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(54) **METHODS AND COMPOSITIONS FOR DETECTION OF TARGETS INVOLVED IN CANCER METASTASIS**

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(57) **ABSTRACT**

The present invention is further directed to methods and compositions for identifying target nucleic acids that are involved in cancer metastasis.

METHODS AND COMPOSITIONS FOR DETECTION OF TARGETS INVOLVED IN CANCER METASTASIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Ser. No. 61/949,927, filed Mar. 7, 2014, the content of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Metastasis is a complex series of steps in which cancer cells leave the original tumor site and migrate to other parts of the body via the bloodstream, the lymphatic system, or by direct extension. The mechanisms underlying metastasis are not fully understood. There is a need for characterization of the genes and proteins involved in metastasis to identify potential targets for therapeutics that inhibit or prevent metastasis.

SUMMARY OF THE INVENTION

[0003] Accordingly, the present invention provides a method of identifying one or more genes that modulate tumor metastasis. The method includes the steps of: (a) transfecting an immortalized cell line with one or more target cDNAs to produce transfected cells, wherein the transfected cells further express a detectable reporter gene; (b) injecting the transfected cells into a non-human mammal; (c) imaging the injected animal model to detect tumor metastasis; (d) isolating tumor cells from the injected animal model; (e) analyzing the isolated tumor cells to identify whether open reading frames from the one or more target cDNAs are integrated into genomes of the tumor cells, thereby identifying one or more genes that modulate tumor metastasis. In certain embodiments, the immortalized cell line that is transfected is a cell line that lacks metastatic potential.

[0004] In certain embodiments, the animal model used in methods of the invention is a mouse model. In further embodiments, the animal model is an immune-deficient mouse that lacks B- and T-cell lineages.

[0005] In further embodiments and in accordance with any of the above, the one or more target cDNAs comprise a library of at least 100 cDNAs.

[0006] In yet further embodiments and in accordance with any of the above, the one or more target cDNAs comprise genes encoding transmembrane proteins.

[0007] In further embodiments and in accordance with any of the above, prior to the isolating step (d), the injected animal model is imaged periodically for at least 50 days.

[0008] In still further embodiments and in accordance with any of the above, the one or more target cDNAs comprises at least 80 pools of genes, wherein each pool comprises at least 40 genes.

[0009] In yet further embodiments and in accordance with any of the above, different cells are transfected with different cDNAs, such that each transfected cell expresses a single target cDNA.

[0010] In further embodiments and in accordance with any of the above, the analyzing is conducted using a method selected from the group consisting of PCR, Southern Blot, and qRT-PCR.

[0011] In further embodiments and in accordance with any of the above, each of the target cDNAs comprises an open reading frame of a gene encoding a transmembrane receptor and a CMV promoter.

DETAILED DESCRIPTION OF THE INVENTION

[0012] The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, phage display, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) Biochemistry (4th Ed.) Freeman, New York, Gait, *"Oligonucleotide Synthesis: A Practical Approach"* 1984, IRL Press, London, Nelson and Cox (2000), *Lehninger, Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry,* 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

[0013] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polymerase" refers to one agent or mixtures of such agents, and reference to "the method" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0014] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing devices, compositions, formulations and methodologies which are described in the publication and which might be used in connection with the presently described invention.

[0015] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

[0016] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to

those skilled in the art have not been described in order to avoid obscuring the invention.

[0017] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the composition or method. "Consisting of" shall mean excluding more than trace elements of other ingredients for claimed compositions and substantial method steps. Embodiments defined by each of these transition terms are within the scope of this invention. Accordingly, it is intended that the methods and compositions can include additional steps and components (comprising) or alternatively including steps and compositions of no significance (consisting essentially of) or alternatively, intending only the stated method steps or compositions (consisting of).

[0018] All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (−) by increments of 0.1. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". The term "about" also includes the exact value "X" in addition to minor increments of "X" such as "X+0.1" or "X−0.1." It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

[0019] A "composition" may include any substance comprising an agent or compound and is also intended to encompass any combination of an agent or compound and other substances, including a carrier, e.g., compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like. Carriers also include pharmaceutical excipients and additives proteins, peptides, amino acids, lipids, and carbohydrates (e.g., sugars, including monosaccharides, di-, tri-, tetra-, and oligosaccharides; derivatized sugars such as alditols, aldonic acids, esterified sugars and the like; and polysaccharides or sugar polymers), which can be present singly or in combination, comprising alone or in combination 1-99.99% by weight or volume. Exemplary protein excipients include serum albumin such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, and the like. Representative amino acid/antibody components, which can also function in a buffering capacity, include alanine, glycine, arginine, betaine, histidine, glutamic acid, aspartic acid, cysteine, lysine, leucine, isoleucine, valine, methionine, phenylalanine, aspartame, and the like. Carbohydrate excipients are also intended within the scope of this invention, examples of which include but are not limited to monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol) and myoinositol.

[0020] The term pharmaceutically acceptable carrier (or medium), which may be used interchangeably with the term biologically compatible carrier or medium, refers to reagents, cells, compounds, materials, compositions, and/or dosage forms that are not only compatible with the cells and other agents to be administered therapeutically, but also are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other complication commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable carriers suitable for use in the present invention include liquids, semi-solid (e.g., gels) and solid materials (e.g., cell scaffolds and matrices, tubes sheets and other such materials as known in the art and described in greater detail herein). These semi-solid and solid materials may be designed to resist degradation within the body (non-biodegradable) or they may be designed to degrade within the body (biodegradable, bioerodable). A biodegradable material may further be bioresorbable or bioabsorbable, i.e., it may be dissolved and absorbed into bodily fluids (water-soluble implants are one example), or degraded and ultimately eliminated from the body, either by conversion into other materials or breakdown and elimination through natural pathways.

[0021] As used herein, the term "patient" or "subject" intends an animal, a mammal or yet further a human patient. For the purpose of illustration only, a mammal includes but is not limited to a human, a simian, a murine, a bovine, an equine, a porcine or an ovine.

[0022] As used herein, the term "oligonucleotide" or "polynucleotide" refers to a short polymer composed of deoxyribonucleotides, ribonucleotides or any combination thereof. Oligonucleotides are generally at least about 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100 or more nucleotides in length. An oligonucleotide may be used as a primer or as a probe.

[0023] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

[0024] The term "isolated" as used herein refers to molecules or biological or cellular materials being substantially free from other materials, e.g., greater than 70%, or 80%, or 85%, or 90%, or 95%, or 98%. In one aspect, the term "isolated" refers to nucleic acid, such as DNA or RNA, or protein or polypeptide, or cell or cellular organelle, or tissue or organ, separated from other DNAs or RNAs, or proteins or polypeptides, or cells or cellular organelles, or tissues or organs, respectively, that are present in the natural source and which allow the manipulation of the material to achieve results not achievable where present in its native or natural state, e.g., recombinant replication or manipulation by mutation. The term "isolated" also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not

naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides, e.g., with a purity greater than 70%, or 80%, or 85%, or 90%, or 95%, 98%, or 99%. The term "isolated" is also used herein to refer to cells or tissues that are isolated from other cells or tissues and is meant to encompass both cultured and engineered cells or tissues.

[0025] A "recombinant" nucleic acid refers an artificial nucleic acid that is created by combining two or more sequences that would not normally occur together. In one embodiment, it is created through the introduction of relevant DNA into an existing organismal DNA, such as the plasmids of bacteria, to code for or alter different traits for a specific purpose, such as antibiotic resistance. A "recombinant" polypeptide is a polypeptide that is derived from a recombinant nucleic acid.

[0026] As used herein, the term "promoter" refers to a nucleic acid sequence sufficient to direct transcription of a gene. Also included in the invention are those promoter elements which are sufficient to render promoter dependent gene expression controllable for cell type specific, tissue specific or inducible by external signals or agents.

[0027] In some embodiments, a promoter is an inducible promoter or a discrete promoter. Inducible promoters can be turned on by a chemical or a physical condition such as temperature or light. Examples of chemical promoters include, without limitation, alcohol-regulated, tetracycline-regulated, steroid-regulated, metal-regulated and pathogenesis-related promoters. Examples of discrete promoters can be found in, for examples, Wolfe et al. Molecular Endocrinology 16(3): 435-49.

[0028] As used herein, the term "regulatory element" refers to a nucleic acid sequence capable of modulating the transcription of a gene. Non-limiting examples of regulatory element include promoter, enhancer, silencer, poly-adenylation signal, transcription termination sequence. Regulatory element may be present 5' or 3' regions of the native gene, or within an intron.

[0029] As used herein, the term "preventing" refers to identifying a subject (i.e., a patient) having an increased susceptibility to a disease but not yet exhibiting symptoms of the disease, and administering a therapy according to the principles of this disclosure. The preventive therapy is designed to reduce the likelihood that the susceptible subject will later become symptomatic or that the disease will be delay in onset or progress more slowly than it would in the absence of the preventive therapy. A subject may be identified as having an increased likelihood of developing the disease by any appropriate method including, for example, by identifying a family history of the disease or other degenerative brain disorder, or having one or more diagnostic markers indicative of disease or susceptibility to disease.

[0030] As used herein, the term "sample" or "test sample" refers to any liquid or solid material containing nucleic acids. In suitable embodiments, a test sample is obtained from a biological source (i.e., a "biological sample"), such as cells in culture or a tissue sample from an animal, most preferably, a human.

[0031] As used herein, the term "substantially identical", when referring to a protein or polypeptide, is meant one that has at least 80%, 85%, 90%, 95%, or 99% sequence identity to a reference amino acid sequence. The length of comparison is preferably the full length of the polypeptide or protein, but is generally at least 10, 15, 20, 25, 30, 40, 50, 60, 80, or 100 or more contiguous amino acids. A "substantially identical" nucleic acid is one that has at least 80%, 85%, 90%, 95%, **96**%, **97**%, 98% or 99% sequence identity to a reference nucleic acid sequence. The length of comparison is preferably the full length of the nucleic acid, but is generally at least 20 nucleotides, 30 nucleotides, 40 nucleotides, 50 nucleotides, 75 nucleotides, 100 nucleotides, 125 nucleotides, or more.

[0032] A population of cells intends a collection of more than one cell that is identical (clonal) or non-identical in phenotype and/or genotype.

[0033] "Substantially homogeneous" describes a population of cells in which more than about 50%, or alternatively more than about 60%, or alternatively more than 70%, or alternatively more than 75%, or alternatively more than 80%, or alternatively more than 85%, or alternatively more than 90%, or alternatively, more than 95%, of the cells are of the same or similar phenotype. Phenotype can be determined by a pre-selected cell surface marker or other marker.

[0034] "Encoded by" refers to a nucleic acid sequence which codes for a gene product, such as a polypeptide.

[0035] Although the present invention is described primarily with reference to specific embodiments, it is also envisioned that other embodiments will become apparent to those skilled in the art upon reading the present disclosure, and it is intended that such embodiments be contained within the present inventive methods.

[0036] The present invention is directed to methods and compositions for detecting the components of signaling pathways that lead to cancer metastasis. In general, the present invention provides screening methods in which metastasis is essentially "rescued" in cells that lack metastatic potential. This rescue is conducted in vivo in an animal model, and in certain embodiments, the animal model is an immune-deficient mouse that lacks B- and T-cell lineages. A unique feature of the screen of the present invention is that it is an in vivo screen in which development of metastasis can be monitored utilizing non-invasive methods.

[0037] In one aspect, the present invention utilizes an immortalized cell line derived from a RhoC knockout mouse (Hakem et al., Genes Dev., 2005, 19(17): 1974-1979). This mouse has decreased in vivo metastatic potential of tumor cells, increased in vivo apoptosis of metastatic cells, and reduced motility and invasiveness of tumor cells in assays conducted in vitro. Cell lines derived from such a mouse thus lack metastatic potential—if injected into an animal model, particularly an animal model lacking B- and T-cell lineages, cells derived from the RhoC knockout mouse will generate tumors, but those tumors will not metastasize. The present screen utilizes such cells to identify target nucleic acids that rescue the metastatic potential of these cells. As discussed in further detail herein, the immortalized cell line from the RhoC knockout mouse can be transfected with one or more target cDNAs and then injected into an animal model, generally a mouse model. If the one or more target cDNAs in the transfected cells are involved in the mechanisms of metastasis, they will, upon injection of the transfected cells, rescue the metastatic potential of the cells and cause metastases to form within the animal model.

[0038] One aspect of the present invention encompasses an immortalized cell line derived from the RhoC knockout mouse. In a further embodiment, the immortalized cell line is produced from mammary tumor cells from the RhoC knock-

out mouse. Such tumors can be dissected from the mouse model and transferred to culture conditions using methods known in the art. In some embodiments, the cells spontaneously immortalize after being maintained in culture for a period of about 3-6 months.

[0039] In one aspect, the present invention provides methods for identifying one or more genes that modulate tumor metastasis, and in particular genes that promote metastasis and are thus targets for inhibiting or preventing metastasis. The screening methods of the present invention generally include the steps of: (a) transfecting an immortalized cell line with one or more target cDNAs to produce transfected cells, wherein the transfected cells further express a detectable reporter gene (which in some embodiments is a luciferase), and wherein the transfected cells lack metastatic potential in the absence of the one or more target cDNAs; (b) injecting the transfected cells into a non-human mammal, wherein the non-human mammal is an animal model that is resistant to tumor metastasis prior to the injecting; (c) imaging the injected animal model to detect tumor metastasis; (d) isolating tumor cells from the injected animal model; (e) analyzing the isolated tumor cells to identify whether open reading frames from the one or more target cDNAs are integrated into genomes of the tumor cells, thereby identifying one or more genes that modulate tumor metastasis.

[0040] In certain embodiments, the animal model used in methods of the invention is a mouse model. In further embodiments, the animal model is an immune-deficient mouse that lacks B- and T-cell lineages.

[0041] In further aspects and in accordance with any of the above, the screening methods of the invention are conducted using pools of target nucleic acids—the bulk of the discussion herein is in term of cDNAs, but as will be appreciated, other nucleic acids can be used for incorporation into cell lines using methods known in the art. In some aspects, one or more pools containing about 10-100 target cDNAs are transfected (or otherwise incorporated into) a cell line for later injection into the animal model. In further embodiments, the pools comprise nucleic acids from a large library divided into parts for efficiency in incorporation into the cell lines. In further embodiments, a library of about 1000-10000, 2000-9000, 3000-8000, 4000-7000, 5000-6000 target cDNAs is divided into about 10-500, 20-450, 30-400, 40-350, 50-300, 60-250, 70-200, 80-150, 90-100 pools for incorporation into the cell lines. The division of the full set of nucleic acids can be equal or non-equal among the different pools. The cells expressing these pools of target genes are, as is described in further detail herein, cells that lack metastatic potential in the absence of these target cDNAs. Once the cells are transfected with the target cDNAs (or the pools of target cDNAs) and then injected into the mouse model, if tumor cells develop and metastasize, those tumor cells can be analyzed to determine whether any of the target cDNAs were incorporated into the genomes of those tumor cells—if any of the target cDNAs are detected, then those target cDNAs are identified as contributing to the development of metastasis. Dividing the target cDNAs into pools prior to transfection increases the throughput of the screen by allowing for multiple potential targets to be "loaded" into the same cells that are subsequently injected into the animal model.

[0042] In further aspects and in accordance with the above, rather than incorporating multiple cDNAs into cells for later injection into the mouse model, the cells instead are treated to express a single target cDNA. Multiple cells incorporating different target cDNAs can then be injected into the animal model to screen for metastasis rescue, but each individual cell will express only a single target cDNA. Such aspects may be of use for the original screen to identify targets for treatment of metastasis. This "single-target" embodiment of the screen may also be used as a second validation screen if multiple potential targets are identified in an original screen in which multiple cDNAs or pools of cDNAs are used to transfect the cells prior to their injection into the animal model.

[0043] As will be appreciated, the target cDNAs used for screens in accordance with the present invention can include any targets that have the potential to participate in the mechanisms underlying metastasis. In certain embodiments, the target cDNAs comprise sequences encoding transmembrane proteins. In further embodiments the target cDNAs screened in methods of the present invention may encode any one or any combination of transmembrane proteins (including without limitation cell surface receptors), cell adhesion proteins, proteinases (including without limitation matrix metalloproteinases), and beta arrestin proteins.

[0044] In further embodiments and in accordance with any of the above, each of the target cDNAs used in methods of the invention comprises an open reading frame of a gene as well as additional elements. These additional elements may include without limitation sequences that identify the target cDNAs (i.e., barcode sequences) during the analysis steps of the screening method. The target cDNAs may further include promoter sequences that help drive expression of the cDNAs in the host cells. In certain embodiments, the target cDNAs further comprise a cytomegalovirus (CMV) promoter. As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a spatially or temporally restricted manner.

[0045] As will be appreciated, any appropriate vectors may be used to introduce the target nucleic acids into host cells using well known techniques, such as infection, transduction, transfection, transvection, electroporation and transformation and accompanying reagents typically used to introduce the compositions into a cell. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into cells. In one embodiment, the vector may be, for example, a phage, plasmid, viral or retroviral. Exemplary viral and retroviral vectors include adenovirus vectors, adeno-associated virus vectors, lentivirus vectors, herpes simplex virus (HSV) vectors, human immunodeficiency virus (HIV) vectors, bovine immunodeficiency virus (BIV), murine leukemia virus (MLV), and the like. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing target host cells. In a preferred embodiment, the vector is a recombinant retroviral vector. A gene delivery vehicle can optionally comprise viral sequences such as a viral origin of replication or packaging signal. These viral sequences can be selected from viruses such as astrovirus, coronavirus, orthomyxovirus, papovavi-

rus, paramyxovirus, parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann et al. (Cell 33: 153, 1983), Cane and Mulligan (Proc. Nat'l. Acad. Sci. USA 81:6349, 1984), Miller et al. (Human Gene Therapy 1:5-14, 1990), U.S. Pat. Nos. 4,405,712, 4,861,719, and 4,980,289, and PCT Application Nos. WO 89/02,468, WO 89/05,349, and WO 90/02,806, the entire contents of which are incorporated herein by reference in their entireties. Numerous retroviral gene delivery vehicles can be utilized in the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Pat. No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart (Cancer Res. 53:3860-3864, 1993); Vile and Hart (Cancer Res. 53:962-967, 1993); Ram et al. (Cancer Res. 53:83-88, 1993); Takamiya et al. (J. Neurosci. Res. 33:493-503, 1992); Baba et al. (J. Neurosurg. 79:729-735, 1993); U.S. Pat. No. 4,777,127; GB 2,200,651; EP 0,345,242; and WO91/02805; the entire contents of which are incorporated herein by reference in their entireties. In specific embodiments, vectors of use in the present invention include lentivirus particles produced in 293FT cells.

[0046] In some embodiments, a vector of the invention may further include reporter genes, including genes encoding fluorescent proteins or enzymes, such as f3-galactosidase and alkaline phosphatase. In certain embodiments, fluorescent reporters may be replaced with alternate fluorescent reporters with shorter or longer protein half-life allowing more precise evaluation of the timing of regulatory control. A reporter may also be replaced by cassettes encoding protein substrates that allow observation (direct or indirect) of response based on cell/biochemical activity, e.g., in screens of chemical libraries to identify potential therapeutic chemical targets/leads. Such reporters may be included in the same vectors containing the target cDNAs or as separate vectors that are then co-transfected with the cDNAs. In other embodiments, the reporters are constitutively expressed in the host cells that are then transfected with one or more target cDNAs, as is discussed in further detail herein.

[0047] In certain embodiments and in accordance with any of the above, the screening methods of the invention include the use of cell lines that are not only transfected with one or more target cDNAs, but these cells also express a detectable reporter. Such detectable reporters allow imaging of the cells in a non-invasive manner as they are incorporated into the animal model and form tumors and/or metastases. Thus, metastases that are triggered by certain target cDNAs within the screen can be observed and followed by periodic imaging of the animal model. This imaging is generally conducted until the animal reaches a human endpoint (due to the continued growth of the metastases) and is sacrificed. In certain embodiments, the injected animal model is imaged periodically for at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 days. In further embodiments, the injected animal model is imaged periodically for about 1-24, 2-22, 3-20, 4-18, 5-16, 6-14, 7-12, 8-10 months. An advantage of the present screening method is that the animals can be maintained for whatever length of time is required for the metastasis to manifest, and thus target nucleic acids that affect and/or trigger slow growing tumors can be screened using the methods of the present invention.

[0048] The reporters used in the screening methods of the invention can include any reporters known in the art. Generally, such reporters are optical reporters, such as bioluminescent proteins (including without limitation luciferases) and fluorescent proteins (including without limitation green or blue fluorescent proteins). Other types of signals include secreted signals that can be detected from samples obtained from the animal, including blood or urine samples. Such secreted signals include without limitation secreted alkaline phosphatase, sialylated neural cell adhesion molecule (NCAM), Pax7 (paired box gene 7), and beta-human chorionic gonadotropin (B-HCG).

[0049] After it is determined that transfected cells injected into the mouse model have metastasized (i.e., the injected cells and/or their progeny have migrated from the site of injection to other points in the body to form tumors), methods of the invention include steps for isolating the tumor cells and analyzing them to detect which target cDNAs have been incorporated into the genome of those tumor cells, thereby identifying the cDNAs that may serve as targets for inhibiting or preventing metastasis. The analyzing of the tumor cells can be accomplished using any methods known in the art for detecting the presence of target nucleic acids within a sample. In certain embodiments, the analyzing is accomplished using a method selected from the group consisting of PCR, Southern Blot, and qRT-PCR.

[0050] In further embodiments, expression of nucleic acids are detected in isolated tumor cells from the animal model by methods including, but not limited to, hybridization (e.g., Southern or Northern analysis), polymerase chain reaction (PCR) (see, for example, U.S. Pat. Nos. 4,683,195; 4,683,202, and 6,040,166; "PCR Protocols: A Guide to Methods and Applications", Innis et al. (Eds.), 1990, Academic Press: New York), reverse transcriptase PCR (RT-PCT), anchored PCR, competitive PCR (see, for example, U.S. Pat. No. 5,747,251), rapid amplification of cDNA ends (RACE) (see, for example, "Gene Cloning and Analysis: Current Innovations, 1997, pp. 99-115); ligase chain reaction (LCR) (see, for example, EP 01 320 30801 320 308), one-sided PCR (Ohara et al., Proc. Natl. Acad. Sci., 1989, 86: 5673-5677), in situ hybridization, Taqman-based assays (Holland et al., Proc. Natl. Acad. Sci., 1991, 88: 7276-7280), differential display (see, for example, Liang et al., Nucl. Acid. Res., 1993, 21: 3269-3275) and other RNA fingerprinting techniques, nucleic acid sequence based amplification (NASBA) and other transcription based amplification systems (see, for example, U.S. Pat. Nos. 5,409,818 and 5,554,527), Qbeta Replicase, Strand Displacement Amplification (SDA), Repair Chain Reaction (RCR), nuclease protection assays, subtraction-based methods, Rapid-Scan™, and the like.

[0051] In some embodiments, analysis of the tumor cells utilizes nucleic acid molecules comprising polynucleotide sequences complementary to all or a portion of the target cDNA, which can then be used for amplification of specific polynucleotide sequences using an amplification method such as PCR (e.g., RT-PCR), followed by analysis of the amplified molecules using techniques known in the art. Suitable primers can be routinely designed by one skilled in the art. In order to maximize hybridization under assay conditions, primers and probes employed in the methods of the invention generally have at least 60%, preferably at least 75% and more preferably at least 90% identity to a portion of the targets.

[0052] In another aspect and in accordance with any of the above, the present invention provides a method of screening for proteins that utilizes a cell line produced from a $RhoC^{-/-}$

knockout mouse. This knockout mouse produces tumors, but the cells of those tumors are show reduced metastatic potential, particularly in the lung. This screen involves identifying targets that "rescue" the metastatic potential of this cell line, thus identifying proteins whose function and/or expression can be manipulated to treat cancer. In this screen, an immortalized cell line produced from tumors from the RhoC$^{-/-}$ mouse is transfected with different cDNA open reading frames (ORFs). The cells further express a luciferase reporter to allow tracing the cells in a living animal. This screening method is of particular use for genes encoding transmembrane proteins. To increase the throughput of the screen, thousands of genes are split into different pools of equal numbers of genes, and the pools of genes are transfected into the cells. After transfection, the cells are IV injected into immune-compromised female NOD-SCID mice. The animals are monitored in an in vivo imaging system to record bioluminescence emitted by the injected cells. This non-invasive assay allows following tumor growth/progression in vivo without having to sacrifice the animal. After 3-18 months post-injection, the mice are sacrificed and examined for tumors that developed from the injected cells. Tumors are analyzed, generally by PCR on genomic DNA extracts, to identify single oncogenic driver genes out of the pools. To further clarify the results of the screen, an additional second screen may be performed in which the pools of genes from the first part of the screen that were positive in terms of tumor development are further filtered according to identify only those pools for which the gene product localized to the surface of the cell or excreted to the extracellular space. Immortalized cells from the RhoC−/− knockout mouse are then transfected with one potential target gene. These transfected cells are then injected into the immune-compromised mice (again, the cells were also expressing luciferase to allow in vivo monitoring of tumor growth). The animals are then sacrificed 3-18 months post-injection and examined for tumors. The tumors are analyzed for oncogenic driver genes, generally by PCR on extracted genomic DNA.

[0053] In some embodiments, genes may be identified by characterizing nucleic acid extracted from a sample. In some embodiments, the nucleic acid comprises a deoxyribonucleic acid or a ribonucleic acid. In some embodiments, the nucleic acid comprises DNA, RNA, mRNA or cDNA. In some embodiments, nucleic acid may be extracted from the sample prior to analysis. In some embodiments, nucleic acid may be extracted from the subject using and extraction method know to those of skill in the art. (see, for example, J. Sambrook et al., "Molecular Cloning: A Laboratory Manual", 1989, 2.sup. nd Ed., Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y.). Most methods of RNA isolation from bodily fluids or tissues are based on the disruption of the tissue in the presence of protein denaturants to quickly and effectively inactivate RNAses. Isolated total RNA may then be further purified from the protein contaminants and concentrated by selective ethanol precipitations, phenol/chloroform extractions followed by isopropanol precipitation or cesium chloride, lithium chloride or cesium trifluoroacetate gradient centrifugations. Kits are also available to extract RNA (i.e., total RNA or mRNA) from bodily fluids or tissues and are commercially available from, for example, Ambion, Inc. (Austin, Tex.), Amersham Biosciences (Piscataway, N.J.), BD Biosciences Clontech (Palo Alto, Calif.), BioRad Laboratories (Hercules, Calif.), GIBCO BRL (Gaithersburg, Md.), and Qiagen, Inc. (Valencia, Calif.). In some embodiments an antibody may be an anti-peptide antibody. In some embodiments an antibody may be recombinantly produced or chemically synthesized, or produced by a hybridoma or other engineered cell line; in some embodiments, an antibody may be obtained from a natural source. In some embodiments an antibody is identified using a display technique such as phage display.

[0054] In some embodiments, hybridization and amplification techniques described herein may be used to assay qualitative and quantitative aspects of expression of nucleic acid molecules comprising polynucleotide sequences coding for genes or gene products described herein. In some embodiments, oligonucleotides or longer fragments from nucleic acids encoding each gene or gene product may be used as targets in a microarray. A number of different array configurations and methods of their production are known to those skilled in the art (see, for example, U.S. Pat. Nos. 5,445,934; 5,532,128; 5,556,752; 5,242,974; 5,384,261; 5,405,783; 5,412,087; 5,424,186; 5,429,807; 5,436,327; 5,472,672; 5,527,681; 5,529,756; 5,545,531; 5,554,501; 5,561,071; 5,571,639; 5,593,839; 5,599,695; 5,624,711; 5,658,734; and 5,700,637). Microarray technology allows for the measurement of the steady-state level of large numbers of polynucleotide sequences simultaneously. Microarrays currently in wide use include cDNA arrays and oligonucleotide arrays. Analyses using microarrays are generally based on measurements of the intensity of the signal received from a labeled probe used to detect a cDNA sequence from the sample that hybridizes to a nucleic acid probe immobilized at a known location on the microarray (see, for example, U.S. Pat. Nos. 6,004,755; 6,218,114; 6,218,122; and 6,271,002). Array-based gene expression methods are known in the art and have been described in numerous scientific publications as well as in patents (see, for example, M. Schena et al., Science, 1995, 270: 467-470; M. Schena et al., Proc. Natl. Acad. Sci. USA 1996, 93: 10614-10619; J. J. Chen et al., Genomics, 1998, 51: 313-324; U.S. Pat. Nos. 5,143,854; 5,445,934; 5,807,522; 5,837,832; 6,040,138; 6,045,996; 6,284,460; and 6,607,885).

[0055] In some embodiments, gene products may be identified by characterizing protein extracted from a sample, including without limitation a tumor sample. In some embodiments, protein may be extracted from the sample prior to analysis. In some embodiments, protein may be extracted without or with limited processing of the sample. For example, protein extract may be prepared from a sample. In some embodiments, a protein extract contains the total protein content. In some embodiments, protein extracts containing one or more of membrane proteins, nuclear proteins, and cytosolic proteins may be prepared. Methods of protein extraction are well known in the art (see, for example "Protein Methods", D. M. Bollag et al., 2.sup.nd Ed., 1996, Wiley-Liss; "Protein Purification Methods: A Practical Approach", E. L. Harris and S. Angal (Eds.), 1989; "Protein Purification Techniques: A Practical Approach", S. Roe, 2.sup.nd Ed., 2001, Oxford University Press; "Principles and Reactions of Protein Extraction, Purification, and Characterization", H. Ahmed, 2005, CRC Press: Boca Raton, Fla.). Numerous different and versatile kits can be used to extract proteins from bodily fluids and tissues, and are commercially available from, for example, BioRad Laboratories (Hercules, Calif.), BD Biosciences Clontech (Mountain View, Calif.), Chemicon International, Inc. (Temecula, Calif.), Calbiochem (San Diego, Calif.), Pierce Biotechnology (Rockford, Ill.), and Invitrogen Corp. (Carlsbad, Calif.). User Guides that describe in great detail the protocol to be followed are usually included

in all these kits. Sensitivity, processing time and costs may be different from one kit to another. One of ordinary skill in the art can easily select the kit(s) most appropriate for a particular situation. After the protein extract has been obtained, the protein concentration of the extract is preferably standardized to a value being the same as that of the control sample in order to allow signals of the protein markers to be quantitated. Such standardization can be made using photometric or spectrometric methods or gel electrophoresis.

[0056] In some embodiments, protein expression levels may be used to identify gene products in a sample, using any suitable method know in the art. (see, for example, E. Harlow and A. Lane, "Antibodies: A Laboratories Manual", 1988, Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y.). In general, protein expression levels are determined by contacting a biological sample obtained from a subject with binding agents for one or more of proteins; detecting, in the sample, the levels of one or more proteins that bind to the binding agents; and comparing the levels of one or more proteins in the sample with the levels of the corresponding proteins in a control sample. In some embodiments, a binding agent may comprise an entity such as a polypeptide or antibody that specifically binds to a protein. An entity "specifically binds" to a polypeptide if it reacts/interacts at a detectable level with the polypeptide but does not react/interact detectably with peptides containing unrelated sequences or sequences of different polypeptides.

[0057] In some embodiments, protein expression levels may be determined using mass spectrometry based methods or image (including use of labeled ligand) based methods known in the art for the detection of proteins. In some embodiments, detection methods include, but are not limited to, 2D-gel electrophoresis and proteomics-based methods. Proteomics, which studies the global changes of protein expression in a sample, can include the following steps: (1) separation of individual proteins in a sample by electrophoresis (1-D PAGE), (2) identification of individual proteins recovered from the gel (e.g., by mass spectrometry or N-terminal sequencing), and (3) analysis of the data using bioinformatics.

[0058] In some embodiments, expression levels may be determined for a sample being analyzed, by comparing its expression level to the expression levels in one or more control samples. Correction may be carried out using different methods well known in the art. For example, the protein concentration of a sample may be standardized using photometric or spectrometric methods or gel electrophoresis (as already mentioned above) before the sample is analyzed. In case of samples containing nucleic acid molecules, correction may be carried out by normalizing the levels against reference genes (e.g., housekeeping genes) in the same sample. Alternatively or additionally, normalization can be based on the mean or median signal (e.g., Ct in the case of RT-PCR) of all assayed genes or a large subset thereof (global normalization approach).

[0059] The screen of the present invention allows identification of new drug targets for prevention and/or inhibition of tumor metastasis. The screens of the present invention can further be used to validate targets identified by other methods to show that the selected targets are indeed involved in the pathway that leads to metastasis. The terms "tumor" or "cancer" refer to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, and certain characteristic morphological features.

EXAMPLE 1

Screening Method

[0060] A 293FT Cell Line: (https://www.lifetechnologies.com/order/catalog/product/R70007) was used to produce lentivirus particles. These cells were originally derived from human embryonic kidney (HEK) cells (Graham et al., 1977; Harrison et al., 1977) and have been genetically engineered to express the SV40 large T-antigen, which allows the episomal replication of transfected plasmids containing the SV40 origin of replication (Naldini et al., 1996). The 293FT cells were cultured in complete D-MEM medium containing 10% FBS supplemented with 1 mM sodium pyruvate, 6 mM L-glutamine and 1% Pen-Strep (antibiotics). Lenti virus production was performed according to guidelines/protocols provided by the RNAi Consortium (http://www.broadinstitute.org/mai/public/). Specifically, 293FT cells were transfected with pLenti-C-myc-DDK-IRES-PURO encoding the gene of interest (LILRB3), psPAX2 (expression of packaging proteins) and pCMV-VSVG (envelope plasmid for producing viral particles) by using the Lipofectamine™ 2000 Reagent (Cat#: 11668-027, Thermo Fisher Scientific Inc.). The lenti virus particles were concentrated using the Lenti-X™ Concentrator (Cat#: 631232, Clontech) prior to the virus transduction of target cells.

[0061] MMTV-PYMT-RhoC−/−_hb cells (murine origin, mammary tumor epithelial cell type) were cultured in DMEM/F12-HAM (1:1) medium supplemented with 10% FBS, 5 ug/mL insulin, 1 ug/mL hydrocortisone, 10 ng/mL EGF, 6 mM L-glutamine at 37° C., 5% CO2. MMTV-PYMT-RhoC−/−_hb cells were transduced with lenti virus particles that contained a cDNA/ORF of interest on the pLenti-C-myc-DDK-IRES-PURO plasmid. The transduced cells were selected using the antibiotic puromycin, until antibiotic resistance was obtained.

[0062] MMTV-PYMT-RhoC−/−_hb cells, stably expressing the cDNA/ORF of interest, were prepared in PBS solution in a concentration of 1×10^6 cells per 500 uL. 1×10^6 cells were intravenously (IV) injected into the tail-vein of one NOD-SCID mouse. 5 female NOD-SCID mice have been IV injected per cell line. MMTV-PYMT-RhoC−/−_hb cells expressing the 'empty' pLenti-C-myc-DDK-IRES-PURO vector were used as controls and were IV injected periodically into NOD-SCID mice. The NOD-SCID strain (http://jaxmice.jax.org/strain/001303.htm) is an immuno deficient mouse strain that lacks B- and T-cell lineages. All animal experiments were approved by the Animal Care and Use Committee of the University Health Network (Toronto, Canada) under the Canadian Council on Animal Care (CCAC).

[0063] Injected mice that reached humane end point, defined by University Health Network (UHN) Animal Care Committee (ACC), were euthanized prior to tumor dissection, tissue isolation and disease documentation. Isolated tumor tissue was preserved by formalin fixation and paraffin embedding, cryopreservation and snap-freezing for further analysis by qRT-PCR, immuno-blot, immuno-histochemistry (IHC) and immuno-fluorescent (IF) staining. Pictures of representative animals and tumor tissues (not shown) showed the severity of the disease and locations of tumor occurrence in

the experimental animals, validating which genes of interest served as an oncogenic driver and a target for therapeutics for inhibiting metastasis and treating cancer.

[0064] The present specification provides a complete description of the methodologies, systems and/or structures and uses thereof in example aspects of the presently-described technology. Although various aspects of this technology have been described above with a certain degree of particularity, or with reference to one or more individual aspects, those skilled in the art could make numerous alterations to the disclosed aspects without departing from the spirit or scope of the technology hereof. Since many aspects can be made without departing from the spirit and scope of the presently described technology, the appropriate scope resides in the claims hereinafter appended. Other aspects are therefore contemplated. Furthermore, it should be understood that any operations may be performed in any order, unless explicitly claimed otherwise or a specific order is inherently necessitated by the claim language. It is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative only of particular aspects and are not limiting to the embodiments shown. Unless otherwise clear from the context or expressly stated, any concentration values provided herein are generally given in terms of admixture values or percentages without regard to any conversion that occurs upon or following addition of the particular component of the mixture. To the extent not already expressly incorporated herein, all published references and patent documents referred to in this disclosure are incorporated herein by reference in their entirety for all purposes. Changes in detail or structure may be made without departing from the basic elements of the present technology as defined in the following claims.

1. A method of identifying one or more genes that modulate tumor metastasis, the method comprising:

(a) transfecting an immortalized cell line with one or more target cDNAs to produce transfected cells, wherein the transfected cells further express a detectable reporter gene, and wherein the immortalized cell line lacks metastatic potential in the absence of the transfected target cDNAs;

(b) injecting the transfected cells into a non-human mammal;

(c) imaging the injected animal model to detect tumor metastasis;

(d) isolating tumor cells from the injected animal model;

(e) analyzing the isolated tumor cells to identify whether open reading frames from the one or more target cDNAs are integrated into genomes of the tumor cells, thereby identifying one or more genes that modulate tumor metastasis.

2. The method of claim **1**, wherein the animal model is a mouse.

3. The method of claim **1**, wherein the animal model is an immune-deficient mouse that lacks B- and T-cell lineages.

4. The method of claim **1**, wherein the one or more target cDNAs comprise a library of at least 100 cDNAs.

5. The method of claim **1**, wherein the one or more target cDNAs comprise genes encoding transmembrane proteins.

6. The method of claim **1**, wherein prior to isolating step (d), the injected animal model is imaged periodically for at least 50 days.

7. The method of claim **1**, wherein the one or more target cDNAs comprises at least 80 pools of genes, wherein each pool comprises at least 40 genes.

8. The method of claim **1**, wherein different cells are transfected with different cDNAs, such that each transfected cell expresses a single target cDNA.

9. The method of claim **1**, wherein the analyzing is conducted using a method selected from the group consisting of PCR, Southern Blot, and qRT-PCR.

10. The method of claim **1**, wherein each of the target cDNAs comprises an open reading frame of a gene encoding a transmembrane receptor and a CMV promoter.

11. The method of claim **1**, wherein the detectable reporter gene encodes a luciferase.

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