Thr His Glu Ile Val Ala Ile Lys Lys Phe Lys Asp Ser Glu
35 40 45

Glu Asn Glu Glu Val Lys Glu Thr Thr Leu Arg Glu Leu Lys Met Leu
50 55 60

Arg Thr Leu Lys Gln Glu Asn Ile Val Glu Leu Lys Glu Ala Phe Arg
65 70 75 80

Arg Arg Gly Lys Leu Tyr Leu Val Phe Glu Tyr Val Glu Lys Asn Met
85 90 95

Leu Glu Leu Leu Glu Glu Met Pro Asn Gly Val Pro Pro Glu Lys Val

100 105 110

Lys Ser Tyr Ile Tyr Gln Leu Ile Lys Ala Ile His Trp Cys His Lys
115 120 125

Asn Asp Ile Val His Arg Asp Ile Lys Pro Glu Asn Leu Leu Ile Ser
130 135 140

His Asn Asp Val Leu Lys Leu Cys Asp Phe Gly Phe Ala Arg Asn Leu
145 150 155 160

Ser Glu Gly Asn Asn Ala Asn Tyr Thr Glu Tyr Val Ala Thr Arg Trp
165 170 175

Tyr Arg Ser Pro Glu Leu Leu Leu Gly Ala Pro Tyr Gly Lys Ser Val
180 185 190

Asp Met Trp Ser Val Gly Cys Ile Leu Gly Glu Leu Ser Asp Gly Gln
195 200 205

Pro Leu Phe Pro Gly Glu Ser Glu Ile Asp Gln Leu Phe Thr Ile Gln
210 215 220

Lys Val Leu Gly Pro Leu Pro Ser Glu Gln Met Lys Leu Phe Tyr Ser
225 230 235 240

Asn Pro Arg Phe His Gly Leu Arg Phe Pro Ala Val Asn His Pro Gln
245 250 255

Ser Leu Glu Arg Arg Tyr Leu Gly Ile Leu Asn Ser Val Leu Leu Asp
260 265 270

Leu Met Lys Asn Leu Leu Lys Leu Asp Pro Ala Asp Arg Tyr Leu Thr
275 280 285

Glu Gln Cys Leu Asn His Pro Thr Phe Gln Thr Gln Arg Leu Leu Asp
290 295 300

Arg Ser Pro Ser Arg Ser Ala Lys Arg Lys Pro Tyr His Val Glu Ser
305 310 315 320

Ser Thr Leu Ser Asn Arg Asn Gln Ala Gly Lys Ser Thr Ala Leu Gln
325 330 335

Ser His His Arg Ser Asn Ser Lys Asp Ile Gln Asn Leu Ser Val Gly
340 345 350

Leu Pro Arg Ala Asp Glu Gly Leu Pro Ala Asn Glu Ser Phe Leu Asn
355 360 365

Gly Asn Leu Ala Gly Ala Ser Leu Ser Pro Leu His Thr Lys Thr Tyr

370 375 380

Gln Ala Ser Ser Gln Pro Gly Ser Thr Ser Lys Asp Leu Thr Asn Asn
385 390 395 400

Asn Ile Pro His Leu Leu Ser Pro Lys Glu Ala Lys Ser Lys Thr Glu
405 410 415

Phe Asp Phe Asn Ile Asp Pro Lys Pro Ser Glu Gly Pro Gly Thr Lys
420 425 430

Tyr Leu Lys Ser Asn Ser Arg Ser Gln Gln Asn Arg His Ser Phe Met
435 440 445

Glu Ser Ser Gln Ser Lys Ala Gly Thr Leu Gln Pro Asn Glu Lys Gln
450 455 460

Ser Arg His Ser Tyr Ile Asp Thr Ile Pro Gln Ser Ser Arg Ser Pro
465 470 475 480

Ser Tyr Arg Thr Lys Ala Lys Ser His Gly Ala Leu Ser Asp Ser Lys
485 490 495

Ser Val Ser Asn Leu Ser Glu Ala Arg Ala Gln Ile Ala Glu Pro Ser
500 505 510

Thr Ser Arg Tyr Phe Pro Ser Ser Cys Leu Asp Leu Asn Ser Pro Thr
515 520 525

Ser Pro Thr Pro Thr Arg His Ser Asp Thr Arg Thr Leu Leu Ser Pro
530 535 540

Ser Gly Arg Asn Asn Arg Asn Glu Gly Thr Leu Asp Ser Arg Arg Thr
545 550 555 560

Thr Thr Arg His Ser Lys Thr Met Glu Glu Leu Lys Leu Pro Glu His
565 570 575

Met Asp Ser Ser His Ser His Ser Leu Ser Ala Pro His Glu Ser Phe
580 585 590

Ser Tyr Gly Leu Gly Tyr Thr Ser Pro Phe Ser Ser Gln Gln Arg Pro
595 600 605

His Arg His Ser Met Tyr Val Thr Arg Asp Lys Val Arg Ala Lys Gly
610 615 620

Leu Asp Gly Ser Leu Ser Ile Gly Gln Gly Met Ala Ala Arg Ala Asn
625 630 635 640

Ser Leu Gln Leu Leu Ser Pro Gln Pro Gly Glu Gln Leu Pro Pro Glu

645 650 655

Met Thr Val Ala Arg Ser Ser Val Lys Glu Thr Ser Arg Glu Gly Thr
660 665 670

Ser Ser Phe His Thr Arg Gln Lys Ser Glu Gly Gly Val Tyr His Asp
675 680 685

Pro His Ser Asp Asp Gly Thr Ala Pro Lys Glu Asn Arg His Leu Tyr
690 695 700

Asn Asp Pro Val Pro Arg Arg Val Gly Ser Phe Tyr Arg Val Pro Ser
705 710 715 720

Pro Arg Pro Asp Asn Ser Phe His Glu Asn Asn Val Ser Thr Arg Val
725 730 735

Ser Ser Leu Pro Ser Glu Ser Ser Ser Gly Thr Asn His Ser Lys Arg
740 745 750

Gln Pro Ala Phe Asp Pro Trp Lys Ser Pro Glu Asn Ile Ser His Ser
755 760 765

Glu Gln Leu Lys Glu Lys Glu Lys Gln Gly Phe Phe Arg Ser Met Lys
770 775 780

Lys Lys Lys Lys Lys Ser Gln Thr Val Pro Asn Ser Asp Ser Pro Asp
785 790 795 800

Leu Leu Thr Leu Gln Lys Ser Ile His Ser Ala Ser Thr Pro Ser Ser
805 810 815

Arg Pro Lys Glu Trp Arg Pro Glu Lys Ile Ser Asp Leu Gln Thr Gln
820 825 830

Ser Gln Pro Leu Lys Ser Leu Arg Lys Leu Leu His Leu Ser Ser Ala
835 840 845

Ser Asn His Pro Ala Ser Ser Asp Pro Arg Phe Gln Pro Leu Thr Ala
850 855 860

Gln Gln Thr Lys Asn Ser Phe Ser Glu Ile Arg Ile His Pro Leu Ser
865 870 875 880

Gln Ala Ser Gly Gly Ser Ser Asn Ile Arg Gln Glu Pro Ala Pro Lys
885 890 895

Gly Arg Pro Ala Leu Gln Leu Pro Asp Gly Gly Cys Asp Gly Arg Arg
900 905 910

Gln Arg His His Ser Gly Pro Gln Asp Arg Arg Phe Met Leu Arg Thr

915 920 925

Thr Glu Gln Gln Gly Glu Tyr Phe Cys Cys Gly Asp Pro Lys Lys Pro
930 935 940

His Thr Pro Cys Val Pro Asn Arg Ala Leu His Arg Pro Ile Ser Ser
945 950 955 960

Pro Ala Pro Tyr Pro Val Leu Gln Val Arg Gly Thr Ser Met Cys Pro
965 970 975

Thr Leu Gln Val Arg Gly Thr Asp Ala Phe Ser Cys Pro Thr Gln Gln
980 985 990

Ser Gly Phe Ser Phe Phe Val Arg His Val Met Arg Glu Ala Leu Ile
995 1000 1005

His Arg Ala Gln Val Asn Gln Ala Ala Leu Leu Thr Tyr His Glu
1010 1015 1020

Asn Ala Ala Leu Thr Gly Lys
1025 1030