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(54) Title: METHODS AND COMPOSITIONS FOR MODULATING CELL MOTILITY AND INHIBITING TUMOR METASTASIS

(57) Abstract: This invention relates to identification of genes that promote motility and migration of tumor cells, e.g., MAP4K4, CDK7, FGFR1, DYRK1B, and SERPINB3. The invention also provides methods of using the pro-migration molecule to screen for compounds that inhibit tumor metastasis. The methods comprise first screening test compounds for modulators that down-regulate one of these pro-migration molecules (its cellular level or enzymatic activities), and then further screening the identified compounds for ability to inhibit migration or motility of a tumor cell. The invention further provides methods and pharmaceutical compositions for inhibiting tumor metastasis in a subject.

METHODS AND COMPOSITIONS FOR MODULATING CELL MOTILITY AND INHIBITING TUMOR METASTASIS

FIELD OF THE INVENTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application Number 60/735,654, filed 09 November 2005. The full disclosure of this application is incorporated herein by reference in its entirety and for all purposes.

[0002] The present invention generally relates to methods for identifying modulators of cell motility, and to therapeutic applications of such modulators. More particularly, the invention pertains to genes that play a role in promoting metastasis of tumor cells, and to methods of using such genes to identify novel compounds that inhibit tumor metastasis.

BACKGROUND OF THE INVENTION

[0003] Cell migration is a fundamental biological process, necessary for the spatial distribution of developing cell types and tissues, wound healing, blood vessel development, immune responses and renewal of cell layers in tissues such as the skin, esophagus and colorectum. The movements that constitute cell migration are complex, requiring the integration and transduction of diverse signaling cues with the mechanical processes of cell movement.

[0004] Tumor cell motility is a critical component of invasion and metastasis, but the regulation of this motility is still poorly understood. The process of tumor metastasis is a multistage event involving local invasion and destruction of the extracellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site and growth in the new location (Fidler et al., Adv. Cancer Res. 28:149-250, 1978; Liotta, et

al., Cancer Treatment Res. 40:223-238, 1988; Nicolson, Biochim. Biophy. Acta 948:175-224, 1988; and Zetter, N. Eng. J. Med. 322:605-612, 1990). Success in establishing metastatic deposits requires tumor cells to be able to accomplish these steps sequentially. Common to many steps of the metastatic process is a requirement for motility. The enhanced movement of malignant tumor cells is a major contributor to the progression of the disease toward metastasis. Increased cell motility has been associated with enhanced metastatic potential in animal as well as human tumors (Hosaka et al., Gann 69: 273-276, 1978; and Haemmerlin et al., Int. J. Cancer 27:603-610, 1981).

[0005] There is a need in the art for better means for modulating motility and migratory activities of various types of cells. In particular, there is a need for more specific and effective methods and compositions for inhibiting tumor cell migration, and for treating diseases and conditions that are caused by or associated with metastasis of tumor cells. The present invention addresses this and other needs.

SUMMARY OF THE INVENTION

[0006] In one aspect, the invention provides methods for identifying an agent that modulates motility of a cell. The methods involve (a) screening test compounds to identify modulating compounds which modulate a pro-migration molecule selected from the group consisting of MAP4K4, CDK7, FGFR1, DYRK1B and SERPINB3; and (b) testing the identified modulating compounds for ability to modulate motility of the cell. In some of these methods, the test compounds are screened for ability to modulate cellular level of the pro-migration molecule. Some of the methods employ MAP4K4, CDK7, FGFR1, or DYRK1B, and the test compounds are screened for ability to modulate the kinase activity of the pro-migration molecule. Some of the methods employ SERPINB3, and the test compounds are screened for ability to modulate the proteinase-inhibiting activity of SERPINB3. Some of the methods are directed to identifying agents that inhibit motility of a tumor cell.

[0007] In a related aspect, the invention provides methods for identifying agents that inhibit tumor metastasis. These methods entail (a) screening test compounds to identify modulating compounds which down-regulate a pro-migration molecule selected from the group consisting of MAP4K4, CDK7, FGFR1, DYRK1B and SERPINB3, and (b)

testing the identified modulating compounds for ability to inhibit migration of a tumor cell. Some of the methods further involve testing the identified modulating compounds for ability to modulate migration of a non-tumor control cell. In some of the methods, the test compounds are screened for ability to down-regulate cellular level of the pro-migration molecule. Some of the methods employ MAP4K4, CDK7, FGFR1, or DYRK1B as the screening target, and the test compounds are screened for ability to inhibit the kinase activity of the pro-migration molecule. Some other methods employ SERPINB3 as the screening target, and the test compounds are screened for ability to inhibit the proteinase-inhibiting activity of SERPINB3. In some methods, the employed pro-migration molecule is MAP4K4, CDK7, or FGFR1, and the tumor cell is selected from the group consisting of an ovarian tumor cell, a breast tumor cell, a melanoma tumor cell, and a prostate tumor cell. In some methods, the employed pro-migration molecule is SERPINB3, and the tumor cell is selected from the group consisting of an ovarian tumor cell, a melanoma tumor cell, and a prostate tumor cell. In some other methods, the pro-migration molecule employed is DYRK1B, and the tumor cell is selected from the group consisting of an ovarian tumor cell and a prostate tumor cell.

[0008] In some of the screening methods, (b) entails comparing migration of a cultured tumor cell in the presence of a modulating compound to migration of the tumor cell in the absence of the modulating compound. In these methods, a significant inhibition of migration of the tumor cell in the presence of the modulating compound relative to migration of the tumor cell in the absence of the modulating compound identifies the modulating compound as an agent that inhibits tumor metastasis. In some methods, migration of the tumor cell is examined with a matrigel Boyden chamber assay. In some other methods, migration of the tumor cell is examined by monitoring closure of a scratch in a culture of the tumor cell.

[0009] In a related aspect, the invention provides methods for inhibiting tumor invasion and metastasis in a subject. These methods involve administering to the subject a pharmaceutical composition that contains an effective amount of an agent which down-regulates a pro-migration molecule. The pro-migration molecule is selected from the group consisting of MAP4K4, CDK7, FGFR1, DYRK1B and SERPINB3. Some of the methods are directed to inhibit metastasis of a tumor selected from the group consisting of an ovarian tumor, a breast tumor, a melanoma tumor, and a prostate tumor. In some of the

methods, the employed agent down-regulates expression of a gene encoding the pro-migration molecule. The agent used in these methods can be, e.g., an short interfering RNA (siRNA), a microRNA (miRNA), and a synthetic hairpin RNA (shRNA), an anti-sense nucleic acid, or a complementary DNA (cDNA). In some other methods, the employed agent is an antagonist antibody that specifically binds to the pro-migration molecule. Some of these methods employ an antibody which specifically inhibits the kinase activity of MAP4K4, CDK7, FGFR1, or DYRK1B. Some other methods employ an antibody which specifically inhibits the proteinase-inhibiting activity of SERPINB3. Preferably, the antagonist antibody employed in these methods is a monoclonal antibody.

[0010] A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Figures 1A-1C show the validation of the automated cell motility assay. Fig. 1A shows the temporal (0, 4, 8, 12 and 16hrs) migration of SKOV-3 cells in the presence and absence of controls. siCON = FITC-conjugated control siRNA; siRAC = a sequence-specific siRNA targeting RAC; DMSO = dimethyl sulfoxide; SRC = c-Src family kinase inhibitor, Compound 43; Fig. 1B displays quantification of SKOV-3 migration. The degree of scratch closure ("migration score") was determined by an automated algorithm, and plotted as a function of time. Low migration scores reflect migratory inhibition; Fig. 1C shows western blot demonstrating knock-down of the RAC protein by the RAC-specific siRNA used in Fig. 1A, compared to a control siRNA (CON) and mock transfected cells (LIPO). The same blot was re-probed with anti-actin antibody to demonstrate equal loading.

[0012] Figure 2 schematically illustrates the SKOV-3 siRNA screen and the associated follow-up studies.

[0013] Figures 3A-3B show the identification and validation of pro-migration genes. Fig. 3A demonstrates validation of migratory inhibition by phenotypic and transcriptional analysis. The migratory inhibition elicited by two independent siRNA duplexes targeting four genes, MAP4K4 (NM_004834), CDK7 (NM_001799), DYRK1B (NM_004714) and SERPINB3 (NM_006919), is shown compared to control siRNA and quantified by the automated algorithm (black bars = migration score; white bars = relative

cellular viability). RT-PCR analysis is shown for each transcript, and the relative transcriptional knockdown was quantified using ImageJ software (from the NIH, Bethesda, MD); Fig. 3B shows that four unique siRNA duplexes (three from the primary screen and one additional sequence) have similar effects on the migration of SKOV-3 cells, reducing motility by up to 75%, with a corresponding knockdown of MAP4K4 transcript, ranging from 64 to 94%.

[0014] Figure 4 shows that inhibition of MAP4K4 affects the motility of multiple carcinoma cell lines. Shown are the affects of the two most potent MAP4K4 siRNAs on the motility of SKOV-3, MDA-MB-231 (MDA-231), DU145, ES-2 and A2058 cell lines. A graphical representation of migratory inhibition relative to control siRNA is shown above an RT-PCR analysis of the MAP4K4 transcript in each of the cell lines.

[0015] Figure 5 shows that down-regulation of MAP4K4 decreases SKOV-3 cell invasion. Transiently transfected cells were subjected to Boyden chamber assay of cell invasion through matrigel. The data were collected from 5 individual consecutive fields of view of each of 4 invasion chambers. Representative photomicrographs are shown beneath the graph.

[0016] Figure 6 displays phosphor-Western Blot graphs showing effect of siRNA knockdown of MAP4K4 on phosphorylation level of JNK, p38 or Erk MAP kinases.

[0017] Figure 7 shows inhibition by a JNK inhibitor of the scratch-closing ability of the SKOV-3 cell. Viability of the cells treated with the JNK inhibitor at different concentration, measured as a percentage of viability of control cells treated with DMSO, is also indicated in the figure. The viability data were the average from cells in quadruplicate wells.

[0018] Figures 8A-8B show that inhibition of FGFR1 decreases SKOV-3 cell motility. Fig. 8A depicts the quantitative results of FGFR1 knockdown by 5 independent siRNAs, as compared to control siRNA transfected cells. RT-PCR was performed to assess transcriptional down-regulation, and quantified using ImageJ; Fig. 8B demonstrates the effect of FGFR1 inhibition on SKOV-3 cell invasion through matrigel. The data on migrating cell number was calculated as described for Figure 5.

[0019] Figure 9 shows that inhibition of FGFR1 affects the motility of multiple carcinoma cell lines. Shown in the figure are the effects of the two most potent FGFR1 siRNAs on the motility of SKOV-3, MDA-MB-231 (MDA-231), DU145, ES-2 and A2058

cell lines. A graphical representation of migratory inhibition relative to control siRNA is shown above RT-PCR analysis of the MAP4K4 transcript in each of the cell lines.

[0020] Figures 10A-10B illustrate validation of the effects of FGFR1 knockdown using small molecule inhibitors of the FGFR1 kinase. Fig. 10A shows the quantitative effects of the FGFR1 inhibitor, PD173074, the c-src inhibitor (compound 43) and a pan-kinase inhibitor, staurosporine. The graph shows the quantitative effects of the inhibitors on motility (bars) and viability (lines); white bars = FGFR1 inhibitor; grey bars = SRC inhibitor; black bars = pan-kinase inhibitor; Fig. 10B shows the effects of these three small molecules on SKOV-3 cellular invasion; representative photomicrographs are shown below the graph.

DETAILED DESCRIPTION

I. Overview

[0021] This invention is predicated in part on the discoveries by the present inventors that several genes not previously known to be involved in cell motility promote migration of cells of a few cultured tumor cell lines. As detailed in the Examples below, the present inventors employed a genetic screen designed to identify components of cancer-associated cell migration using a precision engineered 384-well based wound healing device, coupled with automated microscopy. Specifically, the screen employed a large-scale siRNA library which comprises 10,996 siRNAs targeting human genes in a highly motile ovarian carcinoma cell line, SKOV-3. Known modulators of cell migration were identified alongside other novel cell motility stimulators whose involvement in cell motility has not yet been known in the art. In addition, the migration-promoting activities of five genes identified in the primary screen were validated in secondary assays. These five genes, termed "pro-motility genes" or "pro-migration genes" herein, are MAP4K4 (NM_004834), FGFR1 (NM_000604); CDK7 (NM_001799), DYRK1B (NM_004714) and SERPINB3 (NM_006919). Further, two of the genes, FGFR-1 and MAP4k4, were also subject to additional examination. It was found that diminution of both transcripts inhibits the migration of several carcinoma cell lines of differing anatomic origins, and that abrogation by RNA interference or small molecule inhibition of FGFR-1 inhibits cell invasion through

matrigel. It was also found that MAP4K4 signals through c-Jun N terminal kinase, independent of AP-1 activation and downstream transcription.

[0022] In accordance with these discoveries, the invention provides methods for identifying modulators of cell motility, especially agents that inhibit tumor cell migration and tumor metastasis. Employing compounds (e.g., siRNAs or small molecules) which down-regulate the pro-migration molecules identified by the present inventors, the invention further provides methods for inhibiting cell motility or migration in various therapeutic applications. For example, compounds which inhibit tumor cell migration can be employed for preventing tumor metastasis and for treating various forms of cancers in human or non-human subjects. In addition to compounds which inhibit cell motility, compounds which stimulate cellular migration and motility could also be useful in many therapeutic applications. For example, they can be useful for promoting wound healing and for preventing or treating neurodegenerative diseases.

[0023] The following sections provide more guidance for practicing the methods of the invention, and for making and using the compositions of the invention.

II. Definitions

[0024] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: *Oxford Dictionary of Biochemistry and Molecular Biology*, Smith et al. (eds.), Oxford University Press (revised ed., 2000); *Dictionary of Microbiology and Molecular Biology*, Singleton et al. (Eds.), John Wiley & Sons (3rd ed., 2002); and *A Dictionary of Biology (Oxford Paperback Reference)*, Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). In addition, the following definitions are provided to assist the reader in the practice of the invention.

[0025] The term "agent" or "test agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" can be used interchangeably.

[0026] The term "analog" is used herein to refer to a molecule that structurally resembles a reference molecule but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the reference molecule, an analog would be expected, by one skilled in the art, to exhibit the same, similar, or improved utility. Synthesis and screening of analogs, to identify variants of known compounds having improved traits (such as higher binding affinity for a target molecule) is an approach that is well known in pharmaceutical chemistry.

[0027] As used herein, "contacting" has its normal meaning and refers to combining two or more molecules (e.g., a test agent and a polypeptide) or combining molecules and cells (e.g., a test agent and a cell). Contacting can occur in vitro, e.g., combining two or more agents or combining a test agent and a cell or a cell lysate in a test tube or other container. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

[0028] A "heterologous sequence" or a "heterologous nucleic acid," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that, although being endogenous to the particular host cell, has been modified. Modification of the heterologous sequence can occur, e.g., by treating the DNA with a restriction enzyme to generate a DNA fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous nucleic acid.

[0029] The term "homologous" when referring to proteins and/or protein sequences indicates that they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity is routinely

used to establish homology. Higher levels of sequence similarity, e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more can also be used to establish homology.

[0030] A "host cell" refers to a prokaryotic or eukaryotic cell into which a heterologous polynucleotide can be introduced. The polynucleotide can be introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and/or the like.

[0031] The term "pro-motility molecule" or "pro-migration molecule" encompasses "pro-migration genes" and "pro-migration proteins." It refers to genes or their encoded polypeptides identified by the present inventors that positively regulate cell motility and migration as illustrated in the Examples below. Unless otherwise noted, this term specifically refer to five genes and their encoded polypeptides. These five molecules are MAP4K4, FGFR1, CDK7, DYRK1B and SERPINB3. Accession numbers for a representative polynucleotide sequence encoding each of these molecules are MAP4K4 (NM_004834; SEQ ID NO: 1), FGFR1 (NM_000604; SEQ ID NO: 7); CDK7 (NM_001799; SEQ ID NO: 25), DYRK1B (NM_004714; SEQ ID NO: 29) and SERPINB3 (NM_006919; SEQ ID NO: 35).

[0032] The term "sequence identity" in the context of two nucleic acid sequences or amino acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. A "comparison window" refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are aligned optimally. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482; by the alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443; by the search for similarity method of Pearson and Lipman (1988) Proc. Nat. Acad. Sci U.S.A. 85:2444; by computerized implementations of these algorithms (including, but not limited to CLUSTAL in the PC/Gene program by Intelligentics, Mountain View, CA; and GAP, BESTFIT, BLAST, FASTA, or TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., U.S.A.). The CLUSTAL program is well described by Higgins and Sharp (1988) Gene 73:237-244;

Higgins and Sharp (1989) CABIOS 5:151-153; Corpet et al. (1988) Nucleic Acids Res. 16:10881-10890; Huang et al (1992) Computer Applications in the Biosciences 8:155-165; and Pearson et al. (1994) Methods in Molecular Biology 24:307-331. Alignment is also often performed by inspection and manual alignment. In one class of embodiments, the polypeptides herein are at least 70%, generally at least 75%, optionally at least 80%, 85%, 90%, 95% or 99% or more identical to a reference polypeptide, e.g., a pro-motility molecule described herein, e.g., as measured by BLASTP (or CLUSTAL, or any other available alignment software) using default parameters. Similarly, nucleic acids can also be described with reference to a starting nucleic acid, e.g., they can be 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more identical to a reference nucleic acid, e.g., as measured by BLASTN (or CLUSTAL, or any other available alignment software) using default parameters.

[0033] A "substantially identical" nucleic acid or amino acid sequence refers to a nucleic acid or amino acid sequence which comprises a sequence that has at least 90% sequence identity to a reference sequence using the programs described above (preferably BLAST) using standard parameters. The sequence identity is preferably at least 95%, more preferably at least 98%, and most preferably at least 99%. For example, the BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)). Percentage of sequence identity is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over

at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

[0034] A Boyden chamber assay is a system well known in the art for measuring the ability of cells to pass through an extracellular matrix of coated membranes, such as Matrigel (see, e.g., Kramer et al., Cancer Res 46:1980–1989, 1986; and Albini et al., Cancer Res 47:3239–3245, 1987). Such assay system is commercially available, e.g., the Matrigel transwell chamber assay system from Becton Dickinson & Co. (Franklin Lakes, NJ). The matrigel Boyden chamber assays have been routinely employed by the skilled artisans in the art to study cell motility in general and tumor cell metastasis in particular. See, e.g., U.S. Pat. No. 5,935,850; Kunda et al., J. Cell Biol. 130: 725, 1995; and Parish et al., Int. J. Cancer 52:378-383, 1992.

[0035] The term "metastasis" as used herein refers to the invasion and migration of tumor cells away from the primary tumor site. The invasion and metastasis of cancer is a complex process which involves changes in cell adhesion properties which allow a transformed cell to invade and migrate through the extracellular matrix (ECM) and acquire anchorage-independent growth properties. Metastatic disease occurs when the disseminated foci of tumor cells seed a tissue which supports their growth and propagation, and this secondary spread of tumor cells is responsible for the morbidity and mortality associated with the majority of cancers.

[0036] The term "modulate" with respect to a biological activity of a reference protein (e.g., a pro-migration molecule disclosed herein) or its fragment refers to a change in the expression level or other biological activities of the protein. For example, modulation may cause an increase or a decrease in expression level of the reference protein, enzymatic modification (e.g., phosphorylation) of the protein, binding characteristics (e.g., binding to another molecule), or any other biological (e.g., enzymatic), functional, or immunological properties of the reference protein. The change in activity can arise from, for example, an increase or decrease in expression of one or more genes that encode the reference protein, the stability of an mRNA that encodes the protein, translation efficiency, or from a change in other biological activities of the reference protein. The change can also be due to the activity of another molecule that modulates the reference protein (e.g., a kinase which phosphorylates the reference protein).

[0037] Modulation of a reference protein can be up-regulation (i.e., activation or stimulation) or down-regulation (i.e. inhibition or suppression). The mode of action of a modulator of the reference protein can be direct, e.g., through binding to the protein or to genes encoding the protein, or indirect, e.g., through binding to and/or modifying (e.g., enzymatically) another molecule which otherwise modulates the reference protein.

[0038] The term "subject" includes mammals, especially humans. It also encompasses other non-human animals such as cows, horses, sheep, pigs, cats, dogs, mice, rats, rabbits, guinea pigs, monkeys.

[0039] A "variant" of a reference molecule refers to a molecule substantially similar in structure and biological activity to either the entire reference molecule, or to a fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not identical.

III. Screening for Novel Modulators of Cell Motility

A. Screen scheme

[0040] The pro-migration molecules identified by the present inventors provide novel targets to screen for modulators of cell motility, e.g., small molecule inhibitors. They are particularly suitable for screening test compounds to identify agents that inhibit tumor metastasis. Various biochemical and molecular biology techniques or assays well known in the art can be employed to practice the present invention. Such techniques are described in, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, N.Y., Second (1989) and Third (2000) Editions; and Brent et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (ringbou ed., 2003).

[0041] To identify novel modulators of cell motility, test compounds are typically first assayed for their ability to modulate (e.g., down-regulate) expression or other biological activities of a pro-migration molecule described herein ("the first assay step"). Modulating compounds thus identified are then subject to further screening for ability to modulate (e.g., inhibit) motility or migration of a cell (e.g., a tumor cell), typically in the presence of the pro-migration molecule ("the second testing step"). Depending on the pro-migration molecule employed in the method, modulation of different biological activities of the pro-

migration molecule can be assayed in the first step. As detailed below, test agents can be assayed for binding to the pro-migration molecule. The test agents can be assayed for activity to modulate expression of the pro-migration molecule, e.g., transcription or translation. The test agents can also be assayed for activities in modulating cellular level or stability of the pro-migration molecule, e.g., post-translational modification or proteolysis. If the pro-migration molecule has a known biological or enzymatic function (e.g., kinase activity), the biological activity monitored in the first screening step can be the specific biochemical or enzymatic activity of the pro-migration molecule.

[0042] In both the first assaying step and the second testing step, either an intact pro-migration molecule, or a fragment thereof, may be employed. Polynucleotide and amino acid sequences of the five pro-migration molecules disclosed herein are all known in the art. For example, accession numbers of 3 different transcript variants of human MAP4K4 are NM_004834 (SEQ ID NO:1); NM_145686 (SEQ ID NO:3); and NM_145687(SEQ IUD NO:5). The respective amino acid sequences encoded by the polynucleotide sequences are NP_004825 (SEQ ID NO:2); NP_663719 (SEQ ID NO:4); and NP_663720 (SEQ ID NO:6). Human FGFR1 polynucleotide sequences are shown in, e.g., accession numbers NM_000604 (SEQ ID NO:7); NM_015850 (SEQ ID NO:9); NM_023105 (SEQ ID NO:11); NM_023106 (SEQ ID NO:13); NM_023107 (SEQ ID NO:15); NM_023108 (SEQ ID NO:17); NM_023109 (SEQ ID NO:19); NM_023110 (SEQ ID NO:21); and NM_023111 (SEQ ID NO:23). The corresponding amino acid sequences of FGFR1 isoforms are shown in NP_056934 (SEQ ID NO:8); NP_000595 (SEQ ID NO:10); NP_075593 (SEQ ID NO:12); NP_075594 (SEQ ID NO:14); NP_075595 (SEQ ID NO:16); NP_075596 (SEQ ID NO:18); NP_075597 (SEQ ID NO:20); NP_075598 (SEQ ID NO:22); and NP_075599 (SEQ ID NO:24). Examples of human CDK7 gene accession numbers are NM_001799 (SEQ ID NO:25) and AK130859 (SEQ ID NO:27). The encoded amino acid sequences are respectively shown in accession numbers NP_001790 (SEQ ID NO:26) and AAM77799 (SEQ ID NO:28). Polynucleotide sequences encoding human DYRK1B transcript variants are shown in, e.g., accession numbers NM_004714 (SEQ ID NO:29); NM_006483 (SEQ ID NO:31); and NM_006484 (SEQ ID NO:33). These sequences respectively encode human DYRK1B isoform polypeptide sequences with accession numbers of NP_004705 (SEQ ID NO:30); NP_006474 (SEQ ID NO:32); and NP_006475 (SEQ ID NO:34). Human SERPINB3 is encoded by, e.g., polynucleotide sequence with accession number

NM_006919 (SEQ ID NO:35) and the corresponding amino acid sequence with accession number NP_008850 (SEQ ID NO:36). Any of these pro-migration molecule polynucleotide sequences or polypeptide sequences can be employed to screen test compounds for modulators of the pro-migration molecules.

[0043] In addition to the human polynucleotide or polypeptide sequences, non-human homologs of any of the pro-migration molecules described herein can also be employed to practice the screening methods of the invention. For example, mouse MAP4K4 (NM_008696), FGFR1 (NM_201230), CDK7 (NM_009874), and DYRK1B are all well known and characterized in the art. Homologs of these pro-migration molecules in many other non-human species have been similarly described in the art. Any of these non-human homologs can be used to screen test compounds for modulators. Further, polynucleotide sequences or polypeptide sequences that are substantially identical to the sequence of any of the pro-migration genes or polypeptides disclosed herein can also be employed in the screening methods of the invention. Moreover, analogs or functional derivatives of any of the pro-migration molecules described herein can also be used in the screening. The fragments or analogs that can be employed in these assays usually retain one or more of the biological activities of the pro-migration molecule (e.g., kinase activity if the pro-migration molecule employed in the first assaying step is a kinase). Fusion proteins containing such fragments or analogs can also be used for the screening of test agents. Functional derivatives of a pro-migration molecule usually have amino acid deletions and/or insertions and/or substitutions while maintaining one or more of the bioactivities and therefore can also be used in practicing the screening methods of the present invention. A functional derivative of a given pro-migration molecule can be prepared from a pro-migration molecule by proteolytic cleavage followed by conventional purification procedures known to those skilled in the art. Alternatively, the functional derivative can be produced by recombinant DNA technology by expressing only fragments of a pro-migration molecule that retain one or more of their bioactivities.

B. Test compounds

[0044] Test compounds or agents that can be screened with methods of the present invention include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds,

benzodiazepines, oligomeric N-substituted glycines, oligocarbamates, polypeptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Some test agents are synthetic molecules, and others natural molecules. In some applications, the test agents are nucleic acids. Nucleic acid test agents can be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. In some embodiments, the screening methods are directed to screening inhibitory polynucleotides for agents that specifically down-regulate expression or cellular level of a pro-migration molecule. Such inhibitory polynucleotides include, e.g., short interfering RNAs (siRNAs), microRNAs (miRNAs), synthetic hairpin RNAs (shRNAs), anti-sense nucleic acids, and complementary DNAs (cDNAs).

[0045] Test agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. Combinatorial libraries can be produced for many types of compound that can be synthesized in a step-by-step fashion. Large combinatorial libraries of compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642. Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980). Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts can be obtained from commercial sources or collected in the field. Known pharmacological agents can be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs. Combinatorial libraries of peptides or other compounds can be fully randomized, with no sequence preferences or constants at any position. Alternatively, the library can be biased, i.e., some positions within the sequence are either held constant, or are selected from a limited number of possibilities. Libraries of test agents to be screened with the claimed methods can also be generated based on structural studies of the pro-migration molecules discussed above or their fragments. Such structural studies allow the identification of test agents that are more likely to bind to the pro-migration molecules.

[0046] Test agents also include antibodies that specifically bind to a pro-migration molecule described herein. Some of the antibody agents antagonize (i.e., suppress) a biochemical activity (e.g., kinase) of the pro-migration molecule employed in the screening methods. Some other antibody agents agonize (i.e., stimulate) the biochemical activity of the pro-migration molecule. The antibodies can be monoclonal or polyclonal. Such

antibodies can be generated using methods well known in the art. For example, the production of non-human monoclonal antibodies, e.g., murine or rat, can be accomplished by, for example, immunizing the animal with a pro-migration molecule or its antigenic fragment (see, e.g., Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Press, 3rd ed., 2000). Such an immunogen can be obtained from a natural source, by peptides synthesis or by recombinant expression. In addition, humanized forms of mouse antibodies can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. See Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989) and WO 90/07861. Human antibodies can be produced using phage-display methods. See, e.g., Dower et al., WO 91/17271; McCafferty et al., WO 92/01047.

[0047] In some preferred methods, the test agents are small molecule organic compounds, e.g., chemical compounds with a molecular weight of not more than about 500 or 1,000. Preferably, high throughput assays are adapted and used to screen for such small molecules. Combinatorial libraries of small molecule test agents can be readily employed to screen for small molecule compound modulators of cell motility. A number of assays are available for such screening, e.g., as described in Schultz (1998) Bioorg Med Chem Lett 8:2409-2414; Weller (1997) Mol Divers. 3:61-70; Fernandes (1998) Curr Opin Chem Biol 2:597-603; and Sittampalam (1997) Curr Opin Chem Biol 1:384-91.

C. Screen for compounds that modulate pro-migration molecules

[0048] Test agents can be screened for their ability to either up-regulate or down-regulate expression level or other biological activities of the pro-migration molecule in the first assay step. In some preferred methods, test compounds are first screened to identify modulating compounds which modulate expression or cellular level of one of the pro-migration molecules described herein. Preferably, cell-based assays are used to identify agents that modify expression of genes encoding the pro-migration molecules. For example, each test compound can be contacted with a cell (e.g., a tumor cell). Expression of at least one of the pro-migration molecules (e.g., MAP4K4) is then measured in cells that have been treated with the compounds. A modulating compound is identified if the level of expression of the gene in the cells that have been treated is up-regulated or down-regulated relative to the level of expression of the same gene in cells that have not been treated with the

compound. The level of expression of the gene can be detected by, for example, measuring the level of mRNA transcripts corresponding to or proteins encoded by the pro-migration molecule genes. Standard detection techniques well known in the art for detecting RNA, DNA, proteins and peptides can readily be applied to detect expression levels of the pro-motility genes. Such techniques may include detection with nucleotide probes or may comprise detection of the protein by, for example, antibodies or their equivalent. Types of probe include cDNA, riboprobes, synthetic oligonucleotides and genomic probes. Such techniques are described in, e.g., Sambrook et al., *supra*; and Brent et al., *supra*. In some embodiments, endogenous levels of a pro-migration molecule can be directly monitored in cells normally expressing the pro-migration molecule (e.g., a tumor cell). In some embodiments, expression or cellular level of a pro-migration molecule can be examined in an expression system using cloned cDNA or genomic sequence encoding the pro-migration molecule.

[0049] More preferably, modulation of expression of a pro-migration molecule is examined by monitoring expression of a reporter gene under the control of a transcription regulatory element of a pro-migration molecule. In these methods, modulation of expression of a pro-migration molecule gene is examined in a cell-based system by transient or stable transfection of an expression vector into cultured cell lines. Assay vectors bearing transcription regulatory sequences (e.g., promoter) of a pro-migration molecule gene operably linked to reporter genes can be transfected into any mammalian host cell line for assays of promoter activity. As noted above, genes encoding the pro-migration molecules have been characterized in the art, and their transcription regulatory sequences have all been delineated. See, e.g., Yao et al., J. Biol. Chem. 274:2118-25, 1999; Isacchi et al., Nucleic Acids Res. 18:1906, 1990; Dionne et al., EMBO J. 9:2685-92, 1990; Levedakou et al., Oncogene 9:1977-88, 1994; Wu et al., Oncogene 9:2089-96, 1994; Leder et al., Biochem. Biophys. Res. Commun. 254:474-9, 1999; and Schneider et al., Proc. Natl. Acad. Sci. U.S.A. 92:3147-51, 1995. Constructs containing a transcription regulatory element of a pro-migration molecule gene that is operably linked to a reporter gene can be prepared using only routinely practiced techniques and methods of molecular biology (see, e.g., Sambrook et al. and Brent et al., *supra*).

[0050] General methods of cell culture, transfection, and reporter gene assay have been described in the art, e.g., Brent, *supra*; and Transfection Guide, Promega Corporation,

Madison, WI (1998). Any readily transfectable mammalian cell line may be used to assay A pro-migration molecule promoter function or to express A pro-migration molecule, e.g., CHO, COS, HCT116, HEK 293, MCF-7, and HepG2 cell lines. When inserted into the appropriate host cell, the transcription regulatory elements in the expression vector induces transcription of the reporter gene by host RNA polymerases. Reporter genes typically encode polypeptides with an easily assayable enzymatic activity that is naturally absent from the host cell. Typical reporter polypeptides for eukaryotic promoters include, e.g., chloramphenicol acetyltransferase (CAT), firefly or Renilla luciferase, beta-galactosidase, beta-glucuronidase, alkaline phosphatase, and green fluorescent protein (GFP).

[0051] In some methods, binding of test agents to a pro-migration molecule is determined in the first screening step. Binding of test agents to a pro-migration molecule can be assayed by a number of methods including e.g., labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.), and the like. See, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168; and also Bevan et al., Trends in Biotechnology 13:115-122, 1995; Ecker et al., Bio/Technology 13:351-360, 1995; and Hodgson, Bio/Technology 10:973-980, 1992. The test agent can be identified by detecting a direct binding to the pro-migration molecule, e.g., co-immunoprecipitation with the pro-migration molecule by an antibody directed to the pro-migration molecule. The test agent can also be identified by detecting a signal that indicates that the agent binds to the pro-migration molecule, e.g., fluorescence quenching or FRET. In some embodiments, combinatory library based screening methods are used. In these assay formats, binding of a pro-migration molecule or fragment thereof to a test agent can be determined by, e.g., changes in fluorescence of either the pro-migration molecule or the test agents, or both. Fluorescence may be intrinsic or conferred by labeling either component with a fluorophor.

[0052] Binding of a test agent to a pro-migration molecule provides an indication that the agent can be a modulator of the pro-migration molecule. It also suggests that the agent may modulate cell motility through, e.g., binding to and modulating the pro-migration molecule. Thus, a test agent that binds to a pro-migration molecule can be further tested for ability to modulate motility of a cell, e.g., a tumor cell (i.e., in the second testing step outlined above). Alternatively, a test agent that binds to a pro-migration molecule can be further examined to determine whether it modulates another biological activity (e.g., an

enzymatic activity) of the pro-migration molecule. The existence, nature, and extent of such modulation can be tested by an activity assay. Such an activity assay can confirm that the test agent binding to the pro-migration molecule indeed modulates the pro-migration molecule. More often, such activity assays can be used independently to identify test agents that modulate activities of a pro-migration molecule (i.e., without first assaying their ability to bind to the pro-migration molecule). In general, the methods involve adding a test agent to a sample containing a pro-migration molecule in the presence or absence of other molecules or reagents which are necessary to test a biological activity of the pro-migration molecule (e.g., enzymatic activity if the pro-migration molecule is an enzyme), and determining an alteration in the biological activity of the pro-migration molecule.

[0053] In some embodiments, test compounds are first screened for ability to modulate an enzymatic activity or another biochemical activity of the pro-migration molecule employed in the screen. In some of these embodiments, test compounds are first examined for ability to modulate the kinase activity of the pro-migration molecule (e.g., MAP4K4, FGFR1, DYRK1B, or CDK7). The substrate to be used in the screening can be a molecule known to be enzymatically modified by the enzyme (e.g., a kinase), or a molecule that can be easily identified from candidate substrates for a given class of enzymes. For example, many kinase substrates are available in the art. See, e.g., www.emdbiosciences.com; and www.proteinkinase.de. In addition, a suitable substrate of a kinase can be screened for in high throughput format. For example, substrates of a kinase can be identified using the Kinase-Glo® luminescent kinase assay (Promega) or other kinase substrate screening kits (e.g., developed by Cell Signaling Technology, Beverly, Massachusetts).

[0054] Specific assays for examining the enzymatic or biochemical activities of the pro-migration molecules disclosed herein are also known in the art. These assays can all be employed to practice the screening methods of the invention. For example, compounds modulating (e.g., down-regulate) MAP4K4 kinase activity can be identified by, e.g., monitoring phosphorylation of JNK using an immune complex kinase assay. Such assays are described in, e.g., Su et al., EMBO J. 16:1279–1290, 1997; Yao et al., J Biol Chem, 274:2118-25, 1999; and Machida et al., J Biol Chem. 279:15711-4, 2004. Similarly, compounds modulating the tyrosine kinase activity of FGFR1 can be identified by monitoring their effect on FGFR1 phosphorylation of a substrate (e.g., SNT1) in vitro. Such

assays have been described in the art, e.g., Jin et al., Shi Yan Sheng Wu Xue Bao. 35:184-90, 2002. Alternatively, FGFR1 tyrosine kinase activity may also be examined with in vivo assays as described in Mohammadi et al., Science 276:955-60, 1997; and Chen et al., Proc Natl Acad Sci USA. 101:14479-84, 2004. Kinase activity of CDK7 can be similarly monitored using assays known in the art (e.g., William et al., Arch Biochem Biophys. 314:99-106, 1994; and Rosales et al., Cell Physiol Biochem. 13:285-96, 2003). For DYRK1B, test compounds can be screened for ability to modulate (e.g., inhibit) the serine/threonine kinase activity of DYRK1B. Kinase assays for examining DYRK1B kinase activities are known in the art, e.g., using MBP or recombinant GST-HNF1 as substrate described in Lim et al., J. Biol. Chem. 277:25040-6, 2002. Alternatively, compounds modulating DYRK1B can be identified by examining their effect on DYRK1B binding to RanBPM in a yeast two-hybrid assay as described in, e.g., Zou et al., J. Biol. Chem. 278:49573-81, 2003. Finally, SERPINB3 inhibits cysteine proteinases such as cathepsin S, K, L, and papain. Test compounds can be screened for ability to modulate this biochemical activity of SERPINB3. This can be performed using SERPINB3 (e.g., GST-fused SERPINB3 polypeptide) and any of these cysteine proteinase (e.g., papain or cathepsin L) in enzymatic assays known in the art, e.g., as described in Masumoto et al., J Biol Chem. 278:45296-304, 2003.

D. Screen for agents that modulate motility of a target cell

[0055] Once test agents that modulate the pro-migration molecule are identified, they are typically further tested for ability to modulate (e.g., inhibit) motility or migratory activity of a cell (e.g., a tumor cell). This further testing step is often needed to confirm that their modulatory effect on the pro-migration molecule would indeed lead to modulation of motility or migration of a cell. Typically, this screening step is performed in the presence of the pro-migration molecule on which the modulating agent acts. Preferably, this screening step is performed in vivo using cells that endogenously express the pro-migration molecule. As a control, effect of the modulating agents on the motility of a cell that does not express the pro-migration molecule can also be examined.

[0056] Some of the screening methods of the invention are directed to identifying agents that inhibit motility of tumor cells. In these methods, compounds which modulate (e.g., down-regulate) a pro-migration molecule are further tested for ability to inhibit

motility or migration of a tumor cell. For example, compounds which down-regulate expression level of a pro-migration molecule can be further tested in order to confirm that such modulation can result in suppressed or reduced proliferation of a cell harboring the pro-migration molecule (e.g., a tumor cell such as SKOV-3 cell). Similarly, a test agent which inhibits an enzymatic activity of a pro-migration molecule is usually further examined for ability to modulate motility of a cell. As a control, the modulating agents can be further screened for lack of significant effect on the motility or migration of a normal non-tumor control cell. This additional step could ensure that the agents identified with the screening methods of the invention are specific for tumor cells.

[0057] Many cell lines can be used in this screening step of the methods of the present invention. These include the tumor cell lines described in the Examples herein, e.g., SKOV-3 (ovarian), ES-2 (ovarian), MDA-MB-231 (breast), A2058 (melanoma) and DU145 (prostate) cell lines. In addition, many other tumor or non-tumor cell lines can also be used to screen for compounds that specifically inhibit tumor cell migration and tumor metastasis. Examples of tumor cell lines include human glioblastoma cell line U373 (ATCC); melanoma cell line SK-MEL-2; ovarian cancer cell line OVCAR-4; leukemia lines HL60 and RPMI-8226; lung cancer cell lines NCI-H322M and NCI-H460; colon lines COLO 205 and HCC-2998; brain tumor lines SF-539 and SNB-75; and breast cancer lines MCF7 and HS 578T (Monks et al., Anticancer Drug Des 12: 533-541, 1997; and Boyd and Paull, Drug Dev Res 34: 91-109, 1995). Non-tumor cell lines include, e.g., human embryonic kidney cell line (HEK293); human umbilical vein endothelial cell line (HUVEC); epithelial cell line MCF-10A (Soule et al., Cancer Res. 50: 6075-6086, 1990); colon cell line (CCD-18Co) and ovarian cell line (NOV-31 (Hirasawa et al., Cancer Research 62, 1696-1701, March 15, 2002). In addition, ATCC provides many tumor/normal cell line pairs that are used to elucidate the underlying causes of cancers. They can also be employed to screen modulating agents of the present invention to identify selective anti-tumor agents. These tumor/normal cell line pairs include non-small cell lung cancer cell line (ATCC No. CCL-256) and normal peripheral blood cell line ATCC No. CCL-256.1; adenocarcinoma cell line ATCC No. CRL-5868 and normal peripheral blood cell line ATCC No. CRL-5957; malignant melanoma cell line ATCC No. CRL-1974 and normal cell line ATCC No. CRL-1980; basal cell carcinoma cell line ATCC No. CRL-7762 and normal skin cell line ATCC No. CRL-7761; colorectal adenocarcinoma cell line ATCC No. CCL-228 and normal lymph node cell line ATCC No.

CCL-227; and giant cell sarcoma cell line ATCC No. CRL-7554 and normal bond cell line ATCC No. CRL-7553. Any of these cell line pairs can be used to screen the modulating agents for compounds that selectively inhibit migratory activity of tumor cells.

[0058] There are many available methods for measuring motility or migratory activity of a cell. For example, motility of cells can be assessed with a cell scratching device described in the Examples below. Coupled with automated high-speed microscopy, this system examines motility of cells by assaying a cell's ability to close a uniform wound ("scratch") in an in vitro culture of the cell. The ability to close a scratch by migration in a tissue culture plate is a well-established measure of a cell's migratory capacity. In addition to the cell scratching device, modulation of cell motility by test compounds can be examined with a number of methods known to the skilled artisans. For example, the compounds can be examined for ability to prevent cells from crossing a barrier. One example of such barriers is extracellular matrix coated membranes, such as Matrigel. One commonly used assay is the Boyden chamber invasion assay which measures the ability of the cell to pass through a matrix of basement membrane (BM) (see, e.g., Kramer et al., Cancer Res 46:1980–1989, 1986; and Albini et al., Cancer Res 47:3239–3245, 1987). A similar assay for examining tumor cell invasion in a Matrigel invasion assay system is described in detail by in Parish et al., Int. J. Cancer 52:378-383, 1992. Such assay systems are commercially available, e.g., the Matrigel transwell chamber assay system from Becton Dickinson & Co. (Franklin Lakes, NJ) as described in the Examples below.

[0059] Many other methods for examining cell motility known in the art (e.g., U.S. Pat. No. 5,935,850; and Kunda et al., J. Cell Biol. 130, 725, 1995) can also be used in the practice of the screening methods of the present invention. Kunda et al. employs multiwell chemotaxis chambers overlaid with porosity polycarbonate filters. Migration of cells placed in the upper well toward the lower well was monitored by examining cells that passed through the filters. Cells that passed through the filters and adhered to the lower side were fixed in formalin, and counted under light microscopy. Additional assays for measuring motility of various tumor or non-tumor cells have also been described in the art. See, e.g., In Vitro. Stenn, 16:357-60, 1980; Benestad et al., Cell Tissue Kinet. 20:109-19, 1987; Ratner et al., J Immunol. 140:583-8, 1988; Gorrill et al., Fertil Steril. 55:345-54, 1991; Takaishi et al., Methods Enzymol. 256:336-47, 1995; Scott et al., Anal Biochem. 287:343-4, 2000; Gildea et al., Biotechniques. 29:81-6, 2000; and Pellegrino et al., Differentiation.

71:542-8, 2003. Any of these assays can be readily employed or modified in the present invention to assess effect of compounds on motility or migratory activity of a given cell of interest.

IV. Therapeutic Applications

[0060] The present invention provides novel methods and compositions for modulating cell motility and migratory activity. The methods and compositions of the present invention find therapeutic applications in treating various clinical conditions or disease states by stimulating desired or inhibiting undesired cellular migration. Modulation of cell migratory activities is also useful for preventing or modulating the development of such diseases or disorders in a subject suspected of being, or known to be, prone to such diseases or disorders. Some of the methods are directed to treat various diseases and disorders using therapeutic agents which inhibit cellular migration by down-regulating at least one of the pro-migration molecules. These agents include compounds that can be identified in accordance with the screening methods described above, e.g., small molecule compounds or antibodies (e.g., antagonist antibodies). They also include nucleic acid agents which down-regulate the pro-motility molecules such as antisense nucleotides, ribozymes, double-stranded RNAs, and double-stranded small interference RNAs (siRNAs). As noted above, these agents can be employed to treat or prevent metastatic tumors. In addition, such compounds are also useful for treating and/or preventing disorders associated with inflammation in a subject. Inflammatory disorders and ischemic diseases are characterized by inflammation associated with neutrophil migration to local tissue regions that have been damaged or have otherwise induced neutrophil migration and activation. By specifically inhibiting motility of certain immune or hematopoetic cells, these compounds can be used for preventing inflammation that is associated with immune cell migration and for treating preventing inflammatory disorders and ischemic diseases.

[0061] Some of the therapeutic applications of the invention are directed to inhibiting metastasis of various types of tumors. The tumor types include, e.g., biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer;

sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas. In some preferred embodiments, as exemplified in the Examples below, siRNAs corresponding to at least one of the disclosed pro-motility molecule genes are utilized to interfere with expression of at least one of the genes. Interference with the function and expression of endogenous genes by double-stranded RNA has been shown in various organisms such as C. elegans as described, e.g., in Fire et al., Nature, Vol. 391, pp. 806-811 (1998); drosophilia as described, e.g., in Kennerdell et al., Cell, Vol. 95, No. 7, pp. 1017-1026 (1998); and mouse embryos as described, e.g., in Wianni et al., Nat. Cell Biol., Vol. 2, No. 2, pp. 70-75 (2000). Such double-stranded RNA can be synthesized by in vitro transcription of single-stranded RNA read from both directions of a template and in vitro annealing of sense and antisense RNA strands. Double-stranded RNA can also be synthesized from a cDNA vector construct in which the gene of interest is cloned in opposing orientations separated by an inverted repeat. Following cell transfection, the RNA is transcribed and the complementary strands reanneal. Double-stranded RNA corresponding to the pro-motility genes can be introduced into a cell (e.g., a tumor cell) by transfection of an appropriate construct.

[0062] Compounds which inhibit cellular migration can be used in a subject to treat or prevent cancer metastasis or inflammatory disorders alone or in combination with the administration of other therapeutic compounds for the treatment or prevention of these disorders. There are many anti-cancer and anti-inflammation drugs known in the art, e.g., as described in, e.g., *Cancer Therapeutics: Experimental and Clinical Agents*, Teicher (Ed.), Humana Press (1st ed., 1997); and *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, Hardman et al. (Eds.), McGraw-Hill Professional (10th ed., 2001).

[0063] Compounds which up-regulate the pro-migration molecule and thereby stimulate cellular migration also find therapeutic applications. They can be used to aid tissue regeneration, e.g., in would healing and neuroregeneration. The wound healing process involves a complex cascade of biochemical and cellular events to restore tissue integrity following an injury. Agents which stimulate cell motility are useful for promoting wound healing by promoting cellular migration and thus remodeling. For example, these compounds can be employed for treating a wound to the dermis or epidermis, e.g., a burn or tissue transplant, injury to the skin. For tissue regeneration, these compounds can be useful in the treatment of subjects having or at risk of developing neurodegenerative diseases.

Neurodegenerative disorders are typically associated with a progressive loss of neurons in the peripheral nervous system or in the central nervous system. Examples of neurodegenerative disorders include chronic neurodegenerative diseases such as familial and sporadic amyotrophic lateral sclerosis (FALS and ALS, respectively), familial and sporadic Parkinson's disease, Huntington's disease, familial and sporadic Alzheimer's disease, multiple sclerosis, and etc. Symptoms of these disorders may be treated or alleviated by administering to a subject a compound which promotes neuron cell motility. Other therapeutic compounds for the treatment or prevention of these disorders can be employed concurrently in these therapeutic applications of the present invention.

[0064] The cell motility-modulating compounds of the present invention can be directly administered under sterile conditions to the subject to be treated. They can be administered alone or as the active ingredient of a pharmaceutical composition. Pharmaceutical compositions of the present invention typically comprise at least one active ingredient together with one or more acceptable carriers thereof. Pharmaceutically carriers enhance or stabilize the composition, or to facilitate preparation of the composition. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (*e.g.*, nucleic acid, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. They should also be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the subject. This carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral, sublingual, rectal, nasal, or parenteral. For example, the antitumor compound can be complexed with carrier proteins such as ovalbumin or serum albumin prior to their administration in order to enhance stability or pharmacological properties.

[0065] There are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., Remington: The Science and Practice of Pharmacy, Mack Publishing Co., 20th ed., 2000). Without limitation, they include syrup, water, isotonic saline solution, 5% dextrose in water or buffered sodium or ammonium acetate solution, oils, glycerin, alcohols, flavoring agents, preservatives, coloring agents starches, sugars, diluents, granulating agents, lubricants, and binders, among others. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax.

[0066] The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100% by weight. Therapeutic formulations are prepared by any methods well known in the art of pharmacy. See, e.g., Gilman et al., eds., Goodman and Gilman's: The Pharmacological Bases of Therapeutics , 8th ed., Pergamon Press, 1990; Remington: The Science and Practice of Pharmacy, Mack Publishing Co., 20th ed., 2000; Avis et al., eds., Pharmaceutical Dosage Forms: Parenteral Medications, published by Marcel Dekker, Inc., N.Y., 1993; Lieberman et al., eds., Pharmaceutical Dosage Forms: Tablets, published by Marcel Dekker, Inc., N.Y., 1990; and Lieberman et al., eds., Pharmaceutical Dosage Forms: Disperse Systems, published by Marcel Dekker, Inc., N.Y., 1990.

[0067] The therapeutic formulations can be delivered by any effective means that can be used for treatment. Depending on the specific antitumor agent to be administered, the suitable means include oral, rectal, vaginal, nasal, pulmonary administration, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) infusion into the bloodstream. For parenteral administration, antitumor agents of the present invention may be formulated in a variety of ways. Aqueous solutions of the modulators may be encapsulated in polymeric beads, liposomes, nanoparticles or other injectable depot formulations known to those of skill in the art. Additionally, the compounds of the present invention may also be administered encapsulated in liposomes. The compositions, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surfactants such a diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature.

[0068] The therapeutic formulations can conveniently be presented in unit dosage form and administered in a suitable therapeutic dose. A suitable therapeutic dose can be determined by any of the well known methods such as clinical studies on mammalian species to determine maximum tolerable dose and on normal human subjects to determine safe dosage. Except under certain circumstances when higher dosages may be required, the preferred dosage of an antitumor agent of the present invention usually lies within the range of from about 0.001 to about 1000 mg, more usually from about 0.01 to about 500 mg per

day. The preferred dosage and mode of administration of an antitumor agent can vary for different subjects, depending upon factors that can be individually reviewed by the treating physician, such as the condition or conditions to be treated, the choice of composition to be administered, including the particular antitumor agent, the age, weight, and response of the individual subject, the severity of the subject's symptoms, and the chosen route of administration. As a general rule, the quantity of an antitumor agent administered is the smallest dosage which effectively and reliably prevents or minimizes the conditions of the subjects. Therefore, the above dosage ranges are intended to provide general guidance and support for the teachings herein, but are not intended to limit the scope of the invention.

EXAMPLES

[0069] The following examples are provided to illustrate, but not to limit the present invention.

Example 1. Materials and Methods

[0070] Cell culture: Human carcinoma cell lines were obtained from the American Type Culture Collection (ATCC; www.atcc.org). SKOV-3 (ATCC# HTB-77) and ES-2 (ATCC# CRL-1978) cells were cultured in McCoy's 5A medium, MDA-MB-231 (ATCC# HTB-26) and A2058 (ATCC# CRL-11147) cells were cultured in Dulbecco's Modified Eagle Medium, and DU-145 (ATCC# HTB-81) cells were cultured in RPMI medium. All tissue culture media and supplements were obtained from Invitrogen (Carlsbad, CA).

[0071] Assay hardware: an automated 384-well plate-based cell scratch device was built. The device included a machined aluminum holding block into which 384 orifices had been drilled wide enough to accommodate 12.5 μl pipette tips (Matrix Technologies, Hudson, NH, USA). Sterilized pipette tips were inserted into the holding block, which was placed in a holding block receiving area of the system to suspend the holding block on a vertical tracking arm of a translational mechanism of the system. The holding block was raised against a top plate to stabilize the tip position and prevent any movement upon scratching. 384-well clear bottom tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) were placed on a level platform or container positioning component below the

aluminum block. Once the equipment was initiated, the aluminum holding block was automatically lowered to a point at which the pipette tips touched the bottom of each of the 384 wells. With the pipette tip holding block engaged, the container positioning component was shifted about 3 mm (well diameter = 3.70 mm) by hydraulic pressure, resulting in uniform cellular disruption or "scratches" in each of the 384 wells. Following scratching, the holding block was raised up from the plate, and the container positioning component returned to the start position to allow plates to be manually switched by the user.

[0072] siRNAs: Small interfering (si)RNAs were purchased from Dharmacon (Lafayette, CO) or Qiagen (Valencia, CA, USA), prepared and dispensed into 384-well plates as described (Aza-Blanc et al., Mol. Cell 12:627-637, 2003). The library is comprised of 10,996 siRNAs targeting 5,234 unique genes. Approx. 500 siRNAs in the collection are targeted to known and predicted human kinases as described; the remaining 10,500 siRNAs were designed to target specific families of genes which are considered pharmaceutically tractable, such as proteases, G-protein coupled receptors (GPCRs), cytokines and cytokine receptors, as well as other classes of genes, such as transcription factors, components of the cell cycle and apoptotic machinery.

[0073] 384-well scratch assay: Cells were plated at high density (4,000-5,000 cells per well) in media supplemented with 10% FBS. Cell density was calculated to result in >95% confluence at the time of scratching, accounting for the toxicity of the transfection reagent lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA). Cells were added to a siRNA/transfection reagent cocktail and deposited on the pre-plated siRNAs, resulting in reverse transfection, as described previously (Aza-Blanc et al., Mol. Cell 12:627-637, 2003). For small molecule experiments, compounds were added 12 hours prior to scratching at a final concentration of 0.5% DMSO. Media was changed in all experiments 24 hours after plating. Assay plates were fitted with metal low-evaporation covers and incubated at 37 °C, 5% CO2 in humidified tissue culture incubators. All liquid dispensing steps were performed using a Multidrop 384-well dispenser (Titertek, Huntsville, AL, USA). At 48hrs, confluent monolayers were scratched as described above. Cells were allowed to traverse the wound, typically resulting in closure of control cell wells by 12hrs. Following wound closure, cells were fixed with formaldehyde (Sigma, St. Louis, MO, USA) at a final concentration of 3.7% for 1 hour, washed and stained with the nuclear stain, DAPI (Molecular Probes, Eugene, OR,

USA). Each well of the 384-well plate was photographed by a fluorescent microscope re-tooled by Q3DM Inc (Beckman Coulter, San Diego, CA, USA) to automate image capture. A 4x objective lens was used to capture a majority of the space within each well. Images were collated and quantitatively scored as described below. For display purposes, images were imported into ImageJ (downloaded from the NIH; http://rsb.info.nih.gov/ij/). DAPI-stained nuclei were encircled and the images inverted.

[0074] Cell viability: Cells were plated into a "sister" set of 384-well siRNA assay plates and processed identically to the scratch plates. Viability was measured using Cell Titre Glo (Promega, Madison, WI, USA). The mean luminescent intensity of each plate was calculated, and the percent of the plate mean was calculated for each well. Small interfering RNAs or compounds resulting in an average percent mean of less than 90% were considered to negatively impact viability, and were eliminated from further study.

[0075] Quantitative scoring method: Automated microscopic capture of the assay generates one grayscale image per well (4x magnification). Bright regions represent DAPI-stained nuclei (cells) and black regions represent background; pixel intensities vary. The grayscale image is first converted into a binary black and white mask image, where cells are shown as white pixels and background in black pixels. The presence of contaminants, such as small hairs, etc, show up as unusually large blocks of continuous white regions and can be identified and excluded from our analysis. The initial scratch proceeds from left to right; however, on occasion, a scratch does not start or end beyond the left and right image borders. To avoid incorporating areas of unscratched, confluent cells, the left and right 25% of the original image are cropped.

[0076] An algorithm was implemented using MATLAB 6.5 of Image Processing Toolbox (The MathWorks, Inc., Natick, MA, USA) to quantify the results. The algorithm calculates the number of white pixels for every row in the image; the resultant curve represents cell density as a function of vertical location. The scratched zone contains significantly less white pixels compared to the rest of the image. Given a hypothetical scratch window, the motility score is defined as: $S = AM/AS$, wherein AS is proportional to the number of cells being removed by the scratch, and AM is proportional to the number of cells moving back into the denuded zone as the result of cell migration. A score close to 1 is

assigned to cells with high motility, and a score close to 0 to those with low motility. Since the score is self normalized by cell density, it is comparable across wells and plates.

[0077] The vertical center of the scratch may vary from well to well; therefore the algorithm does not assume a fixed scratch location. The above S score was iteratively calculated with every possible scratch center within a given range. Only the minimal possible S score is reported, and the corresponding location is the optimal guess of the scratch center. As input parameters, the method only takes the width of the scratch window and a possible range of scratch center. It does not require any training data and is insensitive to variations in cell density. Analysis on some randomly selected wells showed good correlation between our S score and visual inspection.

Example 2. Development of an automated motility assay system

[0078] This example describes development of an automated scratch-closing ("wound healing") assay designed to identify genes involved in promoting tumor cell metastasis. The process of monolayer wound healing is a widely used measure of a cell's migratory capacity. Experimentally, this entails scratching the surface of a confluent cell monolayer with a pipette tip or other blunt instrument, and observing the movement of cells across the denuded surface to "heal" the wound. This procedure was automated by developing a "scratch" device as described above. The scratch device creates uniform wounds in confluent monolayers of cells cultured in 384-well microtiter plates. Wound closure is monitored by automated CCD image capture of formaldehyde-fixed and DAPI-stained cells at a time when the wounds are typically "healed" in the presence of negative controls.

[0079] To examine tumor migration activity with the device, tumor cell with migratory potential are plated at high density in 384-well plates in which different siRNAs (or cDNAs) have been pre-plated. For siRNAs, the cells are incubated for 48 hours, scratched, and incubated for a further 12 hours to allow cells to migrate. For assessing the effect of small molecules, cells are plated, grown to confluency, and molecules are added 12 hours prior to scratching. Following scratching, cells are incubated for 12 hours as above. Following the timed post-scratch incubation, cells are fixed with formaldehyde and stained with the nuclear stain DAPI. Each well of the 384-well plate is then photographed by the

Q3DM high content imaging microscope using a 4x objective to visualize a majority of the space of each well. All assays are conducted in duplicate to assess the reproducibility of the results.

[0080] It is possible that a lack of closure of the wound/scratch may be due to a loss of cell viability, leading to the appearance of a specific block in cellular motility. To control for this possibility, a cell viability assay was run in parallel to the scratch assay. The viability plates are processed identically to sister scratch assay plates up until the point of fixation. At this point, the plates destined for the viability assay are incubated with Cell Titre Glo (Promega, Madison, WI), a reagent that measures cell viability through the measurement of ATP metabolism. Following incubation, the luminescent intensities of the wells containing Cell Titre Glo are recorded, with the intensity being proportional to the number of viable metabolizing cells in the well.

[0081] Efficacy of the assay system was first tested by examining migration of a tumor cell line in the presence of known modulators of tumor migration. The temporal migration of SKOV-3 cells, a highly migratory ovarian carcinoma-derived cell line, was monitored in the presence and absence of siRNAs, small molecules and appropriate controls. The efficacy of siRNA-mediated migratory inhibition was assessed using a siRNA against the RhoGTPase Rac1 and compared to a sequence scrambled, FITC-conjugated siRNA control (Figure 1A). Rac1 is an enzyme which integrates pro-migratory signals with dynamic reorganization of the actin cytoskeleton (Ridely et al., Science 302:1704-1709, 2003). The assay also included 2 compounds that target the c-Src kinase: SKI-606 (Golas et al, Cancer Research 65, 5358-5365, 2005) and Compound 43 (2-phenyl-aminoimidazo-[4,5-h]-isoquinolin-9-one; Goldberg et al, J. Medchem 46: 1337-1349, 2003). The activated form of c-Src plays a central role in the motility and invasion of cancer cells, including ovarian cancer. Effects of these compounds on SKOV-3 migratory activity were compared to diluent (DMSO) alone. As shown in Figure 1A, at cell densities ranging from 3,000 to 5,000 cells per well, cells migrated to close the wound typically within 12 hours. In contrast, the addition of Rac siRNA or Src inhibitor significantly inhibited wound closure in the same period of time.

[0082] Migratory inhibition of SKOV-3 cell by the Rac siRNA and the Src inhibitor migration was quantified in Figure 1B. In this figure, the degree of scratch closure

("migration score") was determined by an automated algorithm, and plotted as a function of time. Low migration scores reflect migratory inhibition. Fig. 1C shows western blot demonstrating knock-down of the RAC protein by the RAC-specific siRNA used in Fig. 1A, compared to a control siRNA (CON) and mock transfected cells (LIPO). Also shown in the figure are results of the same blot that was re-probed with anti-actin antibody to demonstrate equal loading.

[0083] In parallel, cell viability was measured in identically treated sister 384-well plates using an ATP-based luminescent assay, to monitor potential toxic effects of siRNA transfection and small molecule inhibition on SKOV-3 cells. The results indicate that in all cases (i.e., the Rac1 and control siRNAs and c-Src inhibition below 3μM), cell viability was comparable to controls (>90%).

[0084] The reproducibility of the assay was tested using a diverse subset of 384 pre-plated siRNAs targeting 192 genes (2 siRNAs per gene plated in duplicate). For these experiments, SKOV-3 cells were reverse transfected on each of three replicate plates, grown to confluency, wounded and incubated for a further 12 hrs. Following image capture, wells from each of the three replicate plates were scored by the quantitative algorithm described above and the score from each individual well in each of the three replicate runs was compared to the mean well score using the Pearson correlation coefficient. In each case, r2 was >0.87, demonstrating a high degree of well-to-well consistency.

Example 3. Screening siRNA library for pro-motility genes

[0085] The automated motility assay system described above was used to screen an siRNA library to identify genes that promote tumor cell motility. The screening employed a pre-plated library of 10,996 siRNAs, targeting 5,234 genes, to identify inhibitors of cellular motility in SKOV-3 cells (Figure 2). The screen was performed in duplicate (approx. 22,000 wells), as described above, and quantitatively scored. Measurement of cell viability was performed in a set of duplicate siRNA library plates and the luminescence of each well was compared to the normalized mean well intensity of each 384-well plate. Based on measurements from multiple controls that did not affect viability in this assay (i.e., control siRNAs), a cut-off of 0.9 (10% deviation from the plate mean) was adopted, below which

siRNAs affecting migration may have resulted from arrested cell growth or cell death and were therefore disregarded.

[0086] The top 5% of wells in which SKOV-3 cells migrated the least (n=532), were chosen for further analysis, based on a statistical review of the screen. Because of the significant potential for off-target effects when considering the phenotypic effects of single siRNAs, only those transcripts targeted by at least two independent siRNA sequences (n=23) were focused on, with the assumption that a similar phenotypic effect observed with two siRNAs would be less likely to occur by chance. To formally test this assumption, we re-synthesized the siRNAs from the library sequences and monitored transcript knockdown by semi-quantitative RT-PCR in parallel with migratory inhibition. Of the 48 siRNAs targeting 23 genes, 36 (74%) which target 17 genes yielded migratory phenotypes similar to that of the primary screen. However, the transcripts of 4 of these 17 genes were significantly diminished by both siRNAs, correlating precisely with the wounding phenotype (Figure 3A). These 4 genes are MAP4K4 (NM_004834), CDK7 (NM_001799), DYRK1B (NM_004714) and SERPINB3 (NM_006919). In addition, fibroblast growth factor receptor 1 (FGFR1) gene (NM_000604), which was shown to have strong effect on motility in the primary screen, was also chosen for additional validation studies.

[0087] We then assessed whether transcriptional inhibition of these genes may have a more general effect on carcinoma cell motility, i.e., whether the effects are cell type specific or reflect more general affects on migration. This was performed by using the most effective siRNAs targeting each of the genes in a small series of other migratory carcinoma cells from different anatomic origins in the 384-well assay. These cell lines include ES-2 (ovarian), MDA-MB-231 (breast), A2058 (melanoma) and DU145 (prostate). The results indicate that RNAi-mediated knockdown of MAP4K4 and CDK7 variably affected the migration of all of the cell types that we tested. In contrast, inhibition of SERPINB3 affected the motility of SKOV3, DU-145 and A2058 cell lines, and inhibition of DYRK1B affected the motility of SKOV3 and DU145 cell lines.

Example 4. Further examination of MAP4K4 and FGFR1 activities in tumor cell motility

[0088] Two of the pro-migration molecules described above, MAP4K4 and FGFR1, were subject to additional studies. The map kinase, MAP4K4, was chosen for further characterization for several reasons. First, siRNAs targeting MAP4K4 variably retarded the

migration of all motile carcinoma cells tested, suggesting a central role for this kinase in cell migration. Second, MAP4K4-deficient mice exhibit specific defects in the migration of neural crest cells during early development (Xue et al, Development 128:1559-1572, 2001), further supporting a migratory role. Third, over-expression of a rare cancer-associated splice variant of MAP4K4 in rat intestinal epithelial cells, was reported to increase cellular invasiveness in the presence of hepatocyte growth factor/scatter factor (HGF/SF), suggesting a role in cancer development (Wright et al, Mol Cell Biol 23:2068-2082, 2003).

[0089] Figure 3B illustrates the quantitative effects of 4 independent siRNAs (three from the primary screen and one additional siRNA, hereafter termed si_1 through si_4) on SKOV-3 migration. The migratory inhibition, which ranged from 50 to 66% relative to control siRNA-transfected cells, was consistent with the degree of transcript knockdown (Figure 3B), which ranged from 64 to 94%. The effect of the two most potent siRNAs (si_1 and si_2) on other highly motile carcinoma cells, and their associated transcriptional inhibition, are depicted in Figure 4. Migratory inhibition is evident in all 4 cell lines relative to control siRNA, with variably potency. We assume that the variability reflects: (1) variable transfection efficiencies, (2) differences in the relative expression of the gene and, (3) the effects of wide-ranging cellular densities on the ability of the automated scoring algorithm to comparably score different cell types relative to SKOV-3.

[0090] Because of the strong relationship between increased cancer cell motility, tissue invasion and metastasis, we next asked whether transient MAP4K4 knockdown could affect cell invasion. SKOV-3 cells were transfected using si_1 and si_2 and invasion monitored using a matrigel (Boyden) chamber assay. The results were compared to those obtained with SKOV-3 cells transfected with scrambled siRNA control. Invasion was inhibited by 76 and 52% with si_1 and si_2, respectively relative to control transfected cells (Figure 5).

[0091] To address the underlying signaling mechanism(s) through which MAP4K4 mediates its effects on cell migration, we asked whether MAP4K4 signaling converges on one or more of the JNK, p38 or Erk MAP kinase pathways in SKOV-3 cells. Knockdown of MAP4K4 by two independent siRNAs had no appreciable effect on the phosphorylation of Erk1/2 (Figure 6). This is consistent with a lack of migratory inhibition observed using the Erk pathway inhibitor, U0126, or siRNAs specific to the MEK1 kinase (data not shown). Similarly, cells transfected with MAP4K4 siRNAs did not show any differences in

detectable levels of phospho-p38 kinase. In contrast, phosphorylation of c-Jun N-terminal kinase (JNK) was significantly decreased in MAP4K4 siRNA transfected cells, consistent with reports showing that MAP4K4 can phosphorylate JNK *in vitro*. We examined the effect of a small molecule JNK-specific inhibitor, SP600125, on migratory activity of the SKOV-3 cells using the automated motility assay system described above. It was found that SKOV-3 migration as measured by scratch-closing ability was inhibited by the presence of SP600125 in a dose dependent manner (from 0.3 μM to 90μM; Figure 7). As a control, effect of treatment with the JNK inhibitor on viability of the cells was also assessed. The results indicate that cell viability was not significantly affected by the presence of SP600125. Thus, it is clear that JNK plays a significant role in mediating MAP4K4 signaling. These results, together with the observation that siRNAs targeting rac1 inhibit SKOV-3 migration, suggest a constitutively active rac1-MEKK1-JNK pathway in SKOV-3 cells. This was validated in part by the showing that exogenous EGF was able to stimulate JNK phosphorylation significantly above basal levels (data not shown).

[0092] Although EGF can activate MEKK1-JNK by phosphorylation of focal adhesion kinase (FAK), validated siRNAs against FAK were inactive in the SKOV-3 migration assay, thus pointing to rac1 as the likely mediator. We next asked whether the effect of JNK on SKOV-3 migration was mediated through activation of AP1 transcription. To first address this, the expression profile of cells transiently transfected with two MAP4K4 siRNAs (si_1 and si_2) was assessed by oligonucleotide microarray hybridization on a 22,500-member array. The only significantly altered probe-sets when compared to control cells were those homologous to the MAP4K4 transcript, strongly suggesting a lack of AP1-mediated transcription (data not shown). Basal c-Jun phosphorylation levels were essentially absent in SKOV-3 cells, confirming the lack of AP1 activation (data not shown).

[0093] In the initial screen, we noted that several siRNAs targeting the fibroblast growth factor receptor, type 1 (FGFR1), led to a moderate retardation of the scratch phenotype, albeit with a migration score just under the 5% cutoff chosen above. Nonetheless, the effect of FGFR1 inhibition on cell motility was studied further.

[0094] First, all of the siRNAs targeting FGFR1 present in the original library were re-synthesized, and a small series of siRNAs targeting FGFR1 from were purchased Dharmacon Incorporated (Lafayette, CO). Of these, we identified 5 independent siRNAs that robustly affected the migration of SKOV-3 cells as compared to cells transfected with

control siRNAs (Figure 8). RT-PCR using primers specific for the FGFR1 transcript was performed on cells trasfected with all 5 siRNAs under identical conditions to the scratch assay. All of the siRNAs diminished the FGFR1 transcript by at least 74% (range 74-86%). We used two of the most potent siRNAs in the Boyden chamber invasion assay to assess whether inhibition of FGFR1 affected the invasive capacity of SKOV-3 cells. Both siRNAs had an appreciable affect in the assay, reducing the numbers of invading cells by ~20 and 30%, respectively, compared to controls ($p<0.05$).

[0095] These two siRNAs were examined for their effect on motility of the other tumor cell lines, MDA-MB-231 (MDA-231), DU145, ES-2 and A2058. Aside from ES-2 cells, the 2 siRNAs reduced motility in the other cell lines by at least 40% (Figure 9). This highly significant result indicates that FGFR1 promotes migratory activities of multiple carcinoma cell lines.

[0096] Finally, we used a small molecule designed against the catalytic domain of FGFR1, PD173074, and assessed its effect in the scratch assay over a range of concentrations (from 3.3uM to 0.04uM). The effects were compared to a SRC inhibitor (described above), as well as a pan-kinase inhibitor, staurosporine, for reference. FGFR1 inhibition reduced motility by at least 40%, even at concentrations as low as 40nM. Notably, PD173074 concentrations as high as 3.3μM did not appreciably affect cell viability (Figure 10A). The small molecules were used to assess SKOV-3 cell invasion in Boyden chambers as above. The results indicate that PD173074 at a concentration of 3μM inhibited invasion by ~50% (Figure 10B).

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[0097] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, patent applications, polynucleotide and polypeptide sequence accession numbers and other documents cited herein are hereby incorporated by reference in their entirety and for all purposes to the same extent as if each of these documents were individually so denoted.

We Claim:

1. A method for identifying an agent that modulates motility of a cell, the method comprising (a) screening test compounds to identify one or more modulating compounds which modulate a pro-migration molecule selected from the group consisting of MAP4K4, CDK7, FGFR1, DYRK1B and SERPINB3; and (b) testing the identified modulating compounds for ability to modulate motility of the cell.

2. The method of claim 1, wherein the test compounds are screened for ability to modulate cellular level of the pro-migration molecule.

3. The method of claim 1, wherein the pro-migration molecule is MAP4K4, CDK7, FGFR1, or DYRK1B, and the test compounds are screened for ability to modulate the kinase activity of the pro-migration molecule.

4. The method of claim 6, wherein the pro-migration molecule is SERPINB3, and the test compounds are screened for ability to modulate the proteinase-inhibiting activity of SERPINB3.

5. The method of claim 1, wherein the cell is a tumor cell.

6. A method for identifying an agent that inhibits tumor metastasis, the method comprising (a) screening test compounds to identify one or more modulating compounds which down-regulate a pro-migration molecule selected from the group consisting of MAP4K4, CDK7, FGFR1, DYRK1B and SERPINB3, and (b) testing the identified modulating compounds for ability to inhibit migration of a tumor cell.

7. The method of claim 6, further comprising testing the identified modulating compounds for ability to modulate migration of a non-tumor control cell.

8. The method of claim 6, wherein the test compounds are screened for ability to down-regulate cellular level of the pro-migration molecule.

9. The method of claim 6, wherein the pro-migration molecule is MAP4K4, CDK7, FGFR1, or DYRK1B, and the test compounds are screened for ability to inhibit the kinase activity of the pro-migration molecule.

10. The method of claim 6, wherein the pro-migration molecule is SERPINB3, and the test compounds are screened for ability to inhibit the proteinase-inhibiting activity of SERPINB3.

11. The method of claim 6, wherein the pro-migration molecule is MAP4K4, CDK7, or FGFR1, and the tumor cell is selected from the group consisting of an ovarian tumor cell, a breast tumor cell, a melanoma tumor cell, and a prostate tumor cell.

12. The method of claim 6, wherein the pro-migration molecule is SERPINB3, and the tumor cell is selected from the group consisting of an ovarian tumor cell, a melanoma tumor cell, and a prostate tumor cell.

13. The method of claim 6, wherein the pro-migration molecule is DYRK1B, and the tumor cell is selected from the group consisting of an ovarian tumor cell and a prostate tumor cell.

14. The method of claim 6, wherein (b) comprises comparing migration of a cultured tumor cell in the presence of a modulating compound to migration of the tumor cell in the absence of the modulating compound; wherein a significant inhibition of migration of the tumor cell in the presence of the modulating compound relative to migration of the tumor cell in the absence of the modulating compound identifies the modulating compound as an agent that inhibits tumor metastasis.

15. The method of claim 14, wherein migration of the tumor cell is examined with a matrigel Boyden chamber assay.

16. The method of claim 14, wherein migration of the tumor cell is examined by monitoring closure of a scratch in a culture of the tumor cell.

17. A method for inhibiting tumor invasion and metastasis in a subject, the method comprising administering to the subject a pharmaceutical composition comprising an effective amount of an agent which down-regulates a pro-migration molecule selected from the group consisting of MAP4K4, CDK7, FGFR1, DYRK1B and SERPINB3.

18. The method of claim 17, wherein the tumor is selected from the group consisting of an ovarian tumor, a breast tumor, a melanoma tumor, and a prostate tumor.

19. The method of claim 17, wherein the agent down-regulates expression of a gene encoding the pro-migration molecule.

20. The method of claim 19, wherein the agent is selected from the group consisting of an short interfering RNA (siRNA), a microRNA (miRNA), and a synthetic hairpin RNA (shRNA), an anti-sense nucleic acid, and a complementary DNA (cDNA).

21. The method of claim 17, wherein the agent is an antibody that specifically binds to the pro-migration molecule.

22. The method of claim 21, wherein the pro-migration molecule is MAP4K4, CDK7, FGFR1, or DYRK1B, and the antibody inhibits the kinase activity of the pro-migration molecule.

23. The method of claim 21, wherein the pro-migration molecule is SERPINB3, and the antibody inhibits the proteinase-inhibiting activity of SERPINB3.

24. The method of claim 17, wherein the antibody is a monoclonal antibody.